THE MYCOTA 5
A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research
Series Editors: Dee Carter · Anuradha Chowdhary
Joseph Heitman · Ulrich Kück

Barry Scott
Carl Mesarich *Editors*

Plant Relationships

Fungal-Plant Interactions

Third Edition

Springer

The Mycota

A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research

Volume 5

The fungi represent a heterogenous assemblage of eukaryotic microorganisms and have become favored organisms for research at the cellular and molecular level. Such research involvement has been stimulated by interest in the biotechnological application of fungi in processes related to industry, agriculture and ecology. Considering both yeasts and mycelial fungi, THE MYCOTA highlights developments in both basic and applied research and presents an overview of fungal systematics and cell structure. Foremost authorities in research on mycology have been assembled to edit and contribute to the volumes.

Barry Scott • Carl Mesarich
Editors

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Series Preface

The Mycota Series—2022

The Mycota is an encyclopaedic book series published by Springer as a comprehensive treatise spanning the Fungal Kingdom through a focus on fungi as experimental systems for basic and applied research. Articles in this series present introductory information in a comprehensive manner to broaden our general knowledge, along with the state-of-the-art information on selected topics in fungal biology. Thus, these articles serve as a timeless reference source for historically relevant discoveries in fungal biology to drive further advances in the field.

The Mycota was founded in 1994 by Karl Esser and Paul Lemke. Since then 15 Volumes have been published, with several now in their third edition. The steadily growing interest in *The Mycota*, reflected in particular by the high number of e-book downloads, encouraged Springer to continue this publication with a new international Series Editorial board beginning in 2022.

Historically, the study of fungi originated as a sub-discipline of botany and was a largely descriptive discipline until the early 19th century. Subsequent experimental research has propelled the field of mycology, and achievements in the genetics and molecular biology of fungi have benefited studies in the related fields of fungal biochemistry and biophysics, plant pathology, medical mycology, and systematics. Ground-breaking research has been carried out using fungi as recognized by the receipt of nine Nobel Prizes, including the revolutionary 1945 Nobel Prize in Physiology and Medicine for the discovery of penicillin and the foundational 2006 Nobel Prize in Chemistry for establishing the molecular basis of eukaryotic transcription. All such studies expand our knowledge of fungi and of fungal processes and improve our ability to understand, utilize, and control fungi for the benefit of humankind.

Fungi have invaded every conceivable ecological niche. Saprobic forms abound, especially in the decay of organic debris. Pathogenic forms exist with both plant and animal hosts. Fungi even grow on other fungi. They are found in aquatic as well as soil environments, and their spores have been found in the

air of extreme environments. Fungi can be variously associated with plants as symbionts in the form of lichens, mycorrhizae, and endophytes and also occur as overt pathogens. Association with animal systems varies; examples include the predaceous fungi that trap nematodes, the micro-fungi that grow in the anaerobic environment of the rumen, and medically important pathogens afflicting humans. Currently, taxonomists have identified about 150,000 species, but it is thought that over 90% of fungal species remain undescribed, with conservative estimates ranging from 2 to 5 million fungal species on earth.

From this perspective, there are new topics in fungal biology to explore, which will expand the current volumes and take them in new directions. Further, there will be new volumes in areas (e.g., Cryptomycota) that were not covered before.

Our understanding of the evolution of fungi is still incomplete and mainly based on species that can be grown in culture. But recent environmental DNA analyses have revealed a highly diverse form of eukaryotic life that branches with the Fungi, and thus the resulting and highly diverse clades were named the Cryptomycota. The discovery of novel intermediate forms will redefine the fungal tree of life. Other topics will consider the adaptation of fungi to climate changes, the occurrence of fungal pathogens in the environment, and the dispersal of fungi during global pandemics.

Fungal constituents of the microbiome have received much less attention thus far, yet recent findings from clinical and animal studies clearly establish fungi as a significant component of the oral, gastrointestinal, pulmonary, and skin microbiomes. Finally, the relevance of fungal biology to society is reflected by the increasing number of fungal related human diseases.

In the history of pharmacology, fungi have always been sources of useful molecules for humans, but the diversity of molecules that can be obtained from fungi is still largely under explored. However, efforts in fungal genomics provide resources for data-driven genome mining and large-scale comparisons to explore the molecular repertoires produced by fungi. The result will be new compounds with applications in the pharmaceutical and agricultural science industries.

Fungi can also help the entire planet and may, for example, be relevant in specific sectors, such as that linked to pollution from plastic. Fungi produce a wide range of enzymes that have the potential to break down the chemical bonds of plastic polymers, and in this context the potential role of marine fungi in plastic degradation may be of major relevance. Finally, new biomaterials from fungal species may open the door to alternatives to fossil-based materials, and thus reduce environmental pollution.

For consistency throughout this series of volumes, the names adopted for major groups of fungi should be followed according to the following paper, which gives an overview of all of the orders in the fungal kingdom: https://pubmed.ncbi.nlm.nih.gov/32660385/

We are grateful to Springer for continuing *The Mycota* series and are especially thankful to all of the Volume Editors in selecting topics and assembling experts from diverse fields of fungal biology.

Sydney, Australia — Dee Carter
Delhi, India — Anuradha Chowdhary
Durham, USA — Joseph Heitman
Bochum, Germany — Ulrich Kück
July 15, 2022

Volume Preface to the Third Edition

Filamentous fungi can form a range of different associations with plants that vary from antagonistic through to mutualistic. However, the outcome of an interaction is very dependent on the stage of development of both plant and fungus, the genetic background of both organisms, and the ecological and environmental conditions under which they interact. Consequently, a given species may have a relatively benign interaction under one set of conditions but be severely pathogenic under a different set of conditions or stage of development. Despite this variability, some fungi are more generally recognized as plant pathogens and others as plant symbionts.

It is now over 10 years since the second edition of The Mycota, Volume V (Plant Relationships) was published. In the two earlier editions, the volume editors commented on the large number of fungi and vascular plants in the *Tree of Life* and the enormous number and complexity of the different interactions that occur in nature. It is therefore challenging in a single volume to capture this range of diversity. However, we hope that through the examples presented in this third edition the reader can gain a sense of the very significant new discoveries that have been made, what key biological questions are being addressed, and where the new frontiers in this field of research lie. To help the reader explore the huge range of research topics and diversity of fungi studied, we have divided the volume into six sections which we think capture the most important research themes within this very active and fast-moving area of research.

The theme of Chaps. 1–3 is *Pathogen Fungus–Plant Interactions*. These chapters provide a good overview of some of the key advances that have been made in understanding the molecular dialogue between pathogen and host, especially in the field of effector biology. Understanding the biological function of the protein effectors has been particularly challenging as most share little homology with functionally characterized proteins. Despite this challenge, very significant advances have been made in identifying the function of many effectors, including in some cases the host target proteins with which they interact. One of the most exciting emerging fields has been the demonstration that small RNAs play a crucial role in the dialogue between fungus and plant. This is, and will remain, a very active field of discovery.

Chapters 4–6 (*Mutualistic Fungus–Plant Interactions*) provide a snapshot of the symbiotic interactions that occur between fungi and plants both below ground (the arbuscular mycorrhiza) and above ground (leaf endophytes). A huge challenge with the former is the inability to culture most of these fungi separate from their host thereby limiting the methodologies that can be used to dissect these interesting associations. Recent advances in long-read DNA sequencing have enabled the complete assembly of a number of the large and complex arbuscular mycorrhizal fungal genomes thereby facilitating for the first time new insights into comparative and evolutionary genetics of this important group of fungi. The mutualistic interaction of *Epichloë* fungi with grasses is now the most well-understood above-ground fungal-plant mutualistic association. Comparative genome studies combined with genetic analysis and new advances in metabolite chemistry reveal a remarkable diversity of alkaloids synthesized by these seed endophytes. Among the more complex fungal-plant mutualistic symbioses are the lichens—associations between nutritionally specialized fungi and green algae and/or cyanobacteria photobionts. These associations are very diverse with approximately 17% of extant fungi species lichenized. The obligate nature of these associations makes it very challenging to study the individual contributions of the partners but advances in microscopy and genome analysis have provided significant new insights into these associations. Genome analysis of the mycobionts has provided important new insights into the lifestyle of these fungi, and in particular their potential to synthesize a remarkable range of secondary metabolites. The use of heterologous expression systems combined with mass spectrometry analysis has led to the identification of a range of novel lichen metabolites. A future challenge will be determining their biological role in nature.

How fungi sense signals from their host and respond through transduction of those signals internally is the theme of Chaps. 8–10 (*Sensing and Signalling in Fungus–Plant Interactions*). Among the most well-characterized signal transduction systems are the Mitogen-Activated Protein kinase (MAPK) pathways. Genetic analyses combined with protein–protein interactions have revealed how fungi respond to external stimuli and regulate infection and developmental processes. These studies demonstrate that there are conserved, as well as species-specific, functions associated with the MAPK pathways. In addition, there is considerable crosstalk among the pathways, a result that highlights the complexity of signalling associated with plant host colonization. Ambient pH is one of the key environmental signals that fungi encounter in their environment. Fungi have an exquisitely tuned system to regulate cytosolic pH in response to changes in external pH. Signalling through MAPK pathways control this system and lead to changes in fungal growth, development, and pathogenicity. Plant volatiles are key signals that control fungal invasion of their hosts. Using the *Trichoderma* system, key volatiles have been identified that control plant colonization as well as plant immunity and development.

The theme of Chaps. 11–14 is *Regulation of Fungal Gene Expression and Development*.

A key layer of gene expression control is epigenetic regulation. Transcriptome analysis demonstrates that there is a dramatic difference in fungal gene expression between the free-living and *in planta* fungal life styles highlighted by the dramatic upregulation of genes encoding effectors and enzymes for fungal secondary metabolite biosynthesis. Distinct changes in the chromatin structure accompany these dramatic changes in gene expression but the underlying metabolic cues and mechanisms that lead to these changes in gene expression remain to be determined. Development of structures such as an appressorium is crucial for some fungi to breach the physical barriers of the plant host. The formation of a large, relatively easy to study, appressorium by *Magnaporthe oryzae* on the surface of rice leaves provides an ideal model experimental system to understand this key developmental process. Powerful cell imaging methodology has demonstrated that a septin-mediated ring forms at the base of the appressorium in response to turgor-induced signalling through key signalling pathways. Besides plant cues, fungi also respond to key environmental cues such as light. Studies in *Botrytis cinerea* provide important new insights into how light controls fungal development and host colonization.

A key resource for understanding the biology and evolution of plant-associated fungi has been the availability of telomere-to-telomere assemblies of many fungal genomes. Two exemplars of the power of these new whole genome approaches are provided in Chaps. 15 and 16 (*Genomes and Evolution*). The first highlights the power of comparative genome analysis in increasing our understanding of how fungal pathogens evolve through such processes as gene flow among populations, genetic exchange between species and the action of transposons, and recombination processes within the genome. The second highlights the role of accessory chromosomes in pathogenicity and host adaptation. The genomes of both *Zymoseptoria* and *Fusarium* are comprised of two components, core and accessory, with each contributing in different ways to the overall genome dynamics of the organism and its ability to adapt to their plant hosts.

The theme of the final section (Chaps. 17 and 18) is *Global Pandemics and Food Security*. The first chapter on the *Puccinia* rust fungi demonstrates the power of genomics in understanding virulence evolution in this group of fungi and the molecular basis for disease epidemics. Novel mechanisms are involved in the diversification of these pathogens, which enable an evolutionary arms race between pathogen and hosts. Maintenance and further development of high-quality genome resources will be crucial in helping manage these devastating epidemics. The second chapter highlights the threat of blast disease to the key staple food, rice. The dynamic structure of the genome of *M. oryzae* enables this pathogen to evolve rapidly and jump from one host to another. To protect key global crops like rice and wheat from this serious pathogen, there is a need for intensive surveillance and diagnostics, use of biological and chemical control measures, and ongoing development of new plant cultivars with resistance genes that provide protection.

We hope that this third volume of *Plant Relationships: Fungal Plant Interactions* will provide a good overview of the recent developments that have occurred in this important field of biology and highlight what remains to

be discovered. The volume should be a good resource for researchers, teachers, and graduate students as well as companies working in the plant pathology and agricultural sectors.

Finally, we would like to thank the many authors that have contributed to the 18 chapters within this volume. We know it was difficult for many to meet the initial deadline due to the impact of the Covid pandemic and the pressure that placed on their routine research, teaching, and administrative duties. To all of you many thanks.

Palmerston North, New Zealand
July 2022

Barry Scott
Carl Mesarich

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Editors and Contributors

About the Series Editors

Dee Carter is Professor of Microbiology at the University of Sydney. She has a degree in microbiology and biochemistry from Otago University, New Zealand, and a PhD from Imperial College, London, UK. After postdoctoral fellowships in Montpellier, France, and UC Berkeley, California, USA, she moved to Australia in 1995 to take up a lectureship at the University of Sydney. Dee's research has encompassed (1) the population genetics and ecology of medically important fungi, including yeast and mould pathogens; (2) responses of fungi to host and antifungal stress, including the production of variant morphological forms; and (3) the transcriptome and proteome response to antifungal therapy, particularly during synergistic interactions between antifungals and natural products. As well as research, Dee teaches microbiology including mycology, epidemiology, and molecular biology at Sydney University.

Anuradha Chowdhary is a Professor of Medical Mycology at the Vallabhbhai Patel Chest Institute, University of Delhi, India. She received her medical degree in 1992 and MD (Microbiology) degree in 1996 from the University of Delhi, India and PhD, from the Faculty of Medical Sciences, Radboud University Medical Center, Netherlands. Dr Chowdhary's research interests include molecular ecology and population genetics of pathogenic fungi, antifungal drug resistance

mechanisms, especially *Aspergillus* and *Candida* spp., and the epidemiology of systemic mycoses. She is currently working on molecular epidemiology of *Candida auris*, terbinafine-resistant dermatophytes, and azole-resistant *Aspergillus fumigatus*.

Joseph Heitman is James B. Duke Professor and Chair, Department of Molecular Genetics and Microbiology, Duke University, Durham, NC, USA. His research studies model and pathogenic fungi addressing fundamental questions of scientific and medical importance. Pioneering studies with Baker's yeast revealed how immunosuppressive natural products interdict signalling cascades via FKBP12-drug complexes and discovered TOR as a globally conserved nutrient sensor targeted by the immunosuppressive, antiproliferative drug rapamycin. His research discovered unisexual reproduction of pathogenic microbes, with implications for pathogen emergence, how sex generates diversity, and how sex evolved. Dr. Heitman's lab has further developed genetic and genomic approaches elucidating molecular principles of fungal virulence, identifying therapeutic targets, and illustrating convergent evolution of fungal mating-type loci with mammalian, insect, and plant sex chromosomes, defined the calcium-activated protein phosphatase calcineurin as a globally conserved fungal virulence factor, and elucidated functions of RNAi in microbial pathogen genome integrity, hypervirulent outbreak lineages, and drug resistance via epimutation.

Ulrich Kück is a Professor of General and Molecular Botany at the Ruhr-University in Bochum, Germany. He is being graduated in Biology and Chemistry and has a long-standing experience in the molecular biology of fungi, algae, and plants. His research with fungi has focussed on two general aspects: first, Genetic engineering of the secondary metabolism of biotechnically relevant filamentous fungi, including functional genomics and proteomics. Second,

molecular genetic analysis of cellular growth in filamentous fungus, with the focus on sexual development. In particular, he is interested in the function of mating type loci and the involvement of conserved signalling complexes, such as the striatin-interacting phosphatases and kinases (STRIPAK) complex, in the control of cellular and developmental processes.

About the Volume Editors

Barry Scott was appointed the inaugural Professor of Molecular Genetics at Massey University in 1985. He was head of the Institute of Molecular Biosciences from 2008 to 2012. Early in his career, he made landmark contributions to the understanding of Rhizobium–legume symbiosis, reported in a seminal Nature paper in 1979. He then turned his attention to the fungal endophyte-grass symbiosis, which is economically important to New Zealand agriculture. His team was responsible for identifying the endophyte genes responsible for the biosynthesis of lolitrems and peramine, secondary metabolites unique to the symbiosis. The other major advance made by Professor Scott and his group was the demonstration that fungal synthesis of reactive oxygen species is essential for stable maintenance of the symbiosis. The results of this work were reported in two landmark papers published in Plant Cell in 2006. This discovery has led to a new and general framework for the study of fungal–plant interactions. Professor Scott is an Emeritus Investigator in the BioProtection Research Centre, a National Centre of Research Excellence. He was elected a fellow of the Royal Society of New Zealand in 2010 and awarded a Humboldt Research Award in 2014.

Carl Mesarich was conferred his PhD in Biological Sciences from the University of Auckland, New Zealand, in 2012. Upon completion of his PhD, he spent four years as a postdoctoral scientist in the laboratories of Professor Pierre de Wit at Wageningen University in the Netherlands (2012–2014) and Associate Professor Matthew Templeton at the New Zealand Institute for Plant and Food Research (2015). Major highlights from his postdoctoral research included the identification of *Avr5*, an avirulence effector gene from the tomato leaf mould fungus, *Cladosporium fulvum*, as well as the characterization of an apoplastic effectorome for this fungus (published in Molecular Plant Microbe Interactions in 2014 and 2018, respectively). Since 2016, Dr. Mesarich has led a research team at Massey University, New Zealand, where he is currently a Senior Lecturer in Plant Pathology, as well as an Associate Investigator for Bioprotection Aotearoa, a national Centre of Research Excellence. Much of his team's research focuses on understanding how the fungal pathogens *C. fulvum* and *Venturia inaequalis* (scab disease of apple), as well as the oomycete *Phytophthora agathidicida* (dieback disease of kauri), cause disease and/or trigger host immunity through the deployment of effector proteins, with the goal of informing disease resistance breeding or selection programs.

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Part I

Pathogenic Fungus–Plant Interactions

Modulation of Host Immunity and Development by *Ustilago maydis*

1

Armin Djamei, Jasper Depotter, Georgios Saridis, Maxim Prokchorchik, Sina Barghahn, Natalia De Sousa Teixeira E. Silva, Weiliang Zuo, Johana Misas Villamil, and Gunther Doehlemann

Abstract

Fungal pathogens are an enormous threat to plants, causing tremendous losses in worldwide crop production. A mechanistic understanding of fungal virulence is crucial to developing novel plant protection strategies in sustainable agriculture. Biotrophic pathogens colonize living plant tissue and reprogram their hosts to stimulate proliferation and development of fungal infection structures. To promote infection, fungal pathogens secrete sets of virulence proteins termed "effectors" which interfere with host metabolism and cellular function.

The maize smut pathogen, *Ustilago maydis*, has been established for decades as a prime model system for understanding the genetics, cell biology and pathology of biotrophic fungi. *U. maydis* colonizes primordia of all aerial parts of the maize plant resulting in a comprehensive reprogramming of organ development and host physiology. The most prominent symptoms of maize smut disease are large galls/tumors, which develop within a week at infection sites and contain the teliospores that in nature are the predominant dispersal stage of *U. maydis*.

Within this chapter, we summarize the current knowledge on the pathogenic development of *U. maydis* with an emphasis on its effector repertoire and how it interferes with the host plant during infection. We discuss evolution and transcriptional activation of the fungal effectome, as well as the known molecular mechanisms of virulence by fungal effectors and the plant processes they modulate. We also highlight gaps in current knowledge, discuss limitations of the system, and provide an outlook on possible strategies to address unsolved research questions.

Keywords

Ustilago maydis · Effectors · Maize · Biotrophy · Maize

1.1 *Ustilago maydis*, the Causal Agent of Maize Smut Disease

Smut fungi are one of the largest groups in the phylum of Basidiomycota with an estimated 1300 species (Bauer et al. 2001). Most smut pathogens have a narrow host range, which is often restricted to one plant species. The host spectrum includes

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important crops like sorghum, sugarcane, barley, wheat and maize, where smuts can cause severe damage if not treated in time (Trione 1982; Thomas 1989; Valverde et al. 1995). The common maize smut fungus, *Ustilago maydis*, has become an outstanding model system for the study of various aspects of biology. Research started with very basic questions of DNA recombination in the 1960s (Holliday 1964), followed by aspects of cell-cell recognition and mating (Bölker et al. 1992) and transcriptional regulation of the dimorphic switch (Kronstad and Leong 1989). The research diversified strongly after the genome sequence of *U. maydis* was published and included work on vesicle and targeted protein and RNA transport, protein secretion, genome biology and many other aspects of *U. maydis* biology (Benevenuto et al. 2018; Kwon et al. 2021; Heimel et al. 2013; Wagner-Vogel et al. 2015). In this chapter, our focus is on the knowledge gained so far by elucidating the details of *U. maydis* as a biotrophic pathogen, with a focus on the underlying molecular mechanisms of virulence.

1.1.1 The Process of Infection

The interaction of *U. maydis* with its host plant, maize, is closely interlinked with the completion of its lifecycle. The starting point is the germination of airborne teliospores that land on the host leaf. During teliospore germination, meiosis is completed and haploid cells (sporidia) of compatible mating type are released. Two unlinked mating-type loci exist in *U. maydis*, namely the "a" locus encoding for a pheromone receptor and a pheromone needed for self/non-self- recognition and the multiallelic "b" locus. The b locus encodes bEast (bE) and bWest (bW) homeodomain transcription factors, needed after respective heterodimerisation with the compatible bE and bW transcription factor after mating, for initiation of the transcriptional program that regulates various aspects of the pathogenic lifecycle by inducing among others the master regulator Rbf1 (Heimel et al. 2010; Brachmann et al. 2001; Kamper et al. 1995). Guided by the pheromone–pheromone receptor system, two compatible haploid sporidia form conjugation tubes and grow towards each other. After conjugation, a dikaryotic filament is formed that grows on the hydrophobic plant surface. Interestingly, like in many other eukaryotes, the mitochondria of *U. maydis* are inherited in a a2-dependent uniparental manner (Fedler et al. 2009). The dikaryon is cell-cycle arrested (Castanheira et al. 2014), then forms an appressorium as a penetration structure. This process is triggered by physico-chemical signals, namely hydrophobicity and specific hydroxy fatty acids found on the leaf surface (Mendoza-Mendoza et al. 2009). Unlike other fungi, the appressorium of *U. maydis* is not melanized and cell wall penetration is likely achieved by a mixture of secreted cell wall-degrading enzymes in conjunction with osmotic pressure at the penetration peg (Lanver et al. 2014).

1.1.2 *Ustilago maydis* Inside the Plant

After penetration of the host cell, the cell cycle block is released and mitotic division and growth of the dikaryotic filaments are observed. The fungus grows intracellularly and is fully encased by the host plasma-membrane. In contrast to many other fungi, *U. maydis* does not form haustoria, but instead the host plasma membrane encases the hyphal surface, thereby serving as an interface to acquire nutrients, exchange signals and send host modifying molecules to the plant cell (Djamei and Kahmann 2012). *U. maydis* colonizes all aerial parts of the maize plant, but employs various genetic programs to cause gall (tumor) formation in a plant-organ-specific manner (Skibbe et al. 2010; Schilling et al. 2014). At 2 days post-infection, the fungus induces hypertrophy (cell enlargement) in leaf mesophyll cells and hyperplasia (cell-division) in leaf bundle-sheet cells (Matei et al. 2018). In anthers, *U. maydis* reprograms primordial cell stages, inducing ectopic periclinal and anticlinal divisions and cell-expansion depending on the developmental stage of the anther or the affected cell-type (Gao et al. 2013). The metabolic and transcriptional

reprogramming of the host cells leads to hypertrophy and hyperplasia in the infected tissue and the formation of galls, which are a prominent symptom linked to *U. maydis* infection.

1.1.3 The Molecular Basis of Biotrophy

Comparative analysis of the transcriptional profiles associated with different *U. maydis*-infected organs revealed that *U. maydis* employs, beside a general set of effectors, organ specific effectors as a higher level of specialisation and host adaptation (Schilling et al. 2014; Redkar et al. 2015; Skibbe et al. 2010).

These secreted molecules form the molecular basis of biotrophy. Effectors are defined by their function and not by their chemical composition (Uhse and Djamei 2018). Therefore, all molecules that are secreted by *U. maydis* in order to manipulate the host plant metabolism or suppress the plant defense system are effectors. Whereas our knowledge on potentially non-proteinaceous effectors of *U. maydis* are limited to fungal-derived phytohormones like indole acetic acid (Reineke et al. 2008; Navarrete et al. 2021a) and cytokinin (Morrison et al. 2017), we have obtained greater insights into proteinaceous effectors. Genome sequencing and gene annotation of *U. maydis* led to the discovery that, similar to biosynthetic gene clusters, many of the candidate effector genes are clustered within the genome and are transcriptionally co-regulated during biotrophy (Kamper et al. 2006). Recent findings demonstrated that nine different effectors encoded by genes on one cluster target the same host defense response pathway, which indicates that there is a higher order of functional organisation than previously thought (Navarrete et al. 2021b). As for every biotrophic organism, *U. maydis* co-evolves, especially in terms of its effectome, with its host. Therefore, an understanding of the plant immune system and the co-evolutionary race of recognition and evasion and/or signal suppression might explain a large part of the effectome complexity, which might not be apparent if the effectors were analysed separately as seen for example by the high rate of single effector mutants without a virulence defect (Uhse et al. 2018).

1.1.4 The Infection Cycle Ends with Spore Formation

Around 8 days after the establishment of an active sink tissue, loss of photosynthetic capacity in the infected tissue and host cell proliferation, *U. maydis* grows within the galls intercellularly and become embedded in an extracellular matrix (Horst et al. 2009; Doehlemann et al. 2008). After karyogamy and hyphal fragmentation, diploid spore precursors are released which further mature to black melanized teliospores that are spread by the wind or water. The developmental program of the fungus is driven by several transcription factors which are regulated by fungal signalling pathways which also integrate host plant-derived cues to ensure respective adaptive responses and an adjusted colonization of the host plant (Tollot et al. 2016; Heimel et al. 2010; Brachmann et al. 2001; Rabe et al. 2016b; Zahiri et al. 2010). The ongoing sensing of the host environment, along with the respective signal integration and adaptive effector response, are likely the basis of the success *U. maydis* has as a pathogen.

1.2 Evolution, Structure, and Features of *U. maydis* Effectors

1.2.1 The Clustered Occurrence of the Effectome

U. maydis was amongst the first fungal plant pathogens to be sequenced (Kamper et al. 2006). Relative to genomes of other filamentous plant pathogens, *U. maydis* has a small haploid genome of around 20 Mb (Kamper et al. 2006; Raffaele and Kamoun 2012). This is in stark contrast to rusts, another important group of basidiomycete plant pathogens, which can have haploid genome sizes much larger than 140 Mb (Wu et al. 2021).

Advances in sequence technologies have made whole genome sequencing cheaper and enabled longer sequence reads to be obtained (Goodwin et al. 2016). Consequently, high quality genome assemblies of a wide range of smut pathogens are currently available (Rabe et al. 2016a; Taniguti et al. 2015; Depotter et al. 2022), as are the sequences of sister species without the ability to infect plants (Eitzen et al. 2021). Genome sizes vary considerably among the smut pathogens, which can mainly be attributed to differences in repeat content, including transposable elements (Depotter et al. 2022). For instance, the genome assembly of the sorghum smut pathogen (*Sporisorium reilianum*) is 18.5 Mb in size and consists of 3.6% repetitive sequences, whereas the 25.8 Mb genome assembly of the barley covered smut pathogen (*Ustilago hordei*) has 35.3% repeats (Depotter et al. 2022). The availability of whole genome sequences has enabled the study of the effectome, i.e. the complete repertoire of effector proteins. However, criteria need to be defined to determine bona fide effector candidates, as experimental verification for all effector candidates is laborious. Moreover, effectors might have very specific or redundant functions, which makes gene knock-out verifications insufficient to exclude candidates (Depotter and Doehlemann 2020; Uhse et al. 2018). Machine learning approaches and gene expression data are often used to predict genes that encode secreted proteins that play a role in host colonization (Depotter et al. 2021a; Sperschneider and Dodds 2022). Using a set of bioinformatic criteria, *U. maydis* has been predicted to have 553 effector candidates (Dutheil et al. 2016). In genome studies, effector genes have been associated with specific genome locations that facilitate fast gene evolution through their accessory and repeat-rich nature (Croll and McDonald 2012; Dong et al. 2015). In smut pathogens, many effector genes are characterized by their clustered appearance in the genome, which is mainly a result of tandem gene duplications (Kamper et al. 2006; Dutheil et al. 2016). However, the degree to which effector genes are clustered in the genome is distinct between different smut species. For instance, the sugarcane smut pathogen, *Sporisorium scitamineum*, has 10 candidate effector gene clusters, which are on average 15 genes in size, whereas the *U. hordei* genome does not contain such clusters (Dutheil et al. 2016). The arms race between plant pathogens and their host is often used as a reason for why effector genes reside together in fast-evolving genome compartments. However, based on comparative analysis, non-pathogenic sister species of smuts have similar genomic features including effector candidate clusters (Dutheil et al. 2016; Schuster et al. 2018a; Lefebvre et al. 2013). For instance, the leaf epiphyte *Anthracocystis flocculosa* has three effector clusters with an average size of 18 genes (Dutheil et al. 2016).

1.2.2 Inter- and Intraspecific Effectome Differences

The predicted effectome size of smut pathogens on monocots is relatively consistent and ranges between 484 and 554 members (Dutheil et al. 2016). However, the gall smut of *Persicaria* species, *Melanopsichium pennsylvanicum*, has 362 predicted effector candidates, a significantly smaller effectome. This is thought to be due to its recent jump from a monocot to a dicot host (Dutheil et al. 2016; Sharma et al. 2014). Host jumps conceivably result in a subset of effectors that do not have a cognate target in the new host, leading to their purge from the effectome (Thines 2019). Secretome sizes can vary significantly between basidiomycete plant pathogens, as the stem rust pathogen, *Puccinia graminis*, has a predicted secretome that is 3.8 times larger than that of *U. maydis* (Krijger et al. 2014). Conceivably, differences in life-style/cycle and effective population sizes of plant pathogens determine how many effectors are needed for optimal colonization of a host. Noteworthy, as smut pathogens infect their host through the formation of dikaryotic hyphae, the complete effectome during infection is the combination of the two haploid parents. Haploid smut strains can be genetically engineered to infect hosts, as the so-called solopathogenic strains (Kamper et al. 2006),

nevertheless, they do not reach the virulence observed for the dikaryotic infections. Possibly, this results from gene-dosage effects of the dikaryon, differences in the mitochondria (a2-derived), and combinatorial effects of virulence-relevant genomic regions linked to the mating loci. Also, the added value of effectome combination is likely negligible for genetically similar parents, which is especially the case for the Brachipodieae grass smut *U. bromivora* that has one of the two mating types being lethal in their haploid state (Rabe et al. 2016a). The haplo-lethal mating-type yeasts can only be rescued by fast mating, which stimulates inbreeding and increases the chance for a homogeneous effectome between the mating parents. In contrast, parents from different populations have likely more heterogeneous effectomes. However, very low genetic diversity was found in a *U. maydis* population from Mexico, which is the presumed center of origin of this species (Schweizer et al. 2021) implicating a constant genetic flow within this population. In a *U. maydis* population with predominantly Chinese isolates, presence/absence polymorphisms were observed for 2% of the conserved effector candidates, whereas this was 18% for maize-specific effectors (Depotter et al. 2016). In general, a more extensive sampling of the global *U. maydis* population is needed to get a comprehensive overview of the pan-effectome of *U. maydis*. One could then also study the effectome diversity between *U. maydis* populations and evaluate the impact of crossings between distinct effectomes on the pathogen's virulence. This impact may even be more pronounced in cases where effectomes from different smut species hybridize (Depotter et al. 2016). Although smut hybrids have hitherto not been found in nature, they were in several instances created in the laboratory (Bosch et al. 2019; Storfie and Saville 2021; Kellner et al. 2011). Hybrids between *U. maydis* and *S. reilianum* are much less virulent than their parents and induce symptoms similar to *S. reilianum* (Storfie and Saville 2021). In contrast to *U. hordei* alone, the *U. bromivora* x *U. hordei* hybrid is able to infect *Brachypodium* species, and cause subtle differences in symptoms compared to *U. bromivora* infections (Bosch et al. 2019). Hybrids have altered expression patterns for effector genes and transcription factors important for pathogenicity (Storfie and Saville 2021). Further investigation of the mechanisms that cause the distinct transcription pattern of hybrids may tell us more about how the two nuclei in smut filaments interact and regulate secretome expression.

1.2.3 Effectome Evolution

Effectors are often collectively considered to be a fast-evolving group of proteins, as they are typically targets for recognition by the host's immune system. However, in *U. maydis*, there are two distinct effector groups with distinct evolutionary speed (Depotter et al. 2021b). The conserved effector group undergoes a similar number of changes to non-effector proteins, whereas the rate of change was higher for the diverged group of effectors. Thus, there is a heterogeneity within the effectome, which is probably related to the different functions of effectors or the presence of functionally redundantly acting effectors. Every disease cycle has several distinct stages, including appressorium formation, host penetration, host colonization and sporulation, which likely involves stage-specific effectors (Brefort et al. 2009). Correspondingly, candidate effector genes often have a peak of expression in a specific disease cycle stage (Lanver et al. 2018). The *U. maydis* effector Tin2 stabilizes the cytoplasmic maize protein kinase ZmTKK1, which activates the anthocyanin biosynthesis pathway (Tanaka et al. 2014). The gene encoding Tin2 has a peak of expression at 6 days post-inoculation, which conceivably means that ZmTKK1 stabilization and the linked metabolic changes are especially important for the pathogen at that specific point of the disease cycle. When pathogens diversify into new lineages or species, genes encoding particular effector groups may encounter more selective pressure than others. Although *U. maydis* and *S. reilianum* can cause smut on the same host, their infection strategy is distinct, as *S. reilianum*

colonizes maize systemically and, in contrast to *U. maydis*, does not induce galls (Zuo et al. 2019; Matei et al. 2018; Martínez-Espinoza et al. 2002). During their divergence, the effector-encoding genes with a peak expression during *in planta* proliferation diverged more than the effector-encoding genes that peak during appressoria formation and host penetration (Depotter et al. 2021b).

1.2.4 Effectors and Their Impact on Pathogen Fitness

Effectors also differ in their contribution to the pathogen's fitness. For instance, *U. maydis* effector Pep1 inhibits a maize peroxidase and is crucial for host penetration (Doehlemann et al. 2009; Hemetsberger et al. 2012). Without Pep1, *U. maydis* is not able to infect plants, which means that *U. maydis* cannot complete its sexual cycle (Brefort et al. 2009). The absence of Pep1 has a higher fitness cost than an effectome without the 24 effectors of the effector gene cluster 19A, as *U. maydis* is in this case still able to complete its life cycle (Brefort et al. 2014). Numerous *U. maydis* effector candidate knock-outs have been generated and display a range of responses regarding the impact on pathogen virulence (Schilling et al. 2014). Remarkably, a deletion of effector cluster 2A actually increased the virulence of *U. maydis*, implying an increase in pathogen fitness through loss of this effector cluster. However, optimal fitness may not correspond to maximal virulence, helping to explain why this cluster is retained. Furthermore, many effector knock-outs do not have an apparent effect on the virulence of *U. maydis*, suggesting they are superfluous, but there are other explanations as well: effectors can have maize-line-specific functions or are specific to a particular maize organ (Stirnberg and Djamei 2016; Schilling et al. 2014; Schurack et al. 2021), effects that are not picked up by particular plant assays in the laboratory. Moreover, a group of effectors can have a similar function which makes their individual function redundant or too small to notice. Multiple effector gene knock-outs are then needed to reveal their contribution to virulence (Navarrete et al. 2021b).

Differences in effector properties make certain effectors more accessory than others. A more accessory nature may allow more variation and faster effector gene evolution. This idea is nicely illustrated by the core effector Pep1, which has high sequence and functional conservation across the smut pathogens (Hemetsberger et al. 2015). That the accessory nature is an important determinant for the evolutionary speed of effector change is also indicated by the correspondence of higher interspecific variation (between *U. maydis* and *S. reilianum*) with higher intraspecific variation for *U. maydis* effectors (Depotter et al. 2021b). Effectors are key targets for the host immune system to recognize the pathogen and induce immunity (Stergiopoulos and Wit 2009). Consequently, conserved effector genes with indispensable functions, like Pep1, pose potentially easy targets for the immune system (Doehlemann et al. 2009). Conserved effectors could be protected from being recognized by the so-called decoy effectors, which do not have functional constraints on their evolution other than interfering in effector perception (Paulus and van der Hoorn 2018; Ma et al. 2017). Decoys are in several cases a product of gene duplication of the component they mimic (van der Hoorn and Kamoun 2008; Ma et al. 2017). As mentioned earlier, effector gene duplication is the main driver in the evolution of effector clusters (Dutheil et al. 2016). Although this has not been experimentally verified in *U. maydis*, gene duplication may provide a source of decoy effector proteins. Another evolutionary driving force of effector gene duplication followed by functional diversification could be host–target diversification and formation of gene families with partially overlapping functions which require a sophisticated manipulation by a set of duplicated co-evolving effectors. More generally, the effectome can be considered as a network, both evolutionary and physically, being highly interconnected and interdependent on its components to generate full functionality, but at the same time as a network also sufficiently

robust to tolerate the loss of single peripheral nodes without losing overall virulence capacity.

1.2.5 The Effector Interactome

Effector proteins can also directly interact with each other and themselves to form protein complexes (Djamei et al. 2011; Alcantara et al. 2019; Ludwig et al. 2021; Sánchez-Vallet et al. 2020). A yeast-two-hybrid screening of almost 300 *U. maydis* effector candidates revealed that 126 interact either with themselves or other effectors, which suggests that effectors are a strongly interacting group of molecules (Alcantara et al. 2019). This is also nicely illustrated by the five unrelated *U. maydis* effectors, including the core effectors Cce1 (Seitner et al. 2018) and Pep1, that have been found to form a stable protein complex together with two membrane proteins (Ludwig et al. 2021) (Fig. 1.1). This complex has been shown to be anchored in the membrane of the pathogen and is proposed to play a key role in effector delivery (see Sect. 1.4.4 for more detail). Independently of this possible function, the formation of a super molecular structure, built of multiple indispensable virulence effectors, is a striking finding that opens up new possibilities in understanding cooperative effector function and thus, potential drivers of effector evolution.

1.3 Transcriptional Regulation of the *U. maydis* Effectome

1.3.1 Early Regulators of Biotrophy

In *U. maydis*, the transcription of its 553 predicted effector genes is orchestrated in waves of transcription during the infection process (Lanver et al. 2018; Dutheil et al. 2016; Lo Presti et al. 2017; Schuster et al. 2018a; Mueller et al. 2008), implicating an infection-stage specific employment of the effector repertoire. The initiation of pathogenicity and the activation of the early effectome are essentially associated with sexual development and filamentous growth. Before the fusion of compatible sporidia, pheromone recognition via the receptors Pra1 and Pra2 triggers a signaling cascade, which leads to the combined action of cAMP and MAPK signaling pathways activating the transcription factor Prf1 (Kaffarnik et al. 2003; Mueller et al. 2008; Dürrenberger et al. 1998). Subsequent to cell fusion, Prf1 induces the expression of the *b* mating-type locus genes, leading to the production of the heterodimeric bE/bW homeodomain transcription factor, the master regulator of *U. maydis* early pathogenic development (Gillissen et al. 1992; Kamper et al. 1995). Among the targets of bE/bW, identified via microarray using strains with induced bE/bW combinations, a number of transcription factors, including Rbf1 and its targets Hdp2, Biz1, and Mzr1, but also 38 effector genes, were found (Heimel et al. 2010; Flor-Parra et al. 2006; Zheng et al. 2008). In a detailed transcriptomics study of the fungal responses at different stages of maize infection, Lanver et al. (2018) suggested a modular development of *U. maydis* effectome expression, that was dependent on the different infection stages: the early biotrophic development and the later tumor formation phase. The transcription factors bE/bW and its targets have been associated with the expression of genes encoding secreted proteins that are expressed pre-tumor formation (Lanver et al. 2018).

1.3.2 Late Regulators of Biotrophy

During the later stages of infection, another group of transcription factors takes over to control the next wave of effectome expression. Upon sporulation and mature tumor formation, the late effectome is activated via Ros1, which attenuates the early effectome wave, hypothetically by blocking the bE/bW cascade, and activating the expression of about 70 other effector genes (Tollot et al. 2016). These findings are in concordance with the data from Lanver et al. (2018), where Ros1-induced effector genes were underrepresented in the pre-tumor phase of expression, while Ros1-repressed effector genes were overrepresented during the early stage of

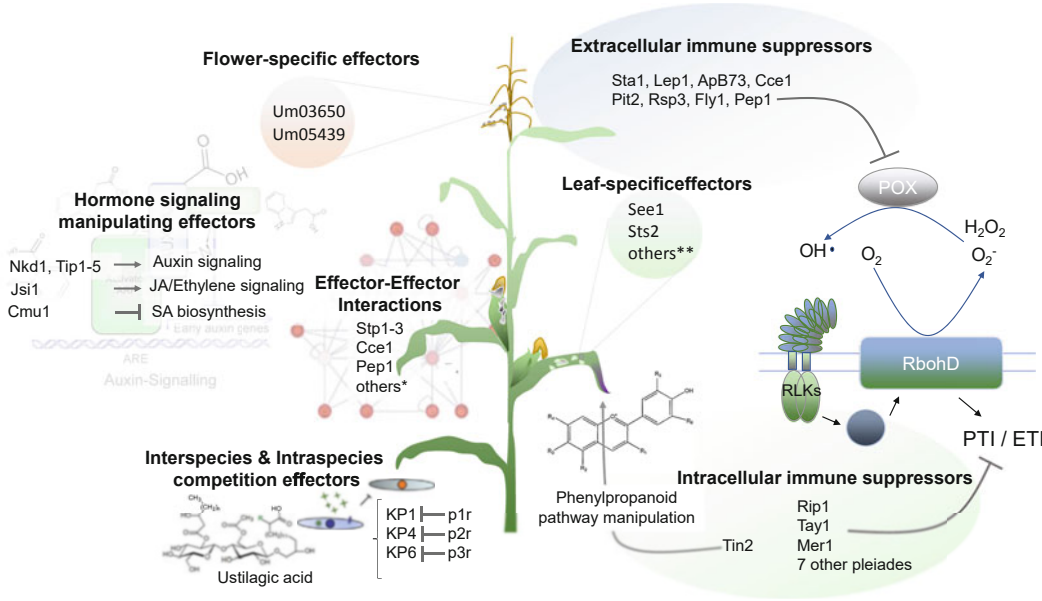

Fig. 1.1 Overview of the biological role of functionally characterized *U. maydis* effectors during the host–pathogen interaction. Additional effectors characterized in: *Alcantara et al. (2019), ** Schilling et al. (2014)

development. In the same study, the transcription factor Nlt1, as well as the previously described Fox1 transcription factor, was identified as key contributors to the expression profile of effectors belonging to the gene set associated with the late wave of effectome expression, upon tumor formation (Lanver et al. 2018; Zahiri et al. 2010). Furthermore, Zfp1, a Zn_2Cys_6 transcription factor, has been linked to the regulation of effectors at various stages of *U. maydis* infection, including the biotrophic phase as well as the tumor formation stage, altering the expression of 112 confirmed effectors and 50 transcription factors, including Ros1 (Cheung et al. 2021). Among the transcriptional regulators studied, expression of novel proteins such as a homeobox domain containing protein, two bHLH, one TEA/ATTS, one HMG, one CCHC, and a winged-helix transcription factors, were found to be correlated with effector gene expression at the early stages of infection. The transcriptional regulation of the late effectome is dominated by yet not characterized transcription factors containing Zn_2Cys_6 (four of them), bHLH (one), C_2H_2 (one), and winged-helix (one) domains (Lanver et al. 2017, 2018). This data indicates that the temporal transcriptional regulation of the effectome might be coordinated by a more complex regulatory network, the details of which still remain to be discovered.

1.3.3 Post-Transcriptional Regulation of the Effectome and Its Transcriptional Feedback

In addition to the regulation of effector genes at the transcriptional level, the high number of secreted proteins expressed during infection challenges the capacity of the ER and the secretory pathway, and therefore requires specific adaptations. Only correctly folded and potentially glycosylated proteins are processed by the secretory system and therefore, increased activity of the proof-reading system is essential (Fernández-Álvarez et al. 2013). The ER glucosidase I, Gls1, and the glucosidases II, Gas1 and Gas2, are thought to target secreted fungal proteins at the

early stages of infection, upon appressorium formation for Gl1, or during intracellular growth for Gas1 and Gas2 (Fernández-Álvarez et al. 2013; Schirawski et al. 2005). In *U. maydis*, some cysteine-rich effectors which are translocated into the apoplast need to form disulfide bonds, in order to develop an active conformation (Lanver et al. 2017; Seitner et al. 2018). Such disulfide bonds are catalyzed by the disulfide isomerase Pdi1, which is regulated post-translationally via *N*-glycosylation, affecting its electrophoretic mobility (Marín-Menguiano et al. 2019). Moreover, the protein products of the linked genes *pit1* and *pit2*, which encode for a membrane protein and a secreted effector, respectively, are *O*-glycosylated by the Pmt4-mediated *O*-mannosyltransferase (Doehlemann et al. 2011; Fernández-Álvarez et al. 2012). Furthermore, the spliced form of Cib1, the core transcription factor orchestrating the unfolded protein response (UPR), a mechanism for detecting and responding to misfolded proteins in the ER, accumulates in the fungal hyphae upon plant penetration (Heimel et al. 2013). Cib1 regulates effectome expression at three levels: (i) transcriptionally, through UPR elements that are found in the promoters of several effector genes, such as *Pep1* and *Pit2*, (ii) indirectly, due to a crosstalk with the bE-bW cascade, via interaction with Clp1, a target of the bE-bW complex that is necessary for clamp formation during cell division and, (iii) via the regulation of the UPR for the correct secretion of effector proteins (Heimel et al. 2013; Hampel et al. 2016).

1.3.4 Spatial Regulation of the Effectome

The complexity of the regulatory system governing the coordinated effectome activity upon *U. maydis* infection is significantly increased when adding the spatial distribution of effector gene expression. *U. maydis* can create tumors in maize leaves, tassels, and flowers, but only a small proportion of about 70 effector candidates (21%) were found constitutively expressed in all maize organs, whereas 118 other effector candidate genes (45%) had organ-specific expression patterns (Skibbe et al. 2010). Of these, 28 effector genes showed specific expression in the leaves of seedlings, 86 in adult leaves, and 4 in tassels, suggesting a genetic adaptation of *U. maydis* to interfere with different host genetic and molecular cues (Skibbe et al. 2010). In a study that utilized mutants with deletions of individual effector gene clusters, specific clusters were shown to be associated with organ-specificity (Skibbe et al. 2010). The gene clusters 5B and 19A were mainly required in seedling leaves, while clusters 2A, 9A, and 10A were linked with symptoms and tumor formation in adult leaves and tassels (Skibbe et al. 2010). These finding were also supported by an organ-specific microarray analysis. A closer look at effectome cell-specificity was conducted in leaves, where a transcriptomic analysis was performed based on Laser dissection confocal microscope to isolate hypertrophic mesophyll tumor cells and hyperplasic bundle sheath tumor cells (Matei et al. 2018). In the hypertrophic tumor cells, 67 effector genes were found that had statistically significant slight to high cell-specific gene expression, 10 of which were exclusively expressed in those cells. On the other hand, expression of 43 effector genes was over represented in hyperplasic tumor cells, with one being strictly expressed in the bundle sheath tumor cells (Matei et al. 2018). The identification and characterization of organ and cell-specific effectors has received increased attention in recent years, and even though a number of novel *U. maydis* effectors have been reported, more studies, focusing on the regulatory network that controls spatial-specific expression of effector genes, could enhance our understanding of the complex effectome regulatory network (Redkar et al. 2015; Schilling et al. 2014).

1.3.5 Host Accession Specific Effector Regulation

The host genome has been identified as another factor that determines effectome regulation in *U. maydis*. Quantitative disease resistance

(QDR) loci in maize are thought to be targets of fungal effectors, as several have been found to determine susceptibility and resistance phenotypes (Pathi et al. 2020; Brefort et al. 2009; Baumgarten et al. 2007). In a detailed transcriptomic study with *U. maydis* infection of six maize lines, with a spectrum of quantitatively distinct susceptibility phenotypes, transcriptional recruitment of effector genes was positively correlated with the infection of resistant lines (Schurack et al. 2021). More specifically, 38 out of the 102 expressed predicted effectors were associated with a resistant line of maize, CML322, while on the other hand, expression of 29 predicted effectors correlated with infection of the more susceptible lines, such as the Tx303 (Schurack et al. 2021). These findings suggest that the host genetic blueprint is essential for the reprogramming of the effectome in *U. maydis*, which is highly active in more resistant lines, highlighting the need for further investigations into genome-to-genome compatibilities in plant–pathogen associations.

1.4 The Molecular Functions of *U. maydis* Effectors

1.4.1 Antimicrobial Effectors and Niche Protection

Microbial competition for nutrient resources and competition for niche occupation and protection are key drivers of evolution that lead to adaptation through diversification. This evolutionary pressure can partially explain the development of many unique metabolic capacities, making fungi an exceptionally important source of antimicrobial substances for medical and biotechnological applications. Plants are the dominating primary source of hydrocarbons, and therefore they harbor myriads of microbes in their rhizo- and phyllospheres, and beside synergistic interactions, there is also competition between putative colonizers (Hassani et al. 2018). Biotrophic pathogens like smuts need to suppress the host immune system, while at the same time prevent opportunistic infections of the host plant by necrotrophs and other competing microbes. Recent work has shown that activity of the small cysteine-rich proteins Ave1 and AMP2 from the hemibiotrophic fungus *Verticillium dahliae*, efficiently inhibit the growth of competing bacteria, especially *Sphingomonads* (Snelders et al. 2020). Another example of microbial antagonism mediated by secreted proteins is the interaction of the epiphytic strain of *Moesziomyces bullatus* (*Moesziomyces bullatus* ex *Albugo* on *Arabidopsis*; MbA), through secretion of MbA (Eitzen et al. 2021). MbA suppresses the infection of *Arabidopsis* by the oomycete *Albugo laibachii* via a GH25 hydrolase, which has lysozyme activity (Eitzen et al. 2021).

1.4.2 Antimicrobial Secondary Metabolites of *U. maydis*

As *Ustilago maydis* is an airborne fungus that infects the aerial parts of maize, it is in competition with the phyllosphere microbiome and the respective endophytes. Additionally, it must prevent superinfections by competing with other pathogens in order to keep its host alive. Secreted secondary metabolites of *U. maydis* thereby play an important role in niche protection. Under nitrogen limitation, *U. maydis* produces large amounts of secreted glycolipids, including the cellobiose lipid ustilagic acid (UA) (Teichmann et al. 2007). Ustilagic acid has a broad range of antibacterial and antifungal activities (Haskins and Thorn 2011). Most prominently, it can efficiently suppress an *U. maydis* competitor, the necrotrophic fungus *Botrytis cinerea* (Teichmann et al. 2007). Ustilagic acid production is controlled by a cluster of 12 genes on chromosome 23, which are regulated by the C_2H_2 zinc finger transcription factor, Rua1 (Hewald et al. 2006; Teichmann et al. 2007, 2010). The complete biosynthetic pathway of ustilagic acid has been elucidated (Teichmann et al. 2007).

A dissection of the dynamics of how *U. maydis* interacts with other microorganisms has been determined by analyzing the biomass, transcriptome, and metabolome while co-cultivating with *Fusarium verticillioides*

(Jonkers et al. 2012). In this particular case, *U. maydis* proliferation seems to be compromised and gene expression of UA and MEL gene clusters is efficiently suppressed by *F. verticillioides* via an unknown mechanism (Jonkers et al. 2012). On the other hand, genes involved in nutrient uptake and defense are significantly upregulated when co-cultivated with *F. verticillioides*, including siderophore peptide synthases and two gene clusters named msum_10 and msum_11 (Jonkers et al. 2012). Functions of msum_10 cluster genes were not described, except for *adr1* (*UMAG_04456*) which is a potential regulator of the process of fungal repopulation after exposure to harsh environmental stresses (Milisavljevic et al. 2018). The msum_11 gene cluster was studied in detail, and shown to be responsible for the biosynthesis of a novel melanin-type that is based on coumarin and pyran-2-one intermediates (Reyes-Fernández et al. 2021). Interestingly, the msum_11 cluster is severely truncated in *U. hordei, Sporisorium scitamineum,* and *Sporisorium reilianum,* demonstrating that this type of melanin biosynthesis is unique to *U. maydis* (Reyes-Fernández et al. 2021). Further research on these gene clusters may help explain the role of the msum_10 gene cluster and the mechanistic basis for unconventional melanin biosynthesis in *U. maydis* niche protection.

1.4.3 Indirect Niche Protection by *U. maydis*?

Transcriptomic and metabolic profile studies with *U. maydis*-infected maize (Doehlemann et al. 2008) demonstrate that the jasmonate (JA)/ethylene (ET)- as well as the auxin and gibberellic acid-regulated genes are upregulated and the salicylic acid signaling pathway is downregulated (Doehlemann et al. 2008). Generally, plants defend themselves from insect herbivores and necrotrophic pathogens by a defense program regulated via the JA/ET signaling. Potentially, host reprogramming promotes biotrophy through repression of SA-regulated plant defense and induction of antagonistic pathways that inhibit potential necrotrophic microbial competitors. Novel *U. maydis* effectors manipulating the host signaling in this manner have been recently discovered and are discussed in more detail elsewhere in this review (Darino et al. 2021; Navarrete et al 2021a).

1.4.4 Intraspecific Competition of *U. maydis*

Symbiotic dsRNA *U. maydis* viruses (UmV) in the cell cytoplasm of some *U. maydis* strains lead to an intraspecific competitive advantage. The carrier of the virus secretes one of the antimicrobial Killer Proteins KP1, KP4, or KP6. These toxins kill surrounding *U. maydis* cells that do not contain the virus (Allen et al. 2013; Park et al. 1996; Koltin and Day 1976). The virus carrier is protected from its toxin by a corresponding recessive nuclear resistance gene *p1r*, *p4r,* or *p6r* (Koltin and Day 1976; Puhalla 1968) respectively. KP1 and KP6 are expressed as pre-protoxins, which are proteolytically processed by the Kex2p protease into alpha and beta polypeptides, both of which are important for activity of KP6 and only the β-polypeptide is important for KP1 activity (Park et al. 1994, 1996; Tao et al. 1990) (Fig. 1.1). KP4 does not share any peptide sequence similarity to KP4 and KP6 and specifically blocks voltage-gated L-type Ca^{2+} channels in both *U. maydis* and mammalian cells (Gage et al. 2001, 2002). The *Cch1* (*Calcium-channel protein 1*) gene product is speculated to be the target of KP4 in fungi, and the fact that plants do not have Cch1 homologs or L-type Ca^{2+} channels may explain how the host plant evades KP4 toxicity (Allen et al. 2013). In contrast, the mode of action of KP6 is not yet clear, but it is clearly different to that for KP4 (Gage et al. 2001). Interestingly *U. maydis* spheroplasts showed insensitivity to KP6, suggesting that its target may be associated with the fungal cell wall. An alternative explanation for this finding could be target membrane receptor degradation by proteases often found in enzyme mixtures used for spheroplast preparation (Steinlauf et al. 1988). Homologs of KP4 were

identified in *Fusarium graminearum*, other fungal species and in a moss, implying a possible interkingdom horizontal gene transfer (Lu and Faris 2019). In *F. graminearum*, a KP4-like protein plays a role in virulence by an unknown mechanism, and like in *U. maydis,* the *KP4-like* genes are located in the genomic DNA of the fungus (Lu and Faris 2019).

In summary, the field of niche protection and microbial competition is just opening up for *U. maydis* research with exciting times ahead.

1.5 Extracellular Plant Immunity Modulation by *U. maydis*

1.5.1 The Apoplastic Battleground

The plant apoplast is a challenging environment for microbes where they face extreme changes in temperature, humidity, and light. It is also the first interface of communication between host and pathogen and has been seen as a battleground for plant–microbe interactions. The plant apoplast is packed with a variety of plant hydrolases and proteases that can cleave microbe-conserved molecules, triggering, upon recognition, the activation of downstream defense responses. It is thought that a substantial number of the proteins secreted by *U. maydis* are effectors targeting extracellular plant components to prevent the activation of immune responses. In order to defend themselves, plants possess innate, as well as inducible defense responses (Zhou and Zhang 2020). One inducible and fast response toward pathogen attack is the local generation of reactive oxygen species (ROS). Production of ROS in response to microbe recognition occurs mainly in the apoplast and is mediated by the plasma membrane-residing NADPH oxidases and cell wall class III apoplastic peroxidases. The ROS generated are toxic to invading microbes, but also act as signaling molecules for the execution of plant immune responses, e.g. cell wall reinforcement (Qi et al. 2017; Bleau and Spoel 2021). An *U. maydis* effector known to interfere directly with ROS generation is Pep1. The *pep1* gene is expressed during the early stages of infection and the encoded effector protein is secreted into the plant apoplast (Doehlemann et al. 2009). Deletion mutants of *pep1* activate plant immune responses including callose deposition, accumulation of ROS around growing hyphae, and induction of pathogenesis-related (PR) gene expression. Also, the maize peroxidase 12 (POX12), a class III peroxidase, was identified to be transcriptionally induced after infection with the *pep1* deletion mutant (Doehlemann et al. 2009; Hemetsberger et al. 2012). Recombinant Pep1 inhibits microbe-associated molecular pattern (MAMP)-induced ROS generation and can directly interact with POX12. Silencing of the maize POX12 results in a partial restoration of virulence of the Δ*pep1* mutant (Hemetsberger et al. 2012). Thus, these data indicate that the effector Pep1 can directly inhibit plant peroxidase activity, which in turn leads to a suppression of plant defense responses (Hemetsberger et al. 2012). In addition to the known function of Pep1, this effector is also part of the cell surface-exposed "Stp effector complex" described above (Ludwig et al. 2021).

1.5.2 Interference with Pathogenesis-Related Plant Proteins

In a second layer of infection, the secretion of PR proteins with antimicrobial activity is a main contributor to plant immunity (van Loon et al. 2006). A number of PR proteins from different plant species hydrolyze the fungal cell wall, e.g. chitinases and β-1,3-glucanases, resulting in fungal growth restriction (van Loon et al. 2006; Gupta et al. 2013). Besides, hydrolysis of the cell wall releases MAMPs such as chitin that can be perceived by plant receptors leading to the activation of downstream defense responses (Pusztahelyi 2018). To counteract plant-derived PR proteins, *U. maydis* protects itself by secreting the effector protein Rsp3. The expression of *rsp3* increases during the early biotrophic stage and secreted Rsp3 attaches to the surface of biotrophic

hyphae through its N-terminal domain. Rsp3 can interact with at least two mannose-binding proteins, AFP1 and AFP2, secreted by the host plant. The DUF26-domain present in AFP1 exhibits antifungal activity toward an *U. maydis* mutant lacking Rsp3, but not toward the wild type strain constitutively expressing *rsp3*. This mutant is also defective in mannose-binding activity (Ma et al. 2018).

Another example of an effector impairing the function of cell wall-degrading enzymes (CWDEs) is the *U. maydis* secreted effector Fly1. In response to *U. maydis* infection, maize genes encoding for chitinases A, B, and C are upregulated (Doehlemann et al. 2008; Ökmen et al. 2018). Fly1 is a secreted fungal lysin metalloprotease which directly interacts and cleaves maize chitinase-A. The Fly1-mediated cleavage of chitinase-A results in the removal of the chitin-binding domain from the catalytic domain which reduces enzyme lytic activity (Ökmen et al. 2018). Thus, reduction of chitinase-A activity by Fly1 might reduce plant immune responses by protecting the fungal cell wall from degradation. Additionally, Fly1 is required for posttranslational cleavage of endogenous fungal chitinases to allow cell separation during the yeast growth phase. Consequently, Fly1 has a dual function: it is required for fungal cell separation, but it also protects *U. maydis* during infection from cell wall degradation by host chitinases (Ökmen et al. 2018).

1.5.3 Fungal Cell-Wall Associated Effectors

There are a few examples of virulence factors that have been suggested to have a shielding function on fungal hyphae. For example, ApB73 (Apathogenic in B73) has been shown to be associated with the fungal cell wall. Interestingly, the virulence defects of ΔapB73 vary strongly between the maize variety B73 and the maize variety EGB, implicating a maize variety specific role (Stirnberg and Djamei 2016). The cysteine-rich core effector 1 (Cce1) is another essential apoplastic core effector of *U. maydis*. It has eight cysteine residues supporting a function in the apoplast and it is needed at the early stage of infection (Seitner et al. 2018). Δ*cce1* mutants, like the Δ*pep1* mutants, are blocked at the penetration stage. Callose deposition occurs as a host defense response that can be observed at the sites of attempted penetration. Cce1 has been recently found to be part of the Stp complex (Ludwig et al. 2021), where it is called Stp4 (Fig. 1.1). Also, the two secreted fungal cell wall proteins Sta1 and Lep1 were shown to be required for virulence (Tanaka et al. 2020; Fukada et al. 2021). Sta1 is upregulated during early stages of infection and is attached to the cell wall of filamentous hyphae. It has been hypothesized to alter the structure of the fungal cell wall to allow hyphae to be located in tissues surrounding the vascular bundle sheath (Tanaka et al. 2020). Similarly, Lep1 is a late effector highly expressed during tumor formation which binds to the cell wall of biotrophic hyphae. Lep1 was suggested to act as a cell wall adhesin, being important for hyphal aggregate formation, and therefore likely to allow proliferation of diploid hyphae (Fukada et al. 2021). However, the plant targets of ApB73, Cce1, Sta1, and Lep1 and their mechanistic role in virulence have not yet been characterized (Fig. 1.1).

1.5.4 Protease Inhibition as a Key Virulence Mechanism

The Pit2 effector is essential for *U. maydis* virulence. It is located in a cluster of four genes that include another essential virulence factor, encoding the membrane-localized protein Pit1 (Doehlemann et al. 2011). Upon secretion, Pit2 localizes to the plant apoplast where it interacts with maize apoplastic papain-like cysteine proteases (PLCPs) (Misas-Villamil et al. 2016). Pit2 inhibits the activity of apoplastic cysteine endo-proteases, but it is a weak inhibitor of CathB, an exoprotease (Mueller et al. 2013). In terms of conservation, Pit2 shares the highest level of sequence similarity across smut fungi at the PID14 motif, a sequence of 14 amino acids

identified to be necessary for the inhibition (Mueller et al. 2013). The PID14 motif is sufficient for the inhibitory function of Pit2 and it has also been identified in different bacteria, making it an interkingdom- and conserved-microbial inhibitor of proteases (cMiP) (Misas Villamil et al. 2019) (Fig. 1.1). Pit2 is a suitable substrate for PLCPs, which process the effector and thereby release the cMIP peptide with inhibitory function. Due to its close proximity to the target, cMIP suppresses the activity of PLCPs, preventing further activation of plant immune responses (Misas Villamil et al. 2019). Therefore, Pit2 acts as a substrate-mimicking molecule that inhibits apoplastic PLCPs.

1.6 *U. maydis* Effectors with Intracellular Virulence Functions

The putative protein effectome of *U. maydis* comprises several hundred candidates which are defined by the presence of a predicted secretion signal and transcriptional upregulation during biotrophy. The challenge is that these candidates largely encode proteins of unknown function. They usually lack sequence homology to any known functional domains. Effectors are molecules (i.e., proteins, peptides, metabolites, small RNAs, and others) secreted during host–microbe interactions to manipulate the host's metabolism and cellular function, thereby increasing the fitness of the invading microbe. Some of these effectors will act solely in the apoplast, whereas others are translocated to the symplast of the host. Considering the subcellular distribution of potentially targeted host pathways or molecules, apoplastic effectors are more likely preventing recognition of the invader, by suppressing extracellular immune receptors, inhibiting systemic immune signals, or detoxifying secreted defense compounds. Translocated effectors are also involved in the manipulation of host metabolism and development by interfering with intracellular signaling pathways, transcription, or metabolic regulation.

1.6.1 Salicylic Acid Signaling Manipulation by *U. maydis*

Hormone signaling regulates the systemic level of plant immunity. Salicylic acid (SA)-associated defense responses and crosstalk of this pathway with other hormone pathways together determine the outcome of *U. maydis* infection. As mentioned above, *U. maydis* regulates the activity of apoplastic cysteine proteases by inducing CC9 expression and by using the Pit2 effector as an inhibitor for PLCPs to prevent the triggering of SA-related defense (van der Linde et al. 2012; Misas Villamil et al. 2019). On the other hand, it is well-known that pathogens can secrete effectors to directly manipulate the production of SA (Djamei et al. 2011; Liu et al. 2014; Bauters et al. 2021). *U. maydis cmu1* encodes a secreted AroQ class of chorismate mutases, which is exclusively expressed during the biotrophic stage (Djamei et al. 2011). In the plant cytoplasm, Cmu1 interacts with the maize cytosolic chorismate mutase ZmCM1 to sequester chorismate and catalyze it into prephenate, a precursor of phenylalanine and tyrosine metabolism, which leads to a decreased level of chorismate for SA biogenesis. The catalytic activity of Cmu1 is not allosterically regulated by the concentration of tryptophan and tyrosine (Djamei et al. 2011; Han et al. 2019) (Fig. 1.1). An analysis by X-ray crystallography revealed that the structure of the Cmu1 protein includes an additional α-helix and an extensive loop region (ELR), which differs from the prototypical chorismate mutase Aro7p from *Saccharomyces cerevisiae* and maize chorismate mutases ZmCM1 and ZmCM2. This additional ELR is recognized by a kiwellin-like protein, ZmKWL1, in maize. In the apoplast, two molecules of ZmKWL1 together recognize one Cmu1 homodimer and abolish the chorismate mutase activity by blocking substrate accessibility to the active site. This inhibition reaction functions in a concentration-dependent, non-competitive, and allosteric manner. In addition, ZmKWL1 may also affect the translocation of Cmu1 into the plant cytosol. KWL proteins are identified in different plant species with the

exception of the Brassicaceae. The maize genome encodes 20 ZmKWL proteins, but only ZmKWL1 is induced during *U. maydis* infection. Furthermore, another ZmKWL1-b protein might be responsible for recognition of Cmu1 in different tissues depending on its tissue-specific expression pattern (Altegoer et al. 2020). The recognition domain in ZmKWL1 is highly diverse among ZmKWL members, which suggests the potential roles of ZmKWLs in recognition of versatile effectors from different pathogens.

1.6.2 Secondary Metabolism Manipulation

Another effector that rewires host defense-related metabolism in *U. maydis* is Tin2 (Fig. 1.1). Tin2 is located in the largest virulence cluster of *U. maydis*, cluster 19A (Brefort et al. 2014). The deletion of cluster 19A dramatically reduces the virulence, but complementation with a single Tin2 effector only partially rescues the virulence defect, indicating that more than one virulence factor is encoded by this cluster (Brefort et al. 2014; Tanaka et al. 2014). However, Tin2 alone completely restores the anthocyanin accumulation phenotype of this mutant, a symptom caused by *U. maydis* during seedling leaf infection. Tin2 interacts with a maize cytoplasmic serine/threonine protein kinase ZmTTK1 (Tin2 targeting kinase 1) and stabilizes it by masking its phosphodegron-like DSGxS motif from being phosphorylated and subsequent recognition by a plant internal ubiquitin ligase complex. The biological function of ZmTTK1 is unknown; however, it is involved in the regulation of anthocyanin synthesis. When the stable kinase mutant of $ZmTTK1_{S279/282A}$ is overexpressed in Δ*tin2*-infected leaves, a weak anthocyanin accumulation is observed. In the phenylpropanoid pathway, anthocyanins and lignin share the same precursor, 4-coumaroyl CoA. Tin2 induces all of the genes involved in the anthocyanin biogenesis pathway, which may redistribute their common precursor, 4-coumaroyl CoA, from lignin biogenesis into anthocyanin synthesis. Consequently, deletion of Tin2 in *U. maydis* causes cell wall fortification in the infected vascular tissue, which may provide a physical barrier to inhibit pathogen growth in the bundle sheath cells. Another interesting finding is that Tin2 orthologs are present in the maize head smut *Sporisorium reilianum*, while they are absent in the barley smut *Ustilago hordei,* as well as in *B. distachyon* smut *U. bromivora*. Deletion of *Srtin2* in *S. reilianum* causes reduced virulence, which results in a reduced leafy structure and spore formation in maize ears (Tanaka et al. 2019). However, attempts to complement the phenotype of the *U. maydis* Δ*tin2* mutant with *Srtin2* was unsuccessful as neither virulence nor anthocyanin induction was restored (Tanaka et al. 2019). Y2H experiments revealed that Srtin2 does not interact with ZmTTK1, but instead with its two maize paralogs, ZmTTK2 and ZmTTK3. ZmTTK2 and ZmTKK3 might be differentially regulated at the post-transcriptional level compared to ZmTTK1, as these proteins are stable when overexpressed in *Nicotiana benthamiana*, which implies a different mechanism for Tin2 orthologs to exploit ZmTTK paralogs. UmTin2 stabilizes ZmTTK1 to make use of its kinase function, whereas SrTin2 interacts with ZmTTK2 and ZmTTK3 to suppress their kinase activities. It was not very surprising to see that the computationally reconstructed ancestral Tin2 protein from six smut fungi functionally resembles SrTin2, as most smut fungi containing a Tin2 ortholog do not induce tumor formation, which suggests that UmTin2 has undergone a neofunctionalization in *U. maydis* during speciation to adapt for its ability to induce tumors in vegetative plant tissue. Indeed, expression of UmTin2 in *S. reilianum* increases its ability to spread in infected leaves. It is noteworthy that *S. relianum* has a different infection strategy and transmits from infection site to floral tissue to express the disease symptoms. It would be interesting to better understand whether ZmTTK paralogs have similar functions in the regulation of the lignin pathway, why *U. maydis* and *S. reilianum* have contrasting needs for the kinase activity of ZmTTKs, and how this is related to the different pathogenesis of two smut fungi. Using

the structure prediction program Alphafold and structure-guided comparative analysis, Tin2-like structure predictions have been identified in several other of the sequence unrelated *U. maydis* effectors indicating a common ancestral origin of these Tin2-structure like effectors (Seong and Krasileva 2022).

1.6.3 Tumorigenic Effectors

In addition to these cytoplasmic plant metabolism manipulators, several tumorigenic effectors are translocated from *U. maydis* into the host cell to directly reprogram host cells to proliferate and undergo hypertrophy. The first tumorigenic effector identified was See1 (Seedling efficient effector1) (Redkar et al. 2015) (Fig. 1.1). It had previously been identified as a leaf-specific virulence factor, being highly induced during leaf infection, but not in the tassel (Schilling et al. 2014). During seedling leaf infection, See1 reactivates DNA synthesis in infected tissue, which is a prerequisite for cell division. Compared to the progenitor strain SG200, the Δ*see1* mutant is strongly impaired in the induction of nuclear division in the maize bundle sheath cells, which results in a reduced number of hyperplasia of tumor cells (Matei et al. 2018). This also explains why See1 is only required during leaf tumor formation, as in tassel, *U. maydis* colonizes the undifferentiated anther cells and maintains their meristematic activity to generate the tumor cells (Gao et al. 2013). Another striking phenotype appears when See1 is overexpressed under the control of the biotrophy-induced promoter of *Pit2*. This resulted in an unexpected formation of tumors in the tassel stem, which is not seen in wild type infections. This indicates that See1 could activate tumor induction in vegetative tissue when expressed ectopically. In maize, See1 interacts with SGT1 (suppressor of G2 allele of skp1), a regulator for yeast cell cycle transition (Kitagawa et al. 1999). In plants, SGT1 is mostly reported to regulate plant immunity (Azevedo et al. 2006; Austin et al. 2002). SGT1-related immune responses require phosphorylation by MAPK, which in turn is suppressed by See1; a virulence mechanism which recently has been also found for effectors from other plant-pathogenic microbes (Yu et al. 2020; Nakano et al. 2020). However, how See1 modifies SGT1 to drive the reactivation of DNA synthesis in tumor formation, is still unknown.

Another effector directly related to tumorigenesis is Sts2 (small tumor on seedlings 2). Sts2 was first identified as a leaf-specific effector (Schilling et al. 2014). However, further transcriptome analysis from different studies suggested that Sts2 is a hypertrophy tumor cell-specific expressed effector and also differentially regulated when introduced into different maize inbred lines/cultivars (Schurack et al. 2021). Furthermore, Sts2 orthologs from *U. maydis* and *S. reilianum* are differentially regulated in the respective pathogens during seedling infection. As SrSts2 orthologs cannot restore the Δ*sts2* virulence defect in *U. maydis*, it is likely they are functionally diverse (Zuo et al. 2021).

1.6.4 Functional Clusters of Effectors

As previously reported, the *U. maydis* effectome is generally organized in gene clusters (Kamper et al. 2006). Recent findings showed that they can display functional partial redundancy (Navarrete et al. 2021b; Bindics et al. 2022). Since host recognition of a given pathogen effector can lead to a high fitness cost, effector redundancy is evolutionarily critical for the pathogen to succeed (Sánchez-Vallet et al. 2018). Thus, hijacking host perception and promoting infection through simultaneously targeting immune hubs improves pathogen adaptation with no or mild costs. Independent of the life-style, some plant molecules and pathways are broadly targeted by unrelated phytopathogens during infection (Carella et al. 2018) and it is now becoming clear that, even within the same genome, co-evolution takes place. The degradation or inhibition of enzymes like chitinases, proteases, and peroxidases, or interference with protein complexes such as the SGT1–RAR1–HSP90 complex, E3 ubiquitin ligases, MAP kinase cascades, with ROS burst and hormonal pathways are examples of

redundant effector target hubs among multiple pathogens (Carella et al. 2018). In the past few years, new findings have shown how *U. maydis* modulates these molecular hubs in a multi-directional way, as we further explain below.

A cluster of 10 effector proteins, called the *pleiades* cluster (cluster 10A), was recently described (Navarrete et al. 2021b) (Fig. 1.1). The cluster is subdivided into three families (A, B, and C) of paralogous genes with low sequence similarity among each other. All of the *pleiades* genes are expressed during the host interaction, but at different times and in different tissues. The proteins encoded by these clusters are translocated from the biotrophic interface to the plant cytoplasm. The full cluster deletion strain SG200Δ*ple* shows strong *in planta* ROS accumulation around the infective hyphae and this leads to massive virulence impairment. The deletion of all the members but Atlas1 (Atl1) also showed strong disease arrest, yet not at the level seen with SG200Δ*ple*. Even when deleting the gene families individually, most show a mild reduction in virulence, with family C being the most effective. Atl1 is the member which mostly contributes to virulence (Erchinger 2017). In terms of individual effects, all of the *Pleiades* proteins, with the exception of Plo1, show strong ROS burst suppression *in planta* when expressed in the plant symplast. Most of the members suppressed apoplastic ROS accumulation independent of the MAMP used, which in this case included either chitin or flg22. However, two of them showed high MAMP-specificity and, while Atl1 just suppressed ROS when challenged with flg22, Cel1 blocked the burst only under chitin perception. The fact that Atl1 is responsive to flg22 suggests that it functions on or downstream of the co-receptor BAK1, which is involved in the perception of multiple MAMPs, but not chitin. Cel1 might act directly on the LysM chitin receptor Cerk1 or other unique parts of the downstream signaling different from the flg22 signaling pathway. The rapid diversification of smut fungi effectors through gene duplication can be an explanation for the specialization of the *pleiades* members. Interestingly, although functionally highly redundant, the family members are potentially targeting multiple maize interactors, yet always somehow suppressing MAMP-based perception. Among the stronger ROS burst suppressors, Tay1 showed activity in the host cytoplasm, while Mer1 is active inside the host nucleus, but not in the cytoplasm. Tay1 and Mer1 are paralogs sharing about 30% sequence similarity and it is important to note that these genes have diversified to be active in different plant compartments but still target the same process. Although the specific host partner of Tay1 still needs to be elucidated, Mer1 directly interacts with multiple members of the RFI2 (RING domain, E3 red and far red insensitive 2) E3 ubiquitin ligase protein family. RFI2 homologs regulate ROS production and are positive regulators of MAMP-triggered immunity (MTI) in the *U. maydis*-maize pathosystem. In vitro experiments show that Mer1 stimulates the RFI2 family capacity of auto-ubiquitination, potentially destabilizing these positive regulators of MTI and thereby repressing ROS production (Navarrete et al. 2021b). Interestingly, RFI2 has been reported in *A. thaliana* to be a negative regulator of flowering and Mer1 overexpression in *A. thaliana* enhances flowering, which are interesting effects for an effector of a smut pathogen that in the field is frequently found to cause major disease symptoms in the floral parts of the plant (Navarrete et al. 2021b).

1.6.5 Maize Lipoxygenase 3, Susceptibility Factor and Effector Target

Another member of the *U. maydis* plethora of ROS burst-suppressing effectors is the recently characterized protein Rip1 (ROS burst interfering protein 1) (Fig. 1.1). Rip1 is conserved among monocot-infecting smuts and has a short C-terminal motif (RIFL) that is essential but not sufficient for ROS-suppressing activity. After translocation to the host cell, the effector surprisingly targets multiple compartments to suppress MTI, which is thought to require several host target proteins to perform this activity (Saado et al. 2022). One host target that has been

identified is the maize lipoxygenase 3 (Zmlox3), previously identified as a susceptibility factor in maize–*U. maydis* interaction (Pathi et al. 2020; Saado et al. 2022). The nuclear localization of Rip1 plays a role in ROS burst-repressing activity of Zmlox3. In this case, it has a cargo capacity and redirects Zmlox3 to the host nucleus which causes a reduction in ROS burst activity *in planta*, partially explaining the ROSburst-suppressive activity of RIP1. Excitingly, the lox3 maize mutant lines showing partial resistance to *U. maydis* infection lose their resistance to Δ*rip1* strains. This implies that Rip1 effector-triggered host resistance reactions are suppressed by Rip1 with the help of its host target Lox3 in the maize nucleus. The exact mechanism as to how Lox3, which is normally cytosolic localized, acts in the nucleus remains to be determined.

1.6.6 Exploiting Growth Versus Defense Antagonism

When plants are under attack from pathogens, there is an antagonistic balance between growth progression and defense responses. Pathogens often exploit and benefit from these modulations and one way is through secretion of effector proteins. In the *U. maydis*-maize pathosystem, the hormonal modulations are also crucial to allow the fungus to maintain a biotrophic lifestyle while at the same time tackling the host immune responses. *U. maydis* is known to cause hormonal imbalances very early during infection. The auxin pathway, for instance, is a pivotal target for smut fungi in suppressing defense and promoting disease. The recently described effector, Naked1 (Nkd1), interferes with maize TOPLESS and TOPLESS-related co-repressors (TPL/TPRs) in the host nucleus leading to incremental auxin signaling and subsequent disruption of the MAMP-triggered ROS burst (Navarrete et al. 2021a). Nkd1 physically interacts with a C-terminal so-called EAR motif (LxLxL)) with maize TPL/TPRs. TOPLESS and TOPLESS-like proteins are involved as negative regulators in many important phytohormone signaling pathways including auxin signaling, JA/ET signaling, brassinosteroid, abscisic acid as well as SA signaling and have recently been shown to be associated with plant immunity in addition to their well-known involvement in plant development (Altmann et al. 2020). In the case of auxin signaling repression during the resting state, the Aux/IAA repressor binds auxin response factors (ARFs) in the nucleus and recruits via an EAR-motif TPL/TPR, which keeps the chromatin in a closed repressive state (Szemenyei et al. 2008). Upon auxin accumulation in the cell, the auxin receptor TIR1/AFB targets the Aux/IAA transcriptional repressor for ubiquitination and degradation and auxin-responsive gene expression is thereby derepressed. Although auxin is well-known for promoting disease-susceptibility in a growth-immune trade-off-dependent manner, the intricate molecular mechanisms require further clarification. The interaction, in this case, takes place with the N-terminal TOPLESS-domain (TPD) of the target protein, which prevents recruitment of the Aux/IAA repressors, leading to auxin signaling de-repression (Fig. 1.1). The *U. maydis* SG200 knock-out strain, Δ*nkd1*, shows a strong virulence defect and Nkd1 suppresses the ROS burst when transiently expressed in maize plants. The ROS burst-suppressive activity of Nkd1 is directly linked to the presence of an EAR motif and its ability to bind to TOPLESS/TPLs. However, the virulence defect of Δ*nkd1* does not depend on this function as Nkd1 complementation constructs without the EAR-binding domain fully restore virulence, suggesting that Nkd1 is also a multifunctional effector (Navarrete et al. 2021a).

The targeting of the N-terminus of TPL/TPR proteins is also underpinned by the Tip- (Topless interacting proteins) effectors (Bindics et al. 2022). The Tips are a family of five effector proteins encoded by genes within cluster 6A, which are involved in promoting virulence. They interact with TPL/TPRs of both maize and dicot model plants and compete with Aux/IAA repressors to induce auxin signaling. The massive targeting of TPL/TPR by *U. maydis* effectors suggests that TOPLESS has a critical role in the maize–*U. maydis* interaction, making it a major effector hub (Navarrete et al. 2021a, Darino et al.

2021; Bindics et al. 2022). Additionally, the *U. maydis* effector JA/ET signaling inducer 1 (Jsi1) also interacts with maize TPL/TPRs in an EAR-dependent manner (Darino et al. 2021) (Fig. 1.1). In contrast to Nkd1, which carries an LxLxL motif and interacts with the N-terminal region of TPL/TPR proteins, Jsi1 binds directly to the second WD40 domain at the C-termini of TPL/TPR proteins through a DLNxxP motif. Using an *in planta* estradiol-inducible system in *Arabidopsis*, Jsi1 expression led to induction of genes related to the ERF branch of the JA/ET signaling pathway as well as inducing other TPL-target genes. Multiple ERF transcription factors (*erf2*, *erf5*, *erf6,* and *erf107*), *acs*s (*acs2*, *acs6*, and *acs11*), the MAP kinase kinase 9 (*mkk9*), and also the defense-related genes *pr5*, *osm34,* and *pdf1.2* were transcriptionally modulated upon Jsi1 overexpression. The antagonism between the JA/ET and SA pathways, although no longer as simplistic as once thought, also seems to be employed in the *Ustilago*-maize pathosystem. The activation of the JA/ET cascade by *U. maydis* increases biotrophic susceptibility, thereby promoting fungal infection. This observation is also supported by previously reported transcriptomic data (Doehlemann et al. 2008) where the authors suggested a manipulation of this pathway. Interestingly, Jsi1 overexpression also increases total SA levels and upregulates SA-responsive genes. Although this is counterintuitive based on the antagonism between both pathways, the authors suggested a more intricate regulation in the case of Jsi1. A similar SA signaling induction behavior upon overexpression was also reported for Nkd1. As effectors in the context of infection do not act alone, it is likely that the SA defense-triggered responses upon overexpression of Jsi1 or other TPL effectors *in planta* will be prevented in *U. maydis*-infected maize, either by other effectors or will not be triggered at all due to overall lower amounts of effectors in the plant cell in natural infections. Moreover, it is possible that TPL/TPRs repression of SA signaling is an intrinsic conserved "guard"-process leading to SA-mediated defenses upon manipulation of this negative key regulator of transcription by pathogens.

1.6.7 A Possible Translocation Machinery for Intracellular Effectors

Although several cytoplasmic effectors are identified in *U. maydis*, it is unknown how effectors are targeted for translocation into the host cells. Unlike the well-characterized needle-like type III secretion structure in bacterial pathogens, the delivery system in fungi is not yet understood. The recent discovery of a cell surface-exposed Stp complex is proposed by the authors to be responsible for the secretion of effectors from *U. maydis* into the host cells (Ludwig et al. 2021). The complex consists of five effectors (Stp1-3, Cce1 (renamed by the authors Stp4) and Pep1) and two transmembrane proteins (Stp5 and 6), which are co-regulated and exclusively expressed during the plant infection process. Single deletions of any members of the complex causes the complete loss of virulence, with the mutants unable to penetrate the plant epidermis (Uhse et al. 2018; Doehlemann et al. 2009; Hemetsberger et al. 2012; Seitner et al. 2018; Ludwig et al. 2021). Targeted effector candidate Y2H screenings revealed frequent effector–effector candidate interactions (Alcantara et al. 2019). Co-immunoprecipitation (IP) followed by mass spectrometry (MS) validated the mutual interactions between Stp1, 3, 6, Cce1 (Stp4), and Pep1; however, Stp2 and Stp5 only interact with Stp5, Stp6, and Stp2, respectively (Ludwig et al. 2021). During host colonization, the Stp complex is anchored in the hyphal membrane to form speckles that protrude into the host cell, which may, in cooperation with the host plasma membrane ATPases, PIP2-type aquaporins, a GPI-anchored lipid transfer protein, and a hydroxyproline-rich glycoprotein, form a portal for effector translocation. Although direct evidence for this mechanism is still absent, the authors suggest that the Stp complex participates in effector delivery. In support of this hypothesis, the "stop" phenotype of *U. maydis* SG200Δcomplex (lacking all seven complex members) can be partially rescued by co-infection with SG200. In addition

SG200Δcomplex fails to induce a non-host resistance in barley, which is a phenotype previously described for the Δ*pep1* mutant in barley infections (Hof et al. 2014). Homologs of all the *Stp* complex genes have been found in all sequenced smut fungi so far, providing further support for this conserved function. This is further supported by the ability of genes encoding orthologs of Pep1, Cce1 (Stp4), and Stp1 from other smut species to complement the *U. maydis stp* mutants (Liang 2012; Hemetsberger et al. 2015; Seitner et al. 2018). However, the biological function of this complex still needs to be confirmed and further evidence, in particular the complex 3-dimensional structure, remains to be determined.

1.7 *U. maydis* as a Model System in the Post-Genomic Era

U. maydis is established as a leading model system for biotrophic plant pathogens. It is easy to grow in axenic culture and highly amenable to reverse genetics, thereby enabling functional studies to be carried out on genes controlling signal perception, signal transduction, and pathogenic development (Brefort et al. 2009). Technical advances in imaging, particularly confocal laser scanning microscopy, and significant improvements in MS-based peptide identification have been instrumental in the successful functional characterization of several effector proteins. The identification of virulence functions of many effectors has also been boosted by the huge expansion of transcriptome and genome data and the use of novel functional genomic approaches (Uhse et al. 2018). The small, compact genome structure of *U. maydis*, and smut fungi in general, has enabled high quality sequence annotation and comparative genome approaches to study the evolution and functional activity of effectors across species (Depotter et al. 2021b; Zuo et al. 2021). The increasing availability of sequence and expression data will allow us to better understand the process of host adaptation and the establishment of different infection styles in various smut pathogens. Integrative analysis of large sequencing data sets can help to predict new virulence functions.

This approach can now be complemented by the use of protein structure prediction via Alphafold 2 (Jumper et al. 2021; Seong and Krasileva 2022). Effector sequences are highly variable, which can result in very low levels of sequence conservation among paralogs (i.e. members of effector gene families within a genome) as well as orthologs in related species. Thus, the prediction of similar (or diverged) structures of effector proteins has an enormous potential to predict functions of individual effectors, but also to recognize potentially similar or cooperative functionality of unrelated effector genes.

A mechanistic understanding of effectors will benefit from the continuously growing toolbox for *U. maydis*. The highly efficient CRISPR/Cas9 system (Zuo et al. 2020; Schuster et al. 2018b) now allows faster genetic modification and also makes *U. maydis*—and smut fungi in general—an excellent platform for the heterologous expression of microbial virulence factors and even for the secretion of maize peptides (van der Linde et al. 2018; Ökmen et al. 2021). However, a comprehensive understanding of a biotrophic microbe–host interaction obviously involves the functional characterization of host processes that are modulated in response to the pathogen. To demonstrate a causal connection between the interference of a fungal effector with its host target, genetic evidence in the host will also be required. Genetic transformation and the use of CRISPR/Cas9-mediated mutagenesis are now well established in maize, as are excellent mutant libraries such as BonnMu (Marcon et al. 2020). Nevertheless, the long generation time and high demands in growth facilities and technical expertise still make the generation of maize knock-out mutants a severe bottleneck in the *U. maydis*–maize pathosystem. This problem has often been circumvented by the use of heterologous plant expression systems, mainly *N. benthamiana* or *Arabidopsis thaliana*. Despite the obvious limitations of heterologous approaches, use of such systems has been crucial for the functional characterization of several

U. maydis effectors that target conserved plant biochemical pathways (Darino et al. 2021; Bindics et al. 2022; Navarrete et al. 2021a, b; Saado et al. 2022). Thus, despite all the technical improvements, it is foreseeable that future studies will continue to involve the use of heterologous test systems to compensate the technical and biological limitations of the maize system.

From the effector biology of the *U. maydis*–maize pathosystem we have learnt that fungal virulence is based on multiple layers of effector functions (Fig. 1.2). Conserved core effectors, which act as suppressors of host innate immunity, are essential virulence factors (Doehlemann et al. 2009; Seitner et al. 2018; Schuster et al. 2018a; Ludwig et al. 2021). Another important level of control is (host)-specific inhibition of pathogen-induced and plant-species-specific defense components. Examples of this control level are the effectors Pit2 and Tin2, whose activities are required for virulence, but whose functions are host-specific (Misas Villamil et al. 2019; Tanaka et al. 2019). Other effectors cause subtle, tissue- and even cell-type-specific modulations of host cellular physiology to induce metabolic or hormonal reprogramming and morphologic transformations, including tumor formation. Such effectors may have evolved during ongoing co-evolution with the host and quantitatively add to the fitness of the pathogen, although not being essential for virulence itself (Fig. 1.2).

Important information which is not generally found in publications is that, in most cases, the deletion of an effector gene does not lead to a visible reduction of virulence (Uhse et al. 2018). These genes, which comprise the majority of *U. maydis* effectors, are often considered as functionally marginal, redundant, or being compensated by other effector activities. Nevertheless, looking at their functional activities can help reveal surprising linkages in the plant immune system and beyond (Navarrete 2021, p. 46; Darino 2021, p. 4; Saado 2022, p. 101). Importantly, the inability to find phenotypes is possibly due to the fact that laboratory conditions only cover a small snapshot of the natural environment in which a pathogen evolves. An example of this is the finding that effectors have maize-line-specific virulence functions (Stirnberg and Djamei 2016; Schurack et al. 2021), with more information likely to be obtained by expanding the tests to different growth and infection conditions. One of the most intriguing challenges will be to understand which factors actually determine fitness of *U. maydis* in the natural environment, and thereby drive its evolution in an ecosystem context. Forthcoming research should therefore not be restricted to the generation of a more and more detailed picture of the (sub)cellular processes taking place during the biotrophic interaction of *U. maydis* with the maize plant. Future work should include a better understanding of the role of fungal dimorphism for fungal fitness: smuts can be found in various environments and associations with other plants and microbes in their haploid form. However, genome sequencing to date of these anamorphic strains has revealed the presence of genes for functional effectors, suggesting that these haploids still have the ability to colonize a plant in the presence of a compatible mating partner (Sharma et al. 2019). On the other side, the investigation of an anamorphic smut yeast grown on *Arabidopsis* showed there was a specific transcriptional activation of secretory genes in response to the microbial phyllosphere (Eitzen et al. 2021). The microbe-induced genes include a GH25, which efficiently inhibits infection of *Arabidopsis* by the oomycete white rust pathogen *A. laibachii*. Such findings suggest that smut fungi are adapted to compete with other microbes, and this might not be limited to the direct context of their host plant. Ultimately, we will only be able to fully understand the evolution of plant pathogens in the context of their complex organismic environment. This will allow us to gain a mechanistic understanding of both the co-evolution with the host plant and the competition with other microbes for their ecological niche. In this challenging future endeavor, *U. maydis* will continue to serve as an excellent model experimental system, delighting scientists with its stunning biology and an outstanding molecular toolbox.

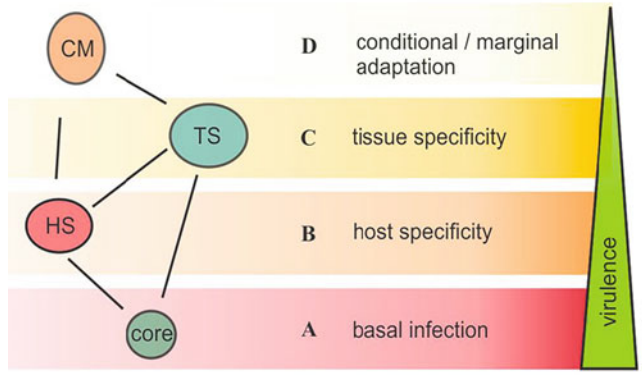

Fig. 1.2 Model of the functional layers occupied by *U. maydis* effectors. Core effectors have an essential role in fungal virulence and are often conserved amongst smut species (core). Effectors with important virulence functions that directly target components of the host immune system undergo strong sequence diversification and thereby have evolved (host) species-specific functions (HS). To colonize different host organs and to induce the specific cellular processes during tumorigenesis, *U. maydis* secretes effectors with tissue- or cell-type-specific activities. These effectors are not essential virulence factors, but quantitatively contribute to symptom formation (TS). A large part of the *U. maydis* effectome does not generally contribute to virulence, but instead, contributes to pathogen fitness in a way that depends on multiple factors ranging from host genotype to environmental conditions. Deletion of the genes encoding such effectors often does not lead to a visible defect virulence in phenotype, unless a condition for its activity is found. Other effectors categorized in this group are likely not targeting the host plant, but contribute to *U. maydis* competitiveness toward other microbes (CM)

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RNA Dialogues in Fungal–Plant Relationships

2

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Abstract

The role of extracellular RNAs in fungal–plant interaction has been neglected for long time due to its assumed instability. Recent advances in isolation and functional analysis of extracellular RNAs, that can overcome the species border between fungi and plants, have shed light on the mechanisms of cross-kingdom RNA communication. In particular, small RNAs inducing cross-kingdom RNA interference and full-length mRNAs encoding for proteins have been discovered in association with extracellular vesicles. These membrane compartments are proposed to facilitate RNA transport between fungal and plant cells across two membranes and two cell walls. Here, we review methods and standards to run extracellular RNA studies in fungal–plant interactions, current concepts of cross-kingdom RNA communication, and RNAi-based biotechnological approaches for crop protection.

Keywords

Extracellular RNAs · Cross-kingdom RNA interference · Extracellular vesicles · RNAi plant biotechnology

2.1 Introduction

2.1.1 Fungal–Plant Communication

Fungi and plants can form a spectrum of relationships. These comprise several serious pathogenic species of plants causing devastating diseases that threaten agricultural and ecological systems (Dean et al. 2012; Fisher et al. 2020), but also form symbiotic interactions that stabilize plant environmental robustness (Rodriguez et al. 2009; Chen et al. 2018; Perez-Alonso et al. 2020). During fungal–plant interactions, communication between the fungus and the host plant takes place by secretion of diverse regulatory molecules. Fungi release extracellular enzymes, protein effectors, and toxins that manipulate plant physiology and immunity, and that can damage or remodel plant cells and tissues for plant infection and for host accommodation. Such fungal molecules often accumulate in the intercellular fungus–plant interaction zone, and some fungal effectors are active inside the plant cell (Giraldo and Valent 2013; Lo Presti et al. 2015).

While research on fungus–plant interactions has been mainly focused on fungal protein

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effectors and the plant immune response, the role of *extracellular RNAs (exRNAs)* in fungal–plant communication has been neglected for a long time, because RNA has been considered to be unstable outside the cell. New lines of evidence have now added exRNAs, in particular regulatory small RNAs (sRNA), to the list of fungal- and plant-released communication molecules. Recent reports have shown that sRNAs can travel across the membrane boundaries between hosts and microbes. In their target cell, they trigger gene silencing *in-trans* in non-related species of different kingdoms, a phenomenon called *cross-kingdom RNA interference* (ckRNAi) (Knip et al. 2014; Weiberg et al. 2014; Weiberg and Jin 2015). Of note, fungi as well as plants produce exRNAs that induce cross-kingdom RNAi for plant colonization or as a defense response against attacking fungal pathogens, respectively.

2.1.2 Regulatory Small RNAs and RNAi

Various sRNAs of 20–30 nucleotides (nt) in length can induce gene silencing, a regulatory mechanism called RNAi, that is highly conserved in the eukaryotic kingdoms, including fungi and plants (Chang et al. 2012; Axtell 2013; Bologna and Voinnet 2014). sRNAs are produced from double-stranded RNA precursors by the conserved Dicer or the Dicer-like (DCLs) type-III RNA endonucleases. MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are common classes of regulatory sRNAs with different origins: miRNAs are cleaved from a single-stranded RNA precursor that forms a hairpin-loop structure, the primary (pri)-miRNA. siRNAs originate from dsRNA molecules, for instance from dsRNA viruses or from transposons. In both cases, the resulting single "guide strand" of the duplex sRNA loads into the conserved gene silencing effector protein, called Argonaute (AGO), to form the RNA-induced silencing complex (RISC). This complex binds targeted RNA or DNA that has a complementary sequence to the sRNA. RISCs can induce silencing at the post-transcriptional level by mRNA cleavage, by translational inhibition of mRNAs, or at the transcriptional level by heterochromatin formation. Remarkably, sRNAs can be mobile and regulate gene expression in a cell non-autonomous fashion (Brosnan and Voinnet 2011; Melnyk et al. 2011; Sarkies and Miska 2014).

2.1.3 Fungal Extracellular RNAs

RNA transport between cells is described for multi-cellular organisms that use RNAs as signaling molecules to orchestrate reactions of specialized cell types, and long-distance transport enables communication between distant organs such as roots and shoots in plants (Kehr and Kragler 2018). In fungi, RNA transport is less well understood. Secretion of RNAs to coordinate cell communities, for instance, during biofilm formation of *Candida albicans* (Zarnowski et al. 2018), comprises the release of molecules, but no targeted transport to the next cell. Drawing a clear line between secretion and targeted extracellular transport for functional purposes remains challenging. However, in recent years, transport of RNA cargo in extracellular vesicles (EVs) has become apparent as an important means of communication also in fungal–plant interactions. Knowledge of RNA dialogues between fungi and plants is modest, but in the mammalian field, EVs from a highly virulent outbreak strain of *Cryptococcus gattii* enhance the intracellular proliferation rate of less virulent strains inside macrophages via their RNA and protein cargo (Bielska et al. 2018).

sRNAs and RNAi in fungi have been reviewed in great detail (Chang et al. 2012; Torres-Martinez and Ruiz-Vazquez 2017; Nicolas et al. 2020). In this chapter, we elaborate on the current scientific concepts of RNA dialogues in fungal–plant relationships, with an emphasis on cross-kingdom RNAi. We describe methodological strategies to identify fungal exRNAs and to experimentally validate cross-kingdom RNAi. We will also touch on how basic research in fungal–plant RNA communication could boost RNAi-based biotechnologies as a new generation of antifungal disease control.

2.2 Cross-Kingdom RNAi in Fungal–Plant Relationships

Fungal sRNA Effectors Silence Plant Genes

Fungal pathogens and symbionts secrete exRNAs to induce ckRNAi in plants during host colonization. In pioneering work, sRNAs of the fungal plant pathogen *Botrytis cinerea* were discovered to translocate into plant cells to support fungal infection (Weiberg et al. 2013); these translocating RNAs are called *sRNA effectors* by analogy to fungal protein effectors. To date, the *B. cinerea–Arabidopsis thaliana* interaction represents the best-characterized cross-kingdom RNAi system; thus, we refer to this interaction as a model to explain fungal–plant RNA communication.

A common strategy to identify sRNA effectors from fungi is *sRNA sequencing (sRNA-seq)*. In a proof-of-principle study, the necrotrophic pathogen *B. cinerea*, the causative agent of gray mold disease, was investigated. In this fungus, 72 sRNAs of 20–24 nt in length were identified, and prediction of plant target genes revealed 315 plant genes in the host species *A. thaliana* and *Solanum lycopersicum* (tomato) (Weiberg et al. 2013). A contribution of *B. cinerea* sRNA effectors (*Bc*-sRNAs) to plant infection was evident, because ectopic expression of individual *Bc*-sRNAs in *A. thaliana* led to constitutive plant target gene suppression and enhanced disease levels when infected with *B. cinerea*. Interestingly, some *Bc*-sRNAs had multiple predicted plant gene targets (Wang et al. 2017b). To confirm which plant genes are targeted by a given *Bc*-sRNA (Table 2.1), *sequence-specific silencing* was verified by transient co-expression of the *Bc*-sRNA and the target gene using an *Agrobacterium tumefaciens*-mediated transient expression assay in *Nicotiana benthamiana*. *N. benthamiana* is easy to transform and offers fast and reliable *in-planta* transient expression of heterologous genes. This *in-planta* assay revealed that *Bc*-sRNAs suppressed plant target gene expression. Expression was monitored at the protein level by western blot analysis and in situ by quantitative microscopy of a fluorescence-tagged target protein. This silencing effect was lost when the *Bc*-sRNA target site was mutated.

Following similar strategies, the 18 nt long *Fusarium graminearum* sRNA *Fg*-sRNA1 was identified to silence a wheat *Chitin Elicitor-Binding Protein* (*TaCEBiP*) (Table 2.1) in a sequence-specific manner (Jian and Liang 2019). *Ta*CEBiP is involved in a chitin-activated plant immune response (Tanaka et al. 2010), and suppression of *TaCEBiP* supports fungal infection. Indeed, *TaCEBiP* was silenced by Barley Stripe Mosaic Virus (BSMV)-induced expression of *Fg*-sRNA1 in wheat. Moreover, *short-tandem target mimic (STTM),* a tool to study the function of endogenous miRNAs (Tang et al. 2012; Wang et al. 2019), has been successfully adapted to investigate sRNA effectors in ckRNAi (Cai et al. 2018; Jian and Liang 2019; Dunker et al. 2020). By expressing a STTM RNA to block *Fg*-sRNA1-induced silencing of *TaCEBiP* in BSMV, an increased resistance phenotype against *F. graminearum* infection was obtained demonstrating the activity of this sRNA in ckRNAi.

A second class of fungal sRNAs inducing cross-kingdom RNAi are miRNA-like RNAs (milRNAs). The term "miRNA-like" reflects that genome-predicted hairpin loop-structured RNA precursors are found (Lee et al. 2010), but evidence for interspecific sequence homology and conserved target gene regulation is lacking, which are definitions of plant and animal miRNAs sensu stricto. The first example of a fungal milRNA effector is the *Pst*milRNA1 of the wheat rust pathogen *Puccinia striiformis* formae speciales (f.sp.) *tritici*. The 20 nt *Pst*milRNA1 is expressed in uredospores and in germ tubes, and induces silencing of the wheat *Pathogenicity-Related 2* (*TaPR2*) gene (Table 2.1) (Wang et al. 2017a). The *A. thaliana PR2* ortholog encodes a β-1,3-glucanase and is implicated in defense responses against plant pathogens. *Pst*milRNA1 was found to be highly conserved among 14 *P. striiformis* f.sp. *tritici* isolates from distinct geographic origins, but is unique to this species, as was not found in any other fungal species, including the cereal

pathogens *P. graminis*, *P. triticina*, and *Fusarium graminearum*. Cleavage of the wheat *PR2* mRNA by *Pst*milRNA1 was evident in a *5` RNA Ligase-Mediated (RLM) Rapid Amplification of cDNA Ends (RACE)-PCR* analysis when transiently co-expressed in tobacco. As a second example of a milRNA effector, the *Fusarium oxysporum* f.sp. *lycopersicum* milRNA1 (*Fol*milRNA1) silences the tomato *CBL-interacting protein kinase* (*CIPK*) *SlyFRG4* (Table 2.1) at the post-transcriptional level when bound to tomato *Sly*AGO4a (Ji et al. 2021). Plant CIPKs are known to regulate calcium signaling for abiotic stress responses (Tang et al. 2020), and *SlyFRG4* loss-of-function in tomato led to enhanced Fusarium wilt disease (Ji et al. 2021). *SlyFRG4* silencing was confirmed by transient co-expression assays in tobacco and cleavage of *SlyFRG4* mRNAs by 5′ RLM RACE-PCR. Of note, the *Fol*milRNA1 and the *Pst*milRNA1 are not homologs.

Recently, cross-kingdom RNAi was reported in a beneficial fungus for the first time. A milRNA of the ectomycorrhizal species *Pisolithus microcarpus* is translocated into root cells of its host plant *Eucalyptus grandis* (Wong-Bajracharya et al. 2022). The milRNA *Pmic*-miR-8 was strongly expressed during ectomycorrhizal colonization (ECM). Remarkably, translocation of *Pmic*-miR-8 into the root cells was demonstrated using probe-specific whole-mount fluorescence in situ hybridization (FISH), providing for the first-time direct visual evidence for a microbial sRNA being translocated into plant cells. To study the biological role of *Pmic*-miR8 in the ECM fungi–root interaction, external application of *synthetic sRNAs* was used. Synthetic *Pmic*-miR8 enhances overall miRNA levels of this species. Antagonistic miRNAs in the form of either antisense *Pmic*-miR-8 with ZEN (N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine) modification (Lennox et al. 2013) or antisense *Pmic*-miR-8 with a bulge designed at position 10/11 (mismatch in RNA–RNA base pairing at this position, which blocks RISC cleavage activity on the target mRNA) inhibited the native *Pmic*-miR-8 activity and thereby served as controls. These studies demonstrate that *Pmic*-miR-8 can suppress a number of *Eucalyptus* genes, including homologs of the *Coiled-coil* (*CC*)-type *Nucleotide-binding leucine-rich repeat* (*NLR*) class of plant immunity receptors and *DNA endonuclease* gene family (Table 2.1). Treatment with enhancing or inhibitory synthetic RNAs increased or reduced the formation of the Hartig net and the level of fungal root colonization, respectively.

So far, only few fungal sRNA effectors have been characterized and confirmed to function in cross-kingdom RNAi (Table 2.1). This is due to the fact that conducting experiments to demonstrate cross-kingdom RNAi is laborious and most of them require transformation of fungi and/or plants. Moreover, analyzing individual sRNA effectors to induce plant target gene suppression and to support plant infection has mostly resulted in quantitative and moderate effects. This is presumably due to fungi secreting many different sRNA effectors that simultaneously affect host plant gene expression for infection. Nevertheless, sRNA effectors are predicted in several more plant-pathogenic fungal species, for instance, in *Blumeria graminis* (Kusch et al. 2018), *P. triticina* (Dubey et al. 2019), *Sclerotinia sclerotiorum* (Derbyshire et al. 2019), *Magnaporthe oryzae* (Zanini et al. 2021), *F. graminearum* (Werner et al. 2021) and symbionts, such as the arbuscular mycorrhiza fungus *Rhizophagus irregularis* (Silvestri et al. 2019).

Retrotransposon as a Source of siRNA Effectors

Shortly after cross-kingdom RNAi was discovered in *B. cinerea*, the question arose as to how *B. cinerea* produces various *Bc*-sRNAs to target genes in diverse plant species. *B. cinerea* is a generalist pathogen that causes gray mold disease in more than 1400 different plant species comprising many important vegetables, fruit crops, and other horticulture crops (van Kan 2006; Elad et al. 2016). Of note, most of the *Bc*-sRNA effectors are encoded by high copy number, long-terminal repeat (LTR) retrotransposon genes

Table 2.1 Small RNA effectors in fungal–plant interactions

Small RNA ID	Producing species	Locus	Target species	Target gene	Sequence specificity	Small RNA oe	Small RNA kd/ko	DCL dependency	AGO-binding	Target cleavage	Reference
Fungal small RNAs targeting plant genes											
Bc-sRNA3.1	*B. cinerea*	Retrotransposon	*A. thaliana*	*AtWAK*	■	■		■	■		Weiberg et al. 2013
Bc-sRNA3.2	*B. cinerea*	Retrotransposon	*A. thaliana*	*AtMPK1, AtMPK2*	■	■		■	■		Weiberg et al. 2013
Bc-sRNA5	*B. cinerea*	Retrotransposon	*A. thaliana*	*AtPRF1IX*	■	■		■	■		Weiberg et al. 2013
Bc-sRNA37	*B. cinerea*	Coding gene	*A. thaliana*	*AtWRKY7, AtFEI2*	■	■			■		Wang et al. 2017b
Fg-siRNA1	*F. graminearum*	Non-annotated	*T. aestivum* (wheat)	*TaCEBiP*	■	■	■				Jian and Liang 2019
Fol-milRNA1	*F. oxysporum fsp. lycopersicum*	*MIRNA* gene	*S. lycopersicum* (tomato)	*SlyFRG4*	■	■	■		■	■	Ji et al. 2021
Pst-milRNA1	*Puccinia striiformis fsp. tritici*	*MIRNA* gene	*T. aestivum* (wheat)	*TaPR2*			■	■		■	Wang et al. 2017a
Pmic-miR-8	*P. microcarpus*	*MIRNA* gene	*E. grandis* (Eukalyptus)	*CC-NLR DNA polymerase*		■	■			■	Wong-Bajracharya et al., 2022
Plant small RNAs targeting fungal genes											
Gh-miR159	*G. hirsutum* (cotton)	*MIRNA* gene	*V. dahliae*	*VdHiC-15*	■					■	Zhang et al., 2016a
Gh-miR166	*G. hirsutum* (cotton)	*MIRNA* gene	*V. dahliae*	*VdClp-1*	■		■			■	Zhang et al., 2016a
TAS2-siRNA453	*A. thaliana*	*TAS1c*	*B. cinerea*	*BcSac1*	■	■	■	■		■	Cai et al., 2018
Tas1c-siRNA482	*A. thaliana*	*TAS1c*	*B. cinerea*	*BcDctn1, BcVps51*	■	■	■	■		■	Cai et al., 2018

(Weiberg et al. 2013). Previously considered as junk DNA, it is now realized that transposons contribute to fungal pathogenicity and expansion of the host range by shaping genome architecture and driving evolution of virulence genes (Möller and Stukenbrock 2017; Fouche et al. 2020). In general, retrotransposon transcripts are often recognized by the cellular RNAi machinery, which leads to massive production of siRNAs for retrotransposon silencing. Comparative analysis of sRNA-seq data from *B. cinerea* in vitro culture and at different time points after plant inoculation revealed that retrotransposon *Bc*-siRNAs are constitutively produced, but showed induced accumulation at early time points of plant infection (Weiberg et al. 2013; Porquier et al. 2021). Upregulation of retrotransposon siRNAs was also found in the rice blast fungus *M. oryzae* (Raman et al. 2017), suggesting a role of transposon-derived siRNAs in virulence in different fungal pathogens. Remarkably, highly virulent *B. cinerea* field isolates that can infect multiple host plant species contain multiple LTR retrotransposon copies. On the contrary, transposon-free *B. cinerea* isolates have been proposed to induce relatively weak disease phenotypes (Martinez et al. 2005). For example, *B. cinerea* strains that lack LTR retrotransposon-derived *Bc*-siRNAs induced only relatively mild disease phenotypes. In a proof-of-principle experiment, transformation of a *gypsy-type LTR retrotransposon* copy into such a *B. cinerea* strain lacking retrotransposon led to *Bc*-siRNA effector production and increased pathogenicity (Porquier et al. 2021). This finding describes for the first time a retrotransposon as a *bona-fide* pathogenicity factor. It further suggests a crucial role of retrotransposons in producing a vast number of highly diverse *Bc*-siRNAs to induce cross-kingdom RNAi in many different plant host species. Such a scenario implies a cross-kingdom RNAi strategy of a broad-host range pathogen by random gene targeting in multiple plant species.

DCLs Produce Fungal sRNA Effectors

A key factor in the biogenesis of sRNAs are DCLs. DCLs are found both as single copy or in a small family of homologs in fungi, and they produce both siRNAs and milRNAs, with functional redundancy of DCLs being reported. DCLs produce fungal sRNA effectors, too, and indeed DCLs contribute to virulence. For example, *B. cinerea* possesses two homologous DCLs, *Bc*DCL1 and *Bc*DCL2, and a Δ*bcdcl1dcl2* double-"knockout," but not Δ*bcdcl* single "knockouts," exhibited loss of *Bc*-sRNA effector accumulation, growth retardation, reduced pathogenicity, and loss of *Bc*-sRNA-induced plant target gene silencing (Weiberg et al. 2013). A similar result was observed, when the single *P. striiformis* f.sp. *tritici DCL* was silenced via virus-induced gene silencing (VIGS) in wheat. *DCL*-VIGS led to reduced *Pst*-milRNA1 effector accumulation, depression of the *Pst*-milRNA1 target gene *PR2* in wheat, as well as reduced disease symptoms (Wang et al. 2017a). Contrasting results are reported for the role of fungal DCLs in *F. graminearum* and *M. oryzae* when infecting different host plants. The outcome seems to depend on the fungal strain, the host species, and the type of infected tissue. For instance, while no alternation of typical scab symptoms in flowering wheat heads, rot symptoms on tomato (Chen et al. 2015), or corn silk infection (Zeng et al. 2018) was observed in *F. graminearum fg-dcl* mutants, Δ*fg-dcl1* showed temporarily reduced infection of wheat ears causing Fusarium head blight (Gaffar et al. 2019), and Δ*fg-dcl1* as well as Δ*fg-dcl1dcl2* caused reduced symptoms on infected barley (*Hordeum vulgare*) and *Brachypodium distachyon* leaves (Werner et al. 2021). In *M. oryzae*, Δ*mo-dcl1*, Δ*mo-dcl2*, and Δ*mo-dcl1dcl2* were less virulent on *B. distachyon* leaves, seedlings, and roots (Zanini et al. 2021), but in contrast *M. oryzae dcl* mutants showed no alteration in virulence on barley leaves (Raman et al. 2017). Moreover, DCLs were reported to be dispensable for virulence in the

fungal plant pathogen *Zymoseptoria tritici* when infecting wheat leaves (Kettles et al. 2019), and cross-kingdom RNAi seems to not play any role in the *Z. tritici*-wheat pathosystem (Kettles et al. 2019; Ma et al. 2019). Therefore, analyzing "knockouts" of fungal *DCL*s as an essential component of sRNA biogenesis can provide valuable information on cross-kingdom RNAi. A limitation of such studies are pleiotropic effects in *dcl* mutants (Weiberg et al. 2013), possibly due to loss of sRNA-directed regulation of endogenous fungal genes. Of note, fungi also produce DCL-independent sRNAs (Lee et al. 2010) that in theory might contribute to cross-kingdom RNAi.

The Plant ARGONAUTE Is a Hub of Fungal sRNA Effectors

Bc-sRNAs, which suppress plant gene expression, share structural features with plant endogenous sRNAs in terms of the size range of 21–22 nucleotides (nt) and the presence of uracil (U) at their 5` termini. Plant sRNAs with such features typically associate with *At*AGO1 in *A. thaliana* (Mi et al. 2008). This could explain why *Bc*-sRNAs, in particular, are recruited to *At*AGO1 in cross-kingdom RNAi. A crucial experiment that supported this notion was *AtAGO/RNA co-immunoprecipitation (AGO co-IP)* from *B. cinerea*-infected plant tissue coupled to sRNA analysis. Performing AGO co-IP, the binding of *Bc*-sRNAs to the *At*AGO1 during infection was confirmed (Weiberg et al. 2013). In support of *At*AGO1 playing a crucial role to induce cross-kingdom RNAi for infection, *A. thaliana atago1* mutants exhibited enhanced resistance against *B. cinerea* and loss of plant target gene silencing. In contrast, enhanced susceptibility was observed in other mutants of the plant canonical RNAi pathway upstream of *At*AGO1, including *A. thaliana atdcl1*. This finding argues that it is not the loss of endogenous plant sRNAs, but the blocking of the fungal *Bc*-sRNA function in plants that is causative for the observed resistance phenotype of *atago1*. Hence, infection phenotypes of plant *ago*s and other RNAi mutant lines, if available, together with plant AGO co-IP coupled to sRNA analysis can provide useful information toward the discovery of cross-kingdom RNAi. AGO co-IP coupled to sRNA-seq is an advanced method of total sRNA-seq analysis in cross-kingdom RNAi studies, and has become the focus for analysis of pathogen sRNAs that bind to a plant AGO/RISC (Dunker et al. 2021). However, AGO co-IP experiments might be limited to species with suitable AGO antibodies available. Alternative AGO purification methods, such as chromatography-based "TraPR" (Trans-kingdom, rapid, affordable Purification of RISCs) (Grentzinger et al. 2020), may be a practical solution to overcoming this limitation.

Verticillium dahliae is another example of a fungal pathogen that delivers sRNAs that bind to the host plant AGO1/RISCs during infection. *V. dahliae* invades the plant roots to systemically colonize the xylem vessels and causes wilt diseases in many plant species (Klimes et al. 2015). In total, 99 *V. dahliae* sRNAs (*Vd*-sRNAs) with 261 predicted plant target genes were found to bind to the *A. thaliana At*AGO1 during infection as revealed by plant AGO co-IP coupled to sRNA-seq analysis. These *Vd*-sRNAs were derived from various coding and non-coding genomic loci (Wang et al. 2016). Similar to *B. cinerea* infections, *A. thaliana atago1* mutant lines showed enhanced resistance to *V. dahliae,* whereas *atdcl*s and other *A. thaliana* RNAi mutant lines showed no difference or enhanced susceptibility to *V. dahliae* (Ellendorff et al. 2009). This finding suggests that *Verticillium* utilizes *Vd*-sRNAs to manipulate host plant genes to establish infection; however, functional data that confirm silencing of plant target genes by *Vd*-sRNAs is missing.

In contrast to *Bc*-sRNAs and *Vd*-sRNAs, *Fol*milRNA1 employs the plant AGO4 instead of AGO1 or AGO2. Plant AGO4 is known to bind endogenous 24 nt siRNAs to mediate transcriptional regulation via RNA-directed DNA methylation (RdDM) and heterochromatin formation (Matzke and Mosher 2014). Typically, AGO4 activity and the RdDM pathway are located in the nucleus; thus, a first hint that fungal

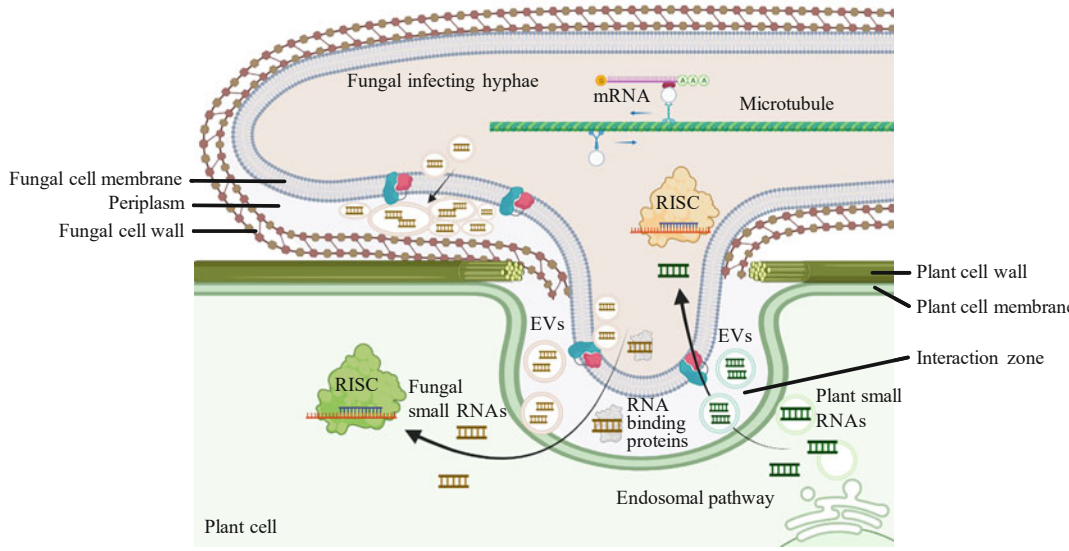

Fig. 2.1 RNA dialogues in fungal–plant relationships. During plant colonization, exchange of extracellular RNAs (exRNAs) between fungi and host plants has been observed. However, the molecular principles of exRNA exchange (from donor cell release, extracellular transport, to recipient cell uptake) at the fungal–plant interface are poorly understood. In this process, RNA translocation between fungal and plant cells has to overcome several obstacles, such as the fungal and plant cell walls and plasma membranes. Of note, when a fungal infecting hypha penetrates into a plant cell, a cell wall-free interaction zone occurs; still, fungal and plant cells remain surrounded by their plasma membranes, so that a cytosolic contact never occurs. In this interaction zone, the level of molecule exchange is increased, most likely including RNAs. Fungal and plant exRNAs have been found encapsulated in extracellular vesicles (EVs), and plant EV-small RNAs (sRNA) were found to induce cross-kingdom RNAi in fungi. Moreover, vesicle-free exRNAs has been proposed in fungi and plants, too. These might be delivered by RNA-binding proteins

sRNAs might reach the plant nucleus to induce cross-kingdom RNAi.

Plant exRNAs Silence Fungal Genes

Cross-kingdom RNAi in fungal–plant interactions is bidirectional (Fig. 2.1). Plants can be genetically engineered to produce regulatory RNAs that exclusively silence genes of an attacking pathogen, known as host-induced gene silencing (HIGS) (Nunes and Dean 2012; Koch and Kogel 2014). Interestingly, it was recently discovered that plants naturally use HIGS: they can produce sRNAs that transfer into infecting pathogens and suppress fungal gene expression related to virulence (Cai et al. 2019). For example, re-isolation of *V. dahliae* from infected cotton tissue and axenic cultivation on agar medium revealed stable accumulation of cotton miRNAs within the fungal mycelium for up to 20 days. The two cotton miRNAs, miR159 and miR166, were confirmed to cleave the *V. dahliae* genes *Isotrichodermin C-15 hydroxylase* (*HiC-15*) and the *Ca2*$^{+}$*-dependent cysteine protease* (*Clp-1*) by 5` RLM RACE-PCR, respectively. Reduced virulence of gene "knockout" strains of *V. dahliae* and enhanced virulence of transgenic strains with target site resistant versions of *HiC15* and *Clp-1* support the concept that both fungal target genes of cotton miRNAs contribute to virulence (Zhang et al. 2016a). In addition to plant miRNAs, *A. thaliana trans*-acting siRNAs (tasiRNAs), secondary siRNAs that derive from primary cleavage of *TAS* transcripts (Axtell 2013), can target fungal genes in *B. cinerea* (Cai et al. 2018). Evidence for translocation of plant sRNAs into *B. cinerea* cells was provided by sequential protoplast formation of plant cells isolated from infected tissue to release *B. cinerea* mycelium followed by removal

of plant protoplasts, *Botrytis* protoplast formation, and extracellular RNAs by RNA nuclease treatment (Cai and Jin 2021). Cleavage of the *B. cinerea* genes *Dctn1*, *Sac1*, and *Vps51* was demonstrated by 5` RLM RACE-PCR, and reduced pathogenicity of gene "knockout" strains suggested that these *A. thaliana* tasiRNAs target genes are involved in pathogenicity (Cai et al. 2018). Remarkably, plant sRNAs targeting *B. cinerea* genes were detected in EVs released by *A. thaliana* (Cai et al. 2018). This result provides a first clue on the mode of RNA translocation between fungi and plants for RNA communication.

Cross-Kingdom RNAi Is Widespread in Plant–Microbial Relationships

Cross-kingdom RNAi is not restricted to fungus–plant interactions. Regulatory sRNAs of the oomycete plant pathogen *Hyaloperonospora arabidopsidis* bind to *A. thaliana At*AGO1 and *At*AGO2 during infection and suppress host gene expression of important defense genes (Dunker et al. 2020). In this study, a new generation of ckRNAi reporters was established in *A. thaliana* that is based on activation of the reporter, if ckRNAi occurs (Dunker et al. 2020). Thereby, ckRNAi was visualized for the first time in infected leaf tissue and appeared in the plant cells under direct contact with the oomycete pathogen. Such a "switch-on" system of the ckRNAi reporter will be very helpful in future to monitor fungal–plant sRNA communication in infection sites at the cellular level and during disease progression. Other types of pathogens, parasites, or symbionts of both plant and animal hosts are proposed to deliver sRNAs into their hosts to manipulate gene expression (Weiberg et al. 2015) as indeed discovered in the insecticidal fungus *Beauveria bassiana*, which deploys milRNA effectors for mosquito infection (Cui et al. 2019), the mouse-infecting parasitic nematode *Heligmosomoides polygyrus* (Buck et al. 2014), the parasitic plant *Cuscuta campestris* (Shahid et al. 2018), and the nitrogen-fixing bacterium *Sinorhizobium meliloti* (Ren et al. 2019). These examples of cross-kingdom RNAi demonstrate that sRNA communication is widespread in fungi and other microbes interacting with different types of host organisms. Herein, examples for different microbial interaction lifestyles—not only in pathogenic biotrophic or necrotrophic, but also in symbiotic, and in different plant organs both at the phyllosphere and the rhizosphere were found.

2.3 RNA Translocation in Fungi and Plants

The phenomenon of RNA communication has been discovered in diverse fungal–plant interactions, but the underlying molecular mechanisms of RNA transport across cellular boundaries are unknown. For translocation of intact and active RNAs from the fungus to the plant or vice versa, several barriers need to be overcome: (i) the fungal plasma membrane, (ii) the fungal cell wall, (iii) protection against degradation outside the cell, (iv) uptake into the plant cell. EVs seem to be an ideal vehicle to overcome these barriers, because the membranous nature of EVs allows release from or fusion with the plasma membrane in source and target cells, respectively. Moreover, cell wall trafficking of liposomes was reported in fungi (Walker et al. 2018), and EVs can protect RNAs against degradation. Nevertheless, EV-free transport either by RNA-binding proteins (RBP) or in the free form has been considered (Fig. 2.1). In the next paragraphs, we highlight current knowledge in mRNA and sRNA transport in fungi and plants, and what we can learn from these studies to understand RNA communication between fungi and plants.

2.3.1 Transport of mRNAs

The organismal organization of fungi is relatively simple compared to the various specialized cells of the different organs of plants, such as

roots vs. shoots vs. flowers. Hence, studies on RNA transport processes in fungi are classically focused on mRNA transport between cells. Some fungal mycelia are aseptate creating multinucleate coenocytes, in which movement is not hindered by barriers, so that transport processes can resemble those in a single cell. By contrast, in a septate mycelium as found in *Ascomycetes* and *Basidiomycetes*, cells are separated by septa. Pores in the septa allow exchange of cytoplasm and organelles, similar to plasmodesmata in plants, and hence RNA exchange is possible (for a comparative review, see (Bloemendal and Kück 2013), but to date the relevant transport studies for mRNAs and sRNAs in fungi are very few (Wang and Dean 2020).

A classical, text-book example for RNA transport in fungi is the movement of the *ASH1* mRNA in *S. cerevisiae* into the daughter cell along the actin filaments during mitosis, where it is translated. Here the transcription factor, ASH1p, prevents mating type switching specifically in the daughter cell only (Beach and Bloom 2001). Interestingly, mRNA transport is mediated by vesicles, more specifically endosomes, moving along microtubules in hyphae of *U. maydis* (Vollmeister et al. 2012) resulting in distribution of mRNAs to their respective target sites. Crucial for transport is the RNA-binding protein, Rrm4, which tethers a large number of mRNAs to the shuttling endosome (Konig et al. 2009; Olgeiser et al. 2019). Some mRNAs, such as those encoding for septins, are translated during transport enabling controlled assembly of the heteromeric structures on the endosomes (Zander et al. 2016). This endosomal transport machinery is widely conserved, and the core components such as Rrm4 and Upa1 found in Basidiomycota and Mucoromycota are active in *U. maydis* (Muller et al. 2019). In *Neurospora crassa*, the RNA-binding protein GUL-1 (the homolog of *Sc*SSD1) shuttles along microtubules (Herold et al. 2019), but it is not yet clear whether this transport is also coupled to endosomes. Similar to Rrm4, it associates with a large number of mRNAs involved in a variety of processes, including cell wall integrity (Herold et al. 2021). While mRNA transport in the cytoplasm of fungi is well-described, less is known about the mechanisms of export of RNAs from fungal cells and extracellular transport routes.

In plants, grafting experiments revealed the mobility of mRNAs between different organs, resulting in spatial separation of gene expression and translation (Thieme et al. 2015). Notably, long-distance transport in the phloem is specific and bidirectional following or opposing the bulk phloem flow, raising the question about transport signals in the mRNA sequence. Polypyrimidine sequences, secondary structure motifs, or RNA modification by methylation (m^5C) can promote long-distance transport from the shoot to the root (Wang et al. 2021). Polypyrimidine sequences are bound by phloem polypyrimidine-binding proteins such as RBP50 from pumpkin or alba-type RBPs from potato (Mahajan et al. 2012). Structural motifs comprise tRNA-like sequences of phloem-enriched tRNAs, which are necessary and sufficient to mediate phloem transport and graft mobility (Zhang et al. 2016b). Methylation promotes phloem transport of mRNAs, for instances, *AtTCTP1* and *AtHSC70.1*, across graft junctions in *A. thaliana* and enhances stability of the mRNAs (Yang et al. 2019), possibly by influencing their interaction with RBPs. During infection, fungal mRNAs secreted by the pathogen might enter this phloem transport pathway and thereby integrate into the plant long-distance regulatory system.

In the extracellular space, mRNAs can be exposed to numerous nucleases, in particular, when they move into the plant apoplast during transport between fungi and plants. Furthermore, apoplastic plant nucleases are induced, for instance, when *Phytophthora spp.* infect tobacco, contributing to efficient defense (Galiana et al. 1997; Hugot et al. 2002). In this regard, the phloem is an ideal transport route also for long-distance transport of fungal RNAs in the plant due to the low level of RNase activity in phloem sap (Sasaki et al. 1998) and its RNase inhibitory activity (Kehr and Kragler 2018). Nevertheless, it is essential to protect the RNAs against degradation and therefore most RNA molecules are bound to RBPs throughout their life-time. For this purpose, the phloem of different plant species

contains numerous RBPs (Kehr and Kragler 2018). This raises a question about the transfer of fungal mRNAs to plant proteins secreted into plants: if an mRNA is bound to a fungal RBP and leaves the fungal cell to migrate into the host, when is it passed over to a plant RBP?

Transport of sRNAs

For free-living fungi, export of RNA into the environment means a loss of nucleotides for the secreting cell, so that it can be viewed as waste in particular for long mRNAs. However, sRNAs can have a role in cell-to-cell communication, for example, in the formation of biofilms similar to bacteria (Chambers and Sauer 2013; Zarnowski et al. 2018). Pathogenic fungi export sRNAs into the host plant as part of their virulence program. For instance, sRNAs can serve as effectors and regulate host gene expression as detailed above, but studies on sRNA transport in fungi are not common (Wang and Dean 2020).

By contrast, in plants the concept of sRNAs as mobile elements has been studied for a long time. Movement of sRNAs was visualized in grafting experiments decades ago: stocks carrying a silencing construct are able to spread silencing in non-silenced scions causing visual phenotypes (Palauqui et al. 1997). This offers the potential for fungi to secrete their sRNAs into the plant and highjack the host transport machinery for fungal–plant communication. However, there is strict control of the mobility of sRNAs throughout the plant symplasm (Skopelitis et al. 2018), which could limit spread of fungal sRNAs in the host plant. Gating occurs at plasmodesmata (Skopelitis et al. 2018). Here, mobility of siRNAs is limited to dsRNA species, and length (21 vs. 24 nt) does not seem to restrict species, while for miRNAs it is less clear whether the precursor, the duplex, or the mature miRNA is a mobile form (Sarkies and Miska 2014). To date, the underlying molecular mechanisms and the gating factors allowing or restricting passage via plasmodesmata remain to be identified. For fungi, another challenge is the entry into the symplasm in the first place.

Long-distance movement of RNA species in plants occurs in the phloem. Connections via plasmodesmata enable loading of cargo into the specialized phloem cells, and hence this is still part of the symplasmic transport pathway. In line with long-distance symplasmic connection, the parasitic plant *Cuscuta* is able to participate via its connecting vascular system in RNA exchange with the host (David-Schwartz et al. 2008). By contrast, stomata that are isolated from the symplasm are excluded from receiving RNAs. An open question remains, whether plant sRNAs are bound to proteins during transport, as it is known for the transport of viral RNAs by the phloem lectin PP2 (Gomez and Pallas 2004). A role of AGO proteins was suggested (Marin-Gonzalez and Suarez-Lopez 2012), but clear evidence for its role in the plant sRNA transport machinery is still missing. To address this question, investigating how fungal sRNAs enter the plant transport system can help. For instance, fungal AGO proteins loaded with sRNA might move in the phloem toward non-colonized plant organs. As an alternative pathway for sRNA transport, plant EVs can bring sRNAs even outside the symplasm and this paves the way for exchanging RNAs with microbes (Hou and Ma 2019). However, working with plant EVs is challenging, since different isolation methods are used, and purity of the EVs, which is critical when the cargo is determined, is not easy to confirm (Rutter and Innes 2020). The two plant EV markers Tetraspanin (TET)8 and the syntaxin PEN1 (Zhang et al. 2020) are able to distinguish pellets obtained at different centrifugal speeds, but whether this reflects functional differences remains to be validated.

2.3.2 Fungal EVs and Their Function in RNA Transport

EVs are small membranous vesicles of 50–1000 nm in diameter which are released by virtually all organisms under different conditions. Based on their origin, they are sub-divided into different subclasses including exosomes, which are released from multi-vesicular bodies, and microsomes, that bud off from the plasma

membrane (Zaborowski et al. 2015). Export of sRNAs into EVs was first discovered in human-pathogenic fungi. Here, milRNA sequences were specifically enriched in purified EVs from *C. neoformans, C. albicans,* and *P. brasiliensis*, but the molecular function in infection or in fungal growth remained unknown and to date still remains largely elusive (Peres da Silva et al. 2015). Recent reviews give an excellent overview of fungal EVs, their relevance to fungal biology and plant–microbe interactions, as well as the open questions in this cutting-edge research field (Rizzo et al. 2020; Cai et al. 2021). At present, EVs are being investigated in a growing number of fungi, and as a first step their cargo is being cataloged (Garcia-Ceron et al. 2021; Kwon et al. 2021). Inventories are essential for the description and categorization of EVs, but we are only at the beginning of understanding the physiological roles of the cargo. The cargo molecules might act in fungal cell wall biosynthesis or remodeling, sensing environmental stimuli, or contribute to pathogenesis when EVs are loaded with protein or RNA effectors (Liebana-Jordan et al. 2021). Global knowledge on EVs is cumulated in the compendia "ExoCarta" (Keerthikumar et al. 2016), "EVpedia" (Kim et al. 2015), and "Vesiclepedia" (Pathan et al. 2019).

To deliver their cargo outside the cell, fungal EVs need to penetrate the fungal cell wall. However, in yeast, a subset of EVs does not penetrate the cell wall, but these vesicles remain in the periplasm where they are also called periplasmic vesicles (Liebana-Jordan et al. 2021). The cargo that has been mostly studied are the proteins and include those involved in the process of gluconeogenesis, where the biogenesis is regulated by nutrient levels. Glucose starvation induces EV formation and hence the enzymes are sequestered outside the cell. They are re-internalized upon addition of nutrients, and this shuttling adds a level of control in addition to cost-intensive adjustment of steady-state protein levels by synthesis and degradation or glucose-dependent regulation of enzymatic activity (Stein and Chiang 2014). Another subset of periplasmic vesicles contributes to cell wall biogenesis and remodeling as they contain several cell wall remodeling enzymes. Formation of these EVs requires the endosomal sorting complex required for transport (ESCRT) machinery, similar to the mammalian system, but the ESCRT components do not remain associated with EVs during secretion, pointing to a divergent mechanism of release (Zhao et al. 2019). Since *S. cerevisiae* has lost the RNAi machinery (Drinnenberg et al. 2009), the cargo cannot contain Dicer-dependent sRNAs, but it will be interesting to identify the other RNA species such as mRNAs, transfer RNAs, ribosomal RNAs, and RNAi-independent sRNAs in yeast EVs in the future.

To date, the only RNA inventory of EVs in a plant-pathogenic fungus comes from the maize smut fungus, *U. maydis* (Kwon et al. 2021), although EV proteomes have been characterized in other phytopathogens. Therefore, we consider these culturable filaments as a model template for EV research for biotrophic fungi. *U. maydis* EVs were purified using a filtration and ultracentrifugation protocol. They contain a wide range of RNAs (Kwon et al. 2021), but since this smut fungus has lost its RNAi machinery (Laurie et al. 2008), canonical sRNAs cannot be addressed in this system. Several mRNAs are enriched in the EVs and are likely to be full-length, suggesting that they can be translated by the maize ribosome, if delivered into host cells. Many of the EV-associated mRNAs encode for metabolic enzymes, but a few transcripts of effector proteins and virulence factors have also been detected (Kwon et al. 2021). Their delivery into the plant cells and translation remain to be shown, and a functional role of EVs for the infection process needs to be validated. In the future, it will be very interesting to analyze the RNA cargo of EVs in a range of phytopathogens. On the one hand, it is important to investigate EV-mediated transport of sRNAs with known function in cross-kingdom RNAs such as *B. cinerea, Fusarium spp.*, or *Puccinia spp.* (Table 2.1). On the other hand, comparing *U. maydis* EVs to other smut fungi, for instance, the *Brassicaceae* smut fungus *Thecaphora thlaspeos*, which infects the model plant *A. thaliana* (Frantzeskakis et al. 2017) will give insight into the contribution of mRNA and sRNA

effectors to smut infection and show whether EV loading mechanisms are evolutionary conserved or convergent features of the transport system.

Overall, EV research is expanding in the field of fungal–plant interactions, with a focus on vesicle cargo selection, targeting, and release of cargo. In addition, a central question for fungal EVs, but also for plant EVs, is how they can pass through cell walls. Brown et al. (2015) proposed three hypotheses: channels or pores enable passage of EVs, cell wall remodeling by enzymes creates such a passage, or turgor pressure enables penetration. A convincing answer to this question, possibly combining non-mutually exclusive explanations, will in the future help to address persistent doubts about the existence of EVs with a biological function in cell wall-containing organisms. Besides the short distance movement through a cell wall to a neighboring cell, EVs have the potential to travel long distances. To address this question, marker lines that label fungal EVs will be very useful.

2.4 RNAi-Based Applications for Antifungal Disease Control in Plants

2.4.1 Host-Induced Gene Silencing in Fungal–Plant Interactions

Since its discovery, RNAi has been successfully applied as a biotechnology tool. In plant biotechnology, HIGS is a transgenic approach to express dsRNAs in a plant that exclusively targets essential genes in a parasite engineering cross-kingdom RNAi, which were initially reported to control insect pests (Baum et al. 2007). Nowara et al. demonstrated HIGS for the first time against the fungal pathogen, *B. graminis* (Nowara et al. 2010). Since then, HIGS has been universally applied as a powerful tool to confer resistance against a plethora of plant-colonizing organisms including bacteria, fungi, oomycetes, and invertebrates (Nunes and Dean 2012; Koch and Kogel 2014; Koch and Wassenegger 2021).

2.4.2 Spray-Induced Gene Silencing in Fungal–Plant Interactions

HIGS requires plant transformation of a dsRNA transgene and is therefore classified as a genetically-modified organism (GMO)-based tool. Alternatively, RNA spray or spray-induced gene silencing (SIGS) has been established in which dsRNA is topically applied in solution on plant surfaces (Wang and Jin 2017). Organisms that come in contact with such plants might take up sprayed RNAs. SIGS approaches were successful to delay *B. cinerea* gray mold on diverse fruits (Wang et al. 2016; Duanis-Assaf et al. 2022) and *F. graminearum* lesion formation on barley leaves (Koch et al. 2016; Werner et al. 2020). The effectiveness of the RNA spray might depend on the efficiency of RNA uptake by parasites. A recently published survey of fluorescently labeled fluorescein-dsRNA uptake in distinct fungi and oomycetes species showed that some had low or no RNA uptake, such as *Trichoderma virens* and *Colletotrichum gloeosporioides*, respectively, or uptake was restricted to particular cell types or growth stage (Qiao et al. 2021). In a similar experiment, fluorescently labeled Cy3-dsRNA was not internalized by *Z. tritici* cells in culture (Kettles et al. 2019). Also, the silencing effect triggered by external dsRNAs in *F. graminearum* negatively correlates with dsRNA size (Hofle et al. 2020).

Important open research questions need to be solved in future to further establish plant RNAi biotechnologies. Although these methods have been shown to be efficient under laboratory conditions, their applicability under glass house or field conditions with more extreme weather, as well as under other environmental condition must be tested (Rank and Koch 2021). By the nature of RNAi, the possibility to silence homologous genes, a so-called off-target effect, must be systematically evaluated, not only in the host plant but also in other non-pathogenic, plant-associated organisms. In particular, RNA spray attracts lots of attention for its huge market potential as a GMO-free approach with high flexibility and 100% biodegradability (Fletcher et al. 2020). As

RNA therapeutics used to be considered very expensive and not feasible for in-the-field application in the past, RNA mass production seems nowadays manageable for the urgent need of a new generation of RNA fungicides and pesticides in worldwide agriculture. As the next steps for optimization, RNA uptake efficiency, environmental RNA stability and target delivery must be improved by engineering RNA nano-carriers and protection materials (Mitter et al. 2017; Demirer et al. 2020). In addition, basic research on RNA transport during natural cross-kingdom RNAi and RNA-based fungal–plant communication has great potential to provide new knowledge to further improve RNAi biotechnologies so they become a sustainable application in plant protection. By learning from nature, liposome-based RNA delivery and RNA stabilization by formulation of ribonucleoprotein complexes that prevent rapid RNA degradation, could be engineered. Moreover, biosafety aspects must be seriously considered, as well. Any chance to induce plant stress response and immune activation by external RNAs (Lee et al. 2016; Niehl et al. 2016) should be minimized. Also, any environmental influence in nature due to massive RNA application must be carefully monitored.

2.5 Conclusions

RNA communication in fungal–plant interactions is a relatively young research field and many aspects are unexplored. Cross-kingdom RNAi is the best-characterized concept in fungal–plant RNA communication, for which the *B. cinerea*–*A. thaliana* pathosystem has served as an excellent model. Inter-kingdom communication via siRNAs is possible due to the high level of conservation among the core RNAi components DCLs and AGOs, and due to the common silencing mechanism through RNA–RNA base pairing. Since many siRNAs derive from non-conserved genomic regions in fungi, high sequence variability helps to rapidly adapt to new target genes and new host species. Transport of EVs between fungi and plants is the likely route of RNA delivery. Here, the only RNA inventory comes from the model smut fungus *U. maydis*, and knowledge from the plant field mostly comes from the model species *A. thaliana.* In the future, more inventories will be provided from a whole range of pathogen EVs, so that a substantial dataset will support research.

2.5.1 Fungal sRNA Effectors

Nowadays, we understand that cross-kingdom RNAi is a mechanism of communication not only in fungus–plant, but also in oomycete–plant, and bacterium–plant interactions. However, only a handful of sRNA effectors have been functionally characterized, with retrotransposons being a common source of *Botrytis* siRNA effectors. But we do not know how many more of the fungal sRNAs simultaneously silence host genes with impact on plant physiology. Despite the high numbers of sRNA effectors predicted to target host plant genes, there might be some "core" sRNA effectors that are conserved among strains and species of pathogenic fungi and target key genes and pathways in host plants. Such conserved sRNA effectors indeed have been suggested in the oomycete *Peronosporales* pathogens (Dunker et al. 2020) and in the parasitic plant genus *Cuscuta* (Johnson et al. 2019).

To induce plant gene silencing, fungal sRNAs from *Botrytis*, *Verticillium*, and *Fusarium* hijack the plant's own AGO1/AGO2/AGO4-RISCs. Post-transcriptional gene silencing, including cleavage of plant mRNAs induced by *Fusarium* and *Puccinia* milRNA effectors, was evident. Thus, a mRNA degradome analysis (Addo-Quaye et al. 2008) seems to be valuable to combine with classical sRNA-seq and mRNA-seq for systemic and more reliable prediction of fungal sRNA effectors and plant mRNA targets. Interestingly, the finding of a *F. oxysporum* milRNA bound to the tomato AGO4a suggests that transcriptional host gene silencing via the RdDM pathway might be also plausible. Hence, it remains interesting to find out how many fungal sRNAs can load to the plant AGO4/AGO6/

AGO9-RISCs clade and where the fungal sRNAs meet these plant nuclear-localized AGOs.

Besides siRNAs and milRNAs, other types of fungal non-coding and coding RNAs have been found outside the cell and are suggested to contribute to inter-kingdom communication. In particular, full-length mRNAs encoding for virulence factors, as found in extracellular vesicles of *U. maydis*, and these could be transferred into plant cells to outsource translation and to bypass the host immune system located at the plant cell surface. Moreover, tRNA-derived RNA fragments (tRFs) are proposed to have regulatory functions and, indeed, bacterial *Sinorhizobium* tRFs have been found to modulate root nodule symbiosis via cross-kingdom RNAi (Ren et al. 2019).

The study of ckRNAi has led to the identification of RNAs and previously unknown genes that play a role in fungal–plant interactions. In future, we foresee that more types of RNAs, such as mRNAs and tRFs, that function as carriers of regulatory information in fungal–plant communication will be uncovered.

2.5.2 RNA Transport

To fully understand ckRNAi, and in view of RNA applications such as SIGS, it is important to not only describe and characterize the function of sRNAs in the recipient cell, but also to unravel the transport mechanisms in or outside EVs, which to date remain obscure. The proteomes of EVs from phytopathogens contain predicted RBPs (Bleackley et al. 2019; Hill and Solomon 2020), but it is unclear whether they really bind RNAs in EVs, which RNA species they bind, and how they contribute to loading of RNAs into EVs. Recently, the *A. thaliana* RBPs AGO1, RNA helicase 11, and RNA helicase 37 were identified in EVs, and they bind to EV-enriched sRNAs (He et al. 2021), but their molecular function in EV loading remains elusive. Hence, with regard to ckRNAi, the contribution of AGO proteins of both partners remains interesting to be studied. Following transport from the fungus to the plant, sRNAs from *B. cinerea* are functionally integrated into plant AGO1, suggesting that if the fungal AGO protein or any other RBP is involved in mediating transport, it would pass over the sRNA to the plant silencing machinery. Gaining a mechanistic understanding in model phytopathogens will reveal similarities and differences to the mammalian field and bring predictive power for comparative studies between different fungi.

Besides EV biogenesis and cargo selection, every EV needs to be targeted to its destination. It can be expected that surface proteins mediate the targeting. In plants, TET8-, PEN1-positive, and EXPO (exocyst-positive organelles) EVs can be distinguished (Cai et al. 2021), but even here the markers describe rather than provide information on the targeting and the final destination. As a next step, identification of appropriate marker proteins that enable visualization and categorization of EVs in fungal phytopathogens is needed.

While targeted transport to the destination would minimize energy costs and maximize the efficiency, for fungal pathogens an alternative could be untargeted release into the interaction zone. During infection, many fungi form haustoria or intracellular hyphae, where fungal cells are in close proximity to the plant plasma membrane (Doehlemann and Hemetsberger 2013). These interaction zones are the area of massive exchange, so that local formation of EVs might be sufficient for targeting solely based on the high concentrations of EVs and diffusion. If this hypothesis is true, pathogens not forming these structures, such as the tomato leaf mold fungus *Cladosporium fulvum*, would not rely on EVs for infection or use a different mechanism. Lastly, upon delivery into the target cell, the cargo needs to be functionally integrated into the cellular system. This has been proven for fungal sRNAs found in plant AGO1, but how fungal sRNAs reached AGO1 is unclear.

In summary, RNA dialogues are an underestimated means of communication in fungal–plant interactions. EV-mediated transport of RNAs for cross-kingdom communication is a growing research field with three central questions on the biogenesis of EVs, the cargo

selection, and the delivery for functional integration into the host system. Besides addressing these mechanisms, it will also be important to consider quantitative aspects. How many RNAs are transported overall? How many RNAs can be co-transported in the same vesicle? This will allow an evaluation of the relative contributions of RNA effectors at a given time point during infection.

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3 The Role of Tox Effector Proteins in the *Parastagonospora Nodorum*–Wheat Interaction

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Abstract

Parastagonospora nodorum is the causal agent of the glume blotch disease on wheat and is responsible for significant yield losses globally. Despite its importance, the mechanisms underpinning the interaction of *P. nodorum* and wheat were until recently poorly understood. Historically, *P. nodorum* was considered a simple pathogen that secreted a battery of lytic enzymes to penetrate the surface of the leaf and ultimately lyse host cells to access nutrients. Seminal studies though over the last two decades have demonstrated that the disease is far more complex and is dependent on a series of gene-for-gene interactions that dictate the outcome of the interaction. In this book chapter, we review the studies that have generated the current model and discuss the evolutionary origins of the effector genes based and conserved structural features despite low sequence similarity. One emerging hypothesis is that these effectors have dual roles in facilitating disease and rarely interact directly with their cognate susceptibility gene. Together these studies show how study of necrotrophic effectors can advance our knowledge of not just the pathogen but also wheat immune signalling.

Keywords

Parastagonospora nodorum · Effectors · *Tox* genes · Wheat

3.1 Introduction

Parastagonospora nodorum (syn: *Stagonospora nodorum*, *Phaeosphaeria nodorum*) is a necrotrophic fungal pathogen of wheat. The biology of *P. nodorum* has been well described in other reviews and we refer those unfamiliar with this pathogen to these resources (Oliver et al. 2012; Solomon et al. 2006a). In this chapter, we will focus on how the characterisation of effectors from *P. nodorum* has advanced our knowledge of plant immunity. Effectors are broadly defined as small molecules or proteins which are secreted by a pathogen to modulate the immune response of the host and facilitate infection (Toruño et al. 2016). It is well understood that *P. nodorum* uses highly specialised, small, secreted proteins, termed necrotrophic effectors (NEs), to induce cell death in wheat (*Triticum aestivum*) (McDonald and Solomon 2018). The NEs of *P. nodorum* interact in a gene-for-gene manner with wheat susceptibility genes. This is a

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dominant susceptibility whereby the NE protein secreted or infiltrated into a wheat cultivar carrying the susceptibility gene leads to leaf necrosis (Faris and Friesen 2020; Oliver et al. 2012). In most instances, the interaction between the NE and the product of its corresponding susceptibility gene is not a direct protein–protein interaction (with one exception), which indicates that these effectors induce cell death through other signalling partners (Faris and Friesen 2020; McDonald and Solomon 2018). The interaction between NEs and their corresponding susceptibility genes has been conceptualised as Effector-Triggered Susceptibility (ETS) and *P. nodorum*–wheat has been used as the model pathosystem to study this immune pathway in plants (Liu et al. 2009).

3.2 The Main Characters, *Tox* Genes and Their Partners

To date five NEs from *P. nodorum* have been cloned and three of these have been investigated further to identify interacting plant proteins/receptors (Kariyawasam et al. 2022; Liu et al. 2009, 2016; Richards et al. 2022; Friesen et al. 2006). These NEs take on the prefix "Tox", a shortening of the word "toxin", attributed to these proteins because of the necrosis they induce when infiltrated/secreted into wheat leaves. However, it is now clear that these proteins are not generic "toxins", but highly specialised proteins that have multiple roles in facilitating disease. The five genes are numbered in order of their first genetic characterisation (in some cases many years prior to cloning of the gene), except for *ToxA,* which was first found in another species *Pyrenophora tritici-repentis* and only later discovered in *P. nodorum* (Friesen et al. 2006; Ciuffetti et al. 1997). *ToxA* is also the only NE where there is evidence of very recent horizontal gene transfer, now found in three different fungal wheat pathogens (McDonald et al. 2018). The remaining cloned *Tox* genes are *Tox1, Tox267, Tox3* and *Tox5* (Kariyawasam et al. 2022; Liu et al. 2009, 2012; Richards et al. 2022). Two additional *Tox* loci have been genetically characterised, *Tox4* and *Tox2AS,* but, to date, not yet cloned (Abeysekara et al. 2009; Phan et al. 2016). *ToxA, Tox1* and *Tox5* each interact with a single susceptibility gene in wheat. These are *Tsn1, Snn1* and *Snn5,* respectively (Faris et al. 2010; Friesen et al. 2012; Shi et al. 2016b). *Tox3* has two susceptibility genes, *Snn3-B1* and *Snn3-D1,* which are homoeologous genes occurring on the B and D genomes of hexaploid wheat (Shi et al. 2016a; Zhang et al. 2011). *Tox267* targets three separate susceptibility genes, which are not homeologs (Richards et al. 2022). Three of the eight wheat susceptibility genes have now been cloned, *Tsn1, Snn1* and *Snn3-D1*. Among these three susceptibility genes, only *Snn1* interacts directly with its corresponding NE, Tox1 (Faris and Friesen 2020; Zhang et al. 2021). Plant cell death induced by NEs is characterised by a massive release of reactive oxygen species and rapid host cell wall collapse (~72 hpi) (Solomon et al. 2006b). This makes the identification of the molecular receptors that trigger this signalling cascade extremely difficult. However, cloning of the individual genes from both the pathogen and host has enabled studies to take place outside of the chaotic environment of a dying wheat cell. In the most recent studies, yeast-2-hybrid was used to identify wheat interaction proteins for ToxA and Tox3 (Breen et al. 2016; Lu et al. 2014). This was then followed by extensive biochemical studies of the two proteins using heterologous protein expression systems. This work has led to the discovery of several plant immune gene targets that are not involved in the gene-for-gene interaction. These data expand our knowledge of the plant immune system, in particular highlighting potentially novel pathways that pathogens can target to overcome plant immunity. We will discuss these studies in detail below and highlight opportunities for future work.

3.3 Evolution and Diversity of *Tox* Genes in Globally Distributed Populations of *P. nodorum*

Before jumping into the details of the molecular signalling partners, we consider the evolutionary origins of this group of effector proteins.

P. nodorum is one of a handful of wheat pathogens that have populations sampled from across the globe. These populations have been used to examine the genetic diversity of both neutral loci and *Tox* genes to determine which population harbours the most. Genetic diversity is used as a proxy for age, whereby the older a population is the more mutations (diversity) it can accumulate over time. Populations that represent the centre of origin of a species are so identified because they harbour the most genetic diversity, private alleles (alleles only found in a single population) and/or the highest number of shared alleles with other populations. For *P. nodorum* the diversity at neutral genetic loci is highest in populations collected from Iran. These studies indicate that this pathogen originates in the ancient Fertile Crescent where wheat was first domesticated (Ghaderi et al. 2020; McDonald et al. 2013).

These same populations have been used to assess the evolutionary origins of the *Tox* genes, where the same logic is applied. Earlier studies suggested that the diversity of the NEs was highest in populations outside of the pathogen's Centre of Origin (McDonald et al. 2012, 2013). However, a more recent analysis conducted with newly sampled Iranian populations showed that the number of private alleles for *ToxA, Tox3* and *Tox1* is highest in these Iranian populations (Ghaderi et al. 2020). Taken together, these new estimates of diversity indicate that the population that harbours the highest levels of diversity at neutral genetic loci is also the population that harbours the highest allelic diversity of NEs (*ToxA, Tox3* and *Tox1*). An additional comparison of shared alleles between all populations showed that, on a pairwise basis, Iranian populations shared more *ToxA* and *Tox3* haplotypes with other populations than any other single population (Ghaderi et al. 2020). For the more recently characterised *Tox267* and *Tox5* genes no assessment of their diversity in populations outside of the USA has yet been made. However, in the 197 US isolates sequenced thus far, a large number of nucleotide haplotypes were discovered: 32 for *Tox267* and 22 for *Tox5* (Friesen et al. 2012; Richards et al. 2022). In comparison, 20 *ToxA* haplotypes, 22 *Tox1* haplotypes and 13 *Tox3* haplotypes have been described in a global collection of ~1200 isolates (Ghaderi et al. 2020; McDonald et al. 2013). An opportunity exists to combine these different population collections and assessing the diversity of all five *Tox* genes would be one of the most comprehensive datasets on effector gene diversity for any pathogen.

The genetic diversity of each of the NEs should also be assessed in the context of its genomic environment. The genomic location of these NEs is important because some, but not all, are found in regions rich in Transposable Elements (TEs). These TEs are often then targeted by genome defence mechanisms, such as repeat-induced polymorphism (RIP), whereby RIP-generated mutations can "leak" into nearby coding genes (Selker 1990; Van De Wouw et al. 2019). *SnToxA* is found in such a region and some sequence haplotypes are the result of "RIP-like" mutations (Friesen et al. 2006; McDonald et al. 2013; Stukenbrock and McDonald 2007). In determining the Centre of Origin of a gene based on measures of diversity, there is an inherent assumption that different populations of the same pathogen will accumulate mutations at the same rate. However, Richards et al. (2019) have demonstrated that populations of *P. nodorum* in the USA do show signs of strong local adaptation at the three effector loci (Richards et al. 2019). While the current evidence still points to a Centre of Origin in the Middle East, we consider it feasible that this signal may again become mixed as more modern isolate collections are included.

Long-read genome assemblies give us the first complete insights into the size of the presence/absence polymorphisms, which reveals quite stark differences between the three best characterised effectors. The deletion polymorphism for *ToxA* is >200 kb, whereas the deletions for *Tox3* and *Tox1* are much smaller at 7 kb and 3 kb, respectively (McDonald et al. 2019). There is some evidence that the frequency of each NE's presence in a given population is correlated with the presence of the corresponding susceptibility gene (Richards et al. 2019). However, in Australia and China, Oliver et al. (2009) found

no correlation between the proportion of isolates carrying *ToxA* and the proportion of wheat cultivars grown in the country that carry the susceptibility allele *Tsn1* (Oliver et al. 2009). In a global survey, the three known effectors at the time, *ToxA, Tox3* and *Tox1,* were found to be segregating as expected based on their allelic frequency within the population (McDonald et al. 2013). In order to assess whether the presence of the wheat sensitivity gene does drive an increase in the proportion of individuals carrying the corresponding NE, multi-year field experiments are required.

P. nodorum belongs to a species complex consisting of over 10 species, which was recently characterised using whole genome data and morphology (Croll et al. 2021). Of these, only *P. nodorum* and *P. pseudonodorum* are found infecting wheat outside of Iran. These are also the only two species that have the *Tox* NEs (only assessed for *ToxA,1,3*) (McDonald et al. 2013; Hafez et al. 2020). This indicates that the evolution of the NEs is an important part of these species ability to cause disease on wheat. The original finding of a *Tox* gene in *P. pseudonodorum* suggested that these genes may have been transferred to this species through a hybridisation event. However, more recent whole genome sequencing did not support this hypothesis and the alternative that the genes were shared between the two species via an ancient introgression event was proposed (Croll et al. 2021). Careful examination of the regions of the genome surrounding the NEs in *P. pseudonodorum* would give more insight into rare recombination events that may have led to these effectors being incorporated into the genome.

3.4 Mechanistic Insights from Detailed Studies of Effector Proteins

3.4.1 Target Wheat Immune Proteins

Each of the *P. nodorum* necrotrophic effector proteins described above induces host cell death in the presence of their cognate susceptibility gene. Significant advances over recent years have provided not only an insight as to the molecular basis of cell death, but also discovered that the majority of these effectors also harbour secondary roles during infection (see Fig. 3.1 for a representation of potential *P. nodorum* effector roles during infection).

The first effector identified in *P. nodorum* was SnToxA. As described above, *SnToxA* has been the subject of a horizontal gene transfer event in three closely related fungi (McDonald et al. 2019). Despite this movement and its demonstrable impact on wheat yields, how SnToxA induces cell death on *Tsn1* wheat lines remains poorly understood. SnToxA is a 13.2 kDa secreted protein that harbours a conserved Arg-Gly-Asp (RGD) motif that is required for host cell death (Ciuffetti et al. 1997; Meinhardt et al. 2002). Initial protein interaction studies indicated that ToxA interacted with plastocyanin, ToxABP1 and a Pathogenesis-Related protein, PR1-5, during infection (Tai et al. 2007; Manning et al. 2007; Lu et al. 2014). Of these, there has been no subsequent characterisation on the interaction of SnToxA with plastocyanin whilst ToxABP1 appears to have a role in necrosis (Manning et al. 2010). Mutagenesis studies were used to show that ToxA interacted with the pathogenicity-related protein PR1-5 to potentially mediate SnToxA-induced necrosis in *Tsn1* wheat cultivars. More recently, yeast-2-hybrid screening demonstrated that ToxA also interacts with the integrin-like protein TaNHL10 during infection. Complementary co-immunoprecipitation and confocal approaches were used to show that SnToxA interacted with the C-terminus of TaNHL10 in the apoplast of the plant (Dagvadorj et al. 2022). Mutagenesis of the SnToxA protein proved that mutants unable to interact with TaNHL10 did not induce Tsn1-mediated necrosis implying that this interaction is required for ToxA-induced cell death (Dagvadorj et al. 2022).

SnTox3 induces cell death on wheat lines that are dominant for the *Snn3* susceptibility gene (Liu et al. 2009). SnTox3 encodes a secreted 25.3 kDa pro-protein which is subsequently processed by a Kex protease to generate a mature and active

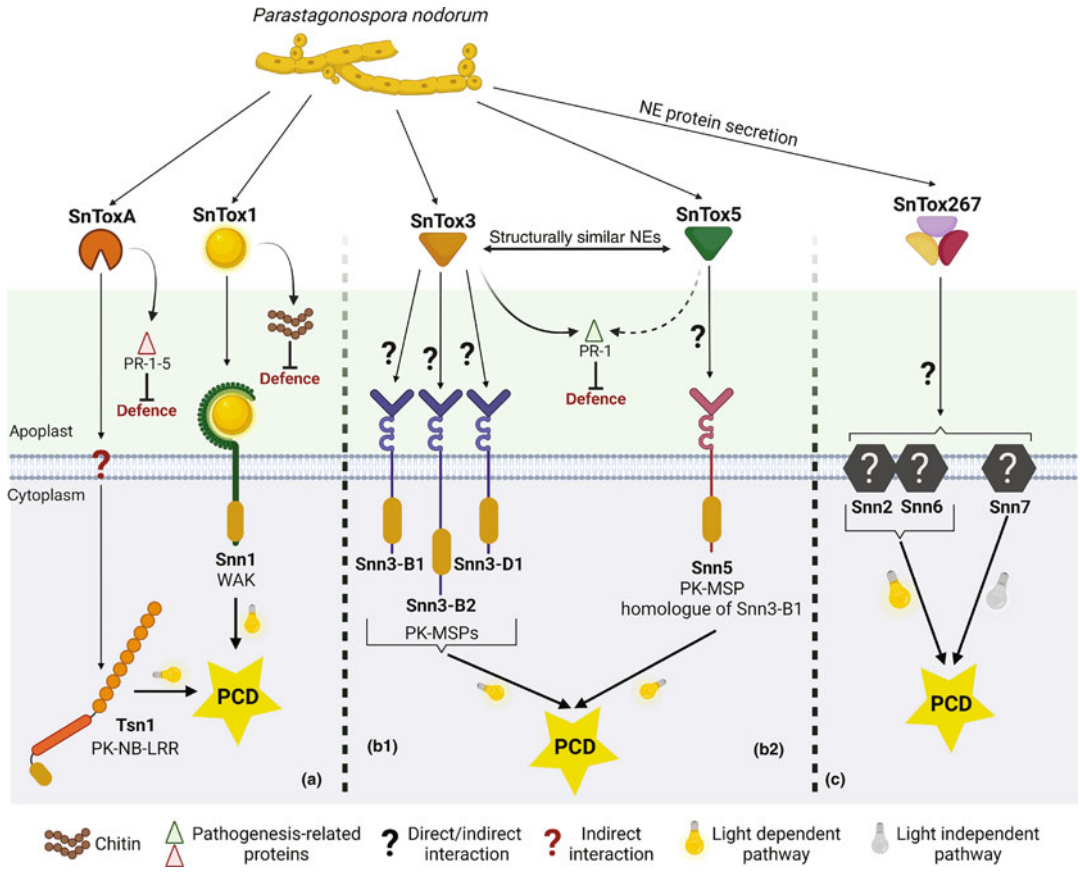

Fig. 3.1 Schematic overview of the various known interactions involving *Parastagonospora nodorum* necrotrophic effector (NE) proteins. (**a**) Different NEs could target different classes of the wheat sensitivity (S) genes; (**b1**) the same NE could target homoeologous and/or paralogous S genes; (**b2**) structurally similar NEs could target homologous S genes; and (**c**) the same NE could target different individual or pairs of S genes that operate in different light-dependent or light-independent pathways leading to programmed cell death (PCD). In addition to targeting specific S genes, either directly like SnTox1 or indirectly like SnToxA, many NEs are also engaged in other interactions often resulting in suppression of host defence particularly during the early stages of plant colonisation. S genes encode receptor-like proteins that belong to different classes, including PK-NB-LRR (protein kinase, nucleotide binding site and leucine-rich domains), WAK (wall-associated kinase) and PK-MSP (protein kinase-major sperm domain protein). A curved dotted arrow indicates the hypothesised interaction (reproduced from Kanyuka et al. 2022)

18 kDa protein (Liu et al. 2009; Outram et al. 2021b). Mechanistic studies on the SnTox3 effector protein have revealed that it directly interacts with PR1 proteins during infection. These interactions were observed with acidic and basic PR1 proteins but not those that harbour a C-terminal extension. Mutagenesis approaches were used to dissect this interaction and a SnTox3 site-directed mutant was generated that was unable to interact with PR1. Heterologous expression of the mutant SnTox3 confirmed that it was unaffected in its ability to induce necrosis on *Snn3* wheat cultivars demonstrating that the interaction with PR1 was not required for necrosis (Breen et al. 2016).

These data raised the question that if this interaction was not required for necrosis, then what was its function? However, to accurately dissect this interaction, an understanding was required as to what the actual role of PR1 proteins was during

disease. To address this, Sung et al. (2021) overcame the genetic intractability of wheat by employing a protein-mediated phenotyping assay whereby purified PR1 proteins were infiltrated into wheat leaves prior to inoculation with *P. nodorum* (Sung et al. 2021). PR1-infiltrated leaves showed significantly less disease implying that PR1 proteins played a role in defence. Gene expression analysis of leaves infiltrated with PR1 proteins demonstrated a strong up-regulation in plant defence genes implying that PR1 proteins played a role in regulating host defence. Importantly, Sung et al. (2021) proved that PR1 proteins were not antimicrobial based on growth assays with different fungi and oomycetes in the presence of varying concentrations of purified PR1 proteins. Co-infiltration of PR1 with SnTox3 prevented the up-regulation of defence genes providing evidence that SnTox3 promoted disease through the repression of PR1-mediated defence signalling. Subsequent studies by Sung et al. (2021) confirmed that SnTox3 directly interacted with PR1 to prevent the release of the CAPE1 peptide from the latter which has been demonstrated to induce plant defence. Importantly, these data demonstrate conclusively that the SnTox3 effector harbours a dual role in inducing cell death and also host defence repression (Sung et al. 2021).

SnTox1 encodes a small secreted protein (10.3 kDa mature protein) that triggers cell death in the presence of the *Snn1* susceptibility gene (Liu et al. 2012). SnTox1 is relatively unique compared to the other *P. nodorum* effectors identified to date in that it harbours 16 cysteine residues yielding a protein rich in disulfide bonds. Interestingly, and unlike the other *P. nodorum* effectors characterised to date, Tox1 can induce cell death by spraying the protein onto the surface of the leaf rather than infiltration into the apoplast (Liu et al. 2016). Localisation studies confirmed that SnTox1 was localised to the outside of epidermal cells up to 72 h post-infection leading to the suggestion that the target of Tox1 is outside of the cell. SnTox1 was postulated to directly interact with the ectodomain of the wall-associated kinase Snn1 in the apoplast of wheat leaves leading to DNA laddering and an oxidative burst that is suspected of being required for penetration of the leaf. Subsequent studies on Snn1 confirmed its identity as a wall-associated kinase supporting the theory that Tox1 does function outside of the cell, possible through a direct interaction with Snn1 (Shi et al. 2016b).

SnTox1 is also reported to have a role in disease independent of inducing cell death (Liu et al. 2016). Initial characterisation studies identified that SnTox1 harbours a C-terminal chitin-binding motif (Liu et al. 2012). Subsequent chitin-binding experiments confirmed that SnTox1 was able to bind to chitin but not chitosan. Growth experiments of different fungi in the presence of specific wheat chitinases confirmed that SnTox1 provided protection against these enzymes as evidenced by the increased growth in the presence of the SnTox1 protein. Localisation studies confirmed that SnTox1 binds to the fungal cell wall therefore prompting the suggestion that the effector provides protection to the pathogen from plant chitinases upregulated as part of the host defence response (Liu et al. 2012, 2016).

More recently, two new effectors were identified which were causal to host cell death in the presence of cognate susceptibility genes. *SnTox5* was identified through a genome-wide association study (GWAS) involving the analysis of 197 isolates of *P. nodorum* (Kariyawasam et al. 2022). The *SnTox5* gene was predicted to encode a secreted protein of 217 amino acids with a 22-amino acid signal peptide and a 49-amino acid pro-domain that was purported to be cleaved by the Kex2 protease. Infiltration of the heterologously produced SnTox5 protein into *Snn5* wheat cultivars resulted in strong cell death and mutants of *P. nodorum* generated that lacked *Tox5* failed to cause necrosis on *Snn5* lines (Kariyawasam et al. 2022). These data were confirmed by transforming the avirulent *P. nodorum* Sn79–1087 strain with *SnTox5* and through demonstrating necrosis on *Snn5* cultivars. However, it is the shared sequence and structural similarity of SnTox5 to the SnTox3 effector which was most interesting. SnTox5 shared 45.1% sequence identity to the mature SnTox3 protein whilst the predicted structure of SnTox5 appeared

almost identical to SnTox3 suggesting a shared intracellular target. Whilst complementation assays (*SnTox5* in a *SnTox3* mutant background) have yet to be undertaken, there is no evidence of cross-specificity (i.e. SnTox3 interacting with *Snn5* and vice versa) as demonstrated through a quantitative trait loci (QTL) analysis using the purified effectors. Functional analysis of SnTox5 was undertaken by infecting wheat leaves with GFP-labelled *P. nodorum* strains. Mutant strains of *P. nodorum* harbouring a disrupted *SnTox5* gene were compromised in their ability to penetrate the leaf surface suggesting that the effector has a role in penetration. Subsequent analysis of disease progression revealed that *P. nodorum* mutants lacking *SnTox5* were unable to colonise the mesophyll or vascular tissue implying that *SnTox5* plays a key role in facilitating pathogen growth *in planta* (Kariyawasam et al. 2022). Of particular interest was that the role of *SnTox5* in promoting growth *in planta* was observed in *snn5* wheat cultivars implying that this role in disease is independent of its interaction with its cognate dominant susceptibility gene.

Another interesting effector recently identified in *P. nodorum* is SnTox267 (Richards et al. 2022). GWAS analysis was initially used to identify the gene *CJJ_13380* and confirm its association with the virulence of *P. nodorum* on the differential wheat lines BG223 (*Snn2*) and ITMI37 (*Snn6*). *CJJ_13380* is predicted to encode an effector protein of 27.4 kDa with no sequence similarity to other known proteins. However, despite this lack of sequence similarity, CJJ_13880 was predicted to harbour a high degree of structural similarity to the SnTox3 crystal structure (Richards et al. 2022; Outram et al. 2021b). Subsequent functional dissection of *CJJ_13380* by a reverse genetics approach confirmed its association with disease in the presence of *Snn2* as demonstrated by the reduced disease symptoms of the mutants on the *Snn2* differential wheat line BG296. These data were further supported by transforming *CJJ_13380* into the avirulent *P. nodorum* isolate 79–1087 and observing a restoration of disease on BG296. Pathogenicity assays using the same mutants on ITMI37 confirmed the role of CJJ_13380 in *Snn6*-mediated disease (Richards et al. 2022). Previous studies had identified that an effector protein of comparable size to CJJ_13380 was responsible for inducing cell death and disease on wheat lines. Subsequent pathogenicity assays with *P. nodorum* 79–10,876 isolates transformed with *CJJ_13380* on *Snn7* differential wheat lines confirmed that along with *Snn2* and *Snn6*, CJJ_13380 also targeted Snn7 to facilitate disease (Richards et al. 2022; Shi et al. 2015). Consequently, *CJJ_13380* was named *SnTox267*.

Genetic approaches were then undertaken to dissect the pathways associated with each of *Snn2*, *Snn6* and *Snn7* in facilitating *SnTox267*-mediated disease. Previous studies had demonstrated that *Snn2-SnTox267* and *Snn6-SnTox267* were light-dependent whilst *Snn7-SnTox267* was light-independent (Shi et al. 2015; Gao et al. 2015). Thus, it was hypothesised that *Snn2* and *Snn6* may function in the same pathway. Subsequent infiltrations of active SnTox267 in culture filtrates into the progeny of crossing sensitive and insensitive lines confirmed that *Snn2* and *Snn6* are both dominant genes that are required for necrosis induced by *SnTox267*. Although not discussed, given that the *Snn7-SnTox267* reaction is light-independent it was assumed that it was independent of *Snn2* and *Snn6*-mediated necrosis.

3.5 Crystal Structures Provide Insights into Conserved Effector Folds and Processing

Like many fungal effectors, the protein sequences of the Tox effectors provide few clues in terms of structure or function, as most lack similarity with protein domains and motifs of known function. The exception being Tox1, which has a verified chitin-binding motif (Liu et al. 2012, 2016). Structural biology and protein biochemistry studies provide an avenue to decipher and characterise effector function. To date, the structures of ToxA and SnTox3 have been solved experimentally using X-ray crystallography.

The ToxA structure was solved by Sarma and colleagues in 2005, and represents one of the first

described protein structures of a fungal effector (Sarma et al. 2005). The structure comprised a single domain protein with a secondary structure dominated by β-strands. The overall β-sandwich fold of ToxA revealed strong structural similarity with the fibronectin type III (FnIII) domain. It also showed that ToxA contained a surface-exposed RGD motif that occupied the same position on the FnIII domain. In animals, RGD motifs are known to mediate interactions with integrin proteins to facilitate endocytosis (Castel et al. 2001). The authors speculated that ToxA may utilise this motif to enter plant cells and it was subsequently demonstrated that a triple alanine substitution at this position prevented ToxA wheat cell internalisation (Manning et al. 2008). To date, the molecular mechanisms that underpin ToxA internalisation remain unknown. Recently, with the discovery that ToxA interacts with the wheat cell-surface receptor NHL10, the RGD motif was targeted for mutagenesis; however, this failed to disrupt the ToxA–NHL10 interaction (Dagvadorj et al. 2022).

Since the description of the ToxA structure, a similar β-sandwich fold has been observed in the protein structures of recognised effectors AvrL567 from flax rust (*Melampsora lini*) and Avr2 from tomato fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*), despite the proteins sharing very low (<20%) protein sequence identity (Di et al. 2017; Wang et al. 2007). This so-called ToxA-like structural family represents one of five conserved effector folds, grouping sequence-unrelated fungal effectors into structural classes (Yu et al. 2021b; Lazar et al. 2021; Praz et al. 2017; Spanu 2017). The conserved fold of ToxA, AvrL567 and Avr2 do not appear to impart conserved function(s), and the conserved structure most likely provides a useful scaffold for their independent activities and functions.

One complication with structural studies of fungal effectors is the production of sufficient quantities of protein required for structural studies. Crystals used to derive the ToxA structure were generated from protein purified from culture filtrates of *Pyrenophora tritici-repentis,* which produces an identical ToxA protein to that secreted by *P. nodorum* (Sarma et al. 2005). Other identified Tox effectors accumulate in culture filtrates of *P. nodorum*, and in some cases heterologous expression has been developed in the methylotrophic yeast, *Pichia pastoris*, however, yields are not sufficient for structure studies (Liu et al. 2009, 2012; Kariyawasam et al. 2022; See et al. 2019). To overcome production issues with Tox3 and Tox1, Zhang and colleagues made use of a modified version of *Escherichia coli*, known as SHuffle, which facilitates the production of correctly-folded disulfide-bonded proteins in the cytosol (Zhang et al. 2017). Recently, this approach was advanced further by co-expression of disulfide-bonded effector with proteins known to facilitate disulfide bonding in eukaryotes (Yu et al. 2021a). For Tox3, the use of a modified prokaryotic expression system produced the yields necessary for structural determination (Outram et al. 2021b).

During the crystallisation and X-ray structure determination of Tox3, Outram and colleagues confirmed that Tox3 was a pro-domain-containing effector protein and showed that in vitro the N-terminal pro-domain could be cleaved by Kex2. SnTox3 was subsequently defined as a Kex2-processed pro-domain (K2PP) effector. The removal of the pro-domain was required for crystallisation and structural determination (Outram et al. 2021b). Interestingly, pro-domain removal increased the potency of the recombinant protein in activating Snn3-dependent cell death in wheat (Outram et al. 2021b). Outram and colleagues subsequently demonstrated that K2PP effectors are likely wide-spread in pathogenic fungi, and include SnToxA, SnTox5 and potentially SnTox1 (Outram et al. 2021a, b).

The Tox3 structure revealed a β-barrel fold, stabilised by three disulfide bonds. The structure is novel among fungal effectors proteins with experimentally determined structures (Outram et al. 2021b). Tox3 does share structural similarity with the cap domains of bacterial pore-forming toxins, however the similarity is linked to the cap domain only. Tox3 did not interact with lipids and it seems unlikely that the protein would disrupt lipid membranes. While the structure of Tox3 did not provide strong functional clues, it

was used to identify regions of the protein required for Snn3 recognition. In addition, residues shown to be important for recognition by Snn3 did not impact the Tox3–PR1 interaction, further supporting the dual functional role that Tox3 plays during infection of wheat (Liu et al. 2009; Outram et al. 2021b).

The structure of Tox1, Tox5 and Tox267 is yet to be determined; however, based on sequence similarities between Tox5 and Tox3 proteins, these structures are likely to be very similar (Kariyawasam et al. 2022). Interestingly, homology modelling of Tox267 revealed that its C-terminal domain also shares structural similarity with Tox3 (Richards et al. 2022). These observations could indicate that the sensitivity proteins that recognise Tox3, Tox5 and Tox267 are also related. To date, the only cloned representative of these is *Snn3*, which encodes a Major Sperm Protein (MSP)-Kinase (Zhang et al. 2021). It will also be interesting to determine if Tox5 and Tox267 also target PR1 proteins.

3.6 Overall Conclusions and Outlook

Taken together these studies demonstrate how quickly the identification of effectors in *P. nodorum* and their contribution towards disease has advanced. It was only in 2006 that the first effector (SnToxA) was identified in *P. nodorum*. Fifteen years later, we now have multiple effectors (and several susceptibility genes) identified and have a basic understanding on how these induce necrosis. Importantly, we also now understand that many of these effectors also harbour roles independent to inducing necrosis, and that these roles are significant in terms of facilitating disease. Whilst once being considered a simplistic pathogen that released a battery of lytic enzymes during infection, we now understand that *P. nodorum* secretes an arsenal of effectors that not only induce necrosis in a gene-for-gene manner, but also operate to subvert plant defence during disease.

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Part II

Mutualistic Fungus–Plant Interactions

Genomes of Arbuscular Mycorrhizal Fungi

4

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Abstract

Arbuscular mycorrhizal fungi (AMF) of the Glomeromycotina subphylum are one of the oldest fungal lineages for which the mechanistic underpinning of genetic diversity is unknown. They are present in all terrestrial ecosystems and interact with the majority of land plants, significantly impacting global nutrient cycling. The study of genomes of AMF is of fundamental importance for understanding their evolutionary history and the molecular bases of symbiosis. Here we summarize the current knowledge of AMF genome organization, regulation, and transmission. We discuss the implications of recent findings in our understanding of AMF biodiversity, adaptation, and evolution.

Keywords

AMF · Genomics; regulation · Evolution · Symbiosis

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4.1 Introduction

Fossil evidence and molecular phylogenies suggest that mycorrhizal symbioses were established with the earliest plants to colonize the earth land surface >450 Mya, initiating long-term co-evolution (Morris et al. 2018; Rich et al. 2021; Selosse and Le Tacon 1998; Strullu-Derrien et al. 2018). While plants and other fungal groups have experienced extinction, radiation events, and diversification, fungi from the Glomeromycotina appear to have poorly diversified morphologically (Kruger et al. 2012). Yet, their ecological success is undeniable since today arbuscular mycorrhizal fungi (AMF) are present on all continents, in environments ranging from tropical forests to Antarctica. Despite their ecological importance (Smith and Read 2008), the molecular mechanisms underlying AMF adaptation and evolution are still elusive. This is partly because AMF defy many of the cellular and molecular approaches that are possible for other eukaryotes.

The definition of the cell in AMF is peculiar. Hyphae form a non-septate mycelium (syncytium) where nuclei and cytoplasmic contents flow bidirectionally. AMF spores contain hundreds of nuclei, and the transition from one 'generation' to the next involves carrying over of multiple nuclear genomes (Ehinger et al. 2012; Jany and Pawlowska 2010; Marleau et al. 2011). The concept of the individual is also blurry: compatible mycelial networks can fuse (a process called anastomosis) (Bago et al. 1999; Cardenas-

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Flores et al. 2011; Purin and Morton 2013; Sbrana et al. 2018), allowing for horizontal exchange of genetic material which can give rise to a network bearing more than one nucleotype (heterokaryon) (Croll et al. 2009; Hijri and Sanders 2005; Ropars et al. 2016; Serghi et al. 2021). At least three AMF species can anastomose (*Rhizophagus irregularis*, *Rhizophagus clarus*, and *Funneliformis mosseae*) although heterokaryosis has been reported so far only in *R. irregularis*. It is unclear whether these processes occur in other fungi of the Glomeromycotina. As obligate biotrophs, AMF are not amenable to genetic transformation approaches that require cultivation outside the host. The AMF lifecycle is dependent on interaction with plants and the provision of fatty acids (Bravo et al. 2017; Jiang et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017). Axenic culture is therefore limited to in vitro spore germination experiments (Dallaire et al. 2021; Kamel et al. 2017; Nadal et al. 2017), which can be extended up to a single next generation of spores by the addition of specific fatty acids and plant-derived hormones (Kameoka et al. 2019; Sugiura et al. 2020). AMF can be grown in co-culture with transformed host hairy roots, a system that is useful for symbiosis-related research, but which likely does not fully recapitulate the metabolic and developmental complexity of symbiotic relationships occurring in nature.

These features make AMF a unique biological mystery, with the unfortunate drawback of being intractable to molecular genetics approaches. Transcriptomic, proteomic, and metabolomic analyses have therefore proven most useful to reveal molecular components involved in AMF biology. The recent accessibility of genome sequencing technologies that can resolve complex repeats and haplotype heterozygosity promises to enable genome-scale analyses of the Glomeromycotina phylum and accelerate our understanding of AMF genetic diversity and evolution. Comparative and population genomics are therefore the next frontier to bridge molecular biology and evolutionary genetics of AMF. In this chapter, we discuss key observations of genome organization, regulation, and transmission in AMF and identify gaps in our understanding of symbiotic genome evolution and lifestyles (Fig. 4.1).

4.2 Organization of the Genome

To date, few AMF species have been isolated and sustainably cultivated. While over 200 AMF species have been defined so far (Davison et al. 2015; Öpik and Davison 2016), full genome sequences are available for only nine AMF species—*Diversispora epigaea*, *R. irregularis*, *R. clarus*, *R. cerebriforme*, *R. diaphanus*, *R. proliferus*, *Gigaspora margarita*, *Gigaspora rosea*, and *Geosiphon pyriformis* (Chen et al. 2018b; Kobayashi et al. 2018; Lin et al. 2014; Malar et al. 2021; Morin et al. 2019; Prasad Singh et al. 2019; Sun et al. 2019; Tisserant et al. 2013; Venice et al. 2020). AMF genomes have been difficult to assemble because of their high content of repetitive sequences. Thanks to long-read sequencing and chromatin proximity-guided scaffolding, this problem is now solved and five strains of the model species *R. irregularis* have been assembled to chromosome-scale (Yildirir et al. 2022). Given their widespread distribution across the globe, considerable genetic diversity remains to be explored, and the community will greatly benefit from large-scale isolation and (re)-sequencing projects.

Fungi from the Glomeromycotina have some of the largest genome sizes in the fungal kingdom (Stajich 2017). The model AMF, *R. irregularis*, has telomeres with the classical (TTAGGG)n sequence (Yildirir et al. 2022), but centromeric structures are still undefined (Friedman and Freitag 2017). In the early-diverging fungal phyla Chytridiomycota and Mucoromycota, the coding space is characterized by the retention of ancestral introns and high intron densities (Lim et al. 2021). Intron retention is proposed to correlate with reduced rates of evolutionary change, whereas fast-evolving lineages experienced more intron loss (Lim et al. 2021). Supporting the

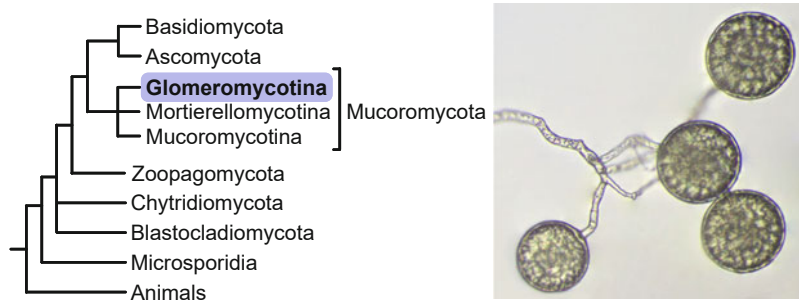

Fig. 4.1 The Glomeromycotina, early-diverging coenocytic (aseptate) fungi

notion that Glomeromycotina fungi are slow evolving, recent analyses of small subunit rRNA gene diversity showed that their speciation rates are one order of magnitude lower than those of other eukaryotes (Perez-Lamarque et al. 2022).

Comparative analyses revealed that AMF have lost genes required for plant cell wall degradation, thiamine biosynthesis, secondary metabolism, and lipid production (Lin et al. 2014; Tisserant et al. 2013; Wewer et al. 2014). These results proved to be physiologically relevant, since it was later discovered that the obligate biotrophy of AMF relies on the provision of lipids by plants (Bravo et al. 2017; Jiang et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017). AMF genomes also contain gene family expansions with predicted protein functions in signalling, protein–protein interaction, and RNA interference (RNAi) (Chen et al. 2018b; Maeda et al. 2018; Morin et al. 2019; Tisserant et al. 2013).

Evidence of within-strain phenotypic and genetic diversity has been accumulating for decades (Angelard and Sanders 2011; Angelard et al. 2014; Boon et al. 2010; Corradi et al. 2007; Corradi and Sanders 2006; Croll et al. 2009; Koch et al. 2004; Mathieu et al. 2018; Ropars et al. 2016; Savary et al. 2018; Wyss et al. 2016). Recently, methods of prokaryotic pangenome analysis have been applied to *R. irregularis* and other fungi to investigate the functional outcome of intraspecific genetic variation. The pangenome is the distinction between a set of "core" genes found among all strains or individuals of a species, and a set of "accessory" genes, found in a subset of, but not all strains of a species. Accessory genes might encode functions that are not essential, but which confer selective advantages, such as adaptation to different niches, resistance to pathogens, or host range expansion (Medini et al. 2005). Knowledge of the content and the dynamics of a pangenome informs on the selective pressures experienced by a population. Evidence for pangenomic structure was found in five species of fungi. The accessory gene sets of the non-pathogenic fungi *Saccharomyces cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* species range between 10% and 20% (McCarthy and Fitzpatrick 2019), while the major wheat pathogen *Zymoseptoria tritici* contains up to 40% accessory genes (Bergstrom et al. 2014; Dunn et al. 2012; Peter et al. 2018; Plissonneau et al. 2018; Song et al. 2015). *In Z. tritici and Fusarium species*, entire chromosomes have been deemed accessory because they show extensive presence/absence variation and rearrangements among strains, and encode accessory virulence factors (Ma et al. 2010; Moller et al. 2018; Plissonneau et al. 2018). A pangenomic analysis of five *R. irregularis* strains revealed up to 50% of accessory coding sequence (Chen et al. 2018b). These accessory genes are part of large family expansions with predicted functions in signalling and signal transduction, and small secreted proteins (Chen et al. 2018b). Since their expression tends to be induced *in planta*, these genes were proposed to play adaptive roles in communication and exchange with host plants (Chen et al. 2018b; Mathieu et al. 2018; Reinhardt et al. 2021). Contrary to *Z. tritici*, the number of *R. irregularis* chromosomes appears stable in strains studied so far (Yildirir et al. 2022), lessening the possibility of extreme genome instability in AMF. Nevertheless, chromosomal

rearrangements have been observed in high-quality long-read-based assemblies (Yildirir et al. 2022), and further analyses of strain-specific structural variation may explain presence/absence variation of accessory genes.

In *R. irregularis*, core and accessory genes have different distributions relative to repetitive elements: accessory genes tend to be sparsely distributed and located next to specific transposable elements (TEs), while core genes form denser clusters in less repetitive regions (Dallaire et al. 2021; Yildirir et al. 2022). In filamentous/eukaryotic plant pathogens, virulence effectors are sometimes embedded into TE-rich regions, and this effectively increases the likelihood of their sequences being reshuffled (Dong et al. 2015; Faino et al. 2016). The increased mutation rate observed in TE-rich regions was therefore proposed to support an evolutionary arms race with correspondingly highly variable immune associated gene sets in the host plant. In AMF, the molecular rate of evolution of TE-linked accessory genes remains to be investigated and may reflect the selective pressures associated with an obligate mutualist lifestyle.

4.3 Regulation of the Genome(s)

Eukaryotic genomic DNA is folded and compacted to form chromatin, which regulates gene expression, as well as DNA replication and repair. Chromatin is divided into euchromatin and heterochromatin, where euchromatin is generally gene-rich, permissive to transcription, and localized towards the periphery of the nucleolus, whereas heterochromatin is typically gene-poor, repeat-rich, and refractory to the transcription machinery. Historically, TEs were the first genetic elements shown to be enriched in heterochromatin (McClintock 1951), a location that restrains their activity and maintains genomic stability. Since then, the presence or absence of specific histone modifications, DNA methylation, pericentromeric regions, and targeting by small RNAs have also been used to distinguish heterochromatin from euchromatin in various organisms, including fungi (Tamaru 2010). In the model AMF *R. irregularis*, DNA folding was assessed using chromosome conformation capture (Hi-C), which revealed a checkerboard pattern consistent with intra- and inter-chromosomal contacts (Yildirir et al. 2022). Two compartments named A and B were detected, which reflect the folding of the chromosomes into euchromatin and heterochromatin, respectively.

Consistent with the fact that the location of DNA sequences within chromatin coincides with particular transcriptional states, genes and TEs of *R. irregularis* were shown to be differentially partitioned into A and B compartments (Dallaire et al. 2021; Yildirir et al. 2022). The euchromatic A compartment preferentially comprises genes with higher expression levels, lower DNA methylation frequencies, and fewer repeats. Strikingly, accessory genes and genes up-regulated during symbiosis were found to be enriched in heterochromatic B compartments, suggesting that their repression by epigenetic mechanisms and chromosome topology might be environmentally-responsive, even perhaps host-responsive. Although the formation of heterochromatin is essential for regulating gene expression and silencing mobile genetic elements, some heterochromatic regions can retain the potential to switch into a euchromatic state following certain cues. Genes that are regulated developmentally or in a cell-type-specific manner are often found in such facultative heterochromatin (Trojer and Reinberg 2007). The regulation of facultative heterochromatin in AMF may involve histone modification, DNA methylation, and small and long non-coding RNAs that modulate boundary formation between different chromatin domains (Cohen and Jia 2014; Freitag et al. 2004; Klocko et al. 2016; Saksouk et al. 2015; Smith et al. 2011).

AMF genomes encode DNA cytosine methyltransferases and an expanded repertoire of RNAi pathway genes (Dallaire et al. 2021; Silvestri et al. 2019, 2020; Tisserant et al. 2013). Whole-genome epigenomic profiling of *R. irregularis* provided direct evidence of 5-methylcytosine (5mC) DNA methylation and

small RNA production which occurs mostly at TE loci, suggesting ongoing epigenetic regulation (Dallaire et al. 2021; Silvestri et al. 2019; Yildirir et al. 2022). In the AMF *G. margarita*, most small RNA-generating loci are intergenic and show similarity to fungal repetitive elements (Silvestri et al. 2020), indicating a conserved contribution of RNAi in suppressing TE activity. In both *G. margarita* and *R. irregularis*, few genes were found to be highly methylated or to be targeted by small RNAs (Dallaire et al. 2021; Silvestri et al. 2020), and in *R. irregularis* these genes did not encode proteins with a clear enrichment for specific domains or functions (Dallaire et al. 2021). The biological relevance of DNA methylation and RNAi in regulating AMF gene expression is therefore still elusive. DNA N6-methyldeoxyadenine (6 mA), a modification with very low abundance and an unclear role in eukaryotes (Zhao et al. 2020), was also investigated as a potential epigenetic mark in *R. irregularis* and other early-diverging fungi and Dikarya (Chaturvedi et al. 2021; Mondo et al. 2017). In *R. irregularis*, the presence of 6 mA on DNA is associated with a subset of transcriptionally active genes with predicted functions in phosphate regulation (Chaturvedi et al. 2021). It is important to consider that in mammalian cells, genomic m6A was shown to originate from the misincorporation of ribo-N6-methyldeoxyadenine from degraded, modified RNA (Musheev et al. 2020). So far, none of the proposed m6A methyltransferase enzymes have been biochemically shown to act as a DNA methyltransferase. Therefore, the detection of genomic m6A on specific sets of genes may be a consequence of their active transcription, which can cause DNA damage and repair with low-level misincorporation of modified nucleotides (Sebastian and Oberdoerffer 2017). While the presence of 6 mA on DNA may have biological consequences, its causative regulatory effect should be carefully interpreted.

In AMF, genome expression and regulation need to be considered in the context of a multinucleate, syncytial state, and occasional heterokaryosis. Throughout the different life stages ranging from spore germination and hyphal growth, to symbiotically engaged mycelial networks, AMF nuclei experience different environments and stresses. Nuclei that are localized at the root interface would likely transcribe different gene sets than those at a foraging hyphal growth tip. However, since AMF nuclei travel freely and coexist in one large cytoplasm, it is puzzling to imagine how gene expression is effectively regulated in space and time. To compartmentalize functions, transcriptionally regulated responses of fungi to either their environment or their host likely need to be coupled with transport of messenger ribonucleoprotein complexes, localized translation, post-transcriptional and post-translational regulation. In filamentous fungi, the subcellular compartmentalization of functions depends on microtubule-dependent mRNP transport for the fast polar growth of hyphae (Becht et al. 2005, Becht et al. 2006; Feldbrugge et al. 2008; Konig et al. 2009), on vesicles and endosomes for RNA transport (Bethune et al. 2019; Haag et al. 2015), and on RNA-binding proteins for post-transcriptional regulation (Hall and Wallace 2022). In other words, the position of a nucleus within a mycelium might dictate the stability and fate of its transcriptional output (Schuurs et al. 1998). In AMF, single-molecule techniques will help identify the subcellular locations where RNAs are transcribed and proteins are synthesized, and will pinpoint the specialized cellular tasks that are required in different fungal structures.

In dikaryotic AMF strains, genome expression and regulation bear an additional layer of complexity since different alleles can be transcribed from different nuclear genomes. Allelic expression can therefore depend on the ratio of nuclei present in the mycelium, as well as differential transcriptional activity from either nucleus. The transcriptional output of both nuclear genomes would be subject to regulation by common components of cytoplasmic regulatory mechanisms (e.g. translation, RNA decay), which can theoretically buffer transcriptional imbalances. In *R. irregularis* dikaryons, the expression ratios of bi-allelic genes largely correspond to the ratios of nuclear genomes present (Robbins et al. 2021). Interestingly, the

expression of a small number of bi-allelic genes deviated from nuclear genotype ratios, suggesting that transcriptional or post-transcriptional mechanisms can differentially impact the output of either nuclear genome.

So far, nuclear ratios of heterokaryotic strains have proven stable over time, indicating compatibility between coexisting genotypes, rather than genomic conflict. In addition, the dikaryotic state was associated with higher growth rates, which might provide a fitness advantage (Serghi et al. 2021). However, while the dikaryotic state and its associated genetic diversity intuitively translate to AMF having greater resilience and ecological success, dikaryotic strains are relatively rare in AMF culture collections (four out of 114 *R. irregularis* strains; (Kokkoris et al. 2021)), and the real prevalence of dikaryons versus monokaryons still needs to be assessed at the population level, in natural systems. Although cooperation might be the most apparent state of so far described dikaryons, it is possible that genomic conflict arising from incompatible genotypes leads to strong selective pressure, and will simply never be observed. Experiments that aim to recreate existing dikaryotic strains from homokaryotic parents, or to create new dikaryotic strains, will inform on the nuclear dynamics that occur at the onset of dikaryosis. Additionally, investigating the existence of dikaryosis in species other than *R. irregularis* will indicate whether this evolutionary strategy is widely used in AMF. Although nuclear ratios of AMF dikaryons are stable under standard laboratory conditions, they can be affected by host shifts (Kokkoris et al. 2020, 2021) and abiotic conditions such as pH, phosphorus concentration and temperature (Cornell et al. 2022). Mechanisms that regulate the relative number of coexisting nuclei are unknown, but could include asynchronous replication and mitosis, and nuclear degradation (Kokkoris et al. 2020). The ability to acquire and maintain more than one genotype may provide functional and adaptive benefits to AMF, but doing so may also create great opportunity for selection. It will be relevant to assess how stable the dikaryotic state is *in planta* and in the wild, as opposed to in vitro culture conditions, in order to understand the parameters acting on AMF adaptation and evolution. Since variations in nuclear ratios can be experimentally triggered, it will be interesting to test whether they are accompanied by corresponding variation in allelic gene expression, and whether these processes are reversible. Such a system could provide an elegant demonstration as to how dikaryotic AMF can make use of available genotypes to adapt to new environmental conditions (Fig. 4.2).

4.4 Transmission of the Genome

In the fungal kingdom, the existence of diverse nucleotypes within one mycelium creates genotypic diversity and possibly phenotypic plasticity (Croll et al. 2009; Maheshwari 2005; Rayner 1991; Roper et al. 2011). The potential for genetic variation to be acquired within the lifetime of a single fungus was proposed to play a role in adaptation to the environment. The coexistence of multiple genotypes within one mycelium can cause competitive and cooperative genome dynamics, and differential segregation and selection of genotypes may also be adaptive (James et al. 2008; Jinks 1952; Roper et al. 2011; Samils et al. 2014). A possible outcome of nuclei coexisting as a heterokaryon is that they can fuse, undergo karyogamy, mitotic recombination, and ploidy reduction, resulting in haploid and aneuploid genomes that are different from the parental ones (Anderson et al. 2019; Forche et al. 2008; Strom and Bushley 2016). This process called parasexuality is independent of sexual reproduction, but retains hallmarks of meiosis and typically yields transiently aneuploid cells which then undergo ploidy reduction, creating de novo genetic diversity (Hickman et al. 2015; Hirakawa et al. 2017; Mishra et al. 2021). In most cases, the heterokaryon stage is expected to precede nuclear fusion, meiosis, and completion of a sexual life cycle. AMF can experience a dikaryotic life stage, express meiosis-related genes, and can recombine genetic material (Chen et al. 2018a; Corradi and Brachmann 2017; Dallaire et al. 2021; Halary et al. 2011; Hofstatter and Lahr 2019; Mateus et al. 2020; Ropars et al. 2016; Yildirir et al.

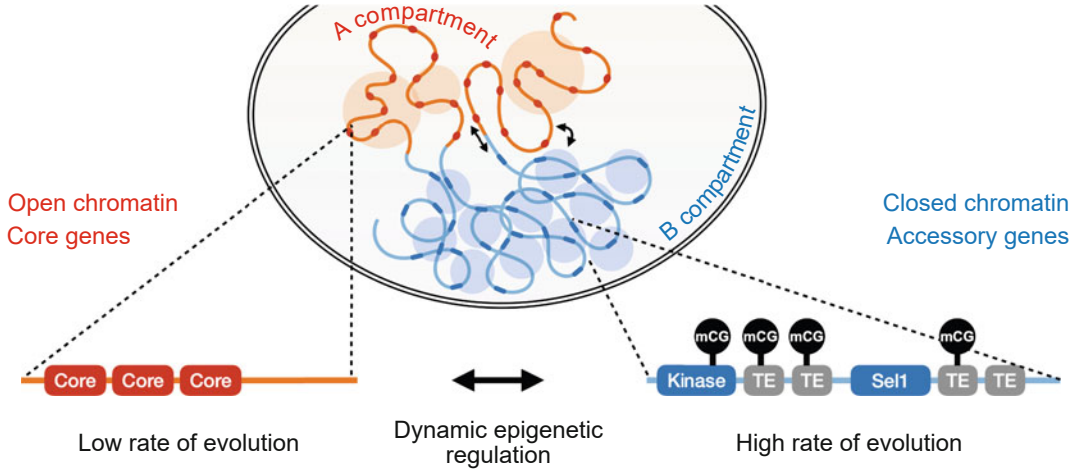

Fig. 4.2 AMF genome compartmentalization and epigenetic regulation. Genes and transposable elements (TEs) are partitioned into euchromatin (A compartments, open) and heterochromatin (B compartments, closed), respectively. Contrary to core genes, accessory genes tend to be located in B compartments, where their expression can be affected by the spreading of heterochromatin, in *cis* or *trans*. Genes in B compartments may experience more sequence disturbance caused by TE activity and proximity, and therefore have higher rates of evolutionary change. Figure inspired by Liang and Fu (2021). mCG, methylated CG dinucleotides

2020). However, since direct evidence of nuclear fusion, karyogamy, meiosis, or a sexual structure is lacking, the current assumption is that AMF are at least partially clonal and may undergo either parasexual or sexual reproduction at a low frequency and under unknown developmental or environmental conditions. Experimental crosses of homokaryotic AMF may reveal signs of genomic cooperation or conflict between nuclear and cytoplasmic (e.g. mitochondria) elements, and if recombination occurs, whether it is accompanied by transient aneuploidy or not (suggesting parasexual or sexual mechanisms, respectively). Limited recombination over long evolutionary timescales is problematic because each generation may generate too little genetic variation to adapt to environmental change, possibly leading to extinction. It is therefore reasonable to expect that low-frequency recombination occurs in AMF, and that alternative mechanisms may also generate the plasticity and genomic heterogeneity required for these fungi to adapt to incredibly varied environments and hosts (Fig. 4.3).

4.5 Perspectives on Adaptation and Evolution

Knowledge of the mechanisms, rates, and consequences of AMF evolution is crucial to understand which functional traits are under selection, and for predicting the capacity of AMF to support ecosystems in the face of rapid environmental change. While AMF display remarkable persistence over long time periods, the mechanisms underlying short-term evolutionary dynamics are still elusive. Are extant AMF in an evolutionary stasis, or diversifying? Is coevolution with plants constraining or promoting evolutionary divergence of AMF, and how can we use this information to predict and support ecosystem functioning? At the molecular level, evolution manifests itself in a variety of changes in DNA sequence, ranging from point mutations to chromosomal rearrangements. Understanding how different mechanisms contribute to genome evolution and quantifying genomic diversity and evolutionary rates will help explain AMF genome organization in adaptive terms.

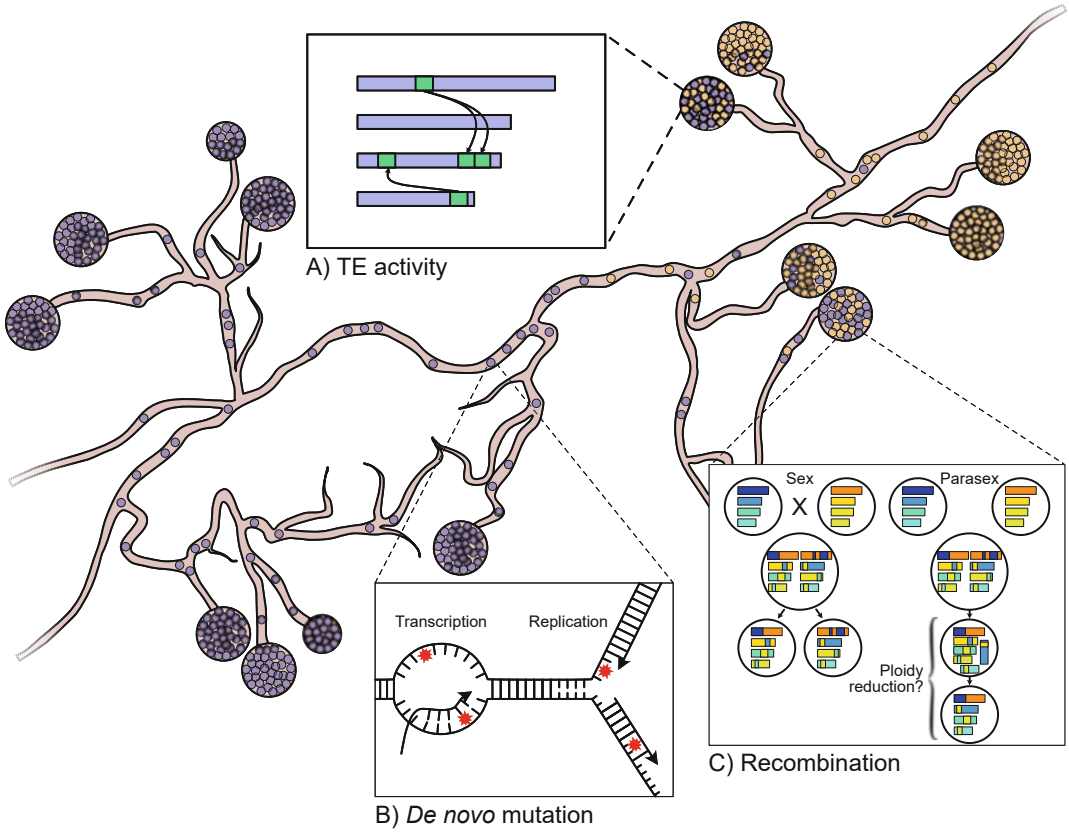

Fig. 4.3 Potential mechanisms contributing to genome evolution in AMF. (**a**) Mobile transposable elements (TEs) can induce duplications, deletions, and chromosome rearrangements. (**b**) Mutagenic mechanisms (shown in red) include errors in DNA replication, errors in DNA repair, but as well as (not shown here) base deamination, oxidative DNA damage, and base methylation. (**c**) Sexual or parasexual recombination can generate genetic diversity, and the latter may be accompanied by asymmetric segregation of chromosomes and transient aneuploidy. Different coloured nuclei illustrate dikaryon formation, which may be associated with TE activity and recombination

AMF genes and TEs tend to be partitioned in different regions of the genome. At present, epigenetic landscapes globally correlate with transcriptional activity within these compartments and may play direct roles in regulating core and accessory gene expression (Kumar et al. 2021). While accessory genes are enriched in heterochromatic compartments and display presence/absence variation, it is still unclear whether they have a higher *molecular rate of evolution* compared to core genes, which would imply ongoing selection pressure. In parallel, since TE expression was detected in *R. irregularis* spores, suggesting ongoing or recent TE mobility (Dallaire et al. 2021; Maeda et al. 2018), *quantifying new TE insertions* in *R. irregularis* strains would indicate which genomic regions were most recently shaped by TEs and are therefore potentially still dynamically evolving. This is important because in the absence of recombination, innovation could still be driven by TE activity or local mutagenesis, rather than sex-dependent recombination. In other fungi, for example, TEs tend to accumulate in accessory chromosomes or accessory compartments in core chromosomes (Croll and McDonald 2012; Sanchez-Vallet et al. 2018), possibly identifying candidate genes or genetic regions with adaptive

potential and evolutionary plasticity, and pointing to regions where TEs are selected against. Another mutagenic mechanism to be considered is deamination of methylated cytosines. In analyses of human somatic mutations, methylated cytosines spontaneously deaminate at a higher rate than non-methylated cytosines, and, when not correctly repaired, result in mutations (Alexandrov et al. 2015; Kong et al. 2012). Excess mutagenicity can therefore be observed at methylated CG dinucleotides. In Ascomycota and certain Basidiomycota fungi, methylation of transposons during meiosis is associated with extremely high rates of C-T transitions and rapid mutagenesis (Gladyshev 2017; Hood et al. 2005; Horns et al. 2012). This process called repeat-induced point (RIP) mutation has however not been detected in the AMF *G. margarita* or in species of the Mucoromycotina (Venice et al. 2020). Nevertheless, the endogenous rate of spontaneous deamination can account for some mutagenesis in AMF, and likely influences point mutation rates.

Quantifying the rate of de novo mutation occurring during AMF vegetative growth would help estimate how much genetic diversity can be expected to arise in the absence of sex, in comparison to rates of molecular evolution in fungi and other eukaryotes (Bezmenova et al. 2020; Hiltunen et al. 2019; Kasuga et al. 2002; Obbard et al. 2012; Wolfe et al. 1987). The combination of mutation rate analysis with intraspecific divergence would help calibrate evolutionary models of species divergence in AMF and provide valuable tools for the reconstruction of their natural history. Since the rate of recombination of a species is critical for estimates of mutation and genomic change, further insights into nuclear dynamics will also be important. *Investigating which of the dikaryotic or monokaryotic states is prevalent in nature and across AMF species, the extent to which inter-nuclear recombination occurs, and whether AMF have an aneuploid stage* may require large numbers of isolates and species to be analysed, but will provide invaluable insights into the frequency of recombination among lineages, and whether clonal lineages arise by recombination.

Until the advent of a stable transformation protocol for AMF, *increasing genomic sampling, both in numbers and in geographical diversity*, is arguably the best way to accelerate our understanding of how AMF genomes work, how genetic variation shapes their phenotypes, how they evolve, and how systems-level patterns in ecological diversity arise. Scaling up AMF biodiversity genomics will improve taxonomic delineation of AMF, which will allow us to tackle major questions about evolutionary selection pressures that shape AMF biodiversity, adaptation, and evolution. Reports on host plants affecting the distribution of AMF communities are accumulating (Alguacil et al. 2019; Croll et al. 2008; Koch et al. 2006; Munkvold et al. 2004; Sanders 2003; Van Der Heijden and Scheublin 2007). Factors selecting AMF and driving their diversification may therefore include a combination of components such as host plant identity, environmental conditions and resources, interaction with microbes (including other AMF), and us (host breeding, agricultural systems). Identifying which functional traits of AMF can be selected and what pressures promote or diminish their diversity in natural communities hold the potential we need for protecting these fungal networks that sustain life on planet Earth.

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Diversity of Seed Endophytes: Causes and Implications

5

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Abstract

The immense importance of microbial symbioses with plants, animals, and other eukaryotes is meeting with ever increasing awareness and interest. Heritable symbionts—those transmitted directly from hosting parents to hosting progeny—are particularly intimate associations with profound ecological, evolutionary, and applied consequences. However, heritable symbioses also tend to be inconspicuous and are often understudied. Heritable fungal symbionts of plants, which we call seed endophytes, have been discovered and rediscovered in a few grass species (family Poaceae) starting well over a century ago, but have been intensively researched only in the last 45 years since their ability to produce antimammalian alkaloids was revealed to cause major toxicoses to livestock. The characterization of those fungal *Epichloë* species has been followed gradually by documentation of other seed endophytes with bioactive alkaloids, such as those found in locoweeds (family Fabaceae) and morning glories (family Convolvulaceae). As the known species diversity of seed endophytes and their hosts has expanded, so too has our knowledge of their alkaloid diversity, defenses against invertebrates, positive and negative effects on host plants, effects on pathogens and beneficial symbionts (e.g., mycorrhizal fungi), protection from abiotic stresses such as drought, and

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cascading population, community, and ecosystem consequences. Recent studies have even revealed endophyte contributions to plant diversity, including an *Epichloë* gene apparently transferred to a host grass in which it confers disease resistance. Here we review the current knowledge of seed-endophyte symbioses with emphasis on their phylogenetic, genetic, and functional diversity.

Keywords

Alkaloids · Bioprotection · Endophyte · Symbiosis · Horizontal gene transfer

5.1 Introduction

Symbiosis, as a word, owes its origin to the early proponent of germ theory, H. Anton de Bary, who also coined the term *endophyte*. The popular notion of symbiosis is equated with mutualism, whereby two interacting partners benefit each other. There are problems with such a perceived equivalence, and biologists can get contentious about whether and when a symbiosis is a true mutualism. We will occasionally touch upon that ambiguity here as we discuss fungal groups that include or are predominantly endophytes which are also heritable: passed on from mother plants to their progeny via the seeds. Suffice to say at the outset that of course these plant-endophyte systems have evolved via natural selection on both the microbes and their hosts, and that the interest of the microbe is neither to be friendly nor destructive to its host, but rather to ensure its own home space, source of nutrition, and means of propagation. Its home is its host, which provides all its nutritional requirements, and its means of propagation is to colonize new host individuals by *horizontal transmission* from one plant to another, *vertical transmission* via seeds, or both. It is the vertically transmissible symbionts that we call *seed endophytes.* A prediction that appears to be borne out in most of seed-endophyte systems is that vertical transmission selects for microbial functions that protect their hosts from the biotic impacts of herbivores, parasites, and pathogens, as well as abiotic stresses such as drought.

An imperative to study mechanisms of endophyte protection of host plants arises from our own need to protect our cultivated plants. For example, as we often rely on chemical controls of herbivores, we can take lessons from the diverse ways that endophytes utilize and deploy their own chemical protectants such as fungal alkaloids. Similarly, their mechanisms of protecting host plants from drought and other stresses can potentially lead to improved strategies in crop production. The potential to establish seed-endophyte symbioses in crop plants is also being explored. As it stands now, much is understood mechanistically about protective alkaloids and the genetics of their production by seed endophytes, while intense study of the basis for enhanced abiotic stress protection is only beginning to reveal candidate genes.

Seed endophytes are diverse phylogenetically (Fig. 5.1) and in their bioprotective mechanisms, of which a remarkable diversity of anti-herbivore metabolites—particularly alkaloids—have been documented. Especially within genus *Epichloë*, and even within individual *Epichloë* species, a variety of alkaloids within each of four major classes have been documented, along with their diverse effects on invertebrates, vertebrates, or both.

Seed-endophyte effects at levels other than chemical antagonism against herbivores are also evident, and recent years have seen a great boon in investigations of the roles they play in tolerance to abiotic stresses, effects on pathogens and foliar endophytes, effects on mycorrhizal fungi, microbiomes, and associated plant species, and multitrophic effects. A summary of the kinds and levels of such interactions studied to date is presented in Fig. 5.2 and is reviewed in this chapter. It is especially notable that many of these effects are variable between genotypes or species, between environmental conditions or

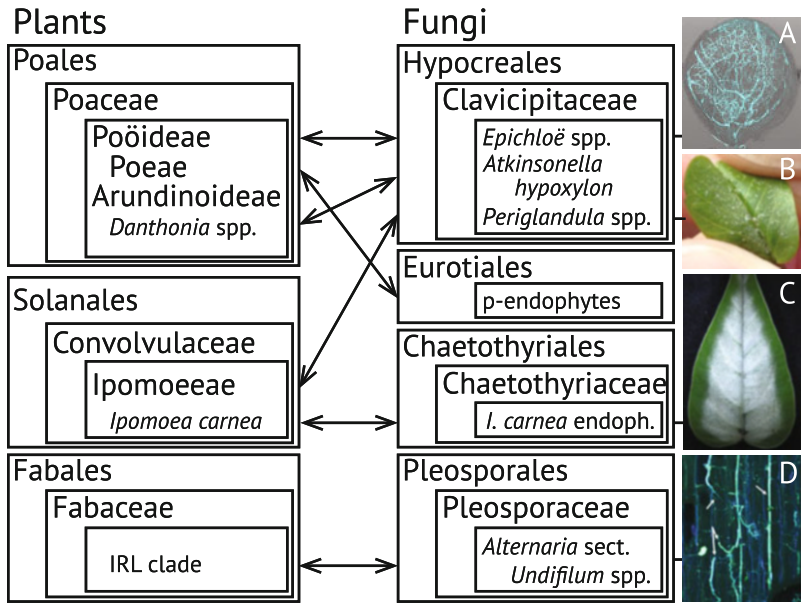

Fig. 5.1 Plant and fungal taxa involved in the best characterized seed-endophyte symbioses. Images at right are (**a**) *Epichloë festucae* expressing cyan fluorescent protein, growing in ovule of *Lolium perenne*, (**b**) young leaf of *Ipomoea pes-caprae* with mycelium of *Periglandula ipomoeae* on the adaxial surface, (**c**) leaf of *Ipomoea carnea* with mycelium of its swainsonine-producing Chaetothyrialean endophyte on the adaxial surface, and (**d**) autofluorescent hyphae of *Alternaria oxytropis* between cells in the rachis of *Oxytropis sericea*

both. Thus, diversity of endophytes results in extensive variation in their effects, and in turn endophytes affect the diversity of life in their ecosystems.

5.2 Taxonomy of Fungal Seed Endophytes and Host Plants

Seed-transmitted fungal endophytes have been identified from several orders of the fungal phylum Ascomycota, and in symbiosis with several orders of flowering plants (An et al. 1993; Cook et al. 2013; Leuchtmann et al. 2014; Reyna et al. 2012). The most well characterized of the symbioses are indicated in Fig. 5.1. There are few surveys of a broad taxonomic range of plants for such endophytes, but indications are that more are to be found (Hodgson et al. 2014), suggesting that seed-endophyte symbiosis is a substantial but largely unexplored component of biodiversity on Earth.

5.2.1 Epichloë, Periglandula, *and Relatives (Order Hypocreales)*

So far, *Epichloë* species (family Clavicipitaceae) have only been observed as symbionts and, in some cases, replacement pathogens of grasses (Poaceae) in the subfamily Poöideae (Leuchtmann et al. 2014), except for a single report in *Juncus* (Kilpatrick et al. 1961) that is thought to be in error (James F. White, personal communication). In keeping with the code of nomenclature prior to 2011, species for which the teleomorphic (sexual) stage was unknown were first classified (along with the mitotic spore state of *Epichloë typhina*) in *Acremonium* sect. *Albo-lanosa*, then as *Neotyphodium* species (Glenn et al. 1996). Phylogenetic analyses indicate that *Epichloë* is a monophyletic genus and is sister to the clade that includes *Claviceps* and *Aciculosporium* species and *Corallocytostroma ornithocopreoides* (Píchová et al. 2018), all of which also associate with grasses. Related to

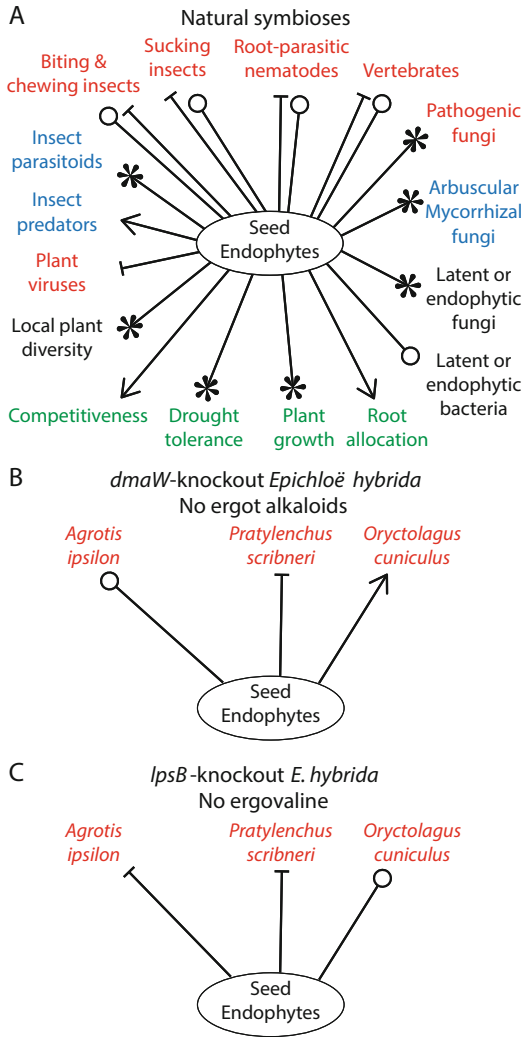

Fig. 5.2 Summary of effects of seed-transmitted endophytes investigated to date. Arrows indicate positive effects, bars indicate negative effects, asterisks indicate variable effects, and open circles indicate no effects. Where more than one symbol is used, results are different in different systems. Factors given in red are generally detrimental to the host plants, those in blue are generally beneficial, those in black are expected to vary in effects on the plants depending on conditions and circumstances, and those in green are direct effects on the plants. (**a**) Effects of natural symbioses. (**b**, **c**) Effects of *Epichloë hybrida* mutants on its host grass *Lolium perenne* compared to endophyte-free plants in tests with sod webworm (*Agrotis ipsilon*), a root-parasitic nematode (*Pratylenchus scribneri*), and rabbits (*Oryctolagus cuniculus*). (**b**) Effects of *E. hybrida* in which *dmaW* is knocked out so that no ergot alkaloids are produced. (**c**) Effects of *E. hybrida* mutants in which *lpsA* is knocked out so that no ergovaline is present, but simpler alkaloids such as ergotryptamine, chanoclavine, and ergine are present

this group is a clade that includes other grass-associated fungi in genera *Atkinsonella, Balansia, Ephelis, Heteroepichloë, Myriogenospora,* and *Parepichloë*, but also *Periglandula* species, which are symbiotic with morning glories (family Convolvulaceae, tribe Ipomoeae) (Píchová et al. 2018; Schardl et al. 2014; Tanaka et al. 2002). Also included in Clavicipitaceae are *Ustilaginoidea* (Zhang et al. 2014) and *Commelinaceomyces* species (Tanaka et al. 2020), which cause false smut disease on monocots, as well as *Metarhizium* and related species best known as insect pathogens. Interestingly, *Metarhizium* species also form symbioses with plant roots (Pava-Ripoll et al. 2011).

Claviceps species in particular, and many other species in the Clavicipitaceae, produce ergot alkaloids, which are infamous as the causative agent of St. Anthony's Fire (ergotism) in humans and similar maladies in livestock (reviewed in Schardl et al. 2006). These alkaloids also serve as starting material for synthesis of lysergic acid diethylamide (LSD). *Epichloë coenophiala* strains in the original US cultivars of the forage grass tall fescue (*Lolium arundinaceum*) produce ergovaline, one of the ergopeptine subclasses of ergot alkaloids. For this reason, these cultivars cause episodic symptoms of ergotism in livestock. In New Zealand and Australia, a syndrome called "ryegrass staggers" is attributable to another class of alkaloids, the indole-diterpenes (reviewed in Saikia et al. 2008), produced by *Epichloë festucae* var. *lolii* in the perennial ryegrass (*Lolium perenne*) originally introduced there by European colonists. Strategies to address these livestock toxicoses are discussed later in this chapter. Also discussed are other alkaloids and products of *Epichloë* species.

Periglandula species are members of the fungal family Clavicipitaceae that are symbiotic with species of family Convolvulaceae, tribe Ipomoeae (Fig. 5.1) and produce ergot alkaloids as well as indole-diterpenes in some instances. Two species, *Periglandula ipomoeae* and *Periglandula turbinae*, are symbiotic with *Ipomoea asarifolia* and *Turbina corymbosa*, respectively (Steiner et al. 2011). Additional

research by Beaulieu et al. (2015) has revealed several more Ipomoeae with symbiotic *Periglandula* species. Interestingly, *Ipomoea* hosts of *P. ipomoeae* all contain the ergot alkaloids and indole-diterpenes, whereas species associated with a *Periglandula* most similar to *P. turbinae* only contain the ergot alkaloids (Cook et al. 2019).

5.2.2 Alternaria *and Relatives (Order Pleosporales)*

A group of Fabaceae (legumes) are called locoweeds by association with locoism suffered by grazing animals, a malady now attributed to the swainsonine produced by symbiotic *Alternaria* sect. *Undifilum* (Ralphs et al. 2008). Host specificity has been identified for North American locoweed endophytes, but is less common for locoweed endophytes in China. *Alternaria oxytropis* has been isolated only from *Oxytropis lambertii* and *Oxytropis sericea* in the western USA (Pryor et al. 2009), whereas the same fungus has been found associated with *Astragalus* and *Oxytropis* species in China. Different species of *Alternaria*—namely, *Alternaria cinerea* and *Alternaria fulva*—have been isolated from *Astragalus mollissimus* varieties and *Astragalus lentiginosus* varieties, respectively, in the western USA (Baucom et al. 2012). However, *Alternaria astragali* has only been identified from *Astragalus adsurgens* (standing milkvetch) in northwest China, and the fungus is a pathogen of its plant host as well (Liu et al. 2016). In Australia, several species of *Swainsona* (also Fabaceae), for which swainsonine was named, contain a species of *Alternaria* sect. *Undifilum* (D. D. Cook, unpublished observation).

Slafractonia leguminicola (Alhawatema et al. 2015) shares characteristics with some *Epichloë* species and with some *Alternaria* section *Undifilum* species. This fungus is not known to produce a sexual stage or even asexual spores and was originally classified in genus *Rhizoctonia*. The fungus is a pathogen of legumes, particularly clovers, and produces a choke that deters plant reproduction. It is seed-borne and infects stems, leaves, and flowers. *Slafractonia leguminicola* produces two indolizidine alkaloids, swainsonine and slaframine (Harris et al. 1988), of which the latter induces a toxicosis called "slobbers" in grazing mammals.

5.2.3 *Morning Glory Endophytes of Order Chaetothyriales*

Among the diverse swainsonine producers is a species of seed endophytes yet to be named, but phylogenetically grouped in the family Chaetothyriaceae (Cook et al. 2017). Hosts of this endophyte include *Ipomoea carnea, Ipomoea riedelii,* and probably—based on the presence of swainsonine—*Ipomoea sericophylla*. These are congeners and close relatives of the hosts of *Periglandula* species. Remarkably, the localization and growth of the Chaetothyrialean endophyte in *I. carnea* and *I. riedelii* is very similar to that of *Periglandula* in other *Ipomoea* and related species (Steiner et al. 2011; Neyaz et al. 2022b), even though the fungi belong to different orders.

5.3 Seed–Endophyte–Host Interactions

5.3.1 Host Compatibility

A series of investigations of *E. festucae* interaction with *L. perenne* have firmly established the role of reactive oxygen species (ROS) in maintaining stable and mutually beneficial symbioses. The fungal NADPH oxidase isoform NoxA is especially important for self-regulation limiting growth in plants. Elimination of the *noxA* gene (Tanaka et al. 2006), as well as genes for several regulators and interactors (Takemoto et al. 2006; Voisey et al. 2016) results in excessive hyphal growth *in planta*, and in many cases, severe plant stunting and other symptoms (reviewed in Scott et al. 2018). Studies in culture indicate that NoxA generates elevated H_2O_2 at and behind the hyphal tip, suggesting that the autoregulation involves inhibition of tip growth

(Tanaka et al. 2006). This is likely to be most important during colonization of young tissues such as near the meristematic zones, since hyphae adjacent to elongating host cells in the leaf blades and sheaths grow mainly by intercalary extension (Christensen et al. 2008).

Another prominent aspect of fungal biology that is key for healthy symbiosis is anastomosis, whereby cells of distinct hyphal strands or branches fuse to allow exchange of nutrients and organelles, even including nuclei. Generally, deletion or disruption of genes required for anastomosis leads to strains that stunt and debilitate their host plants (Charlton et al. 2012b; Green et al. 2016; Shoji et al. 2015).

Similarly, deletion of the *E. festucae sidN* gene for biosynthesis of the siderophore epichloenin A results in altered growth and negative effects on host growth, suggesting that iron homeostasis is also crucial for these symbioses (Johnson et al. 2013).

The observation that at least some symbiotic *Epichloë* species can produce epiphyllous (epibiotic) growth (Moy et al. 2000) has been followed up with documentation of a structure dubbed the *expressorium* (by analogy to the *appressorium* of certain plant-pathogenic fungi) (Becker et al. 2016). Expressoria develop beneath the cuticle in young plant tissues—such as near shoot apical meristems—where endobiotic growth is mainly at hyphal tips (rather than the intercalary growth mentioned above). The cell walls of epiphyllous hyphae are remodeled such that chitin is more readily detectable compared to the cell walls of endobiotic hyphae. Interestingly, Δ*noxA* and Δ*noxR* mutations affect the remodeling process and also affect the host interaction such that an apparent defensive response is elicited by egressing mutant hyphae, a response not observed against the wild-type endophyte.

Although *Epichloë*–host interactions may serve as a model for other endophyte symbioses, a dramatic difference is evident with the epibiotic plant symbionts, including some within the same fungal family (Leuchtmann and Clay 1988; Philipson and Christey 1985; Steiner et al. 2006). Remarkably, the epibiotic growth of *Periglandula* species, which associate with adaxial secretory glands of *Ipomoea* and related species, is very similar to that of the phylogenetically distant Chaetothyrialean symbiont of other *Ipomoea* species. An extensive study of the latter (Neyaz et al. 2022b) indicates that in seeds and plants the fungus grows between tissue layers, but apparently not between cells within tissues. A similar situation is suggested by the growth habit of the Clavicipitaceous epibiont, *Atkinsonella hypoxylon*, in the grass host *Danthonia spicata* (Philipson and Christey 1985). In contrast, *Epichloë* species extensively colonize intercellular spaces of the ovule nucellus pre-anthesis, and following pollination, continue growth intercellularly in the embryonic axis and aleurone layers (Zhang et al. 2017; Liu et al. 2017). Thus, whether fungal growth is largely endobiotic or (almost) exclusively epibiotic follows no definite phylogenetic pattern of either host or symbiont.

Epichloë gene expression and alkaloid production *in planta* differs substantially from in culture (Chujo and Scott 2014; Spiering et al. 2005b) and between asymptomatic tissues and stromata (Berry et al. 2022), as well as over the course of host development (Hettiarachchige et al. 2021; Nagabhyru et al. 2019). Epigenetic control involving histone marks H3K9-m3 and H3K27-m3 and the HepA protein are key to regulation of alkaloid and other genes, and to normal symbiotic development (Chujo and Scott 2014; Chujo et al. 2019).

Interestingly, analysis of gene expression in pre-anthesis ovaries of tall fescue with *E. coenophiala* indicated very little effect of the fungus on host gene expression. However, endophyte genes for protein chaperones and chaperonins, and for ROS-scavenging and trehalose biosynthesis enzymes, dramatically increased in expression in ovaries compared to vegetative tissue (Nagabhyru et al. 2019). An intriguing question is whether these fungal factors help overcome normal stressors in the host ovary or modulate the aforementioned autoregulation system of the fungus.

5.3.2 Host Specificity

In the extensive taxonomic work to date on plant-associated Clavicipitaceae, a high degree of host specificity is common. The *Epichloë* species in general are restricted to the grass subfamily Poöideae, and many but not all of the individual species are restricted to a single tribe, genus, or even species of host (Leuchtmann et al. 2014). Such host specificity is common both for sexual species and apparently asexual species including most interspecific hybrids, but broader host-range species are known for both. *Epichloë typhina* is a genetically diverse sexual species that occurs in sister tribes Agrostideae and Poeae, as well as in Brachypodieae. The hybrid, asexual species *Epichloë tembladerae* is common in Argentina, where it has been identified in sister tribes Bromeae and Hordeeae, as well as Agrostideae, Poeae, and Meliceae (Iannone et al. 2012a, 2015). This hybrid is not known to produce stromata—the structures on which *Epichloë* species develop their sexual state—but its production of a sporogenous hyphal net on leaf surfaces (Moy et al. 2000) may facilitate host jumps. However, *E. tembladerae* is a diploid hybrid of *E. festucae* and *E. typhina* (as discussed below in Sect. 5.5), so another possibility is that it has had multiple origins by hybridization of similar genotypes.

Studies have been undertaken to investigate host specificity of individual *Epichloë* strains. Isolates of *E. coenophiala* and two other hybrids designated FaTG-2 and FaTG-3) (probably from the grass species *Lolium mediterraneum* Banfi et al. 2017) were introduced into *L. perenne* seedlings to produce novel symbioses, as was *E. festucae* var. *lolii* as controls for the natural symbioses with *L. perenne* (Koga et al. 1993). The novel symbioses but not the controls were characterized by higher electron density in the intercellular matrix adjacent to endophyte hyphae, which showed evidence of stress, suggesting that there was a mild defensive response by the host.

Recently, Efe-AfpA, a possible effector protein produced by *E. festucae* strains symbiotic with *Festuca rubra* subsp. *rubra* (Wang et al. 2021), was investigated by CRISPR-based mutation and complementation of its gene. Results indicated that it was required for host compatibility. Since the gene was apparently absent from other *Epichloë* strains, Efe-AfpA may be a host-specificity factor. This appears to be the first report of a host-specificity factor in endophytes, and hopefully will prompt more such studies.

5.3.3 Host Protection Against Herbivory

The vast majority of work on seed endophytes to date has been on the grass-*Epichloë* interactions, for which numerous ecological benefits to a variety of host species have been documented, although in some hosts little benefit has been evident. In all cases, there have been alkaloids—or at least their biosynthesis genes—identified for at least one of the four chemical classes: 1-aminopyrrolizidines such as lolines, ergot alkaloids such as ergovaline, indole-diterpenes such as lolitrem B, and pyrrolopyrazines such as peramine. Alkaloids in each of these classes are known to have activity against insects as feeding deterrents or anti-invertebrate toxins. Additionally, ergovaline and lolitrem B are significantly toxic to livestock and cause major economic losses as a result. In the USA, ergovaline-producing strains of *E. coenophiala* in tall fescue (*L. arundinaceum*) cause hundreds of millions of dollars in losses to the beef industry annually, and perhaps close to one billion dollars in losses to livestock overall (Hoveland 2009). Similarly, major losses from "staggers" of sheep and other livestock are incurred in New Zealand and Australia due to lolitrems and other indole-diterpenes produced (along with ergovaline) by *E. festucae* var. *lolii* in *L. perenne* (Hume et al. 2020). Approaches to solve these problems by identification or generation of "livestock friendly" strains are discussed in the Applications section of this chapter.

Interestingly, very acute toxicity due to indole-diterpenes is common in South America, both due to the morning glory-*Periglandula* symbioses, and to some *Epichloë* species in native grasses

(discussed in Cabral et al. 1999; Lee et al. 2017). Furthermore, the persistence of perennial ryegrass in most of New Zealand is dependent on endophyte-conferred resistance to Argentine stem weevil, a pest introduced from in South America (Lee et al. 2017). In fact, indole-diterpene toxicity appears to be more widespread in the Southern Hemisphere because of these and other plant-endophyte systems such as *Melica decumbens* with *Epichloë melicicola* in South Africa, and *Echinopogon ovatus* with *Epichloë aotearoae* or *Epichloë australiensis* in Australia (Moon et al. 2002).

The known activities of lolines and peramine are against insects and, in the case of lolines, also nematodes (Bacetty et al. 2009a, b). Lolines have been characterized as having a broader anti-insect activity than peramine, but that is in a limited number of studies. Furthermore, variation in the amide-linked substituents that distinguish *N*-acetylnorloline (NANL), *N*-formylloline (NFL), *N*-acetylloline (NAL), and others gives different spectra of activity against insects (Popay et al. 2009).

Other seed-endophyte systems are also known for toxic alkaloid production, particularly swainsonine, a cytotoxic indolizidine which acts as an α-mannosidase II inhibitor and is best known as the cause of locoism in ranging livestock that feed on locoweeds (*Astragalus* and *Oxytropis* species). The phylogenetic distribution of swainsonine producers is very broad within the fungal phylum Ascomycota, but plant symbionts that produce swainsonine belong to at least three distantly related fungal genera: *Alternaria* sect. *Undifilum*, *Metarhizium*, and a yet unclassified fungus in the family Chaetothyriaceae (Cook et al. 2017).

5.3.4 Host Protection Against Abiotic Stresses

As well characterized and widespread are the cases of protection from biotic factors, there is also widespread evidence of enhanced tolerance or protection from abiotic stresses. The most thoroughly studied is the tall fescue—*E. coenophiala* system, with perennial ryegrass—*E. festucae* var. *lolii* also receiving intense scrutiny (Malinowski and Belesky 2000, 2019; Hahn et al. 2008; Decunta et al. 2021).

The most intensely investigated abiotic stress in these systems has been drought or water deficit. Studies with endophyte-symbiotic (E+) plants versus their endophyte-cured (E–) clones indicate that, following acute water deficit the recovery by new tiller growth can be significantly better in the E+ clones than the E– clones. Proline and other amino acids, sugars and sugar alcohols respond to the onset of stress with greater increases in E+ than in E– plants (Nagabhyru et al. 2013). RNA-seq analysis indicates the expected host response to water deficit, but very little effect of the endophyte was observed on transcription of host genes either with or without the stress (Dinkins et al. 2019).

Considering the transcriptome results, we suggest that much of the endophyte enhancement of stress tolerance and metabolite accumulation may be additive, with the endophyte contributing to the L-proline (Pro), sugar (including trehalose), and sugar alcohol pools, as well as enhanced levels of ROS-scavenging enzymes plants (Nagabhyru et al. 2013; Nagabhyru et al. 2022). Additionally, although few host genes are affected, some—such as dehydrin genes—observed to be affected by *E. coenophiala* symbiosis in unstressed plants may serve to prime the host for increased drought tolerance (Dinkins et al. 2019).

5.4 Anti-herbivore Alkaloids

Five classes of fungal alkaloids have so far been associated with seed endophytes. Two of these—ergot alkaloids and indole-diterpenes—are produced by some *Epichloë* and *Periglandula* species (Florea et al. 2017; Saikia et al. 2008). The 1-aminopyrrolizidines (Schardl et al. 2007) and pyrrolopyrazines (Berry et al. 2019) are also produced by some *Epichloë* species. The indolizidine, swainsonine, is produced by the locoweed endophytes (*Alternaria* sect. *Undifilum*) and by the Chaetothyrialean

endophyte of *I. carnea* (Cook et al. 2017). None of these alkaloid classes is restricted to seed endophytes, but most known seed endophytes are capable of producing alkaloids of one or more of these classes. The first four of these five classes are associated with protection of host plants from invertebrate or vertebrate herbivores, but such a role has not been demonstrated for swainsonine.

Epichloë species (Table 5.1) and *Periglandula* species (Cook et al. 2019) exhibit remarkable diversity in their alkaloid profiles. Studies of alkaloid diversity and the underlying genetics in seed endophytes and related fungi have highlighted various evolutionary processes. These include gene and gene cluster duplication, as well as gene neofunctionalization for ergot alkaloids (reviewed in Florea et al. 2017), gene cluster duplication, gene fusion, and neofunctionalization for indole-diterpenes (reviewed in Saikia et al. 2008; Schardl et al. 2013a), and functional diversification and trans-species polymorphism for pyrrolopyrazines (Berry et al. 2015, 2019). Also, loss of some genes or gene domains is associated with massive diversification of all three of those alkaloid classes as well as the 1-aminopyrrolizidines (Schardl et al. 2013a). Even a host plant contribution to loline alkaloid diversity has been documented (Pan et al. 2014b).

Epichoë genome sequencing has also revealed that gene clusters for ergot alkaloids (*EAS*), indole-diterpenes (*IDT*) and 1-aminopyrrolizidines (*LOL*) include extensive AT-rich repetitive sequences that are likely relics of transposable elements (Schardl et al. 2013a, b; Young et al. 2005, 2006). It has been suggested that those repetitive sequences promote gene deletions that help generate alkaloid diversity, providing an evolutionary advantage for such genomic instability (Schardl et al. 2013a).

We also note that in general the genomes apparently encode many more specialized ('secondary') metabolites (Schardl et al. 2013a), and it is reasonable to expect that some of those contribute to host defense. Other factors as well may contribute, such as the anti-insect "makes caterpillars floppy" (MCF) protein produced by some *Epichloë* species (Ambrose et al. 2014), and perhaps the highly diverse epichloëcyclin cyclic peptides (Johnson et al. 2015).

5.4.1 Ergot Alkaloids, Including Ergovaline

The best-known ergot alkaloids are clavines, fumigaclavines, and lysergic acid amides, which include the notoriously toxic ergopeptines. Phylogenomic analysis (Florea et al. 2017) indicates that the ancestor of the major clade of plant-associated Clavicipitaceae produced complex ergopeptines. Furthermore, early in evolution of that clade, a second branch of the pathway evolved to produce simpler lysergyl amides; namely, ergonovine, lysergic acid α-hydroxyamide (LAH), and ergine (lysergic acid amide).

The two biosynthetic branches diverge where lysergic acid is incorporated into amides by the action of nonribosomal peptide synthetase (NRPS) multifunctional proteins (Ortel and Keller 2009). The lysergyl peptide synthetases consist of three polypeptide subunits alternatively paired for each pathway in an apparently unique mechanism of metabolic diversification. The LpsB (= LPS2) subunit has just a single module, with the lysergyl activation and thiol-linkage domains followed by a condensation domain. The LpsA (= LPS1) subunit possesses three modules that each specify an L-amino acid, followed by a C-terminal cyclization domain. The LpsC subunit has one module specifying L-alanine (Ala), followed by a reductase domain. The reason for the branched pathway is that the LpsB subunit can associate either with LpsC for the simpler lysergyl peptide ergonovine or with LpsA for the much more complex ergopeptide lactams. Usually, the lactams are then oxidatively cyclized by the action of EasH to form ergopeptines (Havemann et al. 2014).

Interestingly, phylogenetic analysis of the activation (A) domains of LpsA, LpsB, and LpsC (Fig. 5.3) indicates a close relationship of the LpsC A domain with the LpsA A_1 domain. Furthermore, both LpsC and the likely ancestor of LpsA catalyze Ala addition to the lysergic acid

Table 5.1 Alkaloid genes and most abundant alkaloids produced by *Epichloë* species

				EA[a]		IDT[a]		LOL[a]	
Species (genotype)	Isolate[b]	Ref. or PI[c]	Host (ploidy)[d]	Pre.	Obs.	Pre.	Obs.	Pre.	Obs.
			Haploid *Epichloë* spp.						
Epichloë gansuensis	e7080	Schardl et al. (2013a)	*Achnatherum inebrians*	–	nt	PAX	PAX	–	nt
Epichloë inebrians	MYA-1228	Moon et al. (2007), Schardl et al. (2013a)	*Ach. inebrians*	EN LAH	EN LAA LAH	–	nt	–	–
Epichloë amarillans	ATCC 200744	Schardl et al. (2013a)	*Agrostis hyemalis*	–	–	–	nt	NANL	NANL
E. amarillans	E4668	Schardl et al. (2013b)	*Ag. hyemalis*	ERV	–	–	nt	–	nt
Epichloë brachyelytri	E4804, etc.	Schardl et al. (2013a)	*Brachyelytrum erectum*	CC	CC	–	nt	AcAP	AcAP
Epichloë bromicola	ATCC 200750	Schardl et al. (2013a)	*Bromus erectus*	–	nt	–	nt	–	–
E. bromicola	DAOMC 251834	Shi et al. (2017)	*Bromus dahuricus*	ERV	ERV	TD	TD	–	–
E. poae		Charlton et al. (2014)	*Bromus laevipes*	–	nt	TD	nt	–	nt
Epichloë calamagrostidis	ATCC 200745	Leuchtmann and Schardl (1998, 2022)	*Calamagrostis villosa*	–	nt	–	nt	–	nt
Epichloë aotearoae	MYA-1229	Moon et al. (2002)	*Echinopogon ovatus*	–	nt	TD	nt	NFL	NFL
Epichloë elymi	ATCC 201551	Schardl et al. (2013a)	*Elymus virginicus*	CC	CC	–	nt	–	–
E. festucae	E189	Schardl et al. (2013a)	*Festuca rubra* subsp. *rubra*	ERV	–	LTB	LTB	–	nt
E. festucae	Fl1	Schardl et al. (2013a)	*Festuca trachyphylla*	ERV	ERV	LTB	LTB	–	–
E. glyceriae	E277	Schardl et al. (2013a)	*Glyceria striata*	ERV	ERV	–	nt	AcAP	AcAP
Epichloë mollis	AL9924	Schardl et al. (2013b)	*Holcus mollis*	ERV	nt	–	nt	–	nt
Epichloë festucae	E434	Schardl et al. (2013a)	*Lolium giganteum* (6*x*)	–	nt	–	nt	NFL	NFL
Epichloë typhina	ATCC 200736	(Schardl et al. 2013a)	*Lolium perenne* (2*x*)	–	nt	–	nt	–	–
Epichloë poae	E4646	Schardl et al. (2013b)	*Poa nemoralis*	–	nt	–	nt	–	nt
E. poae	E5819	Schardl et al. (2013a)	*Poa nemoralis*	ERV	nt	–	nt	–	nt
			Polyploid *Epichloë* spp.						
Epichloë chisosa	ATCC 64037	Moon et al. (2007)	*Achnatherum eminens*	–	nt	–	nt	NFL	nt
Epichloë funkii	e4096	Moon et al. (2007)	*Achnatherum robustum*	CC	CC	TD	TD	–	–
Epichloë pampeana	BAFC1623	Iannone et al. (2012b)	*Bromus auleticus*	-	nt	-	nt	NFL	NFL
Epichloë cabralii	BAFC330	Charlton et al. (2014)	*Bromus laevipes*	–	nt	–	nt	NANL	NANL
BlaTG-2		Charlton et al. (2014)	*Bromus laevipes*	ERV	nt	–	nt	–	nt
BlaTG-3 (1)		Charlton et al. (2014)	*Bromus laevipes*	–	nt	TD	nt	–	nt
BlaTG-3 (2, 3)		Charlton et al. (2014)	*Bromus laevipes*	CC	nt	TD	nt	–	nt

E. canadensis	CWR5	Charlton et al. (2012a)	*Elymus canadensis*	ERV	ERV	–	nt	NANL	NANL
E. canadensis	CWR34	Charlton et al. (2012a)	*El. canadensis*	CC	CC	–	nt	AcAP	AcAP
Epichloë coenophiala	e15	Moon et al. (2004)	*Lolium arundinaceum* (6*x*)	ERV	ERV	–	–	NFL	NFL
E. coenophiala	e4309	PI 598903	*Lolium atlantigenum* (8*x*)	–	–	TD	TD	NFL	NANL
E. coenophiala	e4163	PI 422777	*Lolium interruptum* (4*x*)	ERV	ERV	TD	TD	NFL	NFL
FaTG-4	e4305	PI 598863	*Lolium letourneuxianum* (10*x*)	ERV	ERV	TD	nt	–	–
FaTG-2 (G2)	NFe45079	Takach et al. (2012)	*Lolium mediterraneum* (6*x*)	ERV	ERV	LTB	LTB TD	–	nt
FaTG-2 (G3)	NFe45115	Takach et al. (2012)	*L. mediterraneum* (6*x*)	ERV	ERV	TD	TD	–	nt
FaTG-3	NFe1100	Schardl et al. (2013b)	*L. mediterraneum* (6*x*)	–	–	TD	nt	NFL	NFL
FaTG-3	e4074	Moon et al. (2004)	*L. mediterraneum* (6*x*)	–	–	–	nt	NFL	NFL
Epichloë hybrida	Lp1	Campbell et al. (2017)	*L. perenne* (2*x*)	ERV	ERV	TD	TD	–	–
Epichloë siegelii	ATCC 74483	Craven et al. (2001), Moon et al. (2004)	*Lolium pratense* (2*x*)	–	–	PAX	nt	NFL	NFL
Epichloë uncinata	CBS 102646	Schardl et al. (2013a)	*L. pratense* (2*x*)	–	–	–	nt	NFL	NFL
Epichloë occultans	Non-culturable	Moon et al. (2000)	*Lolium* spp. (2*x*)	–	nt	PAX	nt	NFL	NFL
PauTG-2	e55	Moon et al. (2004)	*Poa autumnalis*	nt	nt	nt	nt	NFL	NFL

[a]Pre. = predicted from genome sequence or genetic screen; Obs. = major alkaloids observed. Abbreviations: AcAP = 1-acetamidopyrrolizidine, CC = chanoclavine, EN = ergonovine, ERV = ergovaline, LAA = lysergic acid amide (= ergine), LAH = lysergic acid α-hydroxyethylamide, LTB = lolitrem B, NANL = *N*-acetylnorloline, NFL = *N*-formylloline, nt = not tested, PAX = paxilline, TD = terpendoles. – = none detected

[b]When multiple strains were analyzed, an example is listed. Strains listed with ATCC and MYA prefixes are in the American Type Culture Collection. CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

[c]PI = Plant introduction number, United States Department of Agriculture-Agriculture Research Service

[d]Ploidies of *Lolium* species are indicated as 2*x* = diploid, 4*x* = tetraploids, 6*x* = hexaploid, 10*x* = decaploid

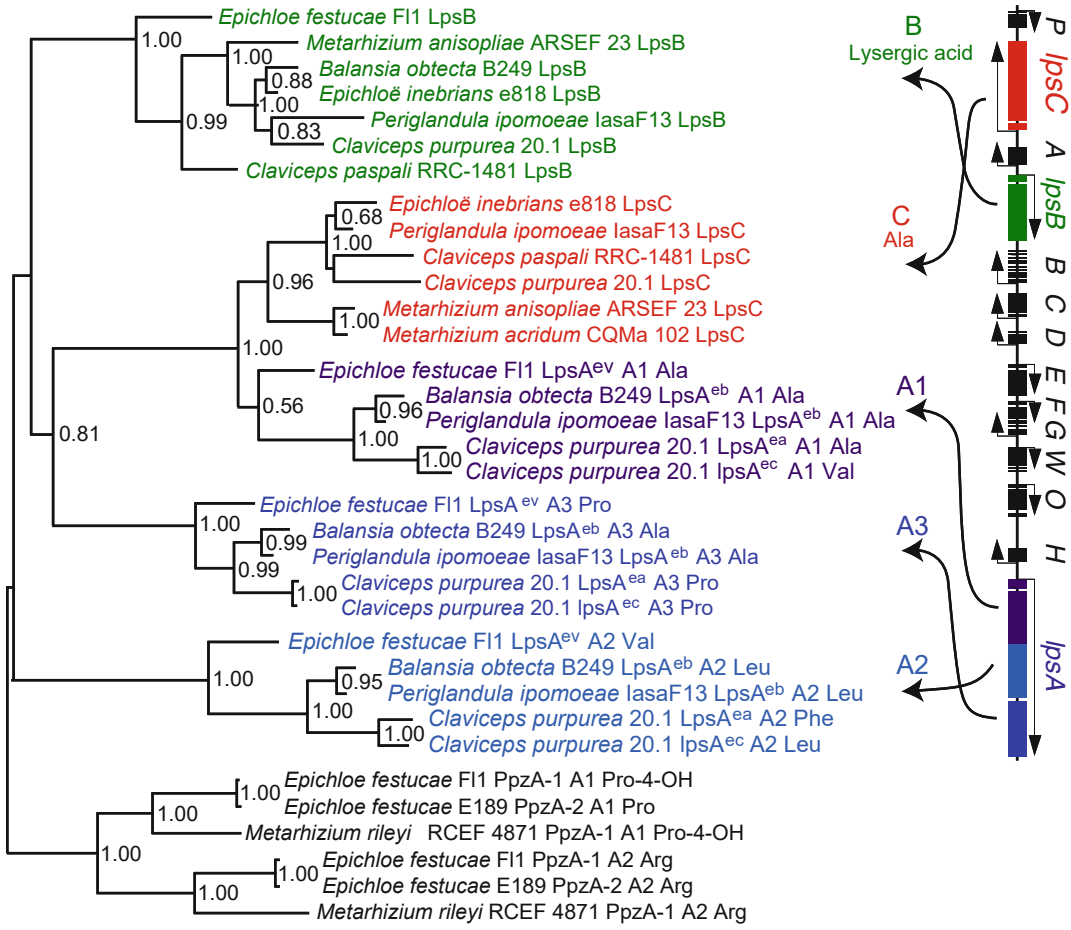

Fig. 5.3 Phylogeny relating A domains of nonribosomal peptide synthetases (NRPSs) involved in biosynthesis of ergot alkaloids and the pyrrolopyrazine peramine. Horizontal branch lengths are proportional to estimated evolutionary times, and vertical branch lengths are arbitrary. Superscripts following "LpsA" indicate the ergopeptine product as ergovaline (ev), ergobalansine (eb), ergotamine (ea), or ergocryptine (ec) (Haarmann et al. 2008; Florea et al. 2017). Substrate specificities of A domains of LpsA are indicated as L-alanine (Ala), L-proline (Pro), L-valine (Val), L-leucine (Leu), or L-phenylalanine (Phe). Substrate specificities of A domains of PpzA-1 and PpzA-2 are indicated as L-arginine (Arg), L-proline (Pro), or 4-hydroxyproline (Pro-4-OH) (Berry et al. 2019). A map of the ergot-alkaloid biosynthesis (*EAS*) gene cluster of *Periglandula ipomoeae* is shown at the right

substituent donated by LpsB. This inferred ancestral substrate specificity is also consistent with the fact that both ergovaline and ergobalansine (as well as many other ergopeptines) have an Ala residue in the corresponding position. It appears, therefore, that LpsC has evolved from the first module of LpsA, but with a reductase domain substituted for the condensation domain.

It is rare for a fungus to contain all genes required for both the ergopeptine branch and the ergonovine (and LAH and ergine) branch, but all such genes are represented in the *EAS* cluster of *P. ipomoeae* p4806 (Schardl et al. 2013a). Gene losses are evident in *Metarhizium* species, *Balansia obtecta*, *Claviceps* species and *Epichloë* species, giving a wide diversity of alkaloid profiles (Fig. 5.4) (Florea et al. 2017). On the other hand, variation in the amino acids specified by the LpsA subunit gives a large variety of ergopeptines. Consequently, there have been

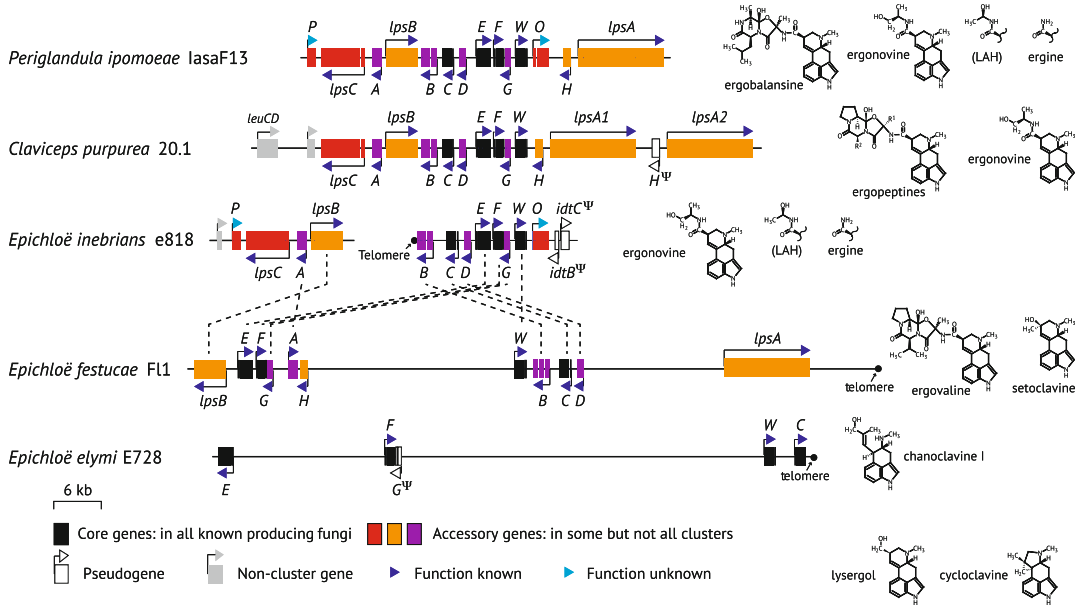

Fig. 5.4 Maps of *EAS* gene clusters in selected fungi, and major end products of their ergot alkaloids pathways. Also shown are lysergol and cycloclavine, products found in some *Ipomoea* species with symbiotic *Periglandula* species (Beaulieu et al. 2015). Names of *eas* genes and *dmaW* are abbreviated with their last letter, and *cloA* is designated *B*. Pseudogenes are indicated by ψ

two main sources of ergot-alkaloid diversification in the Clavicipitaceae: (i) neofunctionalization of genes encoding LpsA variants, mostly within the genus *Claviceps*, account for the vast majority of the 20 natural ergopeptines so far discovered, and (ii) gene loss leading to a buildup of intermediates and spur products.

Chanoclavine I (Fig. 5.4) is the first intermediate common to all ergot-alkaloid producers and is generated from primary metabolites in four enzyme steps catalyzed by 4-γ,-γ-dimethylallyltryptophan synthase (= DMATS) (reviewed in Florea et al. 2017), 4-γ,-γ-dimethylallyltryptophan *N*-methyltransferase, a flavin-containing oxidase, and a catalase. Phylogenetic analysis of the concatenated sequences of the corresponding core genes—*dmaW*, *easF*, *easE,* and *easC*—suggests paralogy due to a duplication of the cluster, and that such an event was associated with movement of the *EAS* cluster from a subterminal location (near a telomere) to a more deeply internal location on a chromosome (Florea et al. 2017). It is worth bearing in mind that translocation in a sexual species effectively generates paralogy. Consequently, one orthologous set of *EAS* clusters has so far only been found in *Epichloë* species, where the complete cluster is associated with ergovaline biosynthesis. The other orthologous set has been found in *Metarhizium*, *Atkinsonella*, *Balansia*, *Periglandula*, *Claviceps* and, rarely, in *Epichloë*, of which *Periglandula* species*, Claviceps* species, *Epichloë inebrians,* and a yet unnamed *Epichloë* species in the grass *Achnatherum robustum* (Shymanovich et al. 2015) can produce ergonovine. Those with *easO* and *easP* genes can also produce LAH and high levels of ergine, the simple amide of lysergic acid. Low levels of ergine are produced by other ergopeptine producers, and in *Epichloë hybrida* ergine production requires *lpsA* (Panaccione et al. 2003). Thus, the basis for ergot-alkaloid diversification in genus *Epichloë* was an instance of *EAS* cluster duplication, and several instances of gene deletion (Florea et al. 2017).

Periglandula species, in their symbioses with plants of tribe Ipomoeeae, also exhibit diversity in ergot alkaloids (Beaulieu et al. 2015) due to presence or absence of some of the above metabolites, as well as sporadic occurrence of lysergol and cycloclavine (Fig. 5.4), both of which are probably produced by simple oxidation of agroclavine. Analysis of *Ipomoea amnicola* in this study revealed a small amount of lysergol, together with chanoclavine I, ergobalansine, and ergine. In contrast, the profile in *Ipomoea hildebrandtii* uniquely included—and was dominated by—cycloclavine. The previous identification of cycloclavine in *Aspergillus japonicus*, a saprophytic member of a different fungal order (Eurotiales), is attributed to its distant homolog of the ergopeptine cyclol-forming enzyme EasH (Jakubczyk et al. 2016). The basis for the occurrence of cycloclavine in *I. hildebrandtii*—whether by convergent evolution or acquisition of a suitable *easH* gene—will be interesting to address in future research.

5.4.2 Indole-Diterpene Alkaloids, Including Lolitrems

Indole-diterpenes (Fig. 5.5) consist of a cyclic diterpene derived from geranylgeranyl diphosphate (GGPP) and an indole moiety likely from a tryptophan precursor such as indole-3-glycerol phosphate. The indole-diterpenes have been observed in members of the order Hypocreales, *Epichloë* (e.g., lolitrem B and epoxy-janthitrems), *Claviceps* (e.g. paspalitrems), *Periglandula* and *Tolypocladium alba* (*Chaunopycnis alba*; e.g., terpendole E) as well as the Eurotiales, *Penicillium* (e.g. paxilline), *Aspergillus* (e.g. aflatrem) (Cook et al. 2019; Motoyama et al. 2012; Saikia et al. 2008). The first indole-diterpene biosynthetic gene cluster (*IDT*) required to produce paxilline was characterized in *Penicillium paxilli* (Scott et al. 2013; Young et al. 2001), followed by identification and characterization of related gene clusters in *Aspergillus flavus* (Nicholson et al. 2009; Zhang et al. 2004), *E. festucae* (Young et al. 2006; Saikia et al. 2012), *Claviceps paspali* (Kozák et al. 2018; Schardl et al. 2013a), and *Periglandula ipomoeae* (Schardl et al. 2013a). Indole-diterpenes have been reported to modulate various ion channels causing toxicity to insects and thermogenicity in mammals (reviewed in Saikia et al. 2008).

The core indole-diterpene pathway starts with synthesis of the GGPP moiety by a pathway-specific GGPP synthase encoded by the *idtG* gene in *Epichloë* species (Young et al. 2005). Most fungi that produce indole-diterpenes have two geranylgeranyl diphosphate synthase copies, one involved in primary metabolism and the other as part of the indole-diterpene biosynthetic gene cluster (Kozák et al. 2018; Schardl et al. 2013a). Biosynthesis of paspaline also requires prenyl transferase IdtC to generate geranylgeranyl indole, followed by epoxidation and cyclization steps catalyzed by FAD-dependent monooxygenase IdtM and protein IdtB of unknown function (Tagami et al. 2013). Diversification of the indole-diterpenes beyond paspaline requires a discontinuous distribution of cluster genes, pseudogenization of pathway-specific genes, and promiscuity of the pathway-specific P450 monooxygenases (IdtP, IdtQ, IdtJ, and IdtK) and prenyl transferases (IdtF and IdtE).

The catalytic versatility and evolutionary diversification of monooxygenases and prenyl transferases results in a metabolic grid providing broad indole-diterpene diversity within the *Epichloë* species (Saikia et al. 2008). So far, only some sequenced *E. festucae* genomes—including *E. festucae* var. *lolii* and some polyploids with an *E. festucae* progenitor—possess the *idtJ* and *idtE* genes required for lolitrem B (Christensen et al. 1993; Takach et al. 2012). Both genes apparently evolved from duplicated *IDT* genes: *ltmJ* from a copy of *idtK*, and *ltmE* by fusion of copies of *idtC* and *idtF* (Schardl et al. 2013a).

Recently, four additional genes—*jtmD* (encodes a prenyl transferase), *jtmO* (encodes an FAD binding oxidoreductase), *jtm01* (encodes a P450 monooxygenase), and *jtm02* (unknown function)—have been identified in *Epichloë* strains that produce epoxy-janthitrems, which share a common indole-diterpene backbone to lolitrem B (Ludlow et al. 2019). The distribution

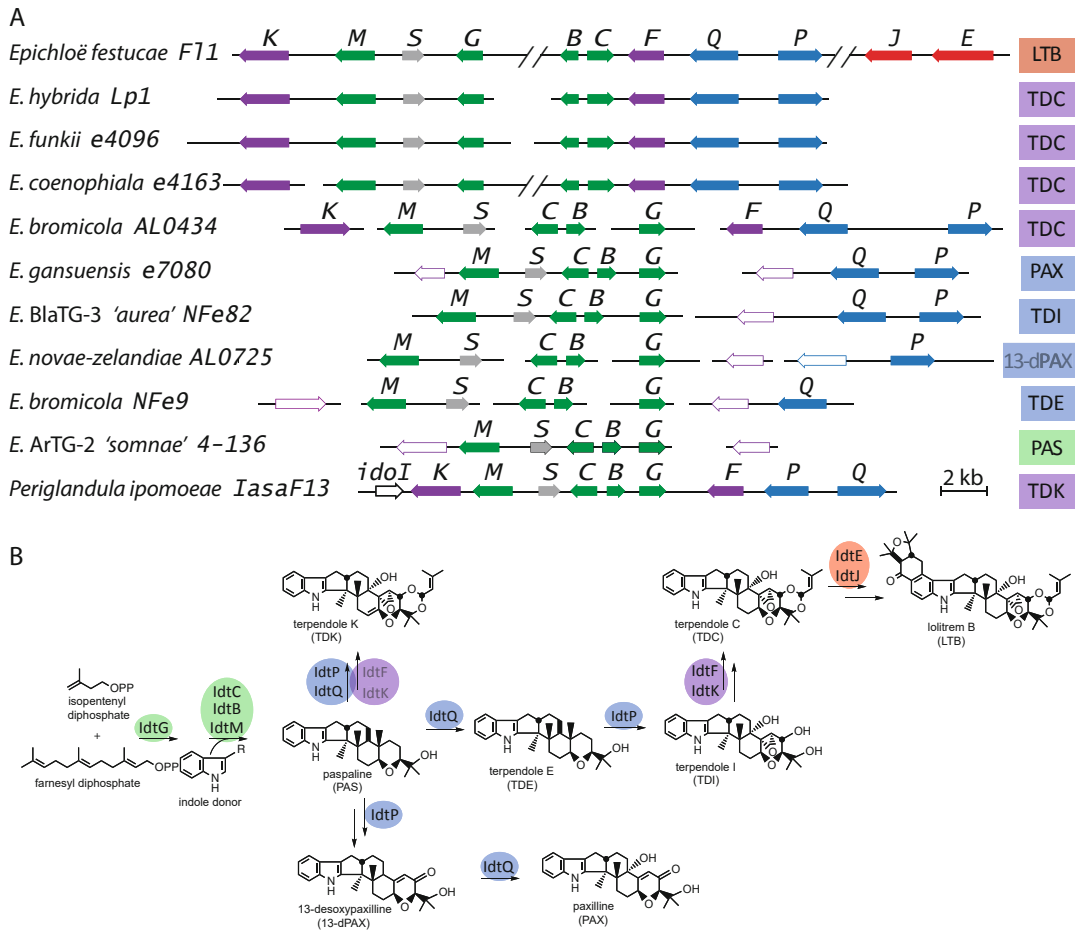

Fig. 5.5 Indole-diterpene gene clusters and pathway summary for selected fungi. (**a**) Gene maps for strains of *Epichloë* species and *Periglandula ipomoeae*, with their major pathway end products indicated at right as LTB (lolitrem B), TDC (terpendole C), PAX (paxilline), TDI (terpendole I), 13-dPAX (13-desoxypaxilline), TDE (terpendole E), PAS (paspaline), and TDK (terpendole K). (**b**) Summary of the pathways for the aforementioned indole-diterpenes, with roles of gene products indicated. Color coding corresponds to genes and products listed in panel A. Names of *idt* genes are abbreviated with their last letter

of the janthitrems-associated genes were thought to be limited to a few *Epichloë* isolates that are associated with perennial ryegrass (Ludlow et al. 2019), but genome sequencing of over 80 *Epichloë* genomes has revealed at least one additional isolate, *Epichloë siegelii* ATCC 74483, with the four *jtm* genes and a few *Epichloë bromicola* isolates have corresponding pseudogenes (C. A. Young and C. L. Schardl, unpublished data).

Functional IDT clusters have been identified in five haploid *Epichloë* species (11 isolates) and 10 polyploid species (15 isolates) representing 32% of the 80 isolates for which genomes have been sequenced. In addition, six isolates have recognizable *IDT* genes present but do not contain a functional gene set for production of indole-diterpenes, which indicates that gene loss has occurred across genus *Epichloë*. The 26 isolates with functional genes produce a diversity of indole-diterpenes, largely explained by various gene presence/absence polymorphisms (Fig. 5.5).

5.4.3 Pyrrolizidine Alkaloids, Including Lolines

Lolines (Schardl et al. 2007) are pyrrolizidines (specifically, 1-aminopyrrolizidines) that are structurally and biosynthetically distinct from the necine class of plant pyrrolizidines, although it is interesting that lolines are produced both by fungi and by certain legumes in which fungal symbionts have not been found. Among fungi, lolines are known from several *Epichloë* species as well as *Penicillium expansum* (Chen et al. 2017). Corresponding *LOL* gene clusters are in these fungi (Ballester et al. 2014; Schardl et al. 2013a) as well as fungi in other orders (C. L. Schardl, unpublished observations). Almost all known diversity of lolines produced by fungi and legumes is based on variations in substituents of the 1-amine (Powell and Petroski 1992).

Three major loline alkaloids *N*-formylloline (NFL), *N*-acetylnorloline (NANL), and *N*-acetylloline (NAL) are variously found in plants with *Epichloë* species and strains possessing the biosynthetic genes (Schardl et al. 2007). Some strains produce 1-acetamidopyrrolizidine (AcAP) as the end product. The associations of *LOL* genes with these alkaloids are shown in Fig. 5.6. The full cluster of 11 genes is required for NFL. Strains lacking all the *LOL* genes except *lolN, lolM,* and *lolP* accumulate NANL, and those also lacking functional *lolO* accumulate AcAP (Schardl et al. 2013a). Curiously, although simple losses of *lolN* or *lolO* should give those respective chemotypes, we consistently see that the *lolM* and *lolP* genes for downstream steps are also absent or dysfunctional in natural *lolN* or *lolO* mutants. This pattern suggests natural selection against genes for enzymes that have come to lack access to their normal substrates. A further mechanism of alkaloid diversification is modification by a plant enzyme. Specifically, some host plant genotypes can convert loline—which has a single methyl group on the 1-amine—to *N*-acetylloline (NAL) (Pan et al. 2014b).

LolO catalyzes both oxidative steps to convert AcAP to NANL by insertion of a strained ether bridge (Pan et al. 2018), which is especially surprising considering that the two methylene carbons involved are non-activated. Also surprising is that several *Epichloë brachyelytri* and *Epichloë canadensis* strains, as well as an *Epichloë* species in *Trisetum spicatum* (Buckley et al. 2019; Charlton et al. 2012a) lack a functional *lolO* gene, and therefore produce AcAP as the pathway end-product (Pan et al. 2014a). This characteristic is shared by *Atkinsonella hypoxylon* B4728, another clavicipitaceous symbiont that is capable of seed transmission in a grass host (Philipson and Christey 1985). These fungal lineages have lost *lolO* and genes for the downstream steps. Coincidentally, they have also lost functional *EAS* genes for steps downstream of chanoclavine, such that both pathways end with simpler compounds that are pathway intermediates in other species. Similarly, some strains of *E. canadensis* are apparently derived as hybrids of *Epichloë elymi* chanoclavine accumulators and *Epichloë amarillans* AcAP accumulators, though chemotypes of *E. canadensis* vary widely (Schardl et al. 2013a; Pan et al. 2014a). The repeated finding of *Epichloë* chemotypes with both chanoclavine and AcAP as pathway end-products warrants further investigation into possible synergistic roles of those two alkaloids in biological protection.

5.4.4 Pyrrolopyrazine Alkaloids, Including Peramine

The basis for diversification of fungal pyrrolopyrazine alkaloids was recently elucidated and constitutes a remarkably complex story considering that biosynthesis of each is based on only a single NRPS protein (Berry et al. 2019). That pyrrolopyrazine synthetase is multifunctional, with between five and seven enzyme domains depending on the allele (Fig. 5.7). All seven domains are required for biosynthesis of peramine, a pyrrolopyrazine with a methyl group attached to one nitrogen and the other amide bond reduced and desaturated (**1a**, Fig. 5.7). The presence of a C-terminal reductase (R_2) domain is consistent with that structure and

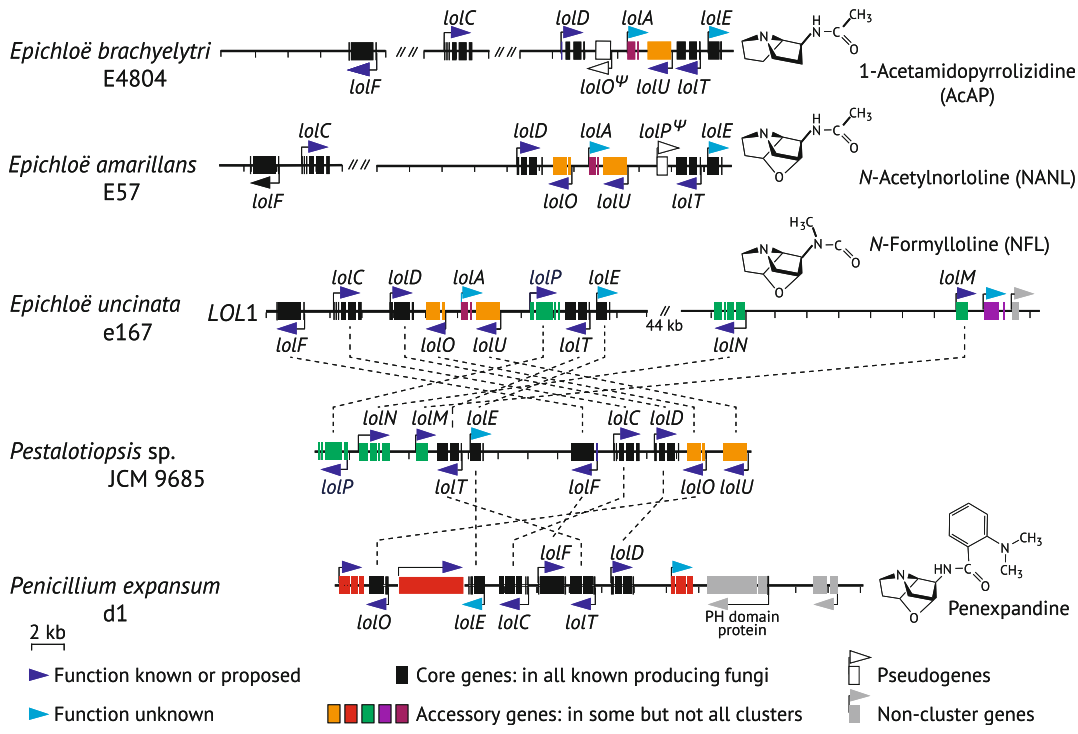

Fig. 5.6 Maps of *LOL* gene clusters in selected fungi and, where known, major end products of their loline alkaloid pathways. Pseudogenes are indicated by ψ

is expected to catalyze reductive cyclization with release of the penultimate intermediate, which is then expected to undergo a spontaneous oxidation to form peramine (Tanaka et al. 2005).

Genome sequencing has revealed that in several *Epichloë* strains homologs of the peramine biosynthesis gene (*ppzA* = *perA*) lack the R_2 domain, apparently due to insertion of an MITE (a small transposable element) (Berry et al. 2015). In all, alleles lacking the R_2 domain (R_2–) have been identified in seven species, and those with the R_2 domain (R_2+) have been identified in nine species (Berry et al. 2015). Remarkably, five species show variation between isolates, with some having complete *ppzA* alleles (*ppzA-1*) and some lacking the R_2 domain (R_2–) (*ppzA-2, ppzA-3,* and *ppzA-4*). Phylogenetic analysis indicates that the R_2+ and R_2– alleles have been maintained through most or all speciation events within the genus *Epichloë*, with multiple recombination events between such alleles (Fig. 5.7; Berry et al. 2015, 2019). Thus, the diversification of *ppzA* alleles is the apparent result of subfunctionalization, recombination, and trans-species polymorphism.

Most NRPS proteins require a terminal domain that facilitates substrate release. However, the presence of proline or hydroxyproline as the penultimate residue allows for spontaneous cyclization that releases the product. Therefore, because peramine is a pyrazine-1-one derived from a dimer of hydroxyproline and arginine, deletion of the R_2 domain has altered, rather than eliminated, functionality of the enzyme (Berry et al. 2019). Subsequent changes in specificity of the A_1 and T_1 domains and in some cases loss of methylation domain function, as well as recombination between alleles, have led to isoforms that favor formation of various pyrazine-1,4-diones (Table 5.2).

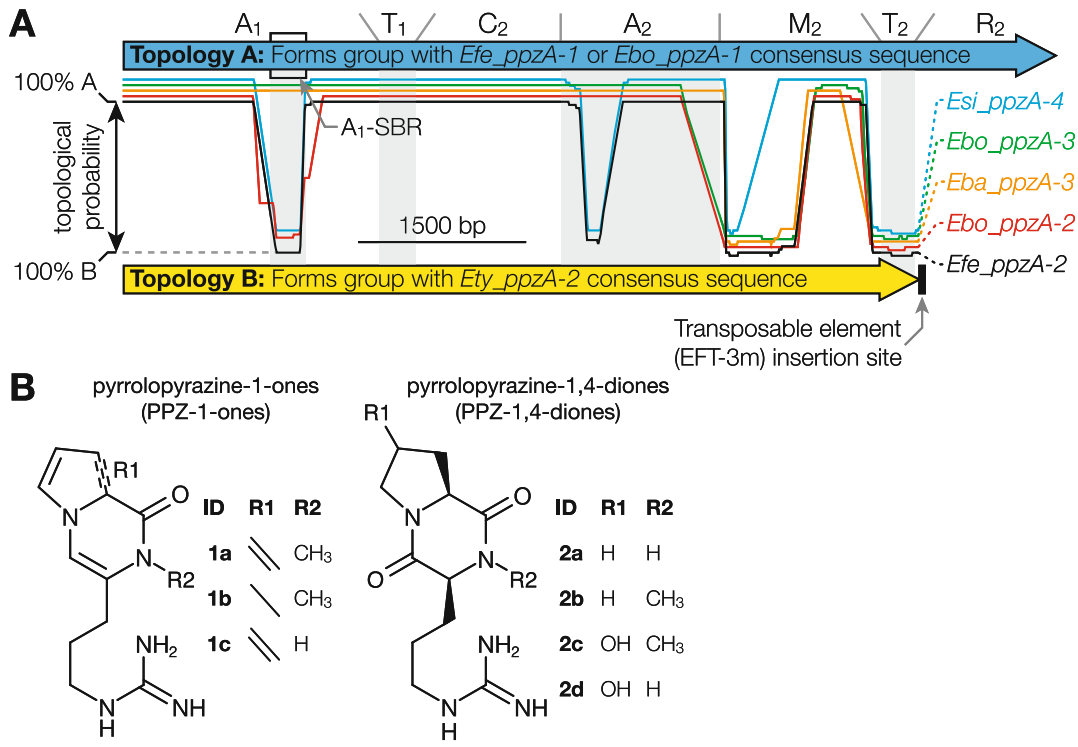

Fig. 5.7 Functionally divergent regions of PpzA-1 and PpzA-2 proteins and pyrrolopyrazine alkaloids. (**a**) Sliding window phylogenetic analysis indicated recombination blocks in *ppzA* alleles 2, 3, and 4. (**b**) Structures of peramine (**1a**) and related pyrrolopyrazines attributable to the corresponding PpzA variants. Adapted from Berry et al. (2019)

5.4.5 Indolizidine Alkaloids Including Swainsonine

Swainsonine (Fig. 5.8) is an indolizidine alkaloid produced by fungi associated with plants as well as fungi that are only associated with mammals (Cook et al. 2017). A related alkaloid, slaframine (Fig. 5.8), is only reported to be produced by *Slafractonia leguminicola,* which also produces swainsonine. The *SWN* cluster of biosynthetic genes was identified by comparative genome analyses of swainsonine-producing fungi in orders Hypocreales, Pleosporales, Onygenales, and Chaetothyriales, and class Leotiomycetes (all in Ascomycota subphylum Pezizomycotina), and contains genes predicted to encode a multi-domain nonribosomal peptide synthetase and polyketide synthase (SwnK), two reductases (SwnN, SwnR), and two dioxygenases (SwnH1, SwnH2). Also present in some but not all are genes for an amino transferase (*Swn*A) and a putative transmembrane transporter (SwnT). The requirement for *swnK* was demonstrated using *Metarhizium robertsii* (an entomopathogen and plant root symbiont) through gene "knockout" and complementation (Cook et al. 2017). Roles have also been demonstrated for SwnA in biosynthesis of the pipecolic acid precursor, and for SwnN, SwnH2, and SwnH1 in biosynthesis of swainsonine (Luo et al. 2020).

Among *Alternaria* sect. *Undifilum* species (locoweed endophytes) there is high conservation within *swnK*, and only 1–3 amino-acid changes in SwnK between species (Noor et al. 2020). Sequence conservation among the other *SWN* genes is much lower. The *swnA* gene has been found in Chaetothyriales (*I. carnea* endophyte), Hypocreales, and Onygenales, but not in

Table 5.2 Pyrrolopyrazine profile of grasses with symbiotic *Epichloë* strains

				Pyrrolopyrazine concentration[a] (μg/g)						
Allele	Endophyte	*n*	Host	[1a]	[1b][b]	[1c][b]	[2a]	[2b][c]	[2c][c]	[2d][c]
ppzA-1	*Epichloë bromicola*	2	*Hordeum bogdanii*	30.5	0.3	nd	nd	nd	1.3	0.2
ppzA-1	*Epichloë festucae* var. *lolii*	1	*Lolium perenne*	19.1	nd	nd	nd	nd	6.0	nd
ppzA-1	*Epichloë poae*	4	*Bromus laevipes*	104.9	0.2	nd	nd	nd	13.5	nd
ppzA-1	*E. poae*	1	*Poa pratensis*	12.5	0.4	nd	6.5	nd	3.0	nd
ppzA-1	*Epichloë typhina*	1	*L. perenne*	347	0.3	LOQ	4.3	4.0	58.3	nd
ppzA-2	*Epichloë festucae*	3	*Festuca rubra*	nd	nd	nd	17.5	0.6	nd	nd
ppzA-2	*E. poae*	3	*Poa nemoralis*	nd	nd	nd	68.1	nd	nd	nd
ppzA-2	*E. typhina*	3	*Dactylis glomerata*	nd	nd	nd	445	nd	nd	0.2
ppzA-3	*Epichloë baconii*	2	*Agrostis tenuis*	nd	nd	nd	5.1	nd	nd	0.3
ppzA-3	*E. baconii*	1	*A. tenuis*	nd	nd	nd	nd	nd	nd	0.4
ppzA-3	*E. bromicola*	2	*H. bogdanii*	nd	nd	nd	nd	nd	nd	5.1
ppzA-3	*E. bromicola*	2	*Hordeum brevisubulatum*	nd	nd	nd	nd	nd	nd	10.4
ppzA-4	*Epichloë siegelii*	4	*Lolium pratense*	nd	nd	nd	2.0	500	0.9	nd
ppzA-5	*Epichloë uncinata*	1	*L. pratense*	nd	nd	nd	nd	nd	2.5	nd
N/A	None	3	*B. laevipes*	nd	nd	nd	nd	nd	nd	nd

[a]Pyrrolopyrazine structures are given in Fig. 5.7. Abbreviations are: nd = absent or below the limit of detection (ca. 0.05 μg/g); LOQ = concentration below the limit of quantitation (0.2 μg/g)
[b]Concentration estimated using the response factor of synthetic **1a**
[c]Concentration estimated using the response factor of synthetic **2a**

Pleosporales, and *swnT* has been found in Hypocreales and Onygenales, but only in *Slafractonia leguminicola* among the Pleosporales (Cook et al. 2017). The functional implications of these differences have yet to be determined. Some members of additional orders of Pezizomycotina—namely, Xylariales, Microthyriales, Caliciales, Patellariales, and Eurotiales—have also been found to have *swnK*, *swnH*2, *swnN*, *swnH*1, *swnR*, and sometimes *swnA* or *swnT* (Neyaz et al. 2022a).

Considering that slaframine is structurally similar to swainsonine, *S. leguminicola* was expected to have another gene related to *swnK*. Surprisingly it actually has two *swnK* paralogs, suggesting that one or more fungal indolizidines have yet to be identified (Cook et al. 2017).

5.5 Evolutionary Relationships, Hybridization, Polyploidy, and Horizontal Gene Transfers

Through phylogenetic and genomic analysis much is known about the ploidies and sometimes complex origins of *Epichloë* species. To date, 20 haploid species have been described (Leuchtmann et al. 2014; Leuchtmann and Schardl 2022; Tadych et al. 2012; Thunen et al. 2022; White 1993) most of which have a known

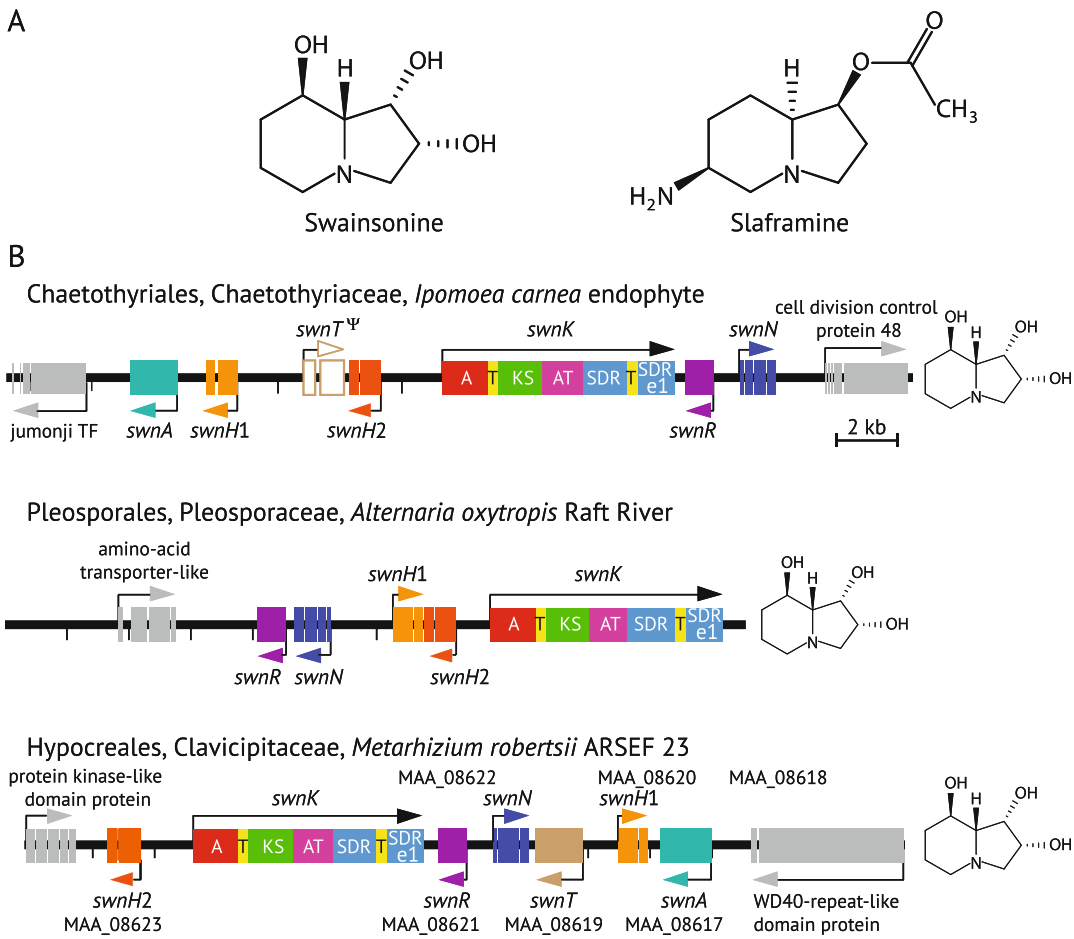

Fig. 5.8 Fungal indolizidine alkaloids and swainsonine biosynthesis gene clusters. (**a**) Structure of swainsonine, characteristic of symbioses of legumes with *Alternaria* sect. *Undifilum* species and plants infected with *Slafractonia leguminicola*, and of slaframine, which is also produced by *S. leguminicola*. (**b**) Maps of *SWN* cluster of swainsonine biosynthesis genes in fungal endophytes of *Ipomoea carnea* and *Oxytropis sericea*, and in *Metarhizium robertsii*. Domains of the multifunctional SwnK enzyme are color coded and described in Cook et al. (2017)

sexual state. Many other *Epichloë* species are diploid or triploid, having arisen by interspecific hybridization. In contrast, only one genome has been sequenced for each of genus *Periglandula*, genus *Alternaria* sect. *Undifilum*, and the Chaetothyrialean endophyte in *Ipomoea* species. The genome of *Periglandula ipomoeae* is apparently haploid (Schardl et al. 2013a), as is that of the Chaetothyrialean endophyte of *I. carnea* (Cook et al. 2017), but the *Alternaria oxytropis* Raft River strain may be diploid or aneuploid (D.D. Cook, R. Creamer, and C.L. Schardl, unpublished data).

5.5.1 *Haploid* Epichloë *Species*

The *Epichloë* species originally described as *E. typhina*, living within cool-season grasses (subfam. Poöideae) can act as replacement pathogens by shutting down seed production on affected host tillers when the fungus initiates its own sexual cycle. In doing so, these fungi exhibit

a remarkable characteristic that they share with many other plant-associated Clavicipitaceae; they persist in most aerial portions of the plant without causing symptoms and only produce a conspicuous external structure—called a stroma—on a specific location of the host. For example, *Balansia henningsiana* and *Balansia epichloë* can produce a stroma on a small segment of each leaf, *Balansia strangulans* produces a stroma on a node of each culm, and *Atkinsonella* species as well as several *Balansia* species produce a stroma engulfing the immature host inflorescence along with associated leaves (White et al. 2002). The *Epichloë* stroma similarly "chokes" an immature inflorescence, overgrowing it and the surrounding leaf sheath but, unlike *Atkinsonella* species, *Balansia cyperi, Balansia obtecta,* and other choking species, the *Epichloë* choke affects little or none of the flag leaf blade (White et al. 1991) (Fig. 5.9).

What sets apart most *Epichloë* species as well as *Atkinsonella hypoxylon* from those other Clavicipitaceae is that some—often most—flowering tillers show no stromata at all, and the endophyte is seed-transmitted instead (Clay and Schardl 2002). The degree to which the fungus manifests choke versus seed transmission is largely a characteristic of the *Epichloë* species, but nevertheless varies by fungus and host genotypes and environmental conditions. Most strains of *E. typhina* (*sensu stricto*) are "strong chokers," producing stromata on nearly every infected tiller. However, seed transmission of *E. typhina* is known in the grass *Puccinellia distans* (Lembicz et al. 2009), and closely related seed-transmissible species are *Epichloë sylvatica* in *Brachypodium sylvaticum*, and *Epichloë poae* in several *Poa* species (Leuchtmann and Schardl 1998; Tadych et al. 2012). Most other *Epichloë* species are seed-transmissible, with the sole exceptions currently known being *Epichloë baconii* in *Agrostis* species, *Epichloë calamagrostidis* in *Calamagrostis* species, *Epichloë glyceriae* in *Glyceria striata*, and *E. bromicola* in *Bromus erectus*, one of its many hosts (Leuchtmann and Schardl 1998, 2022; Schardl and Leuchtmann 1999).

Fig. 5.9 Mature stromata ("chokes") of *Epichloë glyceriae* formed on its host grass *Glyceria striata*

Almost every known sexual species of *Epichloë* grows as a haploid. The sole exception is *Epichloë liyangensis* reported to be a sexual species derived by interspecific hybridization (Kang et al. 2011). Sexual *Epichloë* species are monoecious and have a bipolar mating system governed by the mating-type locus with two idiomorphs, designated *MTA* and *MTB*. The three *MTA* genes are designated *mtAA*, *mtAB,* and *mtAC*, whereas *MTB* has only the gene *mtBA*. When evaluating the population structure of strongly choking *Epichloë* strains, such as that of *E. typhina* on *Dactylis glomerata*, both mating types appear to be in equilibrium (Bushman et al. 2018). Yet, the population structure can be heavily skewed for a single mating type in *Epichloë* species that are predominantly seed transmitted (von Cräutlein et al. 2021). Several other haploid species have been identified for which stroma production has never been observed, and for the most part those have not had mating types analyzed. Considering that the sexual state has rarely been observed for *E. brachyelytri* (Schardl and Leuchtmann 1999)

and *Epichloë gansuensis* (Li et al. 2015), it is possible that in the cases of *E. aotearoae* (Moon et al. 2002), *E. inebrians* (Chen et al. 2015), *Epichloë sibirica* (Zhang et al. 2009b), and *Epichloë stromatolonga* (Ji et al. 2009) the sexual state exists but has not yet been reported.

Even among haploid *Epichloë* species with known sexual cycles, some seem never to form stromata in certain hosts because the endophyte genotypes in those hosts are incapable of, or the host suppresses, stroma development (Brem and Leuchtmann 2003; Tadych et al. 2012). Of those asexual haploids, the best known is *E. festucae* var. *lolii* in *L. perenne*. Prior ubiquity of toxic *E. festucae* var. *lolii* strains in New Zealand and Australia made ryegrass staggers a frequent and major problem (di Menna et al. 2012), but increasingly cultivars with strains non-toxic to livestock are being deployed (Bluett et al. 2005a, b; Fletcher and Sutherland 2009).

Other *Epichloë* species or strains form stromata but rarely exhibit the sexual state. Although *E. poae* is sexual on *Poa nemoralis* and *Poa pratensis* in Europe (Schirrmann et al. 2015), such that they produce perithecia bearing the meiotically-derived ascospores, *E. poae* stromata observed on North American *Poa* species have consistently lacked perithecia (Tadych et al. 2012). Infected plants bear infected seeds, but it is conceivable that conidia (mitotic spores) produced in abundance on unfertilized stromata can also provide for horizontal (contagious) transmission, perhaps by water dispersal. Whether asexual *Epichloë* species ever transmit horizontally is an open question, so it is noteworthy that Oberhofer and Leuchtmann (2014) obtained evidence suggestive of horizontal transmission of an asexual *Epichloë* species between *Hordelymus europaeus* plants, and established symbioses by germinating seeds in fungal cultures, a similar experimental approach as used by Tadych (2014) for *E. poae*.

Species identity may arise by specialization for host, specialization of the symbiotic *Botanophila* species flies, or a combination of both. Several *Botanophila* species lay their eggs on *Epichloë* stromata (Leuchtmann and Michelsen 2016), simultaneously transferring spermatia endozoochorously (via the gut), and thereby cross-fertilizing ("pollinating") the fungus. There is not species-by-species specificity for such interactions. Furthermore, although specific preference of individual flies for *E. typhina* stromata over *E. clarkii* stromata has been documented (Bultman and Leuchtmann 2003), the potential role of flies in reproductive isolation of *Epichloë* species appears to be minor at best (Bultman and Leuchtmann 2008). In fact, although *E. typhina* is interfertile with *E. clarkii*, assortative mating and reduced hybrid fitness is observed, thus demonstrating some pre- and post-zygotic genetic speciation mechanisms (Treindl and Leuchtmann 2019).

5.5.2 *Polyploid* Epichloë *Species*

The majority of *Epichloë* species described to date are polyploid, asexual, and only known to transmit vertically from mother to daughter plant. There is not a strict correlation between sexual versus asexual reproduction, stroma formation, and ploidy. However, many that produce no stromata are likely to be transmitted only vertically and have hybrid genomes that appear to include most of the genomes from two or more haploid species (Leuchtmann et al. 2014, 2019; Moon et al. 2004, 2007; Tian et al. 2020). A dramatic example is *E. coenophiala*, which is triploid with genomes from *E. poae, E. festucae,* and a third contributor in the *Lolium*-associated endophyte (LAE) clade not yet identified among haploids (Tsai et al. 1994).

An interesting consequence of interspecific hybridization is the considerable potential for diversification of chemotypes (Schardl et al. 2013b). For example, different strains of *E. coenophiala* have different chemotypes. Strain e4163 in tetraploid *Lolium interruptum* (Banfi et al. 2017) from Southern France possesses functional *EAS, IDT,* and *LOL* clusters and *ppzA* genes and produces ergovaline, terpendoles, NFL, and peramine as the respective pathway end products (Schardl et al. 2013b). Strain e4309 from *Lolium atlantigenum* lacks the *EAS* cluster and therefore produces no ergovaline or other ergot alkaloids, but it produces terpendoles,

NFL, and peramine (Schardl et al. 2013b; Stephen Lee and D. D. Cook, unpublished data). Strains in hexaploid *L. arundinaceum* from Northern Europe (continental tall fescue), which were widely planted in the USA in the latter half of the twentieth century, lack key *IDT* genes but produce ergovaline, NFL, and peramine (Schardl et al. 2013b). Because of the ergot-alkaloid toxicity of those early continental tall fescue cultivars, Moroccan strains (possibly from *L. atlantigenum*) have been selected and used to replace the toxic strains, generating cultivars that are much more acceptable for livestock (Bouton et al. 2002; Burns and Fisher 2006; Hopkins et al. 2011; Matthews et al. 2005).

Sometimes hybrids have multiple alkaloid gene clusters from multiple ancestors, though in such cases one or more of the clusters may be incomplete. For example, the apparent *E. coenophiala* progenitor inherited *ppzA*-1 copies from all three ancestors, *EAS* clusters from two ancestors, an *IDT* cluster from one ancestor, and a *LOL* cluster from another ancestor (Schardl et al. 2013b). Comparing genome sequence assemblies for strains ATCC 90664 from continental tall fescue and e4163 from *L. interruptum*, each has missing or defective genes in a different *EAS* cluster, which are complemented by the homeologs in the other cluster. In strain ATCC 90664 *lpsB*2 is a pseudogene that is complemented by the subterminal (telomere-associated) *lpsB*1 homeolog (Florea et al. 2016). In contrast, *lpsB*1 and *easE*1 are missing in e4163, apparently having been lost from the chromosome end, but *lpsB*2 and *easE*2 are intact (Schardl et al. 2013b).

A similar situation is evident in *Epichloë uncinata*, which has a *LOL* cluster from each of its ancestors (Spiering et al. 2005b; Pan et al. 2014b). The cluster from *E. bromicola* (Fig. 5.6) is complete in that it has all genes required for NFL production, but the cluster from *E. poae* (not shown) lacks *lolN, lolM,* and *lolP*, which are required for conversion of NANL to NFL. Plants with *E. uncinata* have substantial amounts of NFL, NANL, and NAL, of which NAL production involves enzymes LolN, LolM, and a plant acetyltransferase (Pan et al. 2014b).

Such loss or inactivation of some duplicated alkaloid genes in hybrids may simply be random outcomes of mutations as the lineages diverged, but an intriguing possibility is that mutations that reduce efficiency of the overall pathway are actually beneficial. The rationale proposed by Panaccione (2005) is that pathway inefficiency leads to a buildup of intermediates and spur products in addition to the nominal pathway end-product, thereby providing multiple alkaloids with different specificities and modes of action. This hypothesis supposes that if a cluster is present in two (or more) complete copies the pathway may be especially efficient at producing the end-product. If instead a copy of the gene for a late step in the pathway is lost, the result may be a mixture of products in a bioprotective chemical arsenal. This is an intriguing hypothesis worth further investigation by means of adding or subtracting gene copies, for which new CRISPR-based methods are well suited (Florea et al. 2021).

Epichloë canadensis, the common endophyte of *Elymus canadensis* (Charlton et al. 2012a), presents another example of chemotypic diversification associated with hybridization. The *EAS* cluster is derived from that of *E. elymi*, whereas the *LOL* cluster is from *E. amarillans*. However, different strains either have the clusters for ergovaline and NFL, or clusters with inactivating mutations or loss of some of the genes (Charlton et al. 2012a). All have the *E. elymi* cluster with four *EAS* genes for chanoclavine, but some have a complete *EAS* cluster from *E. amarillans* for ergovaline. Also, the two versions of *LOL*—both from *E. amarillans*—are either sufficient for NANL production or have an inactivating deletion in *lolO* so that they accumulate AcAP (Pan et al. 2014a). Analysis of mating-type idiomorphs suggests multiple origins of different *E. canadensis* strains because some have *E. amarillans MTA* and others have *E. amarillans MTB* (Charlton et al. 2012a).

Another example of multiple hybrids occurring in the same host species is *Bromus auleticus* in Argentina, where different populations have distinct hybrids of *E. festucae* with *E. poae*. Depending on the ancestral *E. poae* genotype as

well as morphology and chemotype, these hybrids are classified either as *E. tembladerae* or *Epichloë pampeana* (Iannone et al. 2009). Interestingly, endophytes occur in Argentine grasses of several tribes and the vast majority of those endophytes (plus one from *Festuca arizonica* in the USA) are *E. tembladerae* (White et al. 2001; Iannone et al. 2013). In some hosts, namely *Festuca fiebrigii* (= *Festuca hieronymi*), *Festuca argentina* and *Poa huecu*, *E. tembladerae* causes tremors and sometimes even death of grazing livestock (Cabral et al. 1999).

Bromus setifolius in Patagonia—the remote southern region of Argentina—also has two distinct endophytes, *E. tembladerae* and a haploid classified as *E. poae* var. *aonikenkana* (Mc Cargo et al. 2014). The latter also appears to be one of the two ancestors to an *E. amarillans* × *E. poae* hybrid (as yet unnamed) found in *Hordeum comosum* from the same region (Iannone et al. 2015), but is phylogenetically distinct from the *E. poae* ancestors of the other Argentine hybrids mentioned above. *Epichloë cabralii* is yet another hybrid from Patagonia, where it is found in *Phleum alpinum* (Mc Cargo et al. 2014). The ancestors of *E. cabralii* are related but phylogenetically distinct from those of the *H. comosum* endophyte. One is *E. poae* and the other has so far defied identification with a known haploid species but has a phylogenetic relationship to both *E. baconii* and *E. amarillans*.

The diversity of the Argentine *Epichloë* species is remarkable considering that the sexual *Epichloë* state has never been observed there, and that grasses in several tribes host polyploids similar enough to be classified together as *E. tembladerae*. These observations suggest a possibility of horizontal transmission of asexual, non-stroma-forming *Epichloë* species among disparate hosts, which would be surprising given their apparent dependence on seed transmission and the usual observation of host specificity among *Epichloë* strains (Christensen et al. 1997). But again, it should be borne in mind that some haploid *Epichloë* species form stromata very rarely, so conceivably such cases may yet exist undetected in that geographical region.

Both *E. tembladerae* and *E. cabralii* are also found in western North America (Charlton et al. 2014; Moon et al. 2004). Interestingly, identical mutations have been identified in key alkaloid biosynthesis genes in *E. cabralii* from *Ph. alpinum* and *B. laevipes* (C. A. Young unpublished data), providing additional support the endophytes are the same species.

Both the broad distribution of some *Epichloë* species and occurrences of multiple *Epichloë* species in individual grass species (even among allopatric plants) are commonly observed (Charlton et al. 2014; Schardl et al. 1994; Leuchtmann and Oberhofer 2013; Oberhofer and Leuchtmann 2012; Shymanovich et al. 2017; Sullivan and Faeth 2004). Considered together with their intraspecific diversity, these seed-borne endophytes represent a dramatic augmentation of the effective diversity of grasses in temperate regions around the world.

5.5.3 Hyphal Fusion and Karyogamy

The occurrence of many hybrids suggests that hyphal fusion followed by karyogamy is facile even between different *Epichloë* species (Schardl and Craven 2003). Indeed, analysis of vegetative compatibility by complementation of nitrate-non-utilizing mutants indicated compatibility even between different species (Chung and Schardl 1997). This observation was confirmed with the visualization of anastomosis in live *Epichloë* cultures, including between *E. festucae* and *E. poae* (Shoji et al. 2015). Strains of the two species were transformed with genes for fusions of histone H2A with fluorescent proteins GFP or TagRFP, respectively, and nuclear migration across anastomosis junctions as well as nuclear fusions was documented by confocal microscopy.

5.5.4 Horizontal Gene Transfer or Not?

Symbiosis is often a major contributor to diversity, vicariously providing host organisms with novel traits that eventually increase their fitness

with respect to their asymbiotic conspecifics and thus drive the evolution of the interacting species. In the case of symbionts transmitted through the germline, analogous to mitochondria and plastids, it is intriguing to consider the possibility of genetic exchange between the partners. So far, transfer of plant genes to endophytic fungi has not been demonstrated. However, *Epichloë* has recently been implicated as the source of several functional genes that have been transferred to grass plant lineages (Wang et al. 2020; Shinozuka et al. 2017, 2020).

Comparative genomics often reveals sporadic distributions of putatively homologous genes with phylogenies that contrast with species phylogenies based on housekeeping genes or rDNA segments. These observations can lead to claims of HGT, but often the results may be explainable instead by gene duplications (paralogy) and losses. In cases involving related species within a genus, divergence analysis can provide further support for HGT, as demonstrated for the fumonisin biosynthetic gene cluster in *Fusarium* (Proctor et al. 2013). However, claims of what we will call *wide HGT*—between families, orders or higher-level taxa—almost always involve homologs that are so divergent as to lack sufficient phylogenetic signal for divergence analysis.

An example of a claimed wide HGT involves the *EAS* clusters in fungal orders Eurotiales and Hypocreales (Marcet-Houben and Gabaldón 2016). However, the analysis included and failed to distinguish between orthologs and paralogs, a recognized flaw in phylogenetic analysis (Thornton and DeSalle 2000). When only functional orthologs are analyzed, the results suggest a single duplication of the *EAS* cluster and numerous losses (Florea et al. 2017). Hypothesizing many losses may seem unparsimonious, but it is clear from remnant genes that deletions of some or all of the *EAS* cluster are very common, and that this is also true of other secondary metabolism gene clusters (Schardl et al. 2013a).

Wide HGT has also been claimed for the *mcf*-like genes in some *Epichloë* genomes. The protein product of the *E. poae* gene (GenBank accession AHY82542.1) has the expected activity against larvae of sod webworm (*Agrotis ipsilon*) (Ambrose et al. 2014). Although it was reported that no homologs were present in other fungi (Ambrose et al. 2014), genes with much better matches than the bacterial genes can now be found, by BLASTp searches, in genome sequence assemblies for six fungal species in three orders of the phylum Basidiomycota (C.L. Schardl, unpublished observation). Conceivably, their distribution may be due to wide HGT between members of Basidiomycota and Ascomycota, but the abundance of other apparent homologs in many genera of both phyla makes this unclear. Nevertheless, the discovery and characterization of *mcf* adds to the catalog of diversity in bioprotective factors encoded in *Epichloë* genomes.

The emergence of questionable claims of wide HGT in eukaryotes illustrates methodological problems (Salzberg et al. 2001; Martin 2017; Salzberg 2017). Such claims most often assert that HGT is the most parsimonious explanation, but this assertion is necessarily based on *assumed* weighting of a so-far mysterious HGT process against the well-known and mechanistically understood phenomena of duplication and loss. If wide HGT is exceedingly rare, a fair analysis should weight such an event very heavily. Unfortunately, searches for putative HGT typically weight it and the alternatives arbitrarily, equally or based on the assumption that HGT of *some* genes have occurred between the taxa in question. Given the circularity of this approach, it would be preferable to find clear examples where the species or genus of origin can be identified based on extremely high sequence identity; a "smoking gun," so to speak.

Interestingly, genus *Epichloë* appears to be such a smoking gun in several recent claims of inter-kingdom HGT into grass genomes. One case has emerged from a search for genes conferring resistance against Fusarium head blight (FHB) in crop wild relatives of wheat, which revealed a gene designated *Fhb7* in *Thinopyrum elongatum* (Wang et al. 2020). A gene highly similar to *Fhb7* was identified in several *Epichloë*

species. The apparent orthologs in *T. elongatum* and *E. aotearoae* are 96.6% identical, encode proteins that are 93.6% identical, have identical translation start and stop positions, and are intronless in both species. This level of identity and the facts that identity extends 16 bp upstream of the coding sequence and that two additional sequences (45 bp and 54 bp) of near identity occur close to the genes in both species (C.L. Schardl, unpublished observation) further support the inference of HGT. Phylogenomic studies suggest that an HGT event occurred in the Triticeae from a relative of *E. aotearoae* to a *Thinopyrum* ancestor 4.7–4.9 million years ago (Ma) (Shinozuka et al. 2020).

Thinopyrum Fhb7 encodes a glutathione *S*-transferase (GST) that catalyzes covalent modification of deoxynivalenol (DON), one of the trichothecenes produced by the FHB pathogen *Fusarium graminearum*. DON is a broad-spectrum cytotoxin of major concern in human food and livestock feed due to its production on diseased ears of cereals such as wheat, oats, and barley. As evidence that DON plays a key role in FHB disease, deletion of the *F. graminearum TRI5* gene renders it unable to produce trichothecenes and results in its significantly reduced virulence to wheat (Maier et al. 2006). The detoxifying activity of the *Fhb7*-encoded enzyme opens the epoxide that is essential for DON activity, and this is the suggested mechanism whereby *Fhb7* enhances disease resistance (Wang et al. 2020). Based on the close sequence identity it is reasonable to hypothesize, and worth testing, that the *Epichloë* orthologs encode enzymes with the same activity. Such an activity may provide a competitive advantage over *Fusarium* DON producers in the seed heads, considering that most *Epichloë* species rely on seeds as their main or only means of dispersal, whereas FHB causes reduced seed production. Although apparent orthologs of *Fhb7* are very common in fungal phyla Ascomycota and Basidiomycota (C.L. Schardl, unpublished observation), the specificity of the enzymes that they encode is unknown. Therefore, it would also be of considerable interest to investigate whether a specific DON-detoxifying activity evolved in the *Epichloë* lineage.

Other fungal genes also seem to have undergone transfer into grass genomes after the evolutionary origin of *Epichloë*-Poöideae symbioses. Interrogation of transcriptomes and genomes from *L. perenne*—a grass species often associated with *E. festucae* var. *lolii* and sometimes with *E. typhina* or *E. hybrida*—revealed three genes designated *Lp*BGNL, *Lp*FTRL, and *Lp*DUF3632 in the nuclear genome of an endophyte-free *L. perenne* genotype, which show high nucleotide-level identity with genes commonly present in *Epichloë* genomes (Shinozuka et al. 2017, 2020). Each is differentially expressed in the tissues of endophyte-free perennial ryegrass plants. The sequence of *Lp*BGNL is similar to a fungal β-1-6-endoglucanase that is secreted into the apoplast of the host plant and functions in nutrition, endophyte branching, and resistance to pathogens (Moy et al. 2002). Orthologous *Lp*BGNL sequences are also found in grasses of the sister subtribes Loliinae and Dactylidinae, and its origin by wide HGT from ancestral *Epichloë* into their common ancestor is estimated at 13–9 Ma (Shinozuka et al. 2017). *Lp*FTRL encodes a putative transcriptional regulatory protein and genes of similar sequence are also present in genome assemblies for several Poöideae including wheat, oat, barley, *Dactylis*, *Phalaris*, *Lolium,* and *Poa* species (Shinozuka et al. 2020). Sequences highly similar to *Lp*FTRL are also present in *Epichloë* and other Ascomycetes. HGT of *Lp*FTRL from a common ancestor of *Epichloë* to a common ancestor of the Poöideae is estimated to have occurred at 39–32 Ma. *Lp*DUF3632 may have been transferred to the common ancestor of the Loliinae at 7.2 Ma. These HGT events may have provided host plants and fungal endophytes with phenotypic innovations that allow them to improve fitness driving the evolution of these symbiotic associations.

5.6 Relationship Between *Epichloë* Symbiosis and Host Plant Interaction with Herbivores

A prominent hypothesis is that the intimate symbioses of grasses with *Epichloë* species evolved under ecological scenarios of high herbivory pressure (Clay 2009). In these symbioses, plants are defended by the fungal endophytes largely by means of alkaloids with anti-herbivore effects (Schardl et al. 2013a; Panaccione et al. 2014), along with other anti-herbivore factors (Ambrose et al. 2014) and possibly induction of plant defenses as well (Bastias et al. 2017a).

Studies suggest that alkaloid levels can respond to activities of some herbivores. Increased production of peramine and lolitrem B is reported in response to biting and chewing by locusts, but not to sap-sucking by aphids (Fuchs et al. 2017). Plant clipping to mimic biting damage is also reported to increase loline alkaloid production by *E. glyceriae* (Gonthier et al. 2008). However, studies of lolines and precursor amino acids in *L. pratense*, and gene expression in *E. siegelii* and *E. uncinata*, indicate that some or all of the clipping effect may be attributable to the presence in young tissues of relatively high levels of the precursors (Zhang et al. 2009a). This apparent control by substrate availability gives an effect that is consistent with the *optimal defense hypothesis* that younger plant tissues tend to rely more on chemical defenses, whereas older tissues rely more on physical defenses.

The differential response to herbivores between endophyte-symbiotic and non-symbiotic plants may drive a prevalence of symbiosis in plant populations. In comparison with herbivore-free treatments, the presence of herbivores is usually associated with a rapid increase in, or higher prevalence of, endophyte-symbiotic plants (Casas et al. 2022; Clay et al. 2005; Rudgers et al. 2016), which can result from both increased performance of symbiotic plants over their non-symbiotic counterparts and increased vertical transmission of the fungus promoted by herbivory (Rudgers et al. 2016; Gundel et al. 2008, 2017, 2021; but see García Parisi et al. 2012). Nevertheless, it has been observed that symbiosis prevalence can increase even when herbivores are excluded (Santangelo et al. 2015). Additionally, endophyte effects can vary depending on the insect and environmental stresses (Bultman and Bell 2003). Thus, the relationship between herbivory pressure and prevalence of endophyte symbiosis may result from complex interactions of multiple physiological effects such as endophyte-mediated stimulation of plant growth and ecological effects such as increased competition and direct and indirect defenses against biotic threats.

The strength of defense a fungal endophyte provides to its host plant can be influenced by the types of alkaloids produced by the endophyte, their concentrations in key plant tissues, and their effectiveness against particular herbivores (Afkhami and Rudgers 2009; Bastias et al. 2017b). Overall, ergot alkaloids and lolitrem B are well known to be effective against small and large mammals, whereas loline alkaloids and peramine are best known as insecticidal or deterrent to insect feeding (reviewed in Schardl et al. 2013a; Panaccione et al. 2014). Despite this big picture, there is variation in the relationship between a given herbivore (species or type) and the alkaloid identity. For example, the same loline-producing endophyte (*E. occultans*) effectively protects *Lolium multiflorum* plants against the aphid *Rhopalosiphum padi* (Bastias et al. 2017b), but not against the aphid *Sipha maydis* (Bastías et al. 2019). Ergot alkaloids well known to affect vertebrates can also be toxic to nematodes and insects (Bacetty et al. 2009b; Graff et al. 2020). Tests with *EAS*-gene knockout mutants of *E. hybrida* (Fig. 5.2, panels B, C) have indicated that ergovaline, ergine, or both contribute some of the endophyte effects against larvae of sod webworm (Potter et al. 2008). In contrast, effectiveness of the "knockout" endophytes against the root nematode *Pratylenchus scribneri* was not significantly different from the wild-type endophyte effects (Panaccione et al. 2006b).

Enhanced resistance to herbivores conferred by fungal endophytes is related to alkaloid concentrations in the plant tissues (Wilkinson

et al. 2000; Graff et al. 2020). However, since the endophyte alkaloids vary among plant organs and plant developmental stages (Gundel et al. 2018; Hewitt et al. 2020; Nagabhyru et al. 2019; Rasmussen et al. 2007; Ueno et al. 2020a; Zhang et al. 2009a), the effectiveness of this defense mechanism may vary accordingly. The heterogeneous distribution of hyphae among host tissues only partly explains local concentrations of alkaloids, since variation in biosynthesis activity and alkaloid mobility are also important factors (Spiering et al. 2005a). Occurrence of some of the alkaloids in roots, where little or no endophyte biomass is evident (Azevedo and Welty 1995; Chakrabarti et al. 2022), suggests their mobility. For example, very little ergovaline distributes to the root, whereas lolines are apparently much more mobile (Popay and Gerard 2007; Popay et al. 2020). Peramine is also highly mobile in the plant (Koulman et al. 2007). Studies of the *Ipomoea tricolor-Periglandula* species symbiosis indicates that chanoclavine, ergine, and other lysergic acid amides are also mobile and can translocate to roots (Durden et al. 2019).

Endophyte-conferred defense has been shown to be inducible upon herbivore attack. For example, it was observed that loline alkaloid concentrations and expression of a loline biosynthesis gene were higher in herbivore-challenged plants (Bultman et al. 2004; Sullivan et al. 2007). Another study (Fuchs et al. 2017) showed that induced chemicals could be specific to the attacking herbivore. Although simulated grazing enhanced levels of lolitrem B, locust damage enhanced levels of peramine. Concentrations of an alkaloid can vary due to environmental conditions differentially affecting how the plant versus the fungus grows, or from variation in the synthesis rate of alkaloid (Bastías et al. 2018; Graff et al. 2020; Rasmussen et al. 2007; Ryan et al. 2014; Simons et al. 2008; Ueno et al. 2020b). At least in the case of lolines, availability of precursor amino acids in the plant tissue may determine levels of alkaloid production (Zhang et al. 2009a).

Certain conditions can impair endophyte-conferred resistance to host plants. For example, since available nitrogen is a key component of alkaloids, its level in the soil can sometimes affect alkaloid levels (Faeth and Fagan 2002). In contrast, enhanced susceptibility to caterpillars observed in endophyte-symbiotic tall fescue plants in soils with high levels of phosphate (Graff et al. 2020) seems to be explained by a phosphate-mediated inhibition of ergot-alkaloid biosynthesis (Malinowski and Belesky 2000). Interestingly, exposure of plants to an ozone-contaminated atmosphere prevented the endophyte from protecting the plants against *R. padi* aphids (Ueno et al. 2016), a condition that persisted after one generation without direct exposure of progeny plants to ozone (Bubica Bustos et al. 2020). Taken together, these studies suggest that the endophyte-conferred protection of host plants is conditional on the ecological context in which the interaction takes place and is especially sensitive to which herbivores are present, resource levels, and environmental stressors.

Attracting predators of attacking herbivores can also be part of a sophisticated mechanism of plant defense; the so-called indirect defense mechanism (Heil 2014). Conceivably, fungal endophytes can affect this process, considering that they have been shown to modulate herbivore-induced plant volatiles (HIPVs) in response to aphids and simulated grazing (Fiorenza et al. 2021; Li et al. 2014). In a mesocosm experiment, the rate of parasitism was higher in aphids feeding on endophyte-free compared to symbiotic plants (Omacini et al. 2001; Bultman et al. 2003). Interestingly, the performance of arthropod parasitoids is usually impaired when their hosts feed on endophyte-symbiotic plants (Bultman et al. 2003). In addition to this direct effect, primary parasitoids can be less successful due to higher rates of hyperparasitism occurring in endophyte-free—not in endophyte-infected—plants (Omacini et al. 2001; Bultman et al. 2003). Future studies should reveal the relative importance of direct or indirect defenses for the performance of host plants in nature dealing with a multitude of different herbivores and associated trophic communities.

5.7 *Epichloë* Control on Host Plant-Associated Symbiotic Microorganisms

As persistent and highly specific symbionts, *Epichloë* species may exert control over host plant-associated microorganisms, either beneficial, commensal or pathogenic. Such endophyte-mediated effects have been mostly studied in relation to plant pathogens (reviewed by Card et al. 2021; Hume et al. 2016; Pérez et al. 2020), and mycorrhizal fungi (reviewed by Mack and Rudgers 2008; Omacini et al. 2012) and to a lesser extent in other symbiotic microorganisms.

5.7.1 Endophyte-Mediated Host Interaction with AMF

Although a review and meta-analysis suggested that *Epichloë* symbioses can inhibit the associations of plants with arbuscular mycorrhizal fungi (AMF) (Omacini et al. 2012), several recent field surveys and experiments in wild grasses show positive or neutral association between endophyte symbiosis and root colonization by AMF. For example, levels of arbuscular mycorrhizae in three South American grasses (*Bromus setifolius*, *Poa bonariensis,* and *Hordeum comosum*) (Novas et al. 2005, 2008; Casas et al. 2022) and in *Poa alsodes* and *Poa sylvestris* from the USA (Kalosa-Kenyon et al. 2018) were positively associated with *Epichloë* presence. Interestingly, the pattern in *H. comosum* was not affected by mean annual precipitation at the site, although aridity impaired the plant association with both types of symbiont (Casas et al. 2022). In a manipulative field experiment, the level of root colonization by native AMF in the American beachgrass *Ammophila breviligulata* was positively affected by symbiosis with *E. amarillans* in one year but negatively in the next one (Bell-Dereske et al. 2017). In *Bromus auleticus*, soil fertility did not affect the positive association between the *Epichloë* symbiosis and the level of root colonization by AMF (Vignale et al. 2016). *Epichloë* symbiosis also increased the abundance and diversity of AMF in the rhizosphere of *Leymus chinensis* (Liu et al. 2021). Interestingly, the effect of *E. gansuensis* on root-associated AMF diversity in *Achnatherum inebrians* was positive under drought conditions but negative when watered (Zhong et al. 2021). Since plant–symbiont interactions depend on the ecological context, it is logical to expect such variability in the *Epichloë* effects on plant–AMF associations. Furthermore, controlled condition experiments are short-term and may not capture the actual variability observed in nature as well as the diversity of AMF capable of interacting with host grasses. In the long term, the multiple benefits provided by both types of symbiont may result in positive feedback between root and foliar fungi. For example, tall fescue, annual ryegrass, and prairie grass (*Bromus catharticus*) exhibited increased mycorrhizal colonization when grown in soils with three-year-old *Epichloë*-infected *B. auleticus* plants (Vignale et al. 2020).

5.7.2 Endophyte-Mediated Host Protection Against Pathogens

Endophyte symbiosis can enhance plant fitness under disease pressure by either reducing incidence of disease or by enhancing the plant's tolerance of infection. Both possibilities are illustrated in endophyte-enhanced protection from viral diseases. The enhanced defense against insects can reduce infection by plant viruses that are vectored by those insects. For example, infection of *L. pratense* by Barley Yellow Dwarf Virus (BYDV) is reported to be reduced in *Epichloë*-symbiotic plants compared to asymbiotic plants (Lehtonen et al. 2006). Alternatively, increased tolerance to infection can result from other fitness enhancements. For example, in tall fescue the endophyte is reported to alleviate the negative effect on plant biomass allocation to root (Rúa et al. 2013).

Numerous reports indicate endophyte-enhanced resistance to fungal diseases (recently reviewed by Card et al. 2021). In plant–fungus

interactions, *Epichloë* symbiosis can affect the plant association with non-systemic fungal endophytes and pathogens through multiple pathways by affecting the defense system of the host, through direct interactions such as competition for resources and production of secondary compounds with fungistatic or antifungal effects, and through ecological mechanisms such as those mediated by volatile organic compounds (VOCs) and root exudates (Fiorenza et al. 2021; Li et al. 2014; Pérez et al. 2020; Steinebrunner et al. 2008; Xia et al. 2018). *In vitro* dual culture assays with various *Epichloë* strains and various plant-pathogenic fungi have shown inhibitory effects on mycelial growth or spore germination of the pathogens (reviewed in Fernando et al. 2020; Hume et al. 2016; Iannone et al. 2012a; Yue et al. 2000; Zhang et al. 2015).

Manipulation and observational experiments at the plant level have shown variable outcomes (negative, neutral, or positive) of the association with *Epichloë* on resistance to fungal pathogens (Hume et al. 2016; Wiewióra et al. 2015). This variability could be attributed to differences in the grass-*Epichloë*-pathogen systems of study and to the environmental conditions in which each assay was conducted. For example, the negative effect of *Epichloë* on fungal pathogens of grasses can depend on the nutritional status of the host and on the pathogen strategy (Pérez et al. 2020).

Compiled evidence suggests that symbiosis with *Epichloë* may increase resistance to pathogens directly via their secondary metabolites, potentially including diketopiperadine alkaloids, sesquiterpenes, phenolics, indole-3-acetic acid, chokol A-G, gamahonolides, and gamahorin (Seto et al. 2005; Steinebrunner et al. 2008; reviewed in Fernando et al. 2020). Additionally, in fine fescues the aforementioned antifungal protein Efe-AfpA, produced by some *E. festucae* strains, has been suggested to play a role in resistance against *Sclerotinia homoeocarpa*, the cause of dollar spot disease (Tian et al. 2017).

Based on literature review and meta-analysis, Bastias et al. (2017a) propose a model for *Epichloë* symbiosis that involves upregulation of jasmonic acid (JA) and down-regulation of salicylic acid (SA) signaling pathways to account for patterns of endophyte-enhanced resistance or susceptibility to insects and fungal pathogens. However, there is no direct evidence that JA or SA levels are affected by the symbioses, and the reported effects on signaling-pathway genes range from extensive (Dupont et al. 2015) to very limited (Dinkins et al. 2017), with a common observation being effects on the expression of WRKY genes that may modulate—possibly decrease—defense responses against biotrophic fungal pathogens. Two fungal genes are particularly intriguing in this respect. One is the *TFF14* gene (GenBank accession CA820680), which is predicted to encode a fatty acid desaturase and may be involved in JA biosynthesis (Johnson et al. 2003). However, the enzymatic function of its product and implication for JA levels have not yet been determined. The other is the *E. festucae shyA* gene (GenBank accession KM400586), a demonstrated salicylate hydroxylase (Ambrose et al. 2015). However, *in planta* levels of SA appear unaffected by *E. festucae*, leaving unresolved the potential role of *shyA* and SA in endophyte compatibility and responses to pathogens. Tests with the respective gene-knockout mutants would help clarify their roles.

Studies of the effects of *Epichloë* symbiosis on floral pathogens have given contrasting results. Field experiments have indicated that *Epichloë* symbiosis significantly reduces the severity ergot, the infamous seed-replacement disease caused by *Claviceps purpurea* (Pérez et al. 2013), as well as systemic smut caused by *Ustilago bullata* (Iannone et al. 2017; Vignale et al. 2013). Effects on ergot can be complicated by the role of arthropods in its spread, and the symbiosis is reported to help repel such spore vectors (Pérez et al. 2017). However, a contrasting observation in *Festuca rubra* indicated no effect of *E. festucae* symbiosis on aphid infestation, and increased incidence of ergot disease (Laihonen et al. 2022). Interestingly, the combination of *Epichloë* and high ergot disease levels resulted in higher resistance of plants to insect herbivores. In future studies, it would be important to integrate the endophyte-mediated mechanisms of plant resistance to

pathogens at an individual level with those modulated ecologically, as well as to ponder the relative impact of pathogens versus herbivores on the differential fitness of endophyte-symbiotic and asymbiotic plants.

5.7.3 Epichloë *Effects on the Plant Association with Non-systemic Fungal Endophytes*

Studies addressing the controls over plant association with non-systemic fungal endophytes and elements of the microbiome are relatively recent. There are a few studies that use classical approaches based on culture and isolation methodologies to explore the association of *Epichloë* symbiosis with non-systemic fungal endophytes. Symbiosis with *E. festucae* was associated with a higher load of non-systemic fungal endophytes in *Festuca rubra* in Arctic meadows but not in riverbanks (Zabalgogeazcoa et al. 2013). In the dioecious grass *Poa bonariensis*, *E. tembladerae* reduced the frequency of *Alternaria* species only in staminate plants (Mc Cargo et al. 2020). Because they are based on culture isolation methodologies, these studies can miss part of the fungal community or microbiome, but nevertheless provide identifications at the genus or species level, facilitating assignment of putative ecological roles of each endophyte.

The advent of sequencing technologies has boosted research aimed at describing and understanding the controls on microbiomes of plants, including roots, shoots, leaves, and seeds (Hawkes et al. 2021). For example, *E. coenophiala* differentially modulates the bacterial and fungal communities in leaves of its host (Nissinen et al. 2019). The endophyte effect on the fungal community is suggested to result from fungus–fungus interaction. Observing no comparable effect on the bacterial community, the endophyte effect on foliar fungal communities was proposed to occur not through changes in the plant immune system but through fungus–fungus interaction.

As vertically-transmitted endophytes, *Epichloë* species may also modulate the seed microbiome. *Epichloë festucae* var. *lolii* modulated the bacterial microbiome of *L. perenne* seeds, a difference that vanished in adult plants (Tannenbaum et al. 2020). *Epichloë occultans*, the most common seed endophyte of annual ryegrasses, was found to increase diversity and evenness of the bacterial communities associated with seeds of *L. multiflorum* (Bastias et al. 2021). It was suggested that the high performance of host seedlings usually ascribed to the *Epichloë* presence could be also explained by the seed-associated bacterial communities.

5.7.4 Epichloë *Effects on the Rhizosphere*

Changes in the rhizosphere microbial community can be associated with plant mechanisms of nutrient acquisition (e.g., phosphorus solubilization), and recruiting beneficial microbes while reducing the presence and action of the negative ones; the so-called cry for help (Rizaludin et al. 2021). In particular, plant symbioses with *Epichloë* species have been found to alter root exudates and VOCs (Guo et al. 2015; Rostás et al. 2015; Van Hecke et al. 2005) and consequently, the microorganisms in the rhizosphere (Omacini et al. 2012). Compared to non-symbiotic plants, different strains of *E. coenophiala* changed the total biomass and relative abundance of fungi in the rhizosphere of tall fescue (Rojas et al. 2016). The relative abundance of main fungal phyla (Ascomycota, Zygomycota, and Basidiomycota) was different between endophyte-symbiotic and non-symbiotic tall fescue (Mahmud et al. 2021). In the same plant species, endophyte impacts on the structure and function of host rhizosphere communities depended on plant development state and environmental conditions such as levels of soil phosphate (Ding et al. 2021). For *Bromus auleticus*, symbiosis with *Epichloë* was associated with increased diversity (not abundance) of phosphate-solubilizing rhizospheric fungi (Arrieta et al. 2015). If *Epichloë* symbioses

modulate the host rhizosphere and thereby promote phosphate availability, it would be interesting to understand how this change modulates interactions between plants and mycorrhizal fungi.

A recent study also considered the impact of dung beetles *Onthophagus taurus* and *Digitonthophagus gazella* on dung from cattle grazing on different endophyte-infected tall fescue pastures (Shymanovich et al. 2020). The cultivars used in this study were infected with different endophyte strains with different capabilities to produce alkaloids, especially ergovaline. Dung beetles performed better on the tall fescue cultivar Texoma MaxQ II, as observed by increase in brood-ball production and reduced development time. The overall impact a single cultivar may have on enhancing dung beetle populations and improvement of pasture ecology needs to be further examined.

5.8 Endophyte Effects on Plant Population Dynamics

Importantly, effects of *Epichloë* symbioses on individual plants can scale up to impact host plant population dynamics. *Epichloë* species are well known to affect many aspects of their host plants' life histories such as mortality, growth rates, phenology (e.g., onset of flowering), and reproductive output. These effects can, in turn, play key roles in population growth rates and population persistence of the host plants. Yet studies often find complex and, at times, conflicting effects of *Epichloë* on plant success at different life history stages, making it challenging to determine how these endophytes shape plant populations. For instance, *Epichloë* symbioses increased survival but reduced the probability of flowering for the rare grass *P. alsodes* (Chung et al. 2015), and strongly reduced survival but increased regeneration of *Cinna arundinacea* (Rudgers et al. 2012). Efforts to integrate ecological plant-endophyte experiments with demographic modeling (matrix models or integral projection models) have made progress providing insight into how endophytes impact population persistence, since these approaches incorporate effects across the life cycle of the plant and account for the relative importance of different life stages to population persistence (Chung et al. 2015; Gibert et al. 2015; Gundel et al. 2008; Rudgers et al. 2012; Yule et al. 2013). These studies have found that endophytic *Epichloë* species often increase host population growth rates and are in some cases required for population persistence. For instance, Chung et al. (2015) found that endophytes increased population growth rates of both a common and rare grass species (by 32% and 18%, respectively), but only populations of the rare grass species were predicted to decline without the endophyte symbiosis. Despite the positive effects these fungi can have on plant population growth rates, the imperfect vertical transmission commonly found in *Epichloë*-grass symbioses (Afkhami and Rudgers 2008) may reduce their effects on plant population dynamics and even cause symbiont extinction in some cases (Gundel et al. 2008; Yule et al. 2013).

It is worth noting that because of the tractability of *Epichloë* symbiosis—due to the ability to manipulate presence or absence in field conditions and their low diversity within each host—we know more about how these fungi influence plant population dynamics than practically all other members of the plant microbiome. Moreover, these studies of endophytes have provided a crucial model for how to test the effects of microbes on plant population dynamics that has recently been utilized (with appropriated modifications) to investigate effects of soil microbiomes (David et al. 2019). This work demonstrated that soil microbiomes were necessary for population persistence of an endangered plant species, but on average had smaller effects on population growth rates than did *Epichloë* symbiosis (13% for soil microbiomes versus 18%–32% for *Epichloë*) (Chung et al. 2015; David et al. 2019; Yule et al. 2013). This was consistent with the expectations of evolutionary theory (Ewald 1987), which predicts stronger selection for microbially-conferred benefits from the vertically-transmitted, systemic symbionts (microbe and host fitness more tightly linked)

than for the more diffuse, horizontally-transmitted soil microbiome. Interestingly, for populations experiencing high stress, the soil microbiome effect on population growth rates was 47%, exceeding what has been documented for effects of *Epichloë* (David et al. 2019). While more studies are needed to disentangle the conditions under which endophyte effects scale up to impact plant populations, taken together population modeling of *Epichloë*-symbiotic plants and the microbiome research inspired by it has already provided some important insights. These studies indicate that both intrinsic properties of the plant–microbe interaction such as the transmission mode and extrinsic properties such as environmental stress can play key roles in determining microbial effects on plant population dynamics.

5.9 *Epichloë* Effects on Communities and Ecosystem Processes

Through "neighborhood effects," symbiotic *Epichloë* species can impact the community ecology of organisms outside those directly participating in the symbiosis (Omacini et al. 2012). Research has shown that *Epichloë*-grass symbioses can alter the species composition, diversity, and functional makeup of plant communities in which they are embedded. For instance, studies have found that *Epichloë*–tall fescue interactions often reduce plant community diversity and abundances of other plant species or functional groups (Clay and Holah 1999; Clay et al. 2005; Rudgers and Clay 2008; Iqbal et al. 2013). Similarly, presence of *E. uncinata* in meadow fescue (*L. pratense*) was associated with higher patch resistance to invasion of weedy species (Saikkonen et al. 2013). These outcomes likely arise from *Epichloë*-conferred benefits increasing the competitive ability and, thereby, dominance of their hosts compared to the rest of the plant community. However, endophyte effects on diversity can vary in strength depending on factors like endophyte genotype and chemotype (Rudgers et al. 2010; Iqbal et al. 2013). In fact, the direction of these effects can also be context-dependent as *Epichloë* species have been shown to increase plant community diversity and reduce invasiveness. For example, in a natural ecosystem an endophyte associating with a non-dominant native grass increases diversity within the surrounding plant community by enhancing the competitive ability of its host, which in turn suppresses the growth of a dominant, invasive congener (Afkhami and Strauss 2016). Likewise, under mowing condition, endophytes in tall fescue have been found to promote plant diversity (Spyreas et al. 2001).

In addition to increasing host competitive abilities, *Epichloë* symbioses can influence plant community functional and species diversity through other pathways. For example, endophyte-generated allelopathy can reduce success of non-hosting competitor plant species through chemical inhibition (Orr et al. 2005; Vázquez-de-Aldana et al. 2013), and endophytes can provide pathogen protection to non-hosting plants in the neighborhood of the *Epichloë* symbiosis (Pérez et al. 2016). These pathways illustrate how endophyte effects on communities can be complex and multifaceted. Further, the fact that endophytes can have strong community-level consequences in these ways is impressive given that they typically make up a relatively small portion of the biomass within the communities. Thus, in cases where endophyte strongly influences biodiversity, community structure, or ecological processes, these hidden players are arguably keystone species (Power et al. 1996; Libralato et al. 2006).

Effects of *Epichloë* symbiosis on community ecology extend beyond plants to organisms that fill diverse roles within ecosystems (Omacini et al. 2001; Lemons et al. 2005; Finkes et al. 2006; Rudgers and Clay 2008; Jani et al. 2010). The fact that *Epichloë* species generally produce anti-herbivore alkaloids leads to the prediction that herbivorous insect community composition and structure should also be impacted, which would cause cascading effects on higher trophic levels. Studies of multitrophic interactions have confirmed that these endophytes can drive meaningful changes in the structure of insect food webs

(Omacini et al. 2001; Fuchs et al. 2013). In a community-wide arthropod study, *E. coenophiala* in tall fescue significantly altered arthropod species composition and reduced arthropod abundance and diversity (by 70% and 20%, respectively), with strongest effect on the herbivorous insects (Rudgers and Clay 2008). However, endophyte-mediated effects can vary depending on environmental conditions and host plant origin (Vesterlund et al. 2011). In fact, in the notoriously toxic population of *A. robustum* near Cloudcroft, New Mexico, the endophyte alkaloids ergine and ergonovine are surprisingly associated with increases in herbivore species richness and abundance, which the authors suggest resulted from the stronger effects of endophytes on natural enemies of the herbivores in this system (Jani et al. 2010). These cascading effects on predators have also been well documented for spiders whose community richness has been shown to decline in successional fields dominated by endophyte-symbiotic tall fescue plants (Finkes et al. 2006). Similarly, because endophytes can change their host plants' litter properties, they have substantial effects on the composition of detritivores communities, such as Collembola (Lemons et al. 2005). Taken together, studies of arthropods have revealed that effects of *Epichloë* symbioses can perturbate through ecosystems to reach communities of herbivores, predators, and decomposers.

Fungal endophytes can affect key community and ecosystem processes from community succession dynamics to nutrient cycling and litter decomposition through a wide range of ecological pathways (Rudgers and Clay 2007; Purahong and Hyde 2010; Omacini et al. 2012; Wolfe and Ballhorn 2020). For instance, previous work showed that fungal endophyte in a dominant grass can suppress secondary succession by reducing tree abundance and size and slowing plant species turnover (Rudgers et al. 2007). This effect was likely due to endophyte-mediated protection of this grass driving a 65% increase in consumption of tree seedlings by voles. Interestingly, fungal endophytes can also modulate fundamental ecological relationships, for example by weakening the relationship between diversity and primary productivity (Rudgers et al. 2004), and even function as ecosystem engineers. For example, Emery et al. (2015) demonstrated that endophyte-associated changes in host plant traits led to increased sand accumulation, a key ecosystem function required for dune building.

5.10 Applications

5.10.1 Natural Strains in Forage Grasses

A range of livestock toxicity symptoms are caused by ergovaline produced by *E. coenophiala* strains in the early tall fescue cultivars such as "Kentucky 31" that were extensively planted in the USA, New Zealand, Argentina, and elsewhere. Ryegrass staggers is mainly caused by indole-diterpenes, including the highly tremorgenic lolitrems (Munday-Finch et al. 1997), produced by *E. festucae* var *lolii* in *L. perenne* introduced to Australia, New Zealand, and the USA by European colonists. Eradication of the endophyte from grass cultivars, originally viewed as the obvious solution, leads to the loss of other benefits such as endophyte-enhanced tolerance to biotic stresses (insects, pests) and abiotic stresses (drought, salinity), and increased plant persistence. Thus, the search for endophytic strains with desirable alkaloids profiles "friendly" to livestock, in order to be artificially introduced in selected cultivars, became a strategy for forage management. Friendly endophytes are unable to produce ergot alkaloids or lolitrems in levels toxic to livestock but promote forage stand persistence and produce the more specifically anti-insect alkaloids.

Artificial or synthetic combinations of endophytes and grasses can be obtained through the inoculation of endophytic mycelium in meristems of seedlings from germinating seeds (Florea et al. 2015; Latch and Christensen 1985) or from callus differentiation (Becker et al. 2018). Due to specificity of the associations, inoculations into new hosts are more likely to succeed when the endophyte comes from the same or a closely related plant species. The endophyte should still

be non-toxic to livestock, beneficial to the cultivar and efficiently transmitted through the seeds to the seedlings of the new host.

Strains *of E. coenophiala* unable to produce toxic levels of ergovaline (e.g., those designated AR542, AR584 by AgResearch New Zealand, and ArkPlus by the University of Arkansas) have been identified from plants found in Morocco and introduced into selected cultivars of tall fescue (Bouton et al. 2002; Watson et al. 2004; Beck et al. 2008). Strains of *E. festucae* var. *lolii* (*e.g.,* AR1, AR37; NEA) unable to produce toxic levels of lolitrems but promoting plant growth and conferring protection against specific pest insects similarly have been identified in natural populations and introduced into perennial ryegrass cultivars (Caradus and Johnson 2020). Though not as toxic as lolitrem B, a level of toxicity to livestock is attributable to epoxyjanthitrems (Fletcher et al. 2017), but these may provide important defense against insects (Popay and Gerard 2007).

Cultivars of tall fescue or perennial ryegrass with endophytes lacking toxicity to livestock are very successful for livestock. The forage quality and the weight gain of cows and ewes grazing on such cultivars are significantly higher than for previous tall fescue or perennial ryegrass cultivars with their original endophytes (Burns and Fisher 2006; Hopkins et al. 2011; Matthews et al. 2005). Such cultivars are commercialized and used mostly in USA, New Zealand, Australia, and South America. In contrast, endophytes especially toxic to vertebrates and invertebrates have been selected for turf grasses in an effort to diminish the abundance of insects and their predator birds in airports (Pennell et al. 2016).

Selected strains from a different host species have been used to provide new traits to grass cultivars in what has been called symbiotically modified organisms (SMOs) (Gundel et al. 2013). For example, two natural symbionts of *L. pratense* (meadow fescue) that produce loline alkaloids were moved into *L. perenne* and *Festulolium* cultivars, respectively, where they confer protection against insects (Caradus and Johnson 2020).

The beneficial effects on plant performance obtained from the combination of friendly endophytes with elite grass cultivars, as well as the level of protection against pests, depend on the cultivar, the endophyte, the insect or pest pathogen to be controlled, and the environment (Gundel et al. 2013). Therefore, it is necessary to have a great diversity of friendly endophytes in order to obtain tailored cultivar-endophyte combinations with specific effects. Current knowledge of the biosynthetic pathways and the genes involved in alkaloid biosynthesis allows the researchers to explore the diversity of endophytes and to predict their potential alkaloid profiles using PCR-based techniques (Charlton et al. 2014). These studies have revealed intraspecific variability in endophytes of the same host species and are useful to select specific candidate endophytes to be inoculated into elite cultivars.

5.10.2 Genetically Altered Strains

An alternative pathway to endophytes friendly to livestock is genetic manipulation to delete the genes involved in the biosynthetic pathways. For example, Panaccione et al. (2001) knocked out the *lpsA* gene and Wang et al. (2004) knocked out the *dmaW* gene in *E. hybrida* strain Lp1 from *L. perenne*. The Δ*lpsA* mutant produced ergotryptamine (Ryan et al. 2015) (previously reported as 6,7-secolysergine) and the clavines chanoclavine and setoclavine (Panaccione et al. 2006a) in re-inoculated plants. The Δ*dmaW* mutant produced no ergot alkaloids *in planta*.

The effects of Δ*lpsA* and Δ*dmaW* endophytes were compared with each other and with wild-type Lp1 in studies of endophyte-enhanced plant protection (Fig. 5.2). Effects on rabbits (Panaccione et al. 2006a) were interesting considering that the Δ*dmaW* endophyte actually enhanced the appeal of the grass. In contrast, Δ*lpsA* gave no enhanced appeal, indicating that one or more of ergotryptamine, chanoclavine, and setoclavine reduced the appeal of the grass. Comparing the effect of wild-type *E. hybrida* with both knockout mutants indicated an appetite-

suppressive effect of ergovaline, which was in keeping with the effect of this ergopeptine as a 5-HT2A receptor agonist (Schöning et al. 2001).

Wild-type *E. hybrida* and its Δ*lpsA* and Δ*dmaW* mutants were also tested for effects on invertebrates (Fig. 5.2). *Pratylenchus scribneri* is a plant-endoparasitic nematode previously shown to be affected by endophyte presence in tall fescue, resulting in reduced reproduction (Timper et al. 2005; Kimmons et al. 1990). A similar effect in perennial ryegrass was observed for the wild-type, Δ*dmaW* and Δ*lpsA* strains, indicating that ergot alkaloids were not responsible for this anti-nematode effect (Panaccione et al. 2006b). This result is unsurprising considering that ergovaline appears to have low mobility in the plant and occurs in low concentration in roots (Popay and Gerard 2007). Although *Epichloë* can be present in roots (Azevedo and Welty 1995) it is apparently sparse there (Hinton and Bacon 1985; Dinkins et al. 2017). We note that Lp1 lacks 1-aminopyrrolizidines (lolines and AcAP) but produces peramine and the indole-diterpene terpendole C (Christensen et al. 1993; Young et al. 2009), which may be candidate anti-nematode factors in that system. However, the possibility should also be considered that plant metabolites such as phenolic acid exudates elevated in endophyte-symbiotic plants (Malinowski et al. 1998), as well as effects on root architecture (Malinowski et al. 1999) could have roles in interactions with root-parasitic nematodes.

Interestingly, ergine and, in much lower amounts, simpler ergot alkaloids (but not ergopeptines) have been reported in *Ipomoea tricolor* with *Periglandula* sp., and that clavicipitaceous endophyte exhibits activity against the root knot nematode *Meloidogyne incognita* (Durden et al. 2019). Whether ergot alkaloids have a role has not been shown, and it should be noted that *I. tricolor* with *Periglandula* sp. also has high levels of indole-diterpenes (Cook et al. 2019). As in the grass system, possibilities remain that other metabolites and physiological effects on roots might be responsible or contribute to endophyte effects on nematodes.

Alteration of ergot-alkaloid profiles of *E. hybrida* in perennial ryegrass is also reported to affect interaction with sod webworm neonates and fifth instars (Potter et al. 2008). The endophyte provided dramatic feeding deterrence, and the effect was reduced slightly but significantly by the *ΔdmaW* and *ΔlpsA* mutations. Those mutations also negated most of the endophyte effect on neonate survival.

All told, the studies of the *ΔdmaW* and *ΔlpsA* mutants of *E. hybrida* suggest that elimination of all ergot alkaloids may result in reduced fitness by unmasking endophyte-enhanced appeal of the grass to small mammals and reduced anti-insect activity. The former issue might be addressed by leaving genes for earlier pathway steps to chanoclavine I and ergine.

In tall fescue, CTE strains of *E. coenophiala* possess two homeologous *EAS* gene clusters. However, at least some have defective or deleted copies of *lpsB* in one or the other cluster (Schardl et al. 2013b; Florea et al. 2016). Early efforts targeted the *dmaW* genes, of which one was removed with difficulty by marker exchange (Florea et al. 2009) and the other only succumbed once a CRISPR-based technique was employed (Florea et al. 2021). The CRISPR/Cas9 approach proved to be facile and versatile, even yielding (from 115 screened colonies) two isolates in which both *EAS* gene clusters were deleted in their entirety without any exogenous DNA being introduced into the genome (Florea et al. 2021).

5.10.3 Symbiotically Modified Cereals

None of the modern cereals such as wheat (*Triticum aestivum*), rye (*Secale cereale*), barley (*Hordeum vulgare*), and oat (*Avena sativa*) are naturally associated with *Epichloë* species (Card et al. 2014), but their phylogenetical relationships with wild grasses that often host such endophytes have encouraged researchers to introduce non-toxic endophytes into some cereal varieties. Endophytes from *Elymus mutabilis* and from *Elymus dahuricus* have been introduced by seedling inoculations into rye and wheat, respectively

(Simpson et al. 2014). In rye the inoculated endophyte reduced the diseases caused by the fungi *Puccinia recondita* and *Cercosporidium graminis* (Card et al. 2021).

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Lichens and Their Allies Past and Present

6

Rosmarie Honegger

Abstract

This chapter gives an overview on (1) lichen-forming fungi, lichen photobionts and peculiarities of lichen symbiosis such as gains and losses of lichenization, species concepts, specificity, morphodemes and morphotype pairs, non-lichen mutualistic fungal interactions with unicellular algae and cyanobacteria and mycophycobioses; (2) the mycobiont–photobiont interface, water relations and gas exchange, mycobiont-derived secondary metabolites and the accumulation of heavy metals or radionuclides; (3) the microbiome of lichen thalli, i.e. the bacteriome (epi- and endolichenic bacteria), lichenicolous and endolichenic fungi, lichenicolous lichens and the virome of lichens and their allies; (4) fossil lichens and their microbiome; (5) lichen–animal interactions such as the micro- and mesofauna of lichen thalli, lichenivory in invertebrates and vertebrates, endo-and epizoochory; (6) lichenomimesis in animals and flowering plants.

Keywords

Lichen symbiosis · Lichen-forming fungi · Lichen photobionts · Endolichenic fungi · Lichenicolous fungi · Lichenicolous lichens · Bacteriome of lichens · Virome of lichens · Fossil lichens · Lichenivory · Lichenomimesis

6.1 Introduction

Lichens are the symbiotic phenotype of nutritionally specialized fungi, ecologically obligate biotrophs which acquire fixed carbon from a population of minute photobiont cells (Honegger 1991a, b). Lichen-forming fungi (also referred to as lichen mycobionts) are, like plant or animal pathogens or mycorrhizal fungi, a polyphyletic, taxonomically diverse group of nutritional specialists, but are otherwise normal representatives of their fungal classes. They differ from non-lichenized taxa by their manyfold adaptations to symbiosis with a population of minute photobiont cells (Honegger 2009). Lichenization is an ancient and very successful nutritional strategy, approximately 17% of extant fungal species being lichenized (Lücking et al. 2017a).

Lichens were the first mutualistic symbiosis discovered. The Swiss botanist Simon Schwendener (1829–1919) realized that the thallus of lichens is built up by a fungus which harbours a population of genetically different, minute green algal or cyanobacterial cells in its thalline interior (Schwendener 1867, 1869; Honegger 2000). Upon closer examination, lichen thalli represent not a dual or triple

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symbiosis of a C-heterotrophic partner, i.e., a lichen-forming fungus (the mycobiont) and a photoautotrophic partner, either a green alga and/or a cyanobacterium (the photobiont, also termed cholorobiont or cyanobiont, respectively), but consortia with an unknown number of participants (Fig. 6.1a–b; Honegger 1991a, b) or complex ecosystems, respectively (Hawksworth and Grube 2020). Lichenicolous and endolichenic fungi and bacterial epi- and endobionts are very common and widespread, their taxonomic affiliation and potential roles for the symbiosis having been intensely studied in the last decades (see Sects. 6.5.1–6.5.5). Endolichenic fungal and actinobacterial symbionts and epithalline bacteria were already present in *Chlorolichenomycites devonicus*, a fossil lichen of the Early Devonian (ca. 415 Ma old, see Sect. 6.6.2; Honegger et al. 2013a).

As pointed out by Hawksworth (2016), there is no need to change the concept of lichen in the light of these findings. The term symbiosis, as defined by de Bary (1879), refers to genetically different organisms living together, neither the outcome of their interaction (parasitic, commensalistic or mutualistic relationships, the term mutualism having been introduced by Van Beneden in 1875) nor the number of organisms involved was defined.

Until the end of the twentieth century, the taxonomy of lichen-forming fungi and their photobionts was based on morphological, chemical and structural characters. In the twenty-first century, molecular tools, bioinformatics and rapidly growing databases facilitated the study of the inter- and intraspecific diversity of lichen-forming fungi and their photobionts, their phylogenies, but also the intrathalline

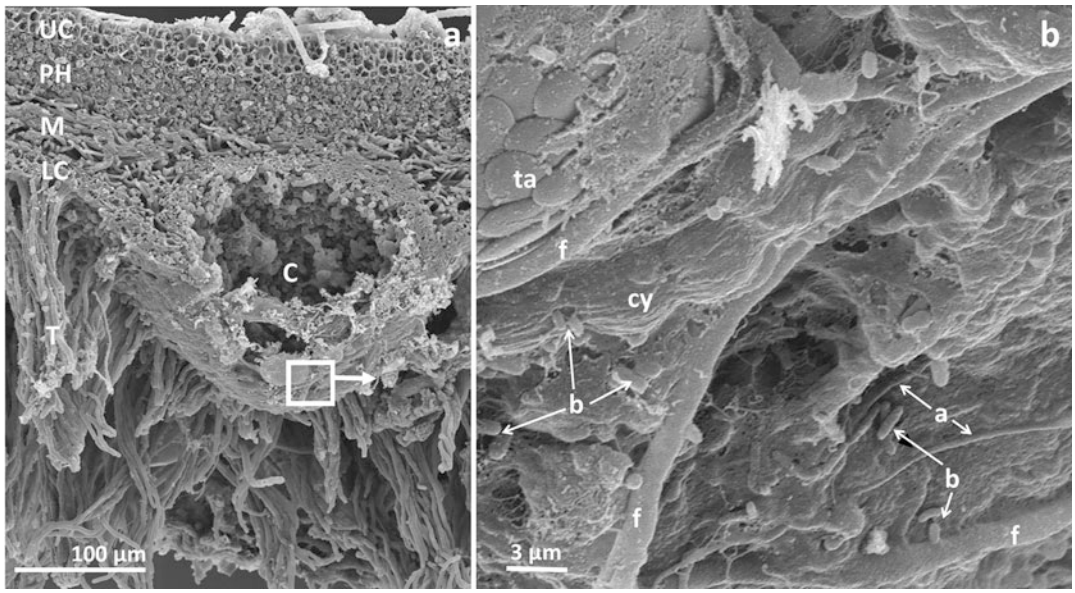

Fig. 6.1 The lichen thallus as a consortium with an unknown number of participants: (**a**, **b**) Scanning electron microscopy (SEM) micrograph of a cross-section of *Sticta sylvatica*, collected in Brittany, with bacterial, fungal, cyanobacterial and protozoan epibionts. A cyphella is tangentially sectioned, details in (**b**). Abbreviations thallus: *C*: cyphella (aeration pore); *UC* upper cortex, *PH* photobiont layer (*Nostoc* sp.); *M* medullary layer, *LC* lower cortex with tomentum (*T*). Abbreviations of epibionts: *a* actinobacteria, *b* bacteria, *cy* cyanobacterial filament, *f* fungal hyphae, *ta* testate amoeba, presumably *Assulina* sp., its shell being composed of self-made siliceous shell-plates (idiosomes)

biodiversity and the pro- and eukaryotic microbiome of lichen thalli.

6.2 Lichen-Forming Fungi (LFF)

6.2.1 Gains and Losses of Lichenization

Lichenization was repeatedly acquired (14–23 lichenization events in the Ascomycota, 6–7 in the Basidiomycota), but also repeatedly lost in favour of a saprotrophic, lichenicolous or parasitic mode of nutrition (Lücking et al. 2017a, b; Nelsen et al. 2020). Examples of relatively recently de-lichenized taxa among the Lecanoromycetes are the lichenicolous *Raesaenenia huuskonenii* (syn. *Protousnea huuskonenii*, Parmeliaceae, Lecanorales; Divakar et al. 2015; Fig. 6.6h–i) or the lignicolous *Xylographa constricta* (Baeomycetaceae, Baeomycetales; Spribille et al. 2014).

6.2.2 Species Concepts and Phylogenies

The genus and species name of a lichen refers to the fungal partner, irrespective of it being symbiotic in nature or axenically cultured apart from its photobiont (Hawksworth 2015). Attempts to refer to the aposymbiotically cultured mycobiont as *. . .-myces* (e.g. *Xanthoriomyces parietinae* for aposymbiotically cultured *Xanthoria parietina*; Thomas 1939) are obsolete. The photoautotrophic partner(s) of lichens have their own names and phylogenies. Traditionally, lichens were described on the basis of morphological (morphospecies) and chemical characters (secondary metabolites: chemospecies). Molecular tools provided novel insights into phylogenies, evolutionary trends and taxonomic relationships in general and in questions related to the delimitation of species in particular (Lücking et al. 2021). Many well-known morphospecies turned out to comprise either cryptic species or morphodemes (see Sects. 6.2.3 and 6.2.4).

In their 2016 classification, Lücking and colleagues listed 19,387 accepted species of lichenized fungi in 995 genera, 115 families, 39 orders and eight classes, the vast majority being ascomycetes; Lecanoromycetes, the largest and most anciently lichenized class among extant ascomycetes, comprises more than 15,100 spp. (Lücking et al. 2017a, b). Only 172 species, 15 genera, five families, five orders in one class belong to the basidiomycetes (Lücking et al. 2017a, b), but molecular data of more than 300 species of basidiolichens were not yet published. In the meantime, new taxa of basidiolichens have been described (Coca et al. 2018; Dal-Forno et al. 2019; Lücking et al. 2022). However, large numbers of species and genera of lichenized asco- and basidiomycetes await molecular analysis.

Based on molecular datasets, large numbers of new species, numerous new genera, families, few orders (e.g., the ascolichen orders Eremithallales; Lücking et al. 2008, Leprocaulales; Lendemer and Hodkinson 2013, Collemopsidales; Pérez-Ortega et al. 2016, or the basidiolichen order Leptostromatales; Hodkinson et al. 2014) and classes (e.g. Lichinomycetes; Reeb et al. 2004; Coniocybomycetes; Prieto et al. 2013) have been described. Within 6 years (2010–16), more than half of all genera of LFF were subjected to changes (Lücking et al. 2017a, b), and this work continues. Intense biodiversity analyses have been performed in areas and ecosystems which have so far been poorly investigated. An example is the TICOLICHEN project in Costa Rica, the major tropical lichen biodiversity inventory initiated in 2002 by Robert Lücking and colleagues (Lücking et al. 2004).

Molecular phylogenies give fascinating insights into evolutionary processes and biogeographic developments. Examples are (1) the calicioid ascomycetes, predominantly crustose species with prototunicate asci whose ascospores achieve maturation in a mazaedium. Their asci disintegrate before the ascospore wall is fully differentiated, spore maturation being completed in a powdery mass, the mazaedium, at the surface of the ascoma (Honegger 1985). Based on this feature, mazaediate ascomycetes were formerly

classified in the order Caliciales within the Lecanoromycetes. Today, mazaediate taxa fall into four classes: Lecanoromycetes, Eurotiomycetes, Arthoniomycetes and Coniocybomycetes (Prieto et al. 2013; Wijayawardene et al. 2020). (2) the leprose lichens with no sexual reproductive stages and morphologically very simple thalli built up by loosely interwoven hyphae which secrete interesting secondary metabolites and are in contact with green algal cells, forming a powdery mass with no stratification. Today leprose lichens of the genus *Lepraria* are classified in the Stereocaulaceae, *Leprocaulon* in the Leprocaulaceae (Lecanoromycetes; Lendemer and Hodkinson 2013), *Botryolepraria* in the Verrucariaceae (Eurotiomycetes; Kukwa and Pérez-Ortega 2010) and *Andreiomyces* in Andreiomycetaceae (Arthoniomycetes; Hodkinson and Lendemer 2013). Moreover, leprose thalli are formed in fertile taxa such as *Chrysothrix* (rarely fertile; Chrysothrichaceae, Arthoniomycetes; Nelsen et al. 2009; Liu et al. 2018) or in *Chaenotheca furfuracea* (syn. *Coniocybe f.*; Coniocybaceae, Coniocybomycetes). (3) Phylogeographic studies provided insights into large-scale population histories, e.g., of *Lobaria pulmonaria* in North America or in the post-glacial re-colonization of the Alps (Lerch et al. 2018; Allen et al. 2021), or of the cosmopolitan *Psora decipiens* (Leavitt et al. 2018).

6.2.3 Species Pairs and Cryptic Species

The species pair concept in lichenology is based on the observation that some morphologically similar lichens differ in their reproductive mode, i.e., are either fertile or asexually reproducing, the fertile stage having been assumed to be the primary species (Crespo and Pérez-Ortega 2009). Many sexual–asexual species pairs turned out to represent a monophyletic lineage. The *Porpidia flavocoerulescens* and *P. melinodes* species pair was hypothesized to maintain asexual reproduction under optimal conditions, but sexual reproduction and re-lichenization for escaping from a suboptimal symbiosis (Buschbom and Mueller 2006). In the presumed *Letharia columbiana* (fertile) and *L. vulpina* species pair *L. columbiana* turned out to comprise five cryptic taxa, hybridization and polyploidization included (Kroken and Taylor 2001; Altermann et al. 2016; Ament-Velásquez et al. 2021).

Many other morphospecies turned out to comprise numerous cryptic species (e.g. *Pseudocyphellaria crocata* comprising 13 spp.; Lücking et al. 2017a, b), the most extreme examples being (1) the lichenized basidiomycete *Dictyonema glabratum* (syn. *Cora pavonia*), which comprises at least 126 taxa (Lücking et al. 2014, 2016; Moncada et al. 2019); upon closer examination these hidden basidiolichen species turned out to be morphologically distinct, although the differences are recognizable to the expert's eye only. (2) the species complex of the cosmopolitan crustose lichenized ascomycete *Lecanora polytropa* comprises up to 103 cryptic species (Zhang et al. 2022).

6.2.4 Morphodemes and Morphotype Pairs (= Photosymbiodemes)

Morphodemes are formed by phylogenetically distinct taxa which differentiate the same morphotype; examples are found in the *Sticta filix* (Ranft et al. 2018) or the *Sticta weigelii* complexes (Moncada et al. 2021). On the other hand, morphologically distinct lichens, some of which had been classified in different genera, turned out to be congeneric or even conspecific. This phenomenon was first observed in tripartite lichens with a morphologically distinct cyanobacterial and a green algal morphotype, i.e., in *Ricasolia amplissima* (lobate chloromorph with cephalodia) and *Dendriscocaulon bolacinum* (fruticose cyanomorph; Dughi 1937), chimaeric forms included (James and Henssen 1976). Photosymbiodemes formed by lobate chloromorphs and fruticose, dendriscocauloid cyanomorphs occur in various peltigeralean genera (*Sticta*, *Lobaria*, *Ricasolia*; Paulsrud

et al. 1998; Magain et al. 2012; Tønsberg et al. 2016; Ranft et al. 2018). Photosymbiodemes with morphologically similar lobate chloro- and cyanomorphs occur in *Peltigera* spp. (Goffinet and Bayer 1997; see Fig. 15.1f in Honegger 2012).

Vastly different morphologies formed by the same fungal species were found in *Endocena* and *Chirleja* spp. (Icmadophilaceae); their coccoid green algal photobiont was not investigated (Fryday et al. 2017). A very peculiar morphotype pair is formed by two crustose lichens with either a trebouxioid or a trentepohlioid green algal photobiont (Ertz et al. 2018). The sterile, sorediate *Buellia violaceofusca* (formerly classified in Lecanoromycetes), which associates with different phylospecies of the genus *Trebouxia* (Trebouxiophyceae), and the fertile *Lecanographa amylacea* (Arthoniomycetes) with a *Trentepohlia* photobiont (Ulvophyceae) turned out to be conspecific. This was the first example of a lichen-forming ascomycete with a trebouxioid and a trentepohlioid morphotype. As in non-lichenized fungi the species name refers to the sexually reproducing stage (teleomorph). *Lecanographa amylacea* most likely captures trebouxioid photobiont cells from adjacent crustose or leprose lichens to form the sorediate (anamorphic) morphotype (Ertz et al. 2018).

6.2.5 Non-lichen Mutualistic Fungal Interactions with Cyanobacteria and Unicellular Green Algae

It is astonishing that no Zygomycetes, Glomeromycetes or Chytridiomycetes are among the extant LFF. However, in each of these classes, which are phylogenetically older than asco- and basidiomycetes, at least one example of a mutualistic symbiosis with either a cynobacterial or green algal partner was reported.

The Glomeromycetes *Geosiphon pyriformis* forms a very ancient endocyanosis with *Nostoc* sp. at the soil surface, the cyanobacterial photobiont being incorporated in hyphae which subsequently differentiate a bladder at the soil surface. The *Nostoc* filaments are kept in membrane-bound vesicles (perialgal vacuoles) in the fungal cytoplasm where they undergo cell division, are photosynthetically active and fix atmospheric N_2. The first glomeromycetan monosaccharide transporter characterized was shown to function at the symbiotic interface of *Geosiphon* with its cyanobacterial partner (Schüssler et al. 2006, 2007; review: Schüssler 2012).

Under certain environmental conditions, mutualistic interactions between non-lichenized fungi and algae are formed without prior co-evolutionary adaptation. The zygomycete *Mortierella elongata* and the unicellular green alga *Nannochloropsis oceanica* are both biotechnologically cultured on large scale to produce lipids for biofuel. In co-culture, both partners interact, the algal cells adhere to the fungal hyphae and finally become internalized, leading to a green mycelium (Du et al. 2019). This situation resembles the *Geosiphon–Nostoc* symbiosis, although a green alga instead of a cyanobacterium is involved.

Chytrids are saprobes or parasites of fungi, algae, plants and amphibians (reviews: Powell 2017; Longcore et al. 2020). Parasites of phytoplankton attack free-living algal species (Van den Wyngaert et al. 2018) and have a devastating effect on commercially grown algal cultures (Hoffman et al. 2008; Longcore et al. 2020). The chytrid *Rhizidium phycophilum* forms a facultative mutualism with a *Bracteacoccus* sp. (Sphaeropleales; syn. Chlorococcales) and can only be cultured in the presence of this zoosporic green algal species. The alga is not parasitized, it even grows larger and more prolific and reveals an up to eight-fold increase in biovolume in co-culture as compared to axenic culture under the same conditions. This is the first report of a mutualistic interaction between a chytrid and a green alga (Picard et al. 2013).

The ascomycetous yeast *Saccharomyces cerevisiae* (Saccharomycotina) and filamentous ascomycetes such as *Aspergillus nidulans* (Eurotiomycetes) form mutualistic interactions with the flagellate unicellular *Chlamydomonas reinhardtii* (Chlamydomonadales,

Chlorophyceae), although with different levels of productivity and without differentiating thallus-like structures as seen in lichen-forming ascomycetes (Hom and Murray 2014) However, *C. reinhardtii* cells are protected by *A. nidulans* from bacteria and their toxins (Krespach et al. 2020). Two extremely halotolerant organisms, the black yeast *Hortaea werneckii* (Teratosphaeriaceae, Capnodiales), which can switch from filamentous growth to the yeast form, and the flagellate unicellular green alga *Dunaliella atacamensis* (Chlamydomonadales), were found to interact in co-cultures (Muggia et al. 2020b). Despite close fungal–algal contacts, neither a mutual benefit, nor an antagonistic interaction was observed. Nevertheless, such heterotroph–autotroph contacts might be the beginning of a stable symbiotic interaction.

6.2.6 Mycophycobioses

Mycophycobioses are mutualistic fungal interactions with quantitatively predominant, multicellular algal hosts. *Mycophycias ascophylli* (Capnodiales incertae sedis, Dothideomycetes; Toxopeus et al. 2011; Wijayawardene et al. 2020) inhabits the intertidal brown algae *Pelvetia canaliculata* and *Ascophyllum nodosum* (Fucales, Phaeophyceae) in the upper littoral of the Atlantic, only its minute ascomata being visible at the surface of the seaweed's receptaculum (gamete-producing structure; Fig. 6.2), their very thin vegetative hyphae being best visible in ultrathin sections (Xu et al. 2008). In fresh water, *Phaeospora lemaneae* (syn. *Leptosphaeria lemaneae;* Verrucariales, Eurotiomycetes) associates with the red alga *Lemanea fluviatilis* (Batrachospermales, Rhodophyta; Brierley 1913; Hawksworth 2000). *M. ascophylli* improves the desiccation tolerance of the zygotes of its brown algal host *A. nodosum* (Garbary and London 1995). Improved desiccation tolerance was also hypothesized for the red algal host *L. fluviatilis* upon infection with *P. lemaneae* (Hill 1992), but experimental data are missing.

About 100 species of fungal endophytes of seaweeds (Phaeophyta, Rhodophyta, Chlorophyta) were identified, some of them producing bioactive secondary metabolites (Noorjahan et al. 2021).

6.2.7 Secondary Metabolites

Lichen thalli are a rich source of interesting polyphenolic secondary metabolites, many of them with bioactive properties. Most of them are produced by the mycobiont, others by endolichenic fungi or by epi- or endolichenic bacteria (see below). Lichen compounds evolved early in the radiation of filamentous fungi (Armaleo et al. 2011). Polyketide synthase (PKS) genes were most likely gained by horizontal gene transfer from actinobacteria before the radiation in Leotiomyceta, which gave rise to the extant crown group of Ascomycota (Schmitt and Lumbsch 2009). PKS genes have been repeatedly lost in non-lichenized ascomycetes but retained and even duplicated in most lichenized ascomycetes (Schmitt and Lumbsch 2009). However, also in lichenized taxa were PKS gene clusters repeatedly lost (Pizarro et al. 2020).

The chemistry of mycobiont-derived secondary metabolites, the so-called lichen products, has attracted considerable interest, with a large body of literature having been published from the late nineteenth century onwards (e.g. Zopf 1896, 1907; Asahina and Shibata 1954; Culberson 1969, 1970; Culberson and Elix 1989; Culberson et al. 1977; Huneck and Yoshimura 1996; Huneck 2001; Elix and Stocker-Wörgötter 2008); a database of high-resolution tandem mass spectrometry (MS/MS) spectra for lichen metabolites is available (Olivier-Jimenez et al. 2019).

It is not known in which form the secondary metabolites are excreted by the fungal cells (possibly as glycosides?). In their soluble form, they are passively translocated into the apoplastic continuum during the wetting and drying cycles and crystallize either at on the surface of ascomata (e.g. bellidiflorin in *Cladonia* spp.; Fig. 6.3a, or haemoventosin in *Ophioparma ventosa*; Fig. 6.3b–c), on the thallus surface (e.g. anthraquinones such as parietinic or

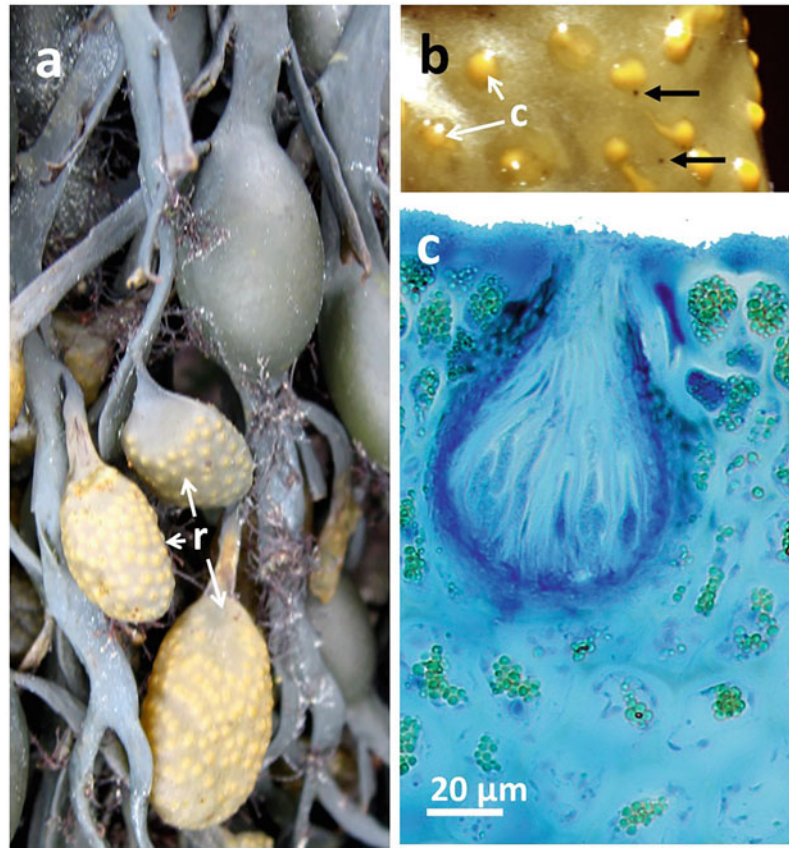

Fig. 6.2 Mycophycobiosis in the knotted kelp (*Ascophyllum nodosum*), a common brown alga (Fucaceae, Phaeophyceae) in the littoral of the Northern Atlantic (**a**); its gametes are produced in conceptacles (c) in stalked receptacles (r). (**b**) Gametes are oozing out of the ostiole of conceptacles. Arrows point to ascomata of *Mycophycias ascophylli* (Mycosphaerellaceae, Capnodiales, Dothideomycetes), recognizable as tiny black dots between conceptacles. (**c**) Stained semi-thin section with ascoma (pseudothecium) of *M. ascophylli*

solorinic acid, pulvinic acid derivatives such as vulpinic or rhizocarpic acid, or depsides such as atranorin), many of them giving the thallus its characteristic colouration; others (e.g. depsidones such as physodic or protocetraric acid) crystallize on the surface of medullary hyphae and even on green algal photobiont cells in the thalline interior (Fig. 6.3d; Honegger 1986b). In soredia, mycobiont and photobiont cells are often heavily loaded with crystalline secondary metabolites which enhance their hydrophobicity (Fig. 6.3e). Crystalline lichen products are almost insoluble in aqueous systems below pH 7. Many mycobiont-derived secondary metabolites are produced in sterile, aposymbiotic culture (Thomas 1939; Yamamoto et al. 1985; Honegger and Kutasi 1990; Culberson and Armaleo 1992; Stocker-Wörgötter et al. 2013; Díaz et al. 2020; Jeong et al. 2021, etc.).

Numerous PKS gene clusters have been characterized (e.g. Miao 1999; Armaleo et al. 2011; Wang et al. 2014; Bertrand and Sorensen 2018; Bertrand et al. 2018; Calchera et al. 2019; Pizarro et al. 2020; Sveshnikova and Piercey-Normore 2021; Gerasimova et al. 2022; Singh et al. 2022), many of them being not expressed in the lichen thallus. Thus, LFF have an unexplored biosynthetic potential (Bertrand et al. 2018; Gerasimova et al. 2022).

As most LFF are slow-growing organisms, lichen-derived PKS genes were transferred into fast-growing non-lichenized ascomycetes. A *Pseudevernia furfuracea*-derived PKS gene was successfully expressed in baker's yeast (*S. cerevisiae*), the depside lecanoric acid being synthesized (Kealey et al. 2021). The transfer of lichen-derived PKS genes in filamentous ascomycetes such as *Aspergillus* spp. lead to successful transcription (Wang et al. 2016), but for unknown reasons, secondary products were often not synthesized (Gagunashvili et al. 2009; Yang et al. 2018; Bertrand and Sorensen 2019a, b).

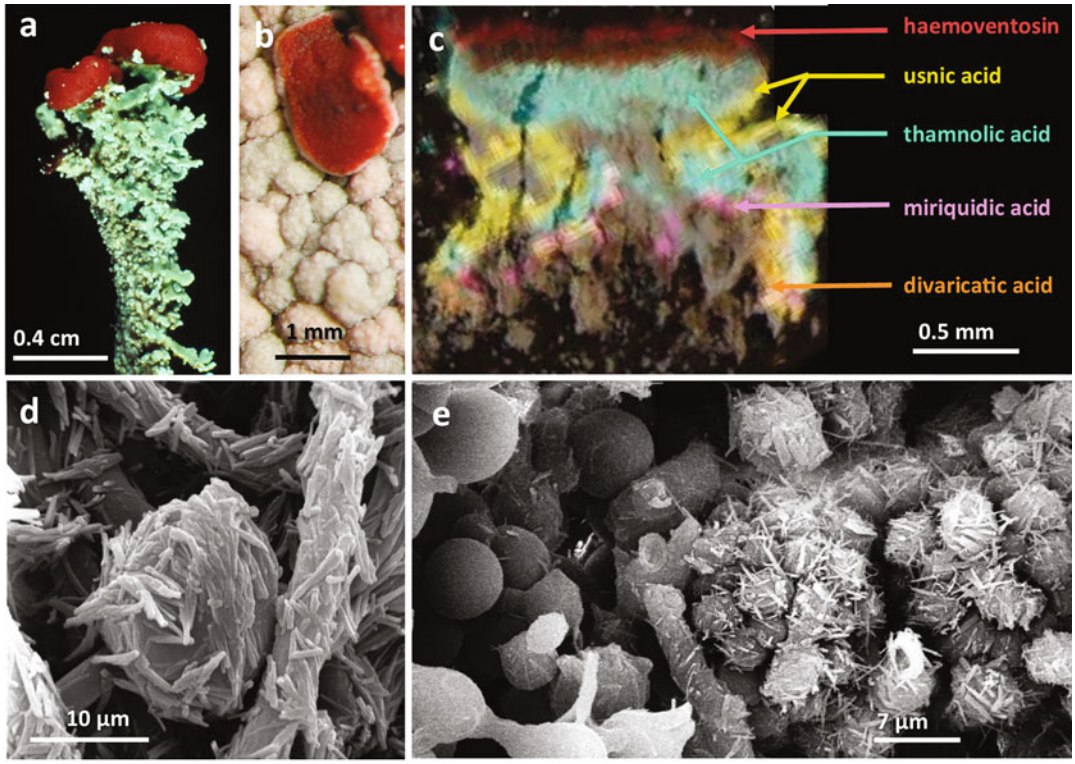

Fig. 6.3 Developmentally regulated biosynthesis of secondary metabolites. (**a**) *Cladonia bellidiflora* with usnic and squamatic acid in the thallus and bellidiflorin in the bright red ascomata. (**b, c**) The saxicolous, crustose *Ophioparma ventosa* with haemoventosin in the bright red ascomata. (**c**) LDI-MSI showing the tissue-specific accumulation of usnic, thamnolic, miriquidic and divaricatic acid in the thallus and haemoventosin in the epihymenial layer of the ascomal disc; from Le Pogam et al. (2016, modified); courtesy of Pierre Le Pogam and Joël Boustie. (**d**) LTSEM (low temperature scanning electron microscopy) of the algal layer in in cryofixed, freeze-fractured *Cetrelia olivetorum*, with crystals of olivetoric acid on fungal hyphae and algal cell. (**e**) LTSEM of the margin of a soralium of *Physcia adscendens*. Secondary metabolites crystallize on hyphae and algal cells of developing soredia, thus increasing the hydrophobicity of their surfaces

Lichens have been used in traditional medicine around the globe (Crawford 2019). In the last decades, a large body of literature was published on interesting pharmaceutical properties of β-glucans such as lichenans (e.g. Caseiro et al. 2022), important cell wall components of Lecanoromycetes (Honegger and Haisch 2001), and of a wide range of lichen-derived secondary metabolites with antibiotic, anti-inflammatory, antiproliferative, antiviral, antineurodegenerative, antioxidant activities, etc. (various authors in Ranković 2019; reviews: Boustie and Grube 2005; Boustie et al. 2011; Bhattacharyya et al. 2016; Studzinska-Sroka et al. 2017; Ingelfinger et al. 2020; Ureña-Vacas et al. 2021). Best investigated is usnic acid (Macedo et al. 2021; Xu et al. 2022), whose antibacterial properties were detected by Stoll et al. (1947).

6.3 Lichen Photobionts

6.3.1 Diversity and Specificity

In her 1988 compilation, Tschermak-Woess listed 44 genera of lichen photobionts: 15 cyanobacterial, 27 chlorophycean, one xanthophycean and one phaeophycean

(Tschermak-Woess 1988). All of these taxa had been described on the basis of morphological characters. From 1989 onwards, molecular tools, applied either to cultured (aposymbiotic) photobiont isolates or to whole thallus DNA preparations, revolutionized our knowledge of lichen photobiont diversity. In 2021, Sanders and Masumoto summarized the current state of the art. They listed 52 genera of lichen photobionts: 13 cyanobacterial, 36 chlorophycean, two xanthophycean and one phaeophycean (Sanders and Masumoto 2021). New photobiont genera had been described (e.g. *Heveochlorella*, Trebouxiophyceae; Sanders et al. 2016, or *Bracteacoccus*, Chlorophyceae; Masumoto 2020).

The Verrucariales (three families, 55 genera, >800 spp.), which comprises terrestrial, but also numerous fresh water and marine species, harbours the highest photobiont diversity. The only phaeophycean (*Petroderma maculiforme* in *Wahlenbergiella tavaresiae*) and xanthophycean photobionts (*Heterococcus* sp. in *Verrucaria* and *Hydropunctaria* spp., *Xanthonema* sp. in *Staurothele clopimoides*) are symbiotic with verrucarialean taxa (review: Sanders and Masumoto 2021). Many photobiont taxa associate exclusively with marine LFF. Examples are the cyanobacterial *Hyella* sp. in *Collemopsidium halodytes* (syn. *Arthopyrenia h.*, Xanthopyreniaceae) or *Rivularia* sp. in *Lichina pygmaea and L. confinis (*Lichinales, Lichinomycetes; see Figs. 15.1.p-r in Honegger 2012). Among marine green algal photobionts of marine Verrucariaceae are the ulvophycean *Blidingia minima* (with *Turgidosculum ulvae*; Pérez-Ortega et al. 2018), *Halophilum ramosum*, *Lithotrichon pulchrum*, *Paulbroadya petersii*, *Pseudendoclonium submarinum* and *Undulifilum symbioticum* (with *Hydropunctaria* and *Wahlenbergiella* spp.), or the trebouxiophycean *Prasiola* spp. in *Mastodia tessellata* (Sanders and Masumoto 2021; Černajová et al. 2022).

The genus *Trebouxia* harbours the most common and widespread unicellular green algal photobionts of LFF; *Trebouxia* spp. are photobionts of Parmeliaceae (>2760 spp.; Lücking et al. 2017a, b), Teloschistaceae (>810 spp.), etc. Tschermak-Woess (1988) listed seven species, Sanders and Masumoto (2021) 27 plus several unnamed phylospecies and clades, whence five had been transferred from *Pseudotrebouxia* (which no longer exists), and 19 species have been newly described from 1989 onwards. In *Asterochloris,* the second-most common genus, Tschermak-Woess (1988) listed one species, Sanders and Masumoto (2021) 18 plus several unnamed phylospecies and clades; four of these *Asterochloris* spp. had been transferred from *Trebouxia* to *Asterochloris* (*A. erici*, *A. glomerata*, *A. italiana*, *A. magna*). *Asterochloris* spp. are photobionts of *Cladonia and Stereocaulon* spp. and many other taxa (Sanders and Masumoto 2021), new phylogenetic lineages having been recently described (Kosecka et al. 2021).

Trebouxia and *Asterochloris* spp. are characterized by their large, lobate chloroplast with central pyrenoid. Chloroplast lobation and its interspecific variation was visualized with Confocal Laser Scanning Microscopy (CLSM; *Trebouxia*: Muggia et al. 2012; Bordenave et al. 2021; *Asterochloris*: Škaloud and Peksa 2008; Moya et al. 2015; Škaloud et al. 2015; Kim et al. 2017).

As in LFF, considerable cryptic diversity was found among lichen photobionts; examples in *Trebouxia* (Singh et al. 2019; Muggia et al. 2020a, b; Kosecka et al. 2022), *Coccomyxa* (Darienko et al. 2015; Malavasi et al. 2016), *Dictyochloropsis* or *Symbiochloris*, respectively (Dal Grande et al. 2014; Škaloud et al. 2016), *Trebouxiophyceae* (Metz et al. 2019) or Trentepohliales (Borgato et al. 2022).

Unfortunately lichen thalli cannot be routinely resynthesized by combining cultured myco- and photobionts. Therefore, the specificity and selectivity of the symbiosis cannot yet be investigated under controlled experimental conditions. Our knowledge about the range of acceptable partners per fungal species or genotype is based on analyses of specimens collected in nature. Ideally, sampling was done over the whole geographic range of the fungal species.

Many (the majority?) of LFF with trebouxioid or trentepohlioid photobionts associate with more

than one algal species or genotype; examples are *Xanthoria parietina* with *Trebouxia decolorans* or *T. arboricola* (Nyati et al. 2014) or cosmopolitan species of *Stereocaulon*, *Cladonia* or *Lepraria*, which reveal low specificity towards their photobiont (Kosecka et al. 2021). In *Cladonia* spp., external factors such as climate or soil chemical properties have an impact on photobiont selection (Pino-Bodas and Stenroos 2021; Škvorová et al. 2022); severe heavy metal pollution even induced a switch from *Asterochloris* to *Trebouxia* spp. (Osyczka et al. 2020).

Based on light microscopy studies, the algal cell population of lichen thalli was assumed to be uniform, but molecular tools revealed intrathalline photobiont diversity. Different photobiont species or genotypes of the same photobiont may grow within the same thallus (for special relationships such as morphotype pairs, see Sect. 6.2.4, or tripartite lichens, see Sect. 6.3.2, respectively). In all thalli of *Ramalina farinacea* were two unnamed *Trebouxia* spp. found with different physiological properties (Casano et al. 2011; del Hoyo et al. 2011). In 45% of *Evernia mesomorpha* were multiple genotypes of one *Trebouxia* species found (Piercey-Normore 2006). In 30 out of 104 thalli of *Tephromela atra* or *Rhizoplaca melanophthalma*, respectively, were co-occurring *Trebouxia* spp. found (de Carolis et al. 2022). Only once were two genotypes of *T. decolorans* found in a thallus of *X. parietina* (Nyati et al. 2014).

Some *Trebouxia* photobionts were found free-living in nature (Bubrick et al. 1984), but they are not common members of aerophilic algal communities. In contrast, photobionts of the genera *Apatococcus*, *Stichococcus*, *Coccomyxa*, *Elliptochloris*, *Myrmecia*, *Symbiochloris*, *Prasiola* (all Trebouxiophyceae) or *Trentepohlia* (Ulvophyceae) are common in the free-living state, many of them having been found in soil samples along an altitudinal gradient (Stewart et al. 2021) or in biological soil crust communities (Flechtner et al. 2013; Borchhardt et al. 2017). This should be kept in mind when exploring microalgal diversity in whole thallus extracts: adhering fragments of symbiotic propagules from other lichens or free-living algae might be included. Ideally, lichen photobionts are isolated from the algal layer of dissected thallus fragments and cultured under sterile conditions prior to genetic analysis (Nyati et al. 2013, 2014).

6.3.2 Tripartite Lichens

(a) Lichens with a mixed photobiont layer comprising green algae and cyanobacteria. In a few lichens, the photobiont layer comprises a green algal and a cyanobacterial partner, both being photosynthetically active. Examples are (a) *Euopsis granatina* with *Trebouxia aggregata* as a green algal and *Gloeocapsa sanguinea* as cyanobacterial photobiont (Büdel and Henssen 1988); (b) *Muhria urceolata* (Jørgensen and Jahns 1987, syn. *Stereocaulon urceolatum*; Högnabba 2006) and (c) various cyanomorphs of *Pseudocyphellaria* spp. (*Ps. rufovirescens*, *Ps. lividofusca*, *Ps. dissimilis*, *Ps. hookeri*, *Ps. crocata*) and *Sticta fuliginosa*, all with *Nostoc* sp. (Henskens et al. 2012). In some of these peltigeralean species the green algal partner was detected when ribitol, the characteristic carbohydrate produced by numerous green algal photobionts (Hill 1976; Honegger 1997), was identified among the photobiont-derived mobile carbohydrates in the thalli (Henskens et al. 2012).

(b) Cephalodiate species are green algal lichens which capture repeatedly a diazotrophic cyanobacterium as a secondary photobiont (see Fig. 15.16 a–k in Honegger 2012), either at the upper (e.g. in green algal *Peltigera* spp.) or at the lower surface (e.g. in *Solorina* spp.) or at both surfaces (*Lobaria pulmonaria*; Cornejo and Scheidegger 2013) and incorporate it in either an external (e.g. *Peltigera aphthosa*, *Stereocaulon ramulosum*) or internal cephalodium (e.g. *Solorina crocea*, *Nephroma arcticum*). Capture of a cyanobacterial partner occurs in young lobes of the green algal lichen, mature cephalodia being found in fully grown

areas of the lobes (Cornejo and Scheidegger 2013). The cyanobacterial photobionts in cephalodia are photosynthetically active, i.e., satisfy their demands of fixed carbon, but are diazotrophic, i.e., reduce atmospheric N_2 into bioavailable ammonium, highest N_2 fixation rates being achieved under microaerobic conditions. In mature cephalodia of Peltigerales with *Nostoc* as the secondary photobiont, the percentage of heterocysts, i.e., the site of N_2 fixation, is elevated (up to 55%) as compared to either cyanobacteria as primary photobiont of cyanolichens (approx. 5–8%) or free-living *Nostoc* spp. (Englund 1977; Hyvärinen et al. 2002). It is particularly interesting to see how lichen mycobionts generate microaerobic conditions, optimal for N_2 fixation, by forming a dense cortex around external cephalodia, which differs anatomically from the thalline cortex (see Fig. 15.16d–f and i–k in Honegger 2012). By 3D imaging using X-ray computed tomography at the microscopy level (Micro-CT, allowing for non-destructive analysis of the 3D-geometry in solid specimens; Elliott and Dover 1982; Hunter and Dewanckele 2021), the cephalodia were quantified (Gerasimova et al. 2021); in *Lobaria pulmonaria* the internal cephalodia amounted for 0.73%, in *Peltigera leucophlebia* (external cephalodia) for 0.97% of thallus volume.

(c) Cephalodiate species are found in all green algal Peltigerales (*Peltigera* and *Solorina* spp. etc. [Peltigeraceae] and *Lobaria*, *Sticta*, *Pseudocyphellaria* spp., etc. [Lobariaceae]), in all representatives of the genera *Stereocaulon* (Stereocaulaceae, Lecanorales), *Placopsis* (Trapeliaceae, Baeomycetales; Ott et al. 1997), but also in some crustose taxa such as *Calvitimela aglaea* (syn. *Lecidea shushanii* or *Tephromela aglaea*, respectively, Tephromelataceae, Lecanorales; Hertel and Rambold 1988; Bendiksby et al. 2015).

6.3.3 Cyanotrophy

Cyanotrophy, i.e., the close contact of lichens with free-living cyanobacterial colonies, first observed in *Bryonora* spp. (Poelt and Mayrhofer 1988), might be common and widespread among saxicolous, epiphytic and soil crust lichens (Elbert et al. 2012; Cornejo and Scheidegger 2016; Gasulla et al. 2020). The contacting lichen benefits from fixed nitrogen, as produced by diazotrophic cyanobacteria, as is also the case in bryophyte interactions with free-living cyanobacteria (Gavazov et al. 2010; Warshan et al. 2017). Epiphytic bryophyte–cyanobacteria associations can serve as a reservoir for lichen cyanobionts (Cornejo and Scheidegger 2016).

6.4 Peculiarities of Lichen Symbiosis

6.4.1 A. Symbiotic vs. Free-Living LFF

In nature, the majority of LFF are symbiotic, but physiologically they do not depend on lichenization. Soon after the discovery of lichen symbiosis by Schwendener (1867, 1869), the first culturing and resynthesis experiments with LFF and their photobionts were carried out (Stahl 1877; Bonnier 1886, 1889). From large numbers of lichen-forming ascomycetes, sterile cultures have been established, starting with either ascospores, soredia or hyphal fragments which had been scratched out of dissected thalli (e.g. Lange de la Camp 1933; Thomas 1939; Ahmadjian 1959, 1962; Honegger and Bartnicki-Garcia 1991; Crittenden et al. 1995; Stocker-Wörgötter 1995, etc.). Single-spore isolates derived from one meiosis (i.e. the content of one ascus) facilitated the analysis of mating type systems (Honegger et al. 2004a; Honegger and Zippler 2007; Scherrer et al. 2005). Thus, LFF are ecologically obligate, but physiologically facultative biotrophs. However, some LFF most likely acquire additional carbohydrates as saprotrophs. Hyphae of *Icmadophila ericetorum*

or *Baeomyces rufus* on decaying wood grow deep into the substratum and penetrate the lignified cell walls (see Figs. 12 and 14 in Honegger and Brunner 1981). In large numbers of Lecanoromycetes, a whole arsenal of genes encoding enzymes for carbohydrate degradation was identified (Resl et al. 2022).

LFF are seldom macroscopically visible in a non-lichenized state in nature. Examples are (1) juvenile developmental stages of *Rhizocarpon* spp. (Rhizocarpales), recognizable as a strongly melanized, black prothallus in search of a compatible photobiont, or (2) the optionally lichenized *Schizoxylon albescens* (Ostropales) on the bark of *Populus tremula*, which grows as a saprobe on dead wood of poplar (Muggia et al. 2011), or (3) the lichenized *Conotrema* spp. and their non-lichenized, saprotrophic stage which had been described as *Stictis* spp., optional lichenization presumably allowing to increase the ecological amplitude (Wedin et al. 2004, 2005).

6.4.2 Morphogenetic Capacity of the Mycobiont

In order to keep their photoautotrophic partner photosynthetically active, LFF have to grow at the surface of the substrate, thus exposing their thallus to temperature extremes, illumination, ultra-violet (UV) radiation included and to continuous fluctuations in water content, i.e. to regular drying and wetting cycles. Many mycobiont-derived cortical secondary metabolites such as parietin or atranorin are autofluorescent, i.e. absorb UV light and transform it into longer wavelengths (Fernandez-Marin et al. 2018); therefore, they were assumed to protect the myco- and photobiont from UV damage and to provide the photobiont cell population with wavelengths matching the absorption spectra of photosynthesis; however, the latter hypothesis was not supported by experimental approaches (Fernandez-Marin et al. 2018). The biosynthesis of secondary metabolites is enhanced by seasonally or experimentally elevated UV radiation (BeGora and Fahselt 2001; Bjerke et al. 2002, 2005; Solhaug et al. 2009). However, there are yet unknown genetic factors causing quantitative differences in cortical secondary metabolites among thalli of the same species growing side by side (Bjerke et al. 2005; Itten and Honegger 2010).

Foliose, band-shaped or fruticose lichen thalli with internal stratification are the most complex vegetative structures in the fungal kingdom. However, less than half of all lichens reveal this morphology and anatomy, the majority forming either microfilamentous, microglobose, crustose, leprose, gelatinous or squamulose thalli with no internal stratification (see Figs. 15.1a–y and 15.8 a–e in Honegger 2012). Many crustose taxa grow within the uppermost zone of the substratum where they meet their photobiont; examples are *Arthopyrenia halodytes* (syn. *Pyrenocollema h.*) with cyanobacterial photobiont (*Hyella* spp.) in the calcareous shell of limpets (*Patella* spp., Mollusca) or barnacles (Cirripedia, Crustacea), *Graphis* spp. with trentepohlioid photobiont in bark, etc. (see Fig. 15.1a–b in Honegger 2012).

6.4.3 The Mycobiont–Photobiont-Interface

In all lichenized asco- and basidiomycetes, the photoautotrophic partner is located outside the contacting fungal hyphae, with different types of mycobiont–photobiont interfaces being differentiated, ranging from simple wall-to-wall apposition to transparietal (formerly termed intracellular) or intraparietal haustoria (Honegger 1986a, b, 1991a; see micrographs in Fig. 15.13 a–l in Honegger 2012). When compared with biotrophic plant pathogenic fungi such as rusts or powdery mildews or with arbuscular mycorrhizal fungi, which form lobate or arbuscular transparietal haustoria, i.e., a large exchange surface in close contact with the plasma membrane of the host cell, the mycobiont–photobiont contact site in lichen is surprisingly simple (see Fig. 1a–f in Honegger 1993): in intraparietal haustoria, as formed by Parmeliaceae, Teloschistaceae, Cladoniaceae, etc., the mycobiont does not pierce the cellulosic cell

wall of the chlorobiont (*Trebouxia* or *Asterochloris* spp., respectively; see Fig. 15.13 a–l in Honegger 2012). A whole range of genes encoding carbohydrate-active enzymes and sugar transporters were identified in 46 representatives of Lecanoromycetes; these LFF would be able to enzymatically degrade the cellulosic cell wall of their photobiont; many of them are likely acquiring additional carbohydrates as saprotrophs (Resl et al. 2022).

Simple wall-to-wall apposition is found in asco- and basidiolichens with *Coccomyxa* or *Elliptochloris* chlorobionts; these incorporate enzymatically non-degradable, sporopollenin-like algaenans in a thin outermost cell wall layer (Honegger and Brunner 1981; Brunner and Honegger 1985; see Fig. 15.3 a–c in Honegger 2012).

6.4.4 Water Relations and Gas Exchange

LFF and their photobionts are poikilohydrous organisms (Greek poikilos: variable; Greek hydror: water) and as such unable to control their water relations. They survive fluctuations in water content between saturation and desiccation (less than 10% water dw^{-1}) unharmed and are metabolically dormant during drought stress events, but recover within few minutes upon rehydration (Scheidegger et al. 1995). During desiccation, the cyanobacterial and green algal cells shrivel and the fungal hyphae cavitate, as seen in Low Temperature Scanning Electron Microscopy preparations of frozen-hydrated specimens, but irrespective of these dramatic changes, the cellular membrane systems remain intact, as visualized in ultrathin sections prepared from cryofixed, freeze-substituted specimens in Transmission Electron Microscopy (Honegger and Peter 1994; Honegger et al. 1996; Honegger and Hugelshofer 2000; see Fig. 16.5a–c in Honegger 2009). Poikilohydrous water relations are also characteristic of lichenicolous and endolichenic fungi and lichen-associated bacteria.

In lichen thalli water and dissolved nutrients are passively taken up at the thallus surface by the conglutinate cortex and/or rhizinae with hydrophilic wall surfaces and passively translocated into the photobiont and medullary layers. As shown with LTSEM techniques, a flux of solutes occurs within the fungal apoplast, mostly in the thick, glucan-rich outer wall layer and underneath the thin hydrophobic wall surface layer (Honegger and Peter 1994); the latter is built up primarily by mycobiont-derived hydrophobin protein (Scherrer et al. 2000, 2002; Scherrer & Honegger, 2003), the characteristic hydrophobic lining of aerial hyphae in the fungal kingdom which self-assembles at the liquid–air interface (Wösten et al. 1993; Wessels 1997; Wösten 2001). Despite being located in the thalline interior, the hyphae of the photobiont and medullary layers are aerial, their hydrophobin layer spreads over the wall surfaces of the photobiont cell population, thus sealing the apoplastic continuum with a hydrophobic coat (Fig. 6.4). The same happens in the lichenized basidiocarp of *Dictyonema glabratum* (syn. *Cora pavonia*; Trembley et al. 2002a, b). The mycobiont-derived hydrophobic wall surface layer plays a crucial role in the functioning of the symbiosis. In co-cultures under sterile conditions, hydrophobin protein is synthesized at an early stage of thallus resynthesis at the contact site of *Usnea hakonensis* and its chlorobiont (*Trebouxia* sp.; Kono et al. 2020).

Traditionally, gas exchange of lichens was measured in the same way as in plant leaves, i.e., outside the thallus which is built up by the heterotrophic partner. However, CO_2 diffusion through the cortex is blocked in fully hydrated thalli; this was interpreted as a depression of photosynthesis at high moisture contents, and water was assumed to fill the intercellular space in the thalline interior (Lange and Tenhunen 1981). In ultrastructural studies, the hydrophobic lining of the wall surfaces in the algal and medullary layers was shown to prevent water from accumulating outside the apoplastic continuum, the algal and medullary layers being gas-filled at any level of hydration (Honegger 1991a, b). The mycobiont of macrolichens amounts to approx. 80% of thalline biomass, the photobiont cell population to approx. 20% or less. The cortex is

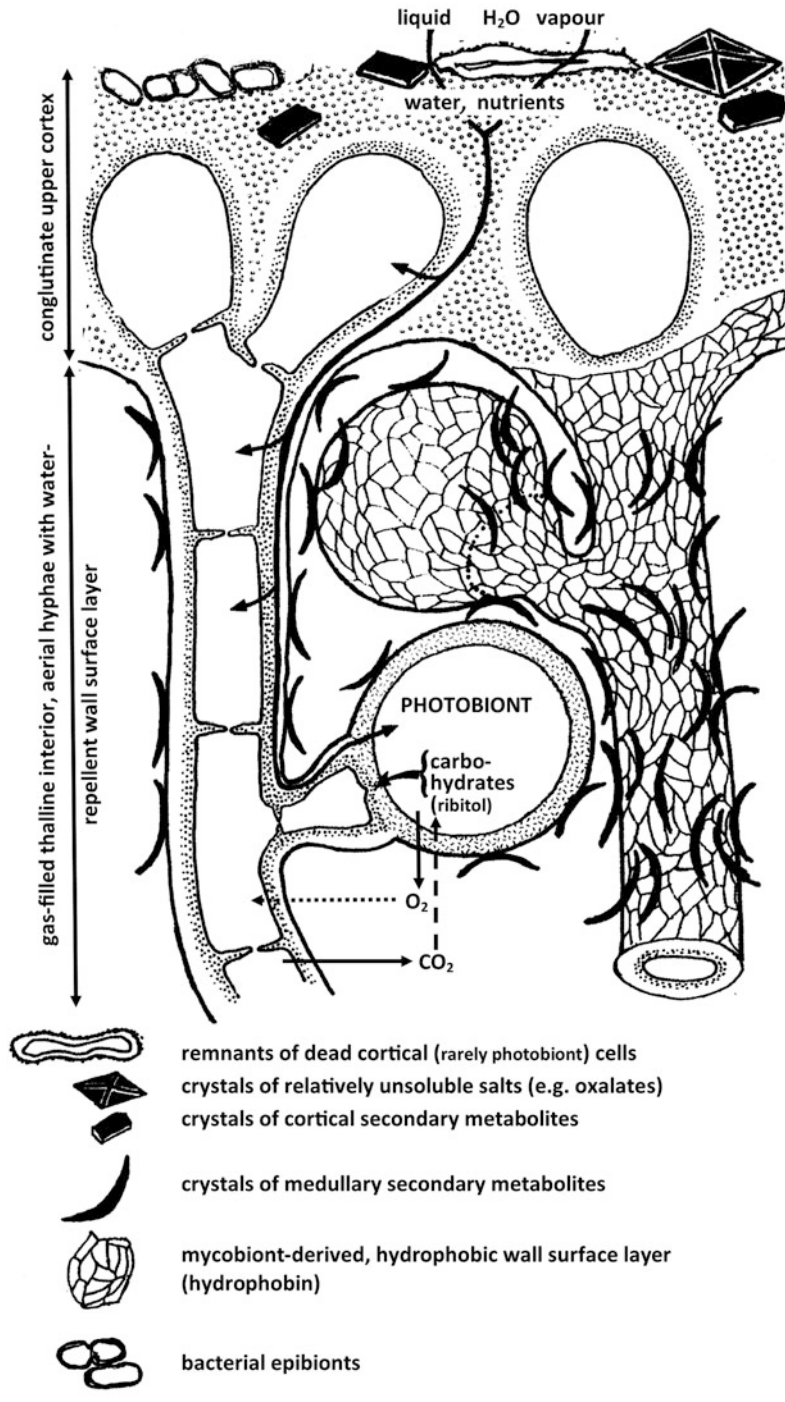

Fig. 6.4 Diagram illustrating the apoplastic continuum and intrathalline gas exchange in Parmeliaceae with *Trebouxia* photobiont. After Honegger (1991a), modified. The mobile carbohydrates, as produced by the different genera of green algal or cyanobacterial photobionts, are summarized by Hill (1976) and Honegger (1997)

translucent when wet, but opaque when dry, and chlorophyll fluorescence is highest in fully hydrated thalli. It was postulated that there should be enough mycobiont-derived respiratory CO_2 in the thalline interior to secure the photosynthetic activity of the photobiont in fully hydrated thalli (Honegger 1991a). By oximetric measurements of electron transport within thalli and fluorimetric

measurements of photosynthesis in *Flavoparmelia caperata* and its *Trebouxia* chlorobiont, ten Veldhuis et al. (2019) showed that photosynthetic O_2 and photosynthates are taken up by the mycobiont and its respiratory CO_2 by the chlorobiont, the algal and fungal energy metabolism being mutually linked, a perfect recycling system.

6.4.5 Heavy Metal and Radionuclides

Lichens absorb not only water and nutrients, but also pollutants, heavy metals and radionuclides included. Thus, they are used in biomonitoring studies in urban and industrial areas (Nimis et al. 2002; Abas 2021). They have also been used as silent chronists of early nuclear weapon testing and of the Chernobyl and Fukushima accidents (Seaward 2002; Saniewski et al. 2020; Dohi et al. 2021; Anderson et al. 2022). Heavy metals are bound in insoluble complexes within the thallus. Characteristic lichen communities develop on heavy metal rich rock substrates. 5000 ppm of copper were found in the crustose thalli of *Lecanora vinetorum*: it grows on wooden supports in vineyards in South Tyrolia which, for decades, were sprayed 12 times per year with copper sulfate as a fungicide to protect the *Vitis* cultivars (Poelt and Huneck 1968); the mycobiont-derived xanthone vinetorin is assumed to form insoluble copper complexes.

6.5 The Microbiome of Lichen Thalli

6.5.1 The Bacteriome: Bacterial Epi- and Endobionts of Lichen Thalli

At least since the Early Devonian (approx. 415 Ma ago), non-photosynthetic bacteria live on the thallus surface of lichens, and filamentous actinobacteria are found on their cortex and in the thalline interior (Fig. 6.5a–b; Honegger et al. 2013b). In the twenty-first century, the taxonomic diversity and potential role of the bacteriome for lichen symbiosis was intensely explored, as reviewed by Grube and Berg (2009), Grube et al. (2012, 2016), and Grimm et al. (2021).

Many lichens live in nutrient-poor habitats such as bare rock surfaces, steppe and desert or oligotrophic boreal-arctic ecosystems. Numerous plant-beneficial bacteria have been identified in the rhizosphere of plants (Burr et al. 1984; Davison 1988); some of them fix nitrogen, others solubilize phosphate or act as antagonists of plant pathogens and thus are important in sustainable agriculture (Romano et al. 2020). Considering the ubiquitous bacterial epi- and endobionts of lichen thalli (Fig. 6.5c–j), the question was: are there any lichen-beneficial bacteria (Honegger 1991b)? Maria Cengia Sambo (1924, 1926) was the first to culture and identify an *Azobacter* associated with lichens and referred to its potential role as a N_2-fixing partner; she introduced the term polysymbiosis. In the last decades, a whole range of nitrogen-fixing representatives of Rhizobiales and *Frankia* have been characterized as associates of lichen thalli (Hodkinson and Lutzoni 2009; Bates et al. 2011; Erlacher et al. 2015; Eymann et al. 2017; Almendras et al. 2018, etc.). Some lichen-associated Rhizobiales synthesize β-carotenes (Pankratov et al. 2020). The most common and widespread bacterial symbionts of lichens are representatives of Alphaproteobacteria. The bacterial community may change upon infection of the thallus by lichenicolous fungi or lichens (Wedin et al. 2016). Chlorolichens carry different bacterial communities than cyanolichens (Almendras et al. 2018).

In non-lichenized fungi, the intimate bacterial–fungal interaction triggers the biosynthesis of secondary metabolites such as archetypal polyketides in *A. nidulans* (Schroeckh et al. 2009) or a cryptic meroterpenoid pathway in *A. fumigatus* (König et al. 2013).

Comparative analyses of the bacteriome of lichens from different ecosystems were performed, e.g., the littoral (Sigurbjornsdottir et al. 2014; Parrot et al. 2015; West et al. 2018), high mountain areas such as alpine soil crust lichens (Muggia et al. 2013), the Andean Paramo

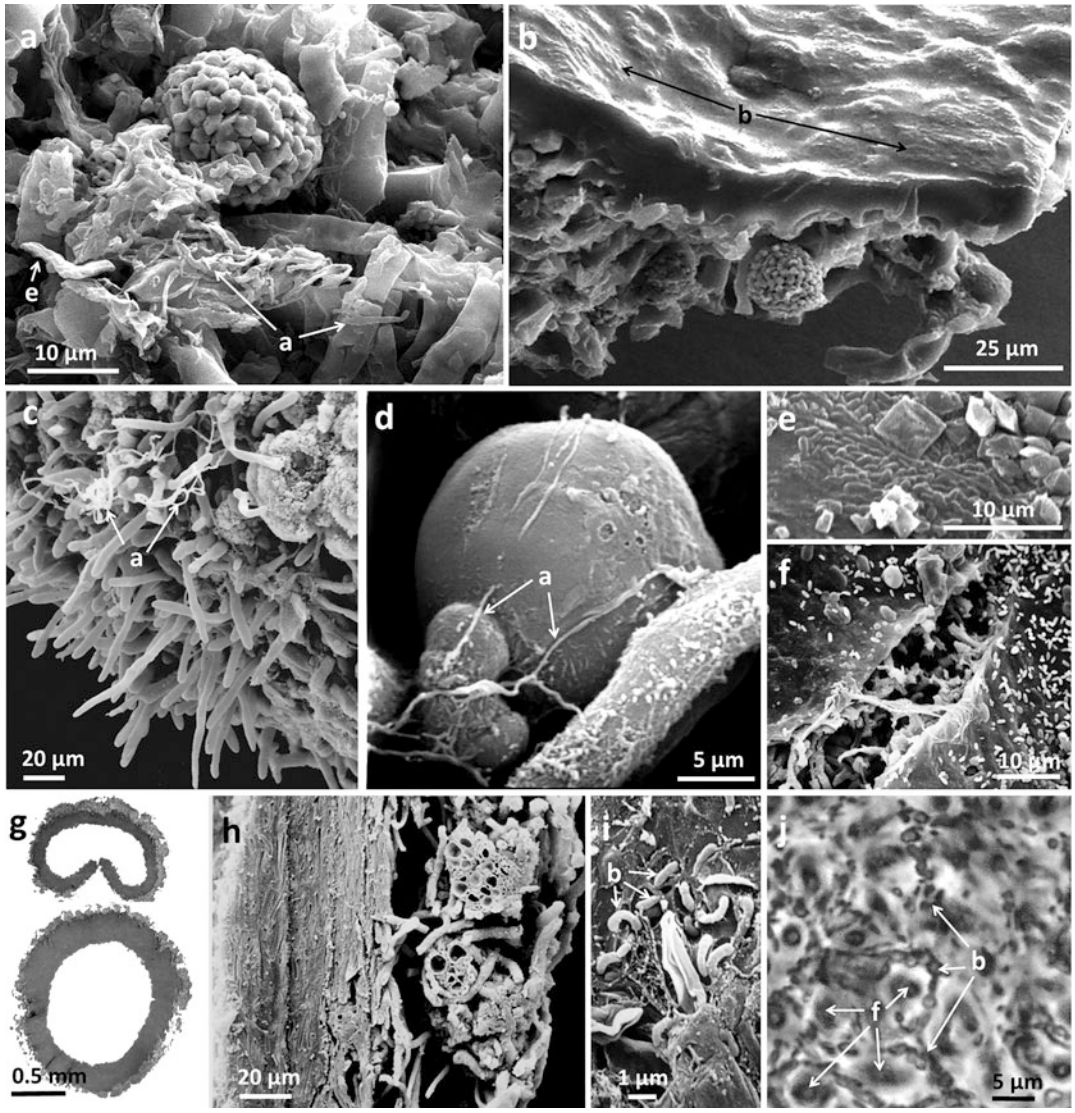

Fig. 6.5 Epi– and endolichenic bacteria past (**a, b**) and present (**c**–**j**). (**a**, **b**) Charcoalified, cross-fractured fragment of the green algal lichen *Chlorolichenomycites salopensis*, 415 Ma old, with endolichenic fungi (*e*) and actinobacterial filaments (*a*) in the thalline interior (**a**) and bacteria (*b*) on the surface of the cortex (**b**). After Honegger et al. (2013b). (**c**) Thallus margin of *Peltigera praetextata* with actinobacterial filaments (*a*) around protruding hyphae (trichogynes?). (**d**) Actinobacterial filaments (*a*) overgrowing the mycobiont–photobiont interface in the algal layer of *Pleurosticta acetabulum*. (**e**, **f**) Bacterial colonies growing on the surface of the upper cortex of *Parmelia sulcata*. (**g**–**j**) The fruticose thallus of the reindeer lichen *Cladonia arbuscula*, a conglutinate, tubiform axis with peripheral photobiont layer and bacterial epi- and endobionts. (**g**) Cross-sections of the young (top) and older part of the thallus. (**h**) SEM of a longitudinal section, the conglutinate axis being built up by thick-walled parallel hyphae. (**i**) Bacteria growing on the inner surface of the axis near the tip of the thallus. (**j**) Cross-section of the basal, old part of the axis with large numbers of bacteria (*b*) growing between the thick walls of the fungal hyphae (*f*)

(Sierra et al. 2020), the Qinghai–Tibet Plateau (Hei et al. 2021) or tropical rainforests (Mara et al. 2011). The best investigated is the microbiome of the lung lichen, *Lobaria pulmonaria*, as reviewed by Grimm et al. (2021).

In the desiccated state, i.e., in herbaria, some bacterial symbionts retain their viability for decades (Cernava et al. 2016), whereas lichen myco- and photobionts die off within a few years of storage at room temperature (Honegger 2003). Thus, herbaria with specimens from remote areas might be a source of interesting bacterial associates of lichens, some of them with biologically interesting properties (Cernava et al. 2016). In nature, bacterial associates of lichen thalli survive drought stress unharmed (Cernava et al. 2018).

Within thalli, the species richness and cell density of lichen-associated bacteria increase from peripheral, young growing zones to older, non-growing areas. In the lobate *Xanthoparmelia*, the highest bacterial diversity was found in the centre (Mushegian et al. 2011), in the fruticose reindeer lichen *Cladonia arbuscula* or in the podetia of *Cladonia squamosa* in basal parts (Cardinale et al. 2008; Noh et al. 2020; Fig. 6.5g–i); some of the bacterial associates of old thallus areas are likely involved in biodegradative processes.

Vertical transmission of the microbiome (except the *Nostoc* cyanobiont of cephalodia) via symbiotic propagules occurs in the lung lichen (*Lobaria pulmonaria*; Aschenbrenner et al. 2014); with high probability isidia and thallus fragments of most other species also carry at least part of the microbiome at their surface, but whether soredia with very hydrophobic surfaces (Fig. 6.3e) carry bacteria remains to be seen. Lichen-inhabiting invertebrates have their own bacteriome on their surfaces (Chasiri et al. 2015).

Lichen-associated bacteria have attracted considerable interest as potential producers of novel secondary compounds with biotechnological potential, the focus being on actinobacteria (González et al. 2005; Parrot et al. 2015, 2016a, b; Suzuki et al. 2016).

6.5.2 Lichenicolous Fungi

Approximately 2320 spp. of lichenicolous asco- and basidiomycetes have been described (Fig. 6.6); 2000 spp. are obligately, 62 spp. facultatively lichenicolous and 257 spp. are lichenicolous lichens (Diederich et al. 2018), new families, new genera and species being continuously described from ecosystems which had not been previously investigated. Examples are representatives of *Pleostigmataceae* and *Melanina* spp. among the Chaetothyriomycetidae in crustose lichens from subalpine areas (Muggia et al. 2021), or *Crittendenia* spp. lichenicolous representatives of Pucciniomycotina (Basidiomycota; Millanes et al. 2021). A well-preserved lichenicolous *Lichenostigma* sp., (Arthoniomycetes, Ascomycota) was found on fossil *Ochrolechia* sp. in palaeogene amber (approx. 24 Ma old; Kaasalainen et al. 2019).

Some lichenicolous fungi have a minor impact on their host (e.g. mycocalicialean *Sphinctrina* spp. on *Pertusaria* spp., Fig. 6.6e), others have a devastating effect, killing the fungal and photoautotrophic partner (e.g. the corticialean basidiomycetes *Marchandiomyces aurantiacus* and *M. corallinus* on *Physcia* and *Parmelia* spp.; Fig. 6.6b). Only a few lichen spp. regenerate after an attack by the athelialean basidiomycete *Athelia arachnoidea* (Fig. 6.6a), whose asexual state is *Rhizoctonia carotae*, a pathogen of carrots (Adams and Kropp 1996), but the free space created by the killing effect is soon occupied by fast-growing crustose taxa such as *Biatora* spp. (Motiejûnaitë and Jucevièienë 2005).

Several lichenicolous fungi stimulate, probably via secretion of hormones, gall formations in their hosts (Hawksworth and Honegger 1994). Examples are the verrucarialean *Telogalla olivieri* on *Xanthoria parietina*, or the parmeliacean *Raesaenenia huuskonenii* on *Bryoria* spp. (Fig. 6.6h–i). The characteristic galls of members of the *Biatoropsis usnearum* complex (Tremellales, Basidiomycota; Fig. 6.6d; Millanes et al. 2016a) on *Usnea* spp. are basidiomata of the parasite. Even hyperparasitism was detected

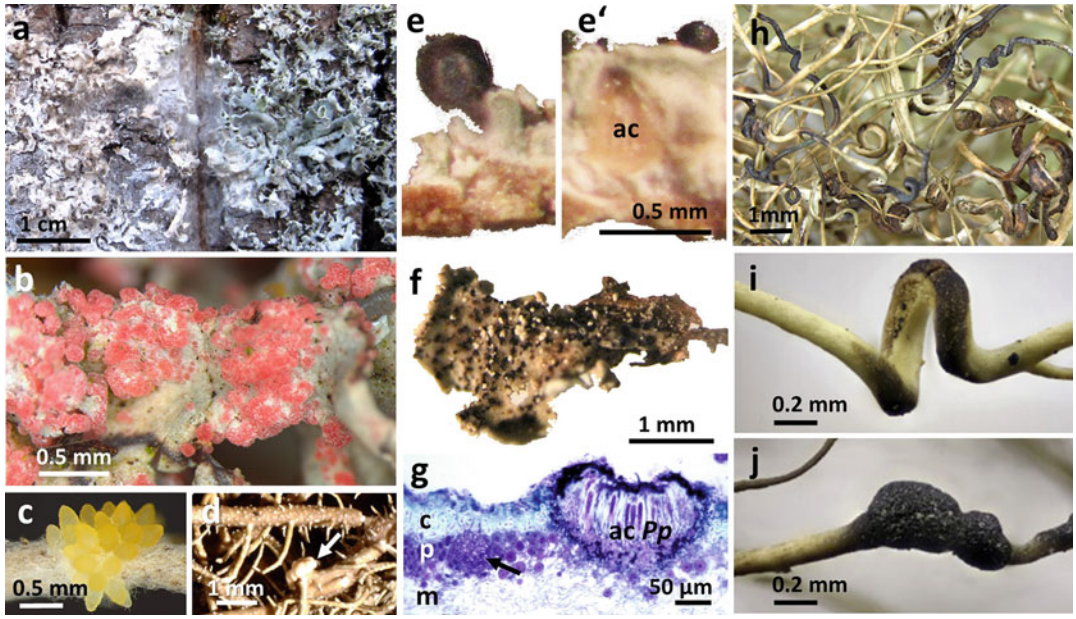

Fig. 6.6 Lichenicolous (parasitic) ascomycetes (**c**, **e**, **f**, **g**, **h**, **i**) and basidiomycetes (**a**, **b**, **d**, **j**). (**a**) *Athelia arachnoidea* (Atheliales, Agaricomycetes) killing *Physcia tenella*. (**b**) *Marchandiomyces corallinus* (Corticiales, Agaricomycetes) on *Physcia stellaris*; courtesy of Erich Zimmermann. (**c**) Ascomata of *Neobarya peltigerae* (Clavicipitaceae, Sordariomycetes) on rhizinae of *Peltigera* sp. From Zimmermann and Feusi (2018), courtesy of Erich Zimmermann. (**d**) Basidioma (arrow) of *Biatoropsis usnearum* (Tremellales, Tremellomycetes) on *Usnea ceratina*. (**e**, **e′**) Mazaediate ascomata of *Sphinctrina turbinata* (Mycocaliciaceae, Eurotiomycetes) on dissected *Pertusaria pertusa*; (ac) ascoma of P. *pertusa*. (**f**, **g**) thallus squamule of *Cladonia macrophylla* invaded by *Phaeopyxis punctum* (Helotiales, Leotiomycetes), black dots in (**f**) are ascomata of the parasite, whitish dots are adhering soredia of the lichen. (**g**) Stained semi-thin section of *C. macrophylla* with cortex (*c*), photobiont (*ph*) and medullary layer (*m*) of the lichen-forming ascomycete and ascoma of the parasite (*ac Pp*); arrows point to the primordium of an ascoma of the parasite with trichogynes protruding through the cortex of the lichen. From Honegger 2012. (**h–j**) Horsehair lichens (*Bryoria fuscescens* in **h**, *B. pikei* in **i–j**) invaded by curled gall-inducing *Raesaenenia huuskonenii* (syn. *Phacopsis* or *Protousnea huuskonenii*, respectively; Parmeliaceae, Lecanoromycetes). (**h**) Infected *Bryoria fuscescens*, from Zimmermann and Feusi (2020), courtesy of Erich Zimmermann. (**j**) basidioma of the hyperparasite *Tremella huuskonenii* (Tremellales, Tremellomycetes) developing on the gall of the lichenicolous *R. huuskonenii* on *B. pikei.* (**i**, **j**) From Lindgren et al. (2015), courtesy of Paul Diederich

(e.g. *Tremella huuskonenii*, parasite of *R. huuskonenii* on *Bryoria* sp., Fig. 6.6j; Lindgren et al. 2015).

Various investigators found basidiomycetous yeasts as symptomless endolichenic fungi on and in lichen thalli, belonging to the Tremellomycetes (Agaricomycotina), Cystobasidiomycetes or Agaricostilbomycetes (Pucciniomycotina; review: Diederich et al. 2018). Examples are *Fellomyces* spp. (Cuniculitremataceae, Tremellales; Prillinger et al. 1997; Lopandic et al. 2005), the *Cyphobasidium usnicola* complex and other *Cyphobasidium* spp. on various macrolichens (Cystobasidiales; Millanes et al. 2016b; Spribille et al. 2016), or *Lichenozyma pisutiana* in *Cladonia* spp. (Cystobasidiales; Černajová and Škaloud 2019). These basidiomycetous yeasts are widespread, but not ubiquitous in lichen thalli (Lendemer et al. 2019). Particularly interesting are tremellalean lichenicolous fungi which produce galls on their host lichen, but switch between a yeast and a hyphal stage, the former being located on and in the cortex of the lichen, as visualized with FISH (fluorescent *in*

situ hybridization techniques; Spribille et al. 2016). Examples are *Tremella lethariae* on *Letharia vulpina* (Tuovinen et al. 2019), *T. macrobasidiata* and *T. varia* on *Lecanora* spp. (Tuovinen et al. 2021).

Some lichenicolous fungi reduce the growth rate and the production of secondary metabolites of their host (e.g. *Plectocarpon* spp. on *Lobaria pulmonaria*; Asplund et al. 2018; Merinero and Gauslaa 2018), others degrade the secondary metabolites of their host (e.g. parietin of *Xanthoria parietina* by the capnodialean *Xanthoriicola physciae*; Edwards et al. 2017, or lecanoric acid by hypocrealean *Fusarium* spp. on *Lasallia* spp.; Lawrey et al. 1999), thus facilitating the attack of opportunistic fungi and/or enhancing the grazing pressure by lichenivorous invertebrates (Lawrey et al. 1999; Asplund et al. 2016). More and different secondary metabolites than in the lichen proper were found in *Cladonia foliacea* upon infection with the lichenicolous *Heterocephalacria bachmannii* (Filobasidiales, Tremellomycetes), the antioxidant potential of the thallus being enhanced (Khadhri et al. 2019).

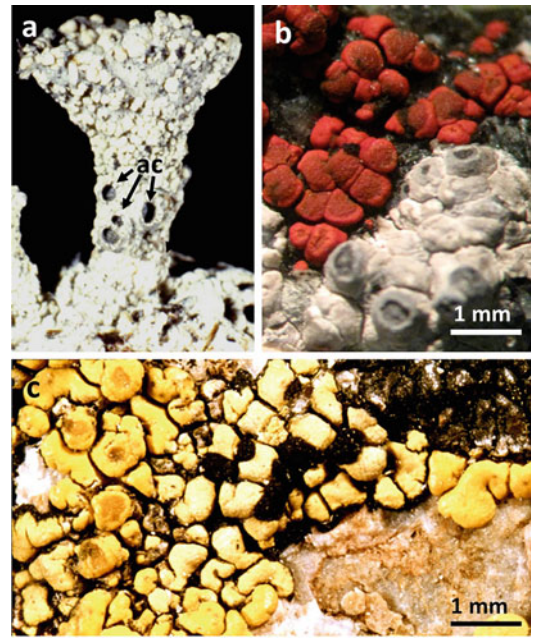

Fig. 6.7 Lichenicolous lichens. (**a**) *Diploschistes muscorum* (Ostropales) invading and overgrowing *Cladonia chlorophaea*. (*ac*) ascomata. (**b**) *Caloplaca anchon-phoeniceon* (Teloschistales) invading *Aspicilia calcarea*. (**c**) *Rhizocarpon effiguratum* (Rhizocarpales) invading *Acarospora oxytona*

6.5.3 Lichenicolous Lichens

Lichenicolous lichens (Fig. 6.7a–c) are a group of specialists whose ascospore-derived germ tubes start their development in the thallus of a host lichen, which they invade; some of them take over the photobiont host, others acquire a different green algal partner (see below). The parasitic lichen overgrows its host and differentiates its own thallus. The first known lichenicolous lichen, *Rhizocarpon lusitanicum*, parasite of *Pertusaria* spp., was described by Nylander in 1865 when lichen symbiosis was not yet defined. In their 2018 compilation, Diederich et al. listed 257 spp. of lichenicolous lichens and their hosts, representatives of 63 genera, 28 families, 16 orders and four classes of ascomycetes. The vast majority are Lecanoromycetes, the most species-rich genera being *Rhizocarpon* (33 spp.), *Acarospora* (27 spp.) and *Caloplaca* (26 spp.; Diederich et al. 2018).

The acquisition of the photobiont by the lichenicolous *Diploschistes muscorum* on *Cladonia* spp. was investigated using light microscopy techniques applied to symbiotic and cultured photobiont cells (Friedl 1987). In the first developmental stage on *Cladonia* sp., *D. muscorum* was shown to associate with *Asterochloris irregularis* (syn. *Trebouxia irregularis*), the unicellular green algal partner of the *Cladonia* host; subsequently algal switching occurred, *A. irregularis* being replaced by *Trebouxia showmanii* (Friedl 1987) and/or other *Trebouxia* spp. (Wedin et al. 2016). *Diploschistes* spp. associate with diverse photobiont species, as concluded from molecular investigations of *D. diacapsis* in Mediterranean soil crust communities, up to four different *Trebouxia* spp. having been found in one thallus (Moya et al. 2020).

6.5.4 Endolichenic Fungi (ELF)

Whereas the diversity of lichenicolous fungi has been intensely investigated from the nineteenth century onwards, the presence of numerous symptomless endolichenic fungi in lichen thalli was first detected towards the end of the twentieth century (Petrini et al. 1990). Today, more than 200 spp. of endolichenic fungi are known, predominantly ascomycetes, few basidio- and zygomycetes, 135 having been identified at species level; they were isolated from 114 lichen species, the majority being parmeliacean macrolichens (Chakarwarti et al. 2020).

Most ELF are saprobic generalists, but also several wood-decay asco- and basidiomycetes were found such as the xylarialean *Xylaria*, *Hypoxylon* and *Daldinia* spp., or the agaricales *Schizophyllum commune* and the russulales *Heterobasidion annosum* (Fig. 6.8). Lichen thalli as a refuge of wood-decay fungi: an unexpected result! Some ELF are plant pathogens, a few are also endophytes of plants (Arnold et al. 2009; U'Ren et al. 2010, 2012). The biology of endolichenic fungi is poorly investigated; they absorb polyols such as mycobiont-derived mannitol from the apoplastic continuum of the lichen thallus (Yoshino et al. 2020).

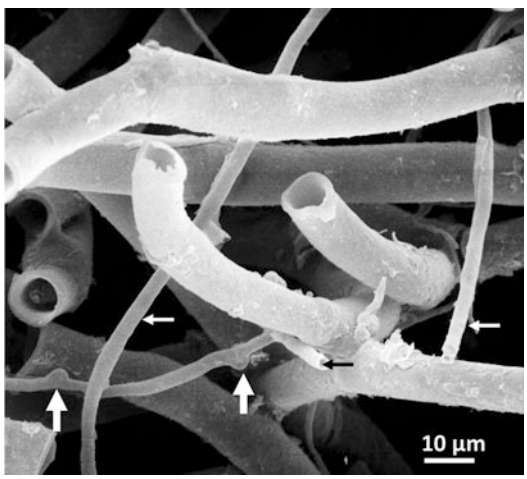

Fig. 6.8 Comparatively thin hyphae of symptomless endolichenic fungi (arrows) growing between the medullary hyphae of *Peltigera leucophlebia*. Broad arrows point to clamp connections of an endolichenic Agaricomycetes. After Honegger (2012)

Upon isolation into sterile culture, ELF turned out to be a rich source of interesting bioactive secondary metabolites. More than 140 novel compounds have been identified, many of them with pharmaceutical potential such as antibiotic, antiviral, antifungal, anti-inflammatory, antiproliferative or antioxidant properties (reviews: Gao et al. 2016; Kellogg and Raja 2017; Tripathi and Joshi 2019; Agrawal et al. 2020; Wethalawe et al. 2021). Considering that only approximately 5% of lichen species have so far been investigated for ELF, there are lots of interesting work ahead.

6.5.5 The Virome of Lichens

Upon isolation into sterile culture, the first mycoviruses in the mycobiome of lichens were detected in the so-called dust lichens, i.e., the leprose thalli of *Lepraria incana* (Stereocaulaceae, Lecanoromycetes; LiCV1) and *Chrysothrix chlorina* (Chrysotrichaceae, Arthoniomycetes; CcCV1). LiCV1 and CcCV1 are classified in the genus *Alphachrysovirus* (family Chrysoviridae; Petrzik et al. 2019) whose host range comprises fungi, plants and possibly insects (Kotta-Loizou et al. 2020). Upon closer examination, i.e., with fluorescent *in situ* hybridization techniques, CcCV1 was located not in the lichen mycobiont proper, but in an associated *Penicillium citreosulfuratum* (Eurotiomycetes; Petrzik et al. 2019).

Lichen photobionts were shown to harbour plant viruses such as the cauliflower mosaic virus (Petrzik et al. 2013). With immunogold labelling techniques applied to ultrathin sections, viruses contained in more than 70 years old sterile cultures of *Trebouxia aggregata*, photobiont of *Xanthoria parietina*, were shown to be located in the algal cytoplasm, not in the chloroplast; upon mechanical transmission to plant host cells these phycobiont-derived viruses were infective (Petrzik et al. 2015).

In a study on virus diversity in the metagenome of the saxicolous *Umbilicaria phaea*, five novel viruses were detected, one of

them, a Caulimovirus, being associated with the *Trebouxia* photobiont and the four others, belonging to the Myoviridae, Podoviridae and Siphoviridae, with bacterial associates of the lichen, but so far, no mycoviruses have been found (Merges et al. 2021).

RNA virus diversity was investigated in an extract of a microcosm, predominantly consisting of *Cladonia* spp. and other Lecanoromycetes and their trebouxiophycean photobionts, overgrowing bryophytes, non-lichenized ascomycetes, cyanobacteria, free-living green algae, fern (spores?) and minute animals living therein, as seen in SSU rRNA sequences (Urayama et al. 2020). A total of 65 operational taxonomic units (OTUs) were achieved, 17 belonging to Partitiviridae (five to the genus *Alphapartitivirus*, one to *Betapartitivirus,* the host range of both genera comprising fungi and plants; Vainio et al. 2018), plus representatives of 10 additional viral families and several unclassified dsRNA and ssRNA viruses (Urayama et al. 2020).

The impact of viral infections on the biology and whether horizontal virus transfers occur between the partners of lichen symbiosis and their allies remain to be investigated.

6.6 Fossil Lichens and Their Microbiome

6.6.1 Fossil vs. Extant Lichens

Fungi colonized terrestrial environments in the Proterozoic, presumably associating with the even more ancient cyanobacteria, the latter having been present already in the Archean (approx. 2400 Ma), but colonized terrestrial habitats from the Proterozoic onwards, as concluded from fossil records and molecular clock analyses (Strother and Wellman 2016; Lutzoni et al. 2018; Demoulin et al. 2019; Garcia-Pichel et al. 2019). Trebouxiophyceae, unicellular green algae, and Trentepohliales, filamentous green algae, were present from the Proterozoic onwards, some extant taxa of both groups being the main photobionts of extant LFF (Lutzoni et al. 2018; del Cortona et al. 2020). Thus, lichenization is assumed to be an ancient fungal lifestyle, but fossil records are very scarce and partly difficult to interpret (Fig. 6.9).

According to molecular phylogenetic datasets, the clades of ascomycetes which gave rise to extant lichens did not radiate before the Silurian (approx. 450 Ma; Lutzoni et al. 2018) or even the Mesozoic (approx. 250 Ma; Nelsen et al. 2019). According to the latter authors ascolichens, as we know them today, are younger than previously assumed, did not predate the evolution of vascular plants and were not among the (extinct) early fungal colonizers of terrestrial habitats. Nevertheless, the lichen lifestyle was most likely established in the common and widespread nematophytes and *Prototaxites* spp. (review: Selosse and Strullu-Derrien 2015). The manyfold problems related to the interpretation of fungal fossils are summarized by Berbee et al. (2020).

6.6.2 Palaeozoic Fossils

An approx. 600 Ma old lichen-like association was preserved in marine phosphorite of the Doushantuo Formation in South China (Yuan et al. 2005). Mats of cyanobacterial or coccoidal algal cells were invaded by extremely thin filamentous hyphae with diameters below 1 micron, a characteristic feature of actinobacteria, not fungi (Honegger 2018); thus, the Doushantuo fossils are not lichens.

Nematophytes, an extinct phylum of presumably lichenized fungi with no modern analogues and thus not represented in molecular phylogenetic trees of living species, were common and widespread constituents of terrestrial cryptogamic ground covers from the late Silurian to the late Devonian. They comprise the families Nematothallaceae (genera *Nematothallus*, *Cosmochlaina, Tristratothallus*; Edwards et al. 2013, 2018) and Nematophytaceae (genera *Nematoplexus*, *Nematasketum* and the enigmatic *Prototaxites*; Edwards and Axe 2012). After long debates about their taxonomic affiliation nematophytes are currently interpreted as presumably lichenized thalloid fungal structures (Edwards et al. 2013, 2018). Cross-fractures of

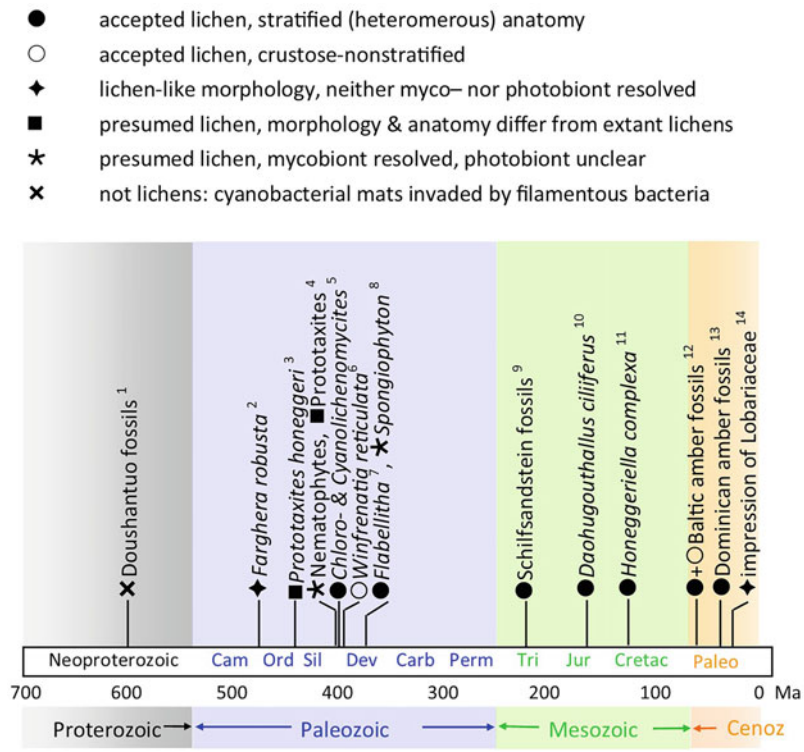

Fig. 6.9 Fossil lichens, an overview. 1. Yuan et al. (2005); 2. Retallack (2009); 3. Retallack (2020); 4. Edwards et al. (2013, 2018); Honegger et al. (2018); 5. Honegger et al. (2013a); 6. Taylor et al. (1995, 1997), Karatygin et al. (2009); 7. Jurina and Krassilov (2002); 8. Stein et al. (1993), Jahren et al. (2003); 9. Ziegler (2001); 10. Wang et al. (2010), Fang et al. (2020); 11. Matsunaga et al. (2013); 12. Mägdefrau (1957), Rikkinen and Poinar (2002), Rikkinen (2003), Hartl et al. (2015), Kaasalainen et al. (2015, 2017, 2020), Rikkinen et al. (2018); 13. Poinar Jr. et al. (2000), Rikkinen and Poinar (2008); 14. MacGinitie (1937), Peterson (2000). Chronostratigraphy (International Commission on Stratigraphy (ICS) chart 2019; Hounslow 2020): Periods in Ma. Neoproterozoic (1000-541); Cam: Cambrian (541-484); Ord: Ordovician (485-443); Sil: Silurian (443-419); Dev: Devonian (419-358); Carb: Carboniferous (358-298); Perm: Permian (298-251): Tri: Triassic (251-201); Jur: Jurassic (201-145); Cretac: Cretaceous (145-66); Paleo: Paleogene (66-23). After Honegger (2018), modified

some nematophyte fossils reveal the same stratification as found in lichens, with a conglutinate peripheral cortex and a medullary layer built up by interwoven hyphae, but photobiont cells are missing; they might have been lost during fossilization. In charcoalifying experiments with extant cyanobacterial lichens, the fungal partner was well preserved, and so were the thick mucilaginous sheaths of the *Nostoc* photobiont, but most of the cyanobacterial cells proper were lost (Honegger et al. 2013a).

Prototaxites spp., enigmatic fossils with erect axes, built up by hyphae of various diameters, ranging from pencil size dimensions as in the mid-Ordovician (Darrivilian, approx. 460 Ma old) *P. honeggeri* (Retallack 2020) to more than 8 metres long stems, as in the mid-Devonian (early Givetian, approx. 385 Ma old) *P. loganii*, were interpreted as presumably lichenized fungi, coccoid photobiont cells having been resolved at the surface of the axes of both species (Retallack and Landing 2014; Retallack 2020). However, it remains unclear how the few coccoid algal cells should have provided enough carbohydrate to nourish these large fungal structures; nematophytes were assumed to be associated with *Prototaxites* (Selosse and Strullu-Derrien 2015). Delicate fruiting structures at the surface

of the axes were seldom retained; luckily the hymenial layer was preserved in a series of petrographic thin sections of *P. taiti* of the Kidston and Lang collection. The ascomata of *P. taiti* combine features of extant ascomycetes, i.e., Taphrinomycotina (Neolectomycetes lacking croziers) and Pezizomycotina (epihymenial layer secreted by paraphyses), but *Prototaxites* is not an ancestor of the latter (Honegger et al. 2018).

The mid-Devonian *Spongiophyton minutissimum* is a presumed lichen, built up by a conglutinate, tissue-like, porate fungal cortex around a central cavity; a photobiont was not resolved (Taylor et al. 2004).

Winfrenatia reticulata is a permineralized fossil lichen, 10 cm long, 2 mm thick, from the Lower Devonian Rhynie chert (approx. 400 Ma old; Taylor et al. 1995, 1997). A presumed mucormycete forms a basal mat and superimposed ridges built up by parallel hyphae which are in contact with coccoid cyanobacterial cells interspersed with a filamentous cyanobacterium (Karatygin et al. 2009). However, *Winfrenatia* might be a fragment of a larger thalloid organism such as a nematophyte (Honegger, unpubl.).

The oldest lichenized ascomycetes with dorsiventally organized, internally stratified thallus so far found are charcoalified fragments of *Cyanolichenomycites devonicus*, a cyanobacterial lichen with *Nostoc*-like photobiont (Fig. 6.10a, b), and *Chlorolichenomycites salopensis*, a presumed green algal lichen whose globular photobiont cells were retained as framboidal pyrite (Fig. 6.10c–e; Honegger et al. 2013a). Both fossils were extracted from a Lower Devonian siltstone from the Welsh Borderland (approx. 415 Ma old). Despite their striking structural similarity with extant Lecanoromycetes lichens, the taxonomic affiliation of these fossils remains unclear. They might either belong to the nematophytes or represent the earliest known members of extant lichenized ascomycetes (Hawksworth 2012; Lutzoni et al. 2018); in the latter case, some molecular clock estimates would require recalibration.

6.6.3 Mesozoic Fossils

Daohugouthallus ciliiferus, a well-preserved, band-shaped lichen resembling extant Parmeliaceae such as *Pseudevernia furfuracea*, was found in mid Jurassic sediments (approx. 165 Ma old) in north-eastern China (Inner Mongolia; Wang et al. 2010; Fang et al. 2020).

Very well preserved is the Mesozoic *Honeggeriella complexa*, a dorsiventrally organized, foliose lichen from the Early Cretaceous of Vancouver Island (Valanginian-Hauterivian boundary, ca. 133 Ma old) with distinct green algal layer and haustorial complexes at the mycobiont–photobiont interface comparable to those found in extant taxa (Matsunaga et al. 2013).

6.6.4 Cenozoic Fossils

Large numbers of well-preserved lichens were found in Cenozoic Baltic and Bitterfeld (approx. 50–35 Ma old) and in Dominican amber (approx. 16 Ma old). Excellent external and internal structural preservation allows to identify taxa similar or closely related to extant species (Hartl et al. 2015), most of them having been epiphytes on the resin-producing trees (e.g. *Pinus* spp. in N-Europe). Among the fruticose-pendulous lichens in Baltic amber are alectorioid taxa and *Usnea* spp. (Kaasalainen et al. 2015, 2020). Among the foliose lichens is *Anzia electra*, common and widespread in Baltic and Bitterfeld amber, but today extinct in Europe (Rikkinen and Poinar 2002; Schmidt et al. 2013). Very well preserved *Parmelia ambra* and *P. isidiiveteris* (Poinar Jr. et al. 2000) and *Phyllopsora dominicanus* (Rikkinen and Poinar 2008) were found in Dominican amber. Among the crustose lichens in Baltic amber are calicioid taxa (Rikkinen 2003; Rikkinen et al. 2018) and *Ochrolechia* spp. (Kaasalainen et al. 2019).

There are many more types of amber worldwide whose fossils await thorough investigation, the oldest ones dating back to the Carboniferous

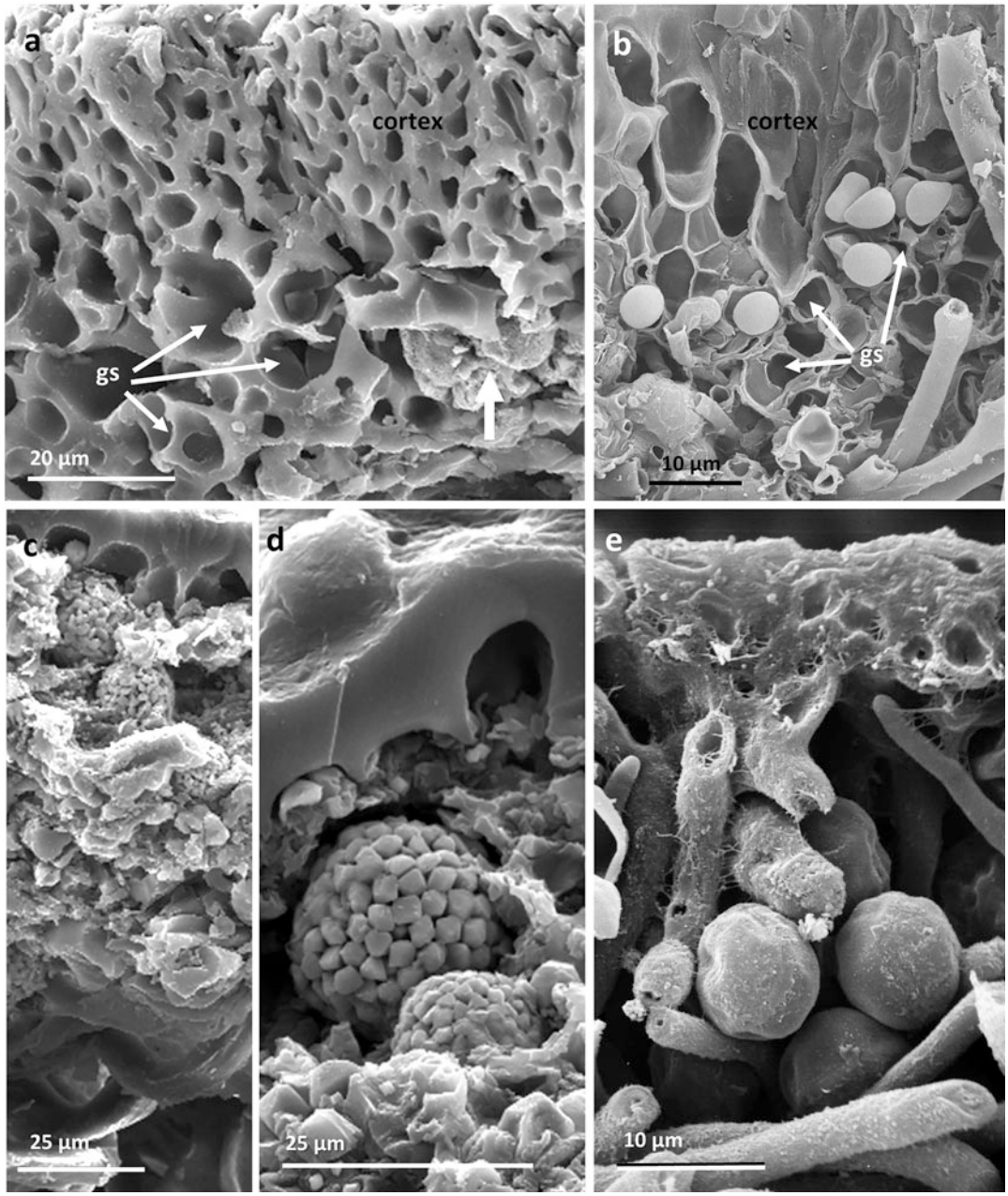

Fig. 6.10 Fossil lichens from the Lower Devonian (ca. 415 Myr old) with dorsiventrally organized, stratified thallus (**a**, **c**–**d**) in comparison with extant Lecanoromycetes (**b**, **e**). (**a**) *Cyanolichenomycites devonicus* with conglutinate cortex and a *Nostoc*-like cyanobiont in the photobiont layer; in contrast to the well-preserved gelatinous sheaths (*gs*) only few cyanobacterial cells (bold arrow) are retained. (**b**) *Peltigera praetextata* (Peltigerales) with *Nostoc* sp. as cyanobiont. (**c**–**d**) *Chlorolichenomycites salopensis* with thin peripheral cortex and globose, presumed green algal photobiont, the latter being preserved as framboidal pyrite. (**e**) *Pleurosticta acetabulum* (Lecanorales); chlorobiont: *Trebouxia* sp. After Honegger et al. (2013a), modified

period (approx. 320 Ma old). DNA has been successfully extracted, amplified and sequenced from invertebrate and plant fossils in Dominican and Lebanese amber, the oldest ones being approx. 120–135 Ma old (Poinar 1994).

6.6.5 The Microbiome of Fossil Lichens

In the dorsiventrally organized thallus of the Lower Devonian *Chlorolichenomycites salopensis* were bacterial colonies growing on the surface of the cortex and actinobacteria and lichenicolous fungi in the thalline interior, comparable to the situation in extant lichens (Fig. 6.5a, b; Honegger et al. 2013b).

Fructifications of *Lichenostigma* sp. (Lichenostigmatales, Arthoniomycetes) were found on *Ochrolechia* sp. in palaeozoic amber (Kaasalainen et al. 2019), *Lichenostigma* spp. being common and widespread lichenicolous fungi of extant lichens (Hafellner and Calatayud 1999; Diederich et al. 2018). A wide range of filamentous fungi were found on decaying crustose lichens embedded in amber, such as well-preserved toruloid taxa, conidiomata of dematiaceous hyphomycetes resembling extant genera such as *Sporidesmium*, *Taeniolella* and *Taeniolina* (Kettunen et al. 2016, 2018).

6.7 Lichen–Animal Relations

6.7.1 The Micro- and Mesofauna of Lichen Thalli

Many interactions of lichens with protozoans, invertebrates and vertebrates have been reported. Among lichen–invertebrate interactions are rotifera, amoebae (Figs. 6.1a, b and 6.11a), ciliates, tardigrades (Fig. 6.11b), mites (Fig. 6.12a, b), spiders, nematodes, collembola and insects (springtails, barklice, lepidopterans, grasshoppers, etc.) which live between and below, some even within lichen thalli (reviews: Gerson 1973; Gerson and Seaward 1977; Sharnoff 1998; Segerer 2009). Epiphytic lichen

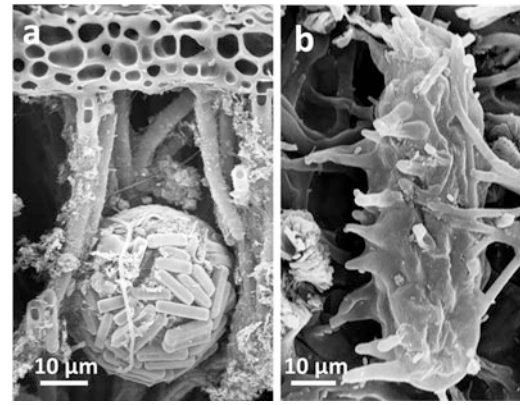

Fig. 6.11 Representatives of the microfauna of lichen thalli. (**a**) Testate amoeba with agglutinate shell, predominantly composed of diatoms, in the tomentum of *Sticta fuliginosa*, collected in La Réunion. (**b**) Tardigrade on the surface of a campylidium (conidioma) of the foliicolous *Lasioloma arachnoideum* collected in Tanzania by Edit Farkaš

biomass on oak correlated significantly with the abundance of arthropods, tardigrades and rotifera in central Maine (Stubbs 1989). The microfauna of one lichen species (*Xanthoria parietina*), collected in Lithuania, comprised 16 protozoan taxa, three tardigrades, two Nemathelminthes and three rotifera (Šatkauskienė 2012). In 26 terricolous lichen samples from Svalbard and Spitzbergen, 23 species of tardigrades were identified (Zawierucha et al. 2017). On *Cladonia rei* growing on heavy metal-contaminated post-smelting dumps in Poland, 50 species of oribatid mites were recorded (Skubała et al. 2014).

Some of these allies of lichen thalli find shelter, others are lichenivorous (see below) or bacterivorous (Liu et al. 2011). Even pest insects of fruit and nut trees find shelter under epiphytic lichens; therefore, agricultural experts recommend fruit growers to control or even prevent lichen growth either mechanically or chemically in their orchards.

6.7.2 Lichenivory: Invertebrates

Lichens have always been part of terrestrial food webs. The Silurian-Devonian nematophytes, presumably lichenized fungi, were grazed by

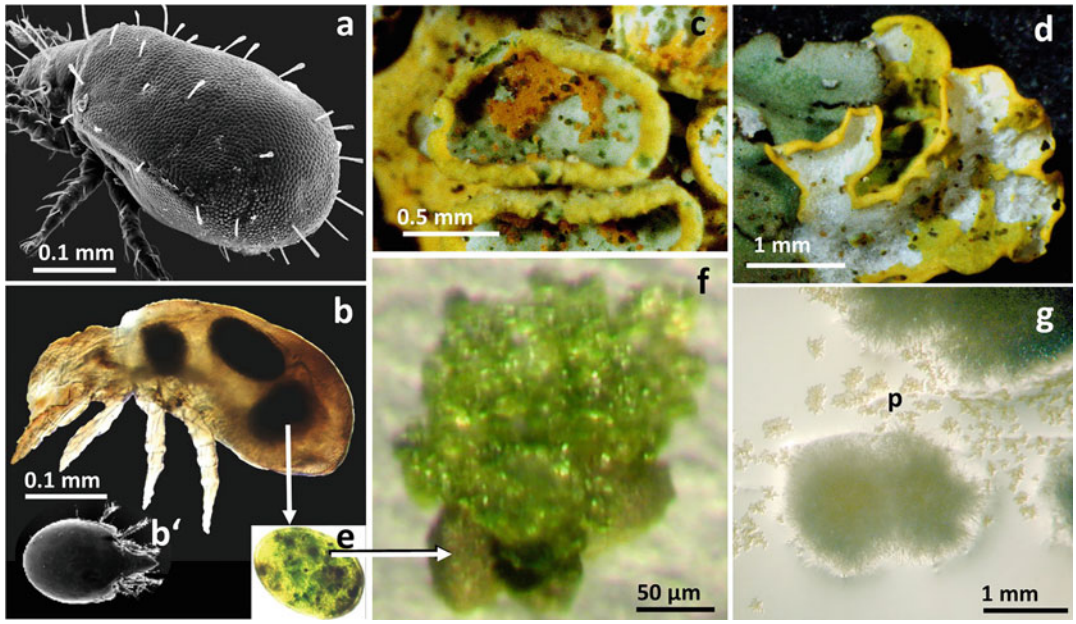

Fig. 6.12 Lichenivory and endozoochory in oribatid mites. In captivity *Trhypochthonius tectorum* (**a**) and *Trichoribates trimaculatus* (**b**, **b′**) were feeding on the hymenium (**c**) or the upper cortex and algal layer (**d**), respectively, of *Xanthoria parietina*; adjacent *Physcia orbicularis* was not grazed. Faecal pellets contained viable fungal and algal cells (*Trebouxia arboricola*), the latter growing out upon incubation on Parafilm (**f**) in a wet chamber; mycelia grew out on agarized non-nutrient mineral medium (**g**). Yellow crystals of parietin (*p*) are formed on and around the mycelium. The identity of the fungal and algal isolates was investigated with molecular tools. After Meier et al. (2002)

arthropods, presumably millipedes, as seen in grazing marks on the thallus surface with adhering coprolites (fossil faecal pellets), some of which contain hyphae or even small fragmentary remains of the thalli (Edwards et al. 1995, 2020). Grazing marks, presumably from arthropods, and faecal pellets were found on fossil foliose lichens in Palaeogene Bitterfeld amber (Schmidt et al. 2022).

There is a large body of literature on lichenivorous invertebrates (Gerson and Seaward 1977; Rambold 1985; Sharnoff 1998; Segerer 2009). In feeding experiments, Arthropoda such as mites (Acari), springtails (Collembola), barklice (Psocoptera) or beetles (Coleoptera) and Gastropoda such as door and pillar snails (Clausiliidae, Cochliopsidae) were all shown to graze the upper cortex and algal layer of *Parmelia* and *Physcia* spp. down to the medullary layer (Schmidt et al. 2022). Lichens were assumed to be protected from lichenivory by their content in secondary metabolites (Stahl 1904). However, many invertebrates consume even those parts of lichen thalli which contain large amounts of secondary products unharmed, crystals of these fungal metabolites passing the digestive tract unchanged (Zopf 1907; Hesbacher et al. 1995; Asplund and Wardle 2013; Gadea et al. 2019). Lichenivorous mites are selective in their food choice, some lichen species being avoided (Reutimann and Scheidegger 1987; Fröberg et al. 2003). In molluscs (slugs and snails), a preference was noted for thallus areas or even species with low contents in secondary metabolites (Benesperi and Tretiach 2004; Asplund 2011). *Arion fuscus* preferentially feeds on the large internal cephalodia of *Nephroma arcticum*, probably because these lack secondary metabolites (Asplund 2010; Asplund and Gauslaa 2010). Feeding experiments with thalli whose

secondary products had been removed by rinsing in acetone were preferentially grazed (Gauslaa 2005; Boch et al. 2011, 2015). Gall-producing lichenicolous fungi (*Plectocarpon* spp.) reduce the growth rate and the production of secondary metabolites in *Lobaria pulmonaria* (Asplund et al. 2016; Merinero and Gauslaa 2018), thus making the infected thallus areas more palatable and increasing the grazing pressure by snails (Asplund et al. 2016).

6.7.3 Lichenivory: Vertebrates

No vertebrate lives exclusively on a lichen diet, but in many regions, lichens are a substantial part of total food intake when other, preferred food such as buds, leaves, tubers and fruits are seasonally unavailable. Mat-forming terrestrial lichens such as *Cladonia* spp., and epiphytic species are a substantial part of the diet of reindeers and caribou (*Rangifer tarandus*) in Arctic tundras (Heggberget et al. 2002), but also of other ungulates such as deer, chamois or alpine *Ibex* in montane and alpine ecosystems of the Northern and Southern Hemisphere (Parrini et al. 2009; Yockney and Hickling 2000), of camelids (guanacos, llamas) in South America (Follmann 1964) and of numerous smaller mammals such as marmots, flying squirrels (Rosentreter et al. 1997), voles, etc. (reviews: Richardson and Young 1977; Sharnoff and Rosentreter 1998; Segerer 2009). Trampling damage occurs in reindeer lichen stands in subarctic and arctic tundras when large herds are feeding in dry weather; reindeer lichens (*Cladonia* spp.), like all other lichens, are elastic and flexible when wet, but very brittle in the dry state, thus being fragmented by the hooves of ungulates, recovery taking more than 30 years (Pegau 1970; den Herder et al. 2003; Théau et al. 2005; Heggenes et al. 2017, 2020).

However, trampling damage and overgrazing are not the only reasons for the decline of lichen heaths in Arctic ecosystems. In response to global warming, the Arctic is greening, deciduous shrubs and graminoids increasingly outcompete the terricolous lichen communities, as observed *in situ* and with remote sensing technologies over time (Myers-Smith et al. 2011, 2019; Fraser et al. 2014; Aartsma et al. 2020; Berner et al. 2020). Increasing summertime temperatures are also causing the decline of cyanobacterial and other lichens in soil crust communities on the Colorado Plateau, where neither grazing nor anthropogenic disturbances are evident (Finger-Higgens et al. 2022).

Large quantities, i.e., 30–75% of total food intake of beard lichens, especially *Usnea longissima* and *Bryoria* spp., are consumed by colobine primates (snub-nosed monkeys; Fig. 6.13) of the genus *Rhinopithecus* (*R. bieti, R. roxellana, R. brelichii*) in Western China and adjacent Tibetan mountain areas (Xiang et al. 2007; Grueter et al. 2009, 2012; Li and Ang 2009; Kirkpatrick and Grueter 2010; Liu et al. 2013; Bissell 2014; Xiang 2014). The rare and endangered lowland *R. strykeri* was shown to eat 15 lichen spp. in captivity, but these amounted to less than 3% of his diet (Yang et al. 2019). Barbary macaques (*Macaca sylvanus*) in high-altitude fir forests in the Ghomaran Rif and other mountain areas in Marocco eat lichens only in harsh winters when no other food is available (Mehlman 1988).

Lichens are rich in easily digestible carbohydrates such as β-glucans in the fungal cell walls such as lichenin (Honegger & Haisch, 2001) but have only low protein content. Reindeer and caribou seasonally feed on mushrooms (Inga 2007), whereas colobines consume mushrooms, invertebrates (Xiang et al. 2007; Yang et al. 2016) and vertebrates such as flying squirrels or bird's eggs (Grueter et al. 2009), and even infanticide followed by cannibalism was observed (Xiang and Grueter 2007).

6.7.4 Endozoochory

Faecal pellets of lichenivorous invertebrates are probably common and widespread symbiotic propagules, but rarely described and experimentally investigated. Jahns (1987) reported on a novel type of symbiotic propagule in *Solorina crocea* but did not realize that these were

Fig. 6.13 Lichenivory in vertebrates. Yunnan black snub-nosed monkey (*Rhinopithecus bieti*) feeding on *Usnea longissima* and other beard lichens. Courtesy of Fabio Nodari

germinating faecal pellets of lichenivorous snails. In light microscopy studies, numerous investigators observed intact fungal and/or algal cells or thallus fragments in the intestines or faecal pellets of lichenivorous invertebrates, e.g., in oribatid mites (Acari; Lawrey 1980; Meier et al. 2002), springtails (Collembola; Lawrey 1980), butterfly larvae (Lepidoptera; Rambold 1985; Brodo et al. 2021), termites (Termitidae; Barbosa-Silva and Vasconcellos 2019; Barbosa-Silva et al. 2019b), and molluscs (snails and slugs, Gastropoda; Baur et al. 2000; Fröberg et al. 2001; Boch et al. 2011, 2015).

However, in only a few lichen–invertebrate interactions was the viability of the cells contained in faecal pellets tested with culturing experiments. Gut transmission of viable mycobiont and photobiont cells was found in oribatid mites which had been feeding on *Xanthoria parietina* (Fig. 6.12a–g); the identity of both partners in these cultures was identified with molecular tools (Meier et al. 2002). *Physcia adscendens* and *Lobaria pulmonaria* were fed to nine gastropod species and the regeneration rate out of their faecal pellets tested. To a varying percentage, *P. adscendens* grew out of the faeces of all nine gastropod species (9/9), *L. pulmonaria* out of eight (8/9; Boch et al. 2011, 2015). No germination was observed in faecal pellets of lichenivorous snails and barklice which had been feeding on foliicolous lichens in a tropical rainforest (Lücking and Bernecker-Lücking 2000).

Various investigators report on partly severe grazing damage in saxicolous and epiphytic lichen communities (e.g. Baur et al. 1995, 2000; Gauslaa et al. 2006; Skinner 2021), but considering the high regeneration rates of lichens out of detached thallus fragments and faecal pellets of lichenivorous invertebrates, there is also a potential for rejuvenation which deserves investigation.

6.7.5 Epizoochory

Epizoochory is probably a very common and widespread mode of dispersal of symbiotic propagules, thallus fragments or faecal pellets of lichenivorous invertebrates, but only few investigations have been published. Only few viable cells of the mycobiont and photobiont are required for regenerating a new thallus. Three types of epizoochory can be distinguished:

1. Lichens growing on animals (invertebrates and vertebrates). Examples are (a) limpets (*Patella* spp., Mollusca) and barnacles (Cirripedia) in the littoral fringe whose calcareous shell is inhabited by *Pyrenocollema halodytes* (syn. *Arthroprenia halodytes, Collemopsidium h.*) and its cyanobacterial photobiont (*Hyella caespitosa*; see Fig. 15.1a in Honegger 2012). (b) foliose lichens growing together with fungi, algae and liverworts on the back of large weevils of the genus *Gymnopholus* in New Guinea (Gressitt et al. 1965; Gressitt 1966), which are getting several years old, as concluded from the lichen growth on their back (Gressitt 1970, 1977). This epizoic cryptogamic community is inhabited by mites, rotifers and nematodes; even a new family, genus and species of oribatid mites (*Symbioribatidae*, *Symbioribates papuensis*) were discovered which live among the cryptogams on the weevils' back (Aoki 1966). Liverworts, lichens and fungi were also found growing on Costa Rican Shield Mantis (*Choeradodis* spp., Mantodea; Lücking et al. 2010). (c) lichens growing on the shell of some, but by far not all Giant Tortoises (*Geochelone elephantopus*) in Galapagos (Hendrickson and Weber 1964).
2. Lichens being actively sampled for nesting or camouflage. Vertebrates: (a) Many birds such as hummingbirds, chaffinks, etc., use lichens for camouflage of their nest, which then seem to be part of the branch on which it is positioned; ideally these lichens continue to grow on the surface of the nest (Graves and Dal Forno 2018). On branches without lichen cover, fragments of bright-grey lichens on the surface of birds nests reflect light, concealment by light reflection being an important mode of camouflage (Hansell 1996). (b) Flying squirrels (*Glaucomys sabrinus*) use lichens as nesting material and food, soft *Bryoria* spp., having low contents of secondary metabolites, are preferentially collected (Rosentreter and Eslick 1993; Hayward and Rosentreter 1994). Invertebrates: (a) Larvae of bagworms (Psychidae, Lepidoptera) mount tiny fragments of the lichens on which they feed to their silk bag as a very efficient camouflage (McDonogh 1939; see Fig. 16.16a, b in Honegger 2009). (b) Barklice cover the body of their nymph with minute fragments of lichens, algae and sand granules (Henderson and Hackett 1986), and so do the larvae of the green lacewing (*Nodita pavida*; syn.), which become invisible under their load of tiny lichen fragments (Slocum and Lawrey 1976). (c) Several species of lichenivorous, terrestrial snails of the genus *Napaeus* (Gastropoda, Pulmonata) actively paste fragments of lichens to their shell (Allgaier 2007; Holyoak et al. 2011).
3. Passive sampling. Soredia, isidia and other symbiotic propagules, tiny thallus fragments or faecal pellets adhere to animal or human vectors and thus get dispersed. Examples are (a) mites carrying soredia (Stubbs 1995). (b) lichenivorous termites most likely carry fragments of lichens to their nest where a species-rich lichen community develops (Aptroot and Cáceres 2014; Barbosa-Silva et al. 2019a). (c) Woodpeckers are neither lichenivorous nor do they collect lichens for camouflage or nest building, but were found to carry vegetative propagules of lichens on their feet, chest and tail feathers in samples in a Finnish Natural History Museum (Johansson et al. 2021). (d) humans carry propagules or fragments of lichens on their clothes, as first described in cases of woodcutters disease, an allergic reaction to usnic acid containing lichens, in housewives in contact with their husbands clothes (Mitchell and Champion 1965; Aalto-Korte et al. 2005). The load of

plant seeds, moss and lichen propagules on clothes of visitors of the Antarctic was thoroughly investigated, largest amounts having been found on socks of field working scientists (Huiskes et al. 2014). However, most of these lichen propagules on human clothes likely end up in the washing machine rather than invading Antarctic ecosystems.

Long-distance anthropogenic dispersal of epiphytic lichens on economic or ornamental plants is probably quite common (Bailey 1976). An example is *Xanthoria parietina*, which was not native to Australia, but is now growing around wineries in the Barossa Valley and elsewhere. In comparative RAPD-analyses of sterile cultured isolates of the mycobiont, some of the isolates from Australia and New Zealand clustered with those from southern France and Spain (Honegger et al. 2004a, b). It could be that *X. parietina* was brought to these areas with plants or cuttings of grape wine (*Vitis vinifera*). Similarly, Brodo et al. (2021) concluded that *X. parietina* was anthropogenically transported from coastal to inland areas in Canada, presumably with trees from nurseries.

6.8 Lichenomimesis

6.8.1 Lichenomimesis in Animals

Large numbers of invertebrates mimick lichens and are thus not easily detected by potential predators (Figs. 6.14a–e). The earliest report of lichenomimesis in insects is a moth lacewing, *Lichenipolystoechotes angustimaculatus* (Neuroptera, Ithonidae), co-occurring with the mesozoic *Daohugouthallus ciliiferus*, which beautifully mimicked the pattern of the lichen on its wings (Fang et al. 2020). Extant examples are (1) lichen moths (Arctiinae, Lepidoptera) in their caterpillar (e.g. *Catocala ilia*; Fig. 6.14c) and/or adult stage such as *Cleorodes lichenaria*, the Brussels lace moth, whose lichenivorous caterpillar and moth stage mimick epiphytic lichens; (2) various species of bush crickets (Tettigoniidae, also termed katydids, in the genera *Lichenomorphus*, *Lichenodracula*, *Markia,* etc.; Braun 2011; Cadena-Castañeda 2011, 2013; Fig. 6.14a); one of them, *Anapolisia maculata*, even mimics foliicolous lichens in a tropical rainforest (Fig. 6.14b; Lücking and Cáceres 2002). (3) stick insects (Phasmatidae, syn. Phasmidae, such as *Extatosoma tiaratum*), or (4) spiders such as the North American Giant Lichen Orb Weaver (*Araneus bicentenarius*; Fig. 6.14d).

Among vertebrates, many frogs mimic lichens, such as the N-American *Dryophytes versicolor* (syn. *Hyla versicolor*), the Brazilian *Bokermannohyla pseudopseudis* (Leite et al. 2012) or the Australian tree frog *Litoria genimaculata* (Vanderduys 2012); the colouration may vary depending on the habitat. Foliicolous lichen communities are mimicked by tree frogs in Tanzanian (*Leptopelis ulugurensis*; Farkas and Pocs 1989) and S-American rainforests (*Boana rufitela,* syn. *Hyla rufitela*; Lücking and Cáceres 2002). Lichenomimesis occurs also in reptiles such as *Rhampholeon spinosus* (Chamaeleonidae) or *Uroplatus sikorae* and other *Uroplatus* spp. (Gekkonidae).

6.8.2 Lichenomimesis in Members of the Araceae (Flowering Plants)

A unique type of lichenomimesis occurs in petioles (leaf stalks) of some members of the *Arum* family (Araceae, monocotyledonous flowering plants). Representatives of several genera have spots on their petioles which resemble lichens (Fig. 6.15a–f); particularly interesting are *Amorphophallus* spp. (Barthlott 1995; Hejnowicz and Barthlott 2005; Claudel et al. 2019), among others *A. gigas*, the Giant Arum, which forms the largest inflorescence in the plant kingdom. One giant leaf per growing season grows out of the large tuber of these Araceae; it is fleshy and thus the vulnerable petiole resembles the stem of a lichen-covered tree, various types of crustose lichens being mimicked (Figs. 6.15a–f). This optical signal is obviously perceived by potential herbivores which assume these leaf stalks to be inedible wooden stems.

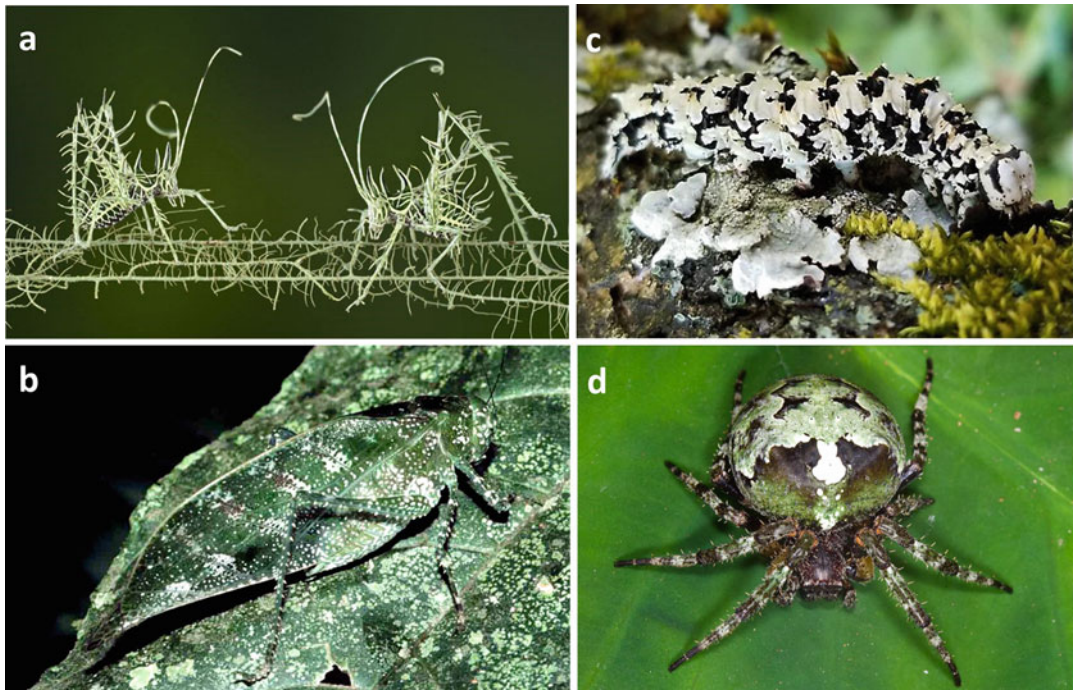

Fig. 6.14 Lichenomimesis in insects (**a**–**c**) and spiders (**d**). (**a**–**b**) Katydids (Tettigoniidae, bush crickets). (**a**) Nymphs of *Lichenodraculus matti* on beard lichen (*Usnea* sp.); courtesy of David Weiller. (**b**) *Anapolisia maculata* mimicking foliicolous lichens in a tropical rainforest in Costa Rica; courtesy of Andrea Bernecker, in Lücking and Cáceres (2002). (**c**) Caterpillar of the Ilia Underwing Moth (*Catocala ilia*); courtesy of Erik Adams. (**d**) Giant Lichen Orb Spider (*Araneus bicentenarius*); courtesy of Jim Petranka and Becky Elkin

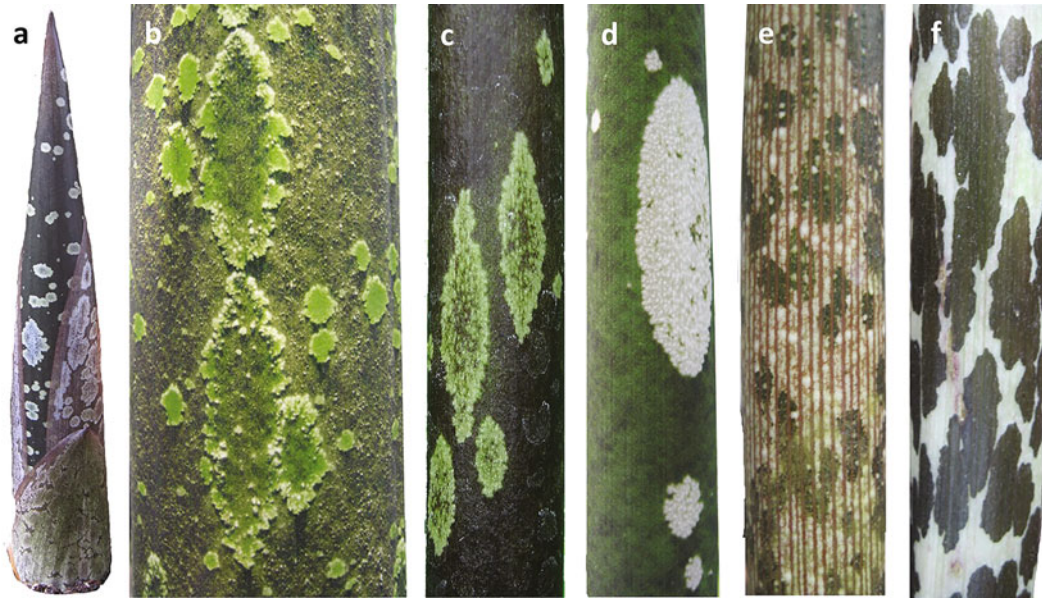

Fig. 6.15 Lichenomimesis in petioles (leaf stalks) of Araceae with characteristic patterns mimicking crustose lichens. (**a**) the bud of *Amorphophallus gigas* is protected by three ephemeral bracts. (**b**) Detail of a mature petiole of *A. gigas*. (**c**) *A. dactylifer*, mimicking representatives of the genus *Graphis*. (d) *A. decus-silvae*, mimicking *Pertusaria* spp. (**e**) *A. pseudoharmandii* (syn. *Pseudodracontium harmandii*). (**f**) *Typhonium venosum*. Images taken in the Botanical Garden of the University of Zürich

Lichenomimesis is also seen in other Araceae such as *Typhonium venosum* (voodoo lily; Fig. 6.15f). The inflorescences of these Araceae emit a remarkable scent, reminiscent of decaying meat, partly intermixed with dung (e.g. horsedung in *T. venosum*), which attracts copro-necrophagous insects, some of them acting as pollinators (Claudel 2021). These plants of the *Arum* family successfully mimic elements of the fungal (i.e. lichens) and of the animal kingdoms.

6.9 Conclusions and Outlook

Molecular genetics have revolutionized our view on the biodiversity of LFF, their photobionts and their microbial associates. These investigations will continue at a global scale.

Lots of fossils, lichens and lichen-like organisms, from the Palaeozoic to the Cenozoic await thorough investigation.

The search for bioactive compounds, as produced by lichen-forming and endolichenic fungi and their bacterial associates will be intensified, several promising components having already been found. Examples are (1) usnic acid with antimicrobial, antiproliferative and antiviral properties (Macedo et al. 2021), as produced by various representatives of the Parmeliaceae in the lichenized and axenically cultured state (Yamamoto et al. 1985; Stocker-Wörgötter et al. 2013; Xu et al. 2022) and by endolichenic *Streptomyces cyaneofuscatus* (Parrot et al. 2016a); (2) antiproliferative cyaneodimycin from the same isolate of *S. cyaneofuscatus* (Parrot et al. 2016a) or (3) uncialamycin, a potent antibiotic with antiproliferative activity produced by *Streptomyces uncialis*, an endolichenic actinobacterium in *Cladonia uncialis* (Nicolaou et al. 2007, 2021; Parrot et al. 2016b). It will be interesting to see whether some of these products with pharmaceutical potential will reach the clinical trial phases. The large-scale production of mycobiont-derived secondary metabolites, either by heterologous expression in fast-growing fungi or by chemical synthesis, will be a challenge. *S. uncialis*-derived uncialamycin is chemically synthesized (Nicolaou et al. 2007).

Many bacterial associates of lichen thalli are in close contact with mycobiont-derived secondary metabolites with antibiotic properties, and some bacterial associates of lichens were shown to synthesize antibiotics (review: Grimm et al. 2021); an example is *Streptomyces cyaneofuscatus*, producer of usnic acid, which was isolated from the marine lichen *Lichina confinis* which does not synthesize usnic acid (Parrot et al. 2016a). The biotransformation of usnic acid into a compound with lower antibiotic activity was observed in several lichen-associated bacteria (Noël et al. 2021). The question is: are some of the epi- and endolichenic bacteria resistant to antibiotics? Bacterial antibiotic resistance emerged in the pre-antibiotic era; an example is the co-evolutionary adaptation of methicillin-resistant *Staphylococcus aureus* on hedgehogs whose skin is infected with antibiotic producing dermatophytes (Larsen et al. 2022).

Lichen-invertebrate and -vertebrate interactions deserve a closer examination as regards the dispersal of lichens and their allies. It would be interesting to see what birds carry on their feet and feathers in terms of symbiotic propagules (soredia, isidia, blastidia) or minute thallus fragments as well as faecal pellets of lichenivorous invertebrates or the minute lichenivorous mites proper. Examining the load of lichen propagules on the feathers and feet of birds with annual bipolar migration such as South Polar Skuas (*Catharacta maccormicki*, syn. *Stercorarius m.*) or Arctic Terns (*Sterna paradisaea*), which are assumed to be vectors of lichens with bipolar distribution (review: Garrido-Benavent and Pérez-Ortega 2017), would be particularly interesting. But also lichen dispersal by smaller birds, which are regularly landing on lichen-covered surfaces, should be investigated, for example, in collaboration with ornithologists during their ringing activities (Bailey and James 1979).

To conclude: there are lots of interesting work ahead, both in the field and laboratory.

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7 Lichen Fungal Secondary Metabolites: Progress in the Genomic Era Toward Ecological Roles in the Interaction

Harman Gill, John L. Sorensen, and Jérôme Collemare

Abstract

Lichens are complex miniature ecosystems that comprise a fungal partner, the mycobiont, and an algal and/or cyanobacterial partner, the photobiont. Lichens have been extensively used for the colors and secondary metabolites they produce, with more than 1000 reported compounds, mostly belonging to the polyketide chemical family. Studying lichens is challenging because of their slow growth rate and the difficulty of reconstituting their ecosystem under controlled conditions. While lichen secondary metabolites are well characterized for their biological activities, their functions in the establishment and survival of the ecosystem are poorly documented. In the last decade of the genomic era, only a few mycobiont genomes have been sequenced, yet they have revealed the presence of an abundance of diverse polyketide pathways that will likely give rise to a host of novel compounds. The use of heterologous expression in recent years to elucidate lichen secondary metabolite biosynthetic pathways is still in its infancy but is opening up a new era in which many new lichen-specific compounds will be discovered. The elucidation of new lichen biosynthetic pathways, together with progress in reconstituting lichen ecosystems, is leading to exciting times where it will be possible to characterize the roles lichen secondary metabolites play in these unique ecosystems.

Keywords

Heterologous expression · Genomes · Biosynthetic gene clusters · Polyketides · Natural products · Lichen fungi

7.1 Introduction

Lichens are slow-growing, dual organisms, formed by a mutual relationship between fungi (mycobiont) and algae and/or cyanobacteria (photobiont) (Lutzoni and Miadlikowska 2009; Grimm et al. 2021). The lichen association itself does not have a name, but the taxonomic name of the mycobiont is typically used to also refer to the whole structure. For example, the name *Cladonia uncialis* refers to both the lichenized fungus alone or in association with photobiont algae. Lichens are actually miniature ecosystems, as the lichen thallus (and its surface) hosts a complex and mostly specific microbiota of bacteria (Grube et al. 2009; Bates et al. 2011; Sierra et al. 2020) and the so-called lichenicolous fungi (Spribille

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et al. 2016; Diederich et al. 2018; Mark et al. 2020), which can either contribute to the symbiosis for nutrient acquisition and stress resistance (Grube et al. 2009; Bates et al. 2011; Sigurbjörnsdóttir et al. 2015; Grube et al. 2015) or behave as lichen pathogens (Diederich et al. 2018; Merinero and Gauslaa 2018). These unique communities have colonized all terrestrial habitats on earth, from the mildest conditions in our home gardens to the extremely harsh environmental conditions found in the arctic tundra or desert, and thus constitute one of the most important components of biodiversity. Such adaptation to extremely diverse environmental conditions has occurred despite the fact that they are poikilohydric organisms, *i.e.* they lack the ability to maintain homeostasis of cells and tissues (Matos et al. 2015). Lichens are commonly used as bio-indicators to monitor air pollution, sulfur dioxide deposition, global warming, and for the sensitive detection of metals and radionuclides (Bačkor and Loppi 2009; Ohmura et al. 2013; Matos et al. 2015). Due to industrialization and subsequent pollution, many lichens have been pushed to extinction or their geographical distribution has been highly restricted.

The organization of the lichen thallus is not random, and each partner and compartment exhibit complementary functions (Fig. 7.1). The mycobiont forms a cortical layer of dense fungal hyphae, which provides the photobiont partner with mechanical stabilization and shelter (Honegger 1997). The photobiont in the medulla layer provides carbon nutrients to the other partners through photosynthesis (Honegger 1997). Cyanobacteria in some lichens are responsible for nitrogen fixation (Seneviratne and Indrasena 2006). In addition, the lichen microbiome is also not homogenous, with differences between the center and edges of the lichen (Mushegian et al. 2011), as well as vertically between the surface and substrate (Noh et al. 2020).

Lichen communities have been established several times during evolution in distant fungal lineages. Mycobionts have mostly been reported to belong to the subphylum Pezizomycotina of the Ascomycota (http://liaslight.lias.net). While the vast majority of lichenized species belong to the Lecanoromycetes class, the Arthoniomycetes, Lichinomycetes, Coniocybomycetes, and Candelariomycetes classes nearly exclusively comprise mycobionts. In contrast, lichenized species form a minority in the large Dothideomycetes, Eurotiomycetes, and Leotiomycetes classes, in which they are restricted to a few orders, mainly in Monoblastiales, Strigulales, Trypetheliales, Mycocaliciales, Pyrenulales, and Verrucariales. A few Basidiomycota genera also form lichens, including *Multiclavula* (Cantharellales order) and *Lichenomphalia* with *Coccomyxa* green algae (Lawrey et al. 2009; Liu et al. 2017), and *Dictyonema* with *Scytonema* cyanobacteria (Dal-Forno et al. 2013; Lücking et al. 2014). We recommend recent reviews for more in-depth information about the biology of lichens (Grube and Wedin 2016; Asplund and Wardle 2017; Grimm et al. 2021; Grube 2021).

Lichenologists have employed various techniques to measure thallus growth and report an average growth of 0.5–8 mm per year depending on the species and environmental conditions (Hale 1973). Due to this slow growth rate and difficulties in reconstituting the partnership under controlled conditions, lichens are difficult to study and little is known on how these communities are established and sustained in diverse habitats. Bioactive molecules, also known as secondary metabolites (SM) or natural products, produced by the mycobiont likely play important roles in lichen mutualism; for example, in protection against light, desiccation, or against herbivorous predators. However, the study of lichen SMs is hampered by bottlenecks inherent to the biology of lichens and by the strict regulation of the underlying biosynthetic pathways. Indeed, fungal SMs are typically produced in low quantities and under specific conditions that are difficult to reproduce in the laboratory, with lichen communities one of the most striking examples of this limitation. In this chapter, we will first provide an overview of the correlation between the wide array of mycobiont SMs and their production controlled by biosynthetic gene clusters (BGCs), and then discuss how the

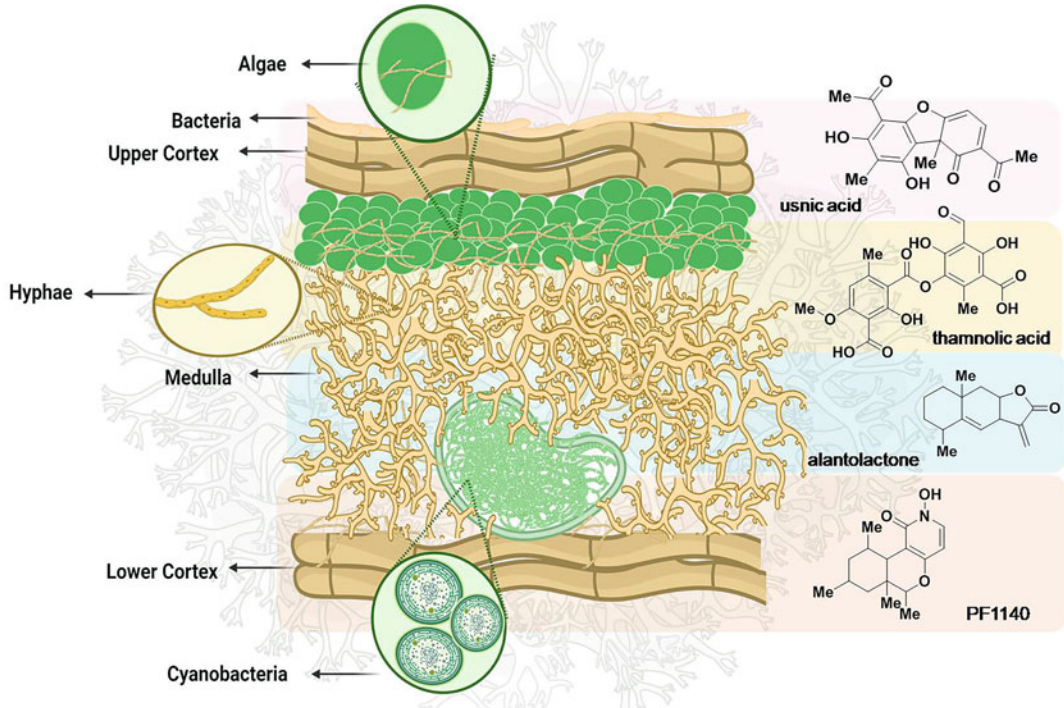

Fig. 7.1 Complex organization of the lichen thallus. Lichen are a symbiotic association between photosynthetic (photobiont) and heterotrophic (mycobiont) partners. The algal and/or cyanobacterial photobionts are surrounded by fungal hyphae of the mycobiont. Lichens also host a microbiome consisting of bacteria, lichenicolous fungi, and Basidiomycota yeasts. Secondary metabolites produced by the mycobiont or lichenicolous fungi exhibit a specific localization that is likely related to their biological functions. Examples of metabolites produced in the lichens *Ophioparma ventosa* (Le Pogam et al. 2016) and *Peltigera* (Garg et al. 2016) are shown on the right, with the colored squares indicating their localization

combination of genomic knowledge with advances in synthetic biology is giving a new horizon to uncover the chemical diversity of lichens and understand their biological functions in such complex ecosystems.

7.2 Lichen Mycobionts Produce a Unique Diversity of Bioactive Secondary Metabolites

Lichens produce a plethora of low molecular weight aliphatic and aromatic compounds that are derived from primary or secondary metabolism. Primary metabolites are the intracellular molecules that are required to carry out the basic functions of life and belong to different chemical classes, including amino acids, polysaccharides, proteins, lipids, carotenoids, and vitamins (Nayaka and Haridas 2020). In lichen symbiosis, both the mycobiont and photobiont partners are responsible for the production of these primary metabolites. In contrast, SMs are mostly synthesized by lichenized fungi. These SMs generally contribute between 5 and 10%, and up to 20% of the dry thallus weight (Molnár and Farkas 2010). Most of the reviews about lichen SMs focus on their biological activities, especially for antimicrobial and anticancer applications, but little is known about their biological functions in the lichen ecosystem.

7.2.1 Lichen Secondary Metabolites Have Been Used for Their Biological Activities

More than 1000 SMs are known from lichenized fungi, many of which are exclusively found in lichens (Shrestha and Saint Clair 2013). Indeed, metabolomics has suggested that lichenized fungi in the Lecanoromycetes class exhibit a unique chemical space compared to other non-lichenized fungal classes (Robey et al. 2021). Thanks to the production of this plethora of unique SMs with diverse biological activities, lichens have been used for thousands of years for their medicinal and dye properties. For example, *Evernia prunastri* and *Pseudevernia furfuracea* are known as "perfume lichens" which are extensively used in the fragrance industry (Lutzoni and Miadlikowska 2009; Calchera et al. 2019). Usnic acid is commonly produced by lichens from the *Usnea* and *Cladonia* genera and this SM, and semisynthetic derivatives, have been well studied because of their diverse biological activities (Araújo et al. 2021). For example, they are used as preservatives and photoprotectants in cosmetics (Galanty et al. 2021). Lichen SMs have been extensively screened for antimicrobial (permelabdone), antiprotozoal (usnic acid), antifeedant (vulpinic acid), or antioxidant (lecanoric acid) activities (Molnár and Farkas 2010). The lichen SMs virensic acid, norlobaric acid, salazinic acid, parellic acid, and physodic acid have been reported for anti-HIV activity (Neamati et al. 1997), while pannarin, 10-chloropannarin, and sphaerophorin are known for their high cytotoxic potential against rat lymphocytes as compared to colchicines (Correché et al. 2005). These examples show the great potential of lichen SMs in diverse applications.

7.2.2 Biological Functions of Lichen Secondary Metabolites

Because it is difficult to study lichens, assigning their SMs with a function in their ecosystem is not trivial. A few functions have been reported. The most well-documented and validated function of fungal SMs is certainly protection from UV-light, desiccation, and extreme temperatures, provided by pigments through light absorption or reflection. Lichens appear particularly resistant to light, and the production of photoprotective SMs contributes to their high resistance to harsh environments (Nguyen et al. 2013). In *L. pulmonaria*, the production of melanin is responsive to light and likely protects against UV-light (McEvoy et al. 2007). However, the production of depsidones in this lichen is not regulated by light and a role in protection against herbivores has been suggested. In contrast to melanin and parietin that absorb light, it was found that atranorin instead protects the lichen from UV-light through reflection (Solhaug et al. 2010). Different SMs protect lichens from different light wavelengths, and they exhibit synergistic properties as antioxidants to protect the lichen thallus and photosystem of the photobiont, as demonstrated with vulpinic acid, pinastric acid, and usnic acid in *Vulpicida pinastri* and *Letharia vulpina* (Legouin et al. 2017; Phinney et al. 2019). Because of these properties, lichen SMs are also prospected for new natural photoprotective agents.

Another suggested function is to protect the lichen ecosystem from herbivorous predators (Pöykkö et al. 2005). However, this function is not clear, as the ability of the predator to defend itself against lichen SMs seems to play a more significant role than the presence of SMs *per se* (Gauslaa 2005). Similarly, nutrient availability appears to be more important as to whether snails eat the lichen *Usnea taylorii* than the presence of SMs (Gadea et al. 2019). Thus, it still remains unclear if protection from herbivorous predators is a primary biological function of lichen SMs. Other less documented functions are related to heavy metal tolerance and allelopathy, as lichen SM extracts inhibit the growth of mosses (Molnár and Farkas 2010; Goga et al. 2017).

7.2.3 How Lichen Mycobionts Produce Secondary Metabolites

Lichen SMs typically comprise one or more phenolic rings and are categorized into various classes based upon the kind of chemical bonds linking the aromatic rings. Single aromatic rings are classified as orcinol-type with orsellinic acid being the primary example of this class. The related depsides, such as lecanoric acid, feature one (or more) aromatic rings joined by a simple ester linkage. The depsidones, such as salazinic acid and grayanic acid, feature a biphenyl ether linkage in addition to an ester bond, forming a central seven-membered ring. Other structural classes of SMs are dibenzofurans, with usnic acid being the most well-known of this class. Quinones such as parietin, and diphenyl butenolides, exemplified by vulpinic acid (Nayaka and Haridas 2020) are also among the common structural classes encountered. The aromatic phenolic core, with a pattern of alternating oxygenation, suggests that the majority of lichen SMs belong to the polyketide chemical family (Fig. 7.2).

Compounds synthesized *via* the polyketide pathway are formed by the successive condensations of acetate and malonate by polyketide synthases (PKS). The carbon core of these SMs is formed by the PKS and typically contains a single aromatic ring with orsellinic acid being a common product. The higher oxidation states such as found in depsides, depsidones, and dibenzofurans are the result of modifications of the PKS product by accessory or tailoring enzymes like cytochrome P450 or methyltransferases. The depsides and depsidones are mostly located in the medulla of lichens.

Dibenzofurans such as usnic acid are located in the cortex of many lichen species and originate from the oxidative coupling of methylphloroacetophenone (Fig. 7.3). All other colored pigments like anthraquinones, xanthones, and chromones are produced by intramolecular condensation followed by aromatization of polyketides units rather than an oxidative coupling step. Other lichen SMs are synthesized by the other major mevalonate terpene and shikimic acid pathways (Goga et al. 2018).

The mevalonate pathway is also known as the isoprenoid or HMG-CoA reductase pathway and it leads to the production of isopentenyl pyrophosphate and dimethylallyl pyrophosphate which serve as precursors for higher terpenes, as well as carotenoids, and steroids. In this pathway, two acetyl-CoA molecules undergo a Claisen condensation followed by the addition of a third acetyl-CoA *via* an aldol reaction to yield a HMG-CoA (Fig. 7.4a). Next, mevalonic acid is formed by the reduction of the thioester which, after introduction of the pyrophosphate, undergoes decarboxylative elimination to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Both IPP and DMAPP act as precursors for the terpene class of compounds such as limonene (Kahrïman et al. 2011), phytol (Rajab et al. 1998), lutein (Czeczuga and Czeczuga-Semeniuk 2003), and lichesterol (Safe et al. 1975). Each of these terpenes have been reported in lichen fungi however a specific role has yet to be elucidated.

The shikimic acid pathway involves seven biosynthetic steps that lead to the formation of aromatic amino acids and folates. Shikimic acid is formed by the fusion of phosphoenolpyruvate (PEP) and erythrose-4-phosphate *via* an aldol reaction and subsequent elimination. A second PEP is condensed onto shikimate acid *via* chorismic acid and a Claisen rearrangement that generates the aromatic amino acids L-tyrosine and L-phenylalanine. Tryptophan is also a shikimate derived amino acid with the extra carbons coming from glyceraldehyde 3-phosphate. The known lichen SMs polyporic acid and thelephoric acid are derived from L-phenylalanine and belong to the triphenyl quinone class of natural products. One of the more ubiquitous lichen natural products pulvinic acid is produced by further oxidative modification of polyporic acid (Fig. 7.4b). SMs produced by the shikimate pathway are widely found in the Stictaceae family of lichens.

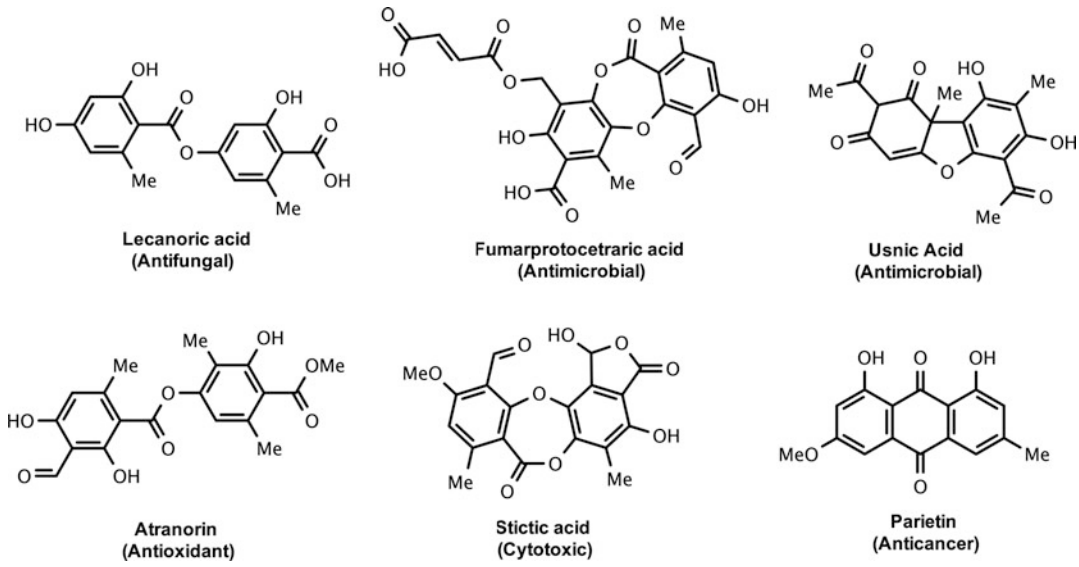

Fig. 7.2 Examples of lichen secondary metabolites and reported bioactivities

7.3 The Genomes of Lichenized Fungi Have Revealed a Unique Potential for Polyketide Production

Despite the large number of SMs isolated from lichen, and the examination of their medicinal properties and potential applications, conclusive links between chemical structures and the corresponding biosynthetic gene clusters (BGCs) remain very limited. This is due in large part to the challenges with cultivating the lichen assemblage or the corresponding mycobiont in the laboratory. This low amenability hampers carrying out the assignment of function by using gene deletions to examine effects on SM production. The application of next-generation sequencing has represented a significant advance in our ability to examine SM production in lichen fungi. The whole-genome sequencing for a significant number of lichen has been carried out and openly available online software such as antiSMASH (Blin et al. 2021) has facilitated the annotation of SM BGCs. What these efforts have revealed is that the number of BGCs present in a mycobiont genome is often far greater than the number of metabolites detected by chromatographic methods such as HPLC and LC-MS. This diversity of BGCs suggests that the full potential of lichen SMs have yet to be fully revealed. The vast majority of these cryptic BGCs comprises a gene that codes for a PKS. However, there is a significant amount of variability in the accessory genes that flank the PKS genes, suggesting a high degree of structural diversity in the final SM structures. Other BGCs contain other core genes encoding terpene cyclases and non-ribosomal peptide synthases, suggesting that other structural classes of SMs may also be hidden in these genomes. Some of the ongoing efforts to unlock this hidden potential are discussed below.

7.3.1 A Decade of Mycobiont Genome Sequencing

Lichenized fungi are found scattered in several taxonomic classes, but the Lecanoromycetes class comprises most of the described mycobiont species, including the well-studied *Cladonia* species, *Usnea florida* and *Xanthoria parietina*. However, although many fungal species have been described as mycobionts, genomic resources are available for a very limited number of species. While more than 1000 non-redundant genomes

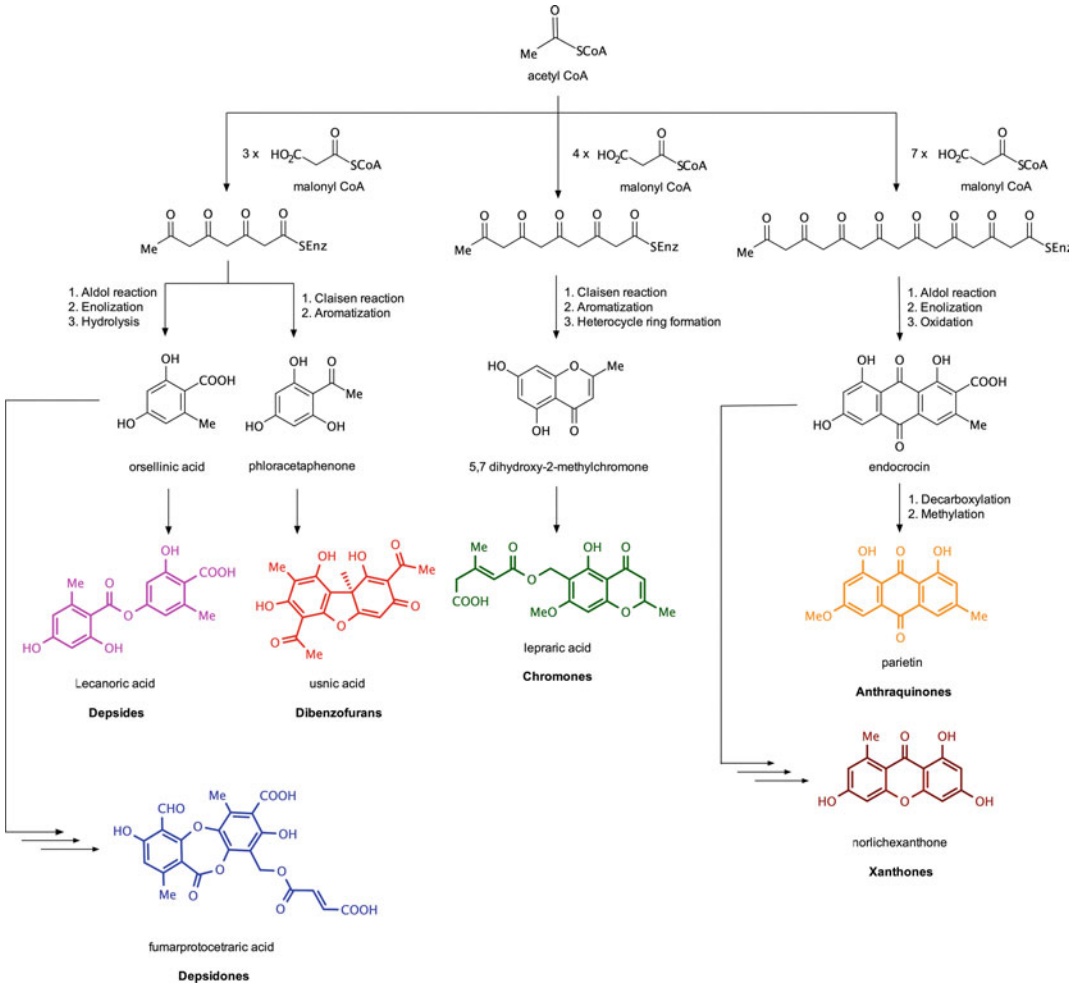

Fig. 7.3 Biosynthesis of lichen secondary metabolites through the acetyl–polymalonate pathway. Aromatic polyketides produced through this route differ by the number of malonyl CoA molecules incorporated and the type of cyclization. Additional modifications, including dimerization, increase the diversity of lichen compounds. The colors indicate different classes of compounds

are available at NCBI for non-lichenized fungi, only 28 genomes are available for mycobionts, of which 18 are from Lecanorales species (Table 7.1). Twenty of these genomes were only generated in the last 4 years. In addition, eight additional genome assemblies are available at the Joint Genome Institute MycoCosm repository (Table 7.1; Grigoriev et al. 2014). These numbers represent significant progress compared to 2009 when the first two mycobionts, *X. parietina* and *Cladonia grayi*, were sequenced (Lutzoni and Miadlikowska 2009). However, lichenized fungi lag behind in the global genome sequencing effort because 1.3 to 2.5% of non-lichenized fungi in the best-studied classes (Dothideomycetes and Eurotiomycetes) have been sequenced, but only 0.1 to 0.9% of mycobionts belonging to these classes have been sequenced (Table 7.2). In Lecanoromycetes and Arthoniomycetes, the sequencing effort also remains behind with just 0.2–0.3% of the reported mycobiont species currently being sequenced (Table 7.2). These low numbers are likely due to the difficulties of growing mycobionts under laboratory conditions and

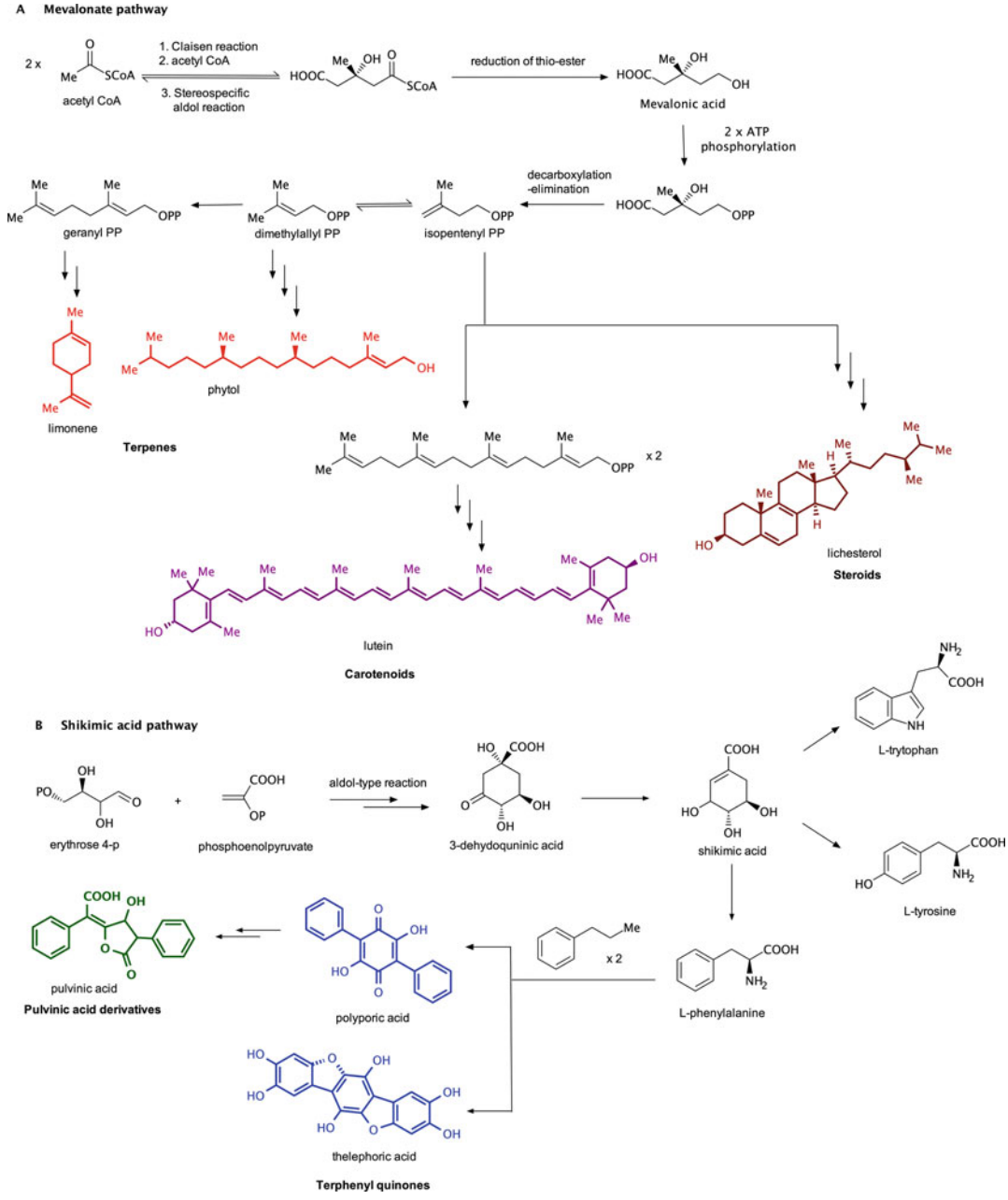

Fig. 7.4 Biosynthesis of lichen secondary metabolites *via* the mevalonate and shikimic acid pathways. (**a**) Lichen terpenes, carotenoids, and steroids are synthesized from isoprene units. (**b**) Lichen terphenyl quinones and pulvinic acid derivatives are synthesized from L-phenylalanine. The colors indicate different classes of compounds

obtaining good quality genomic DNA. With advanced sequencing technologies, performing whole-genome sequencing from environmental samples, *i.e.* from the lichen community rather than the mycobiont alone, will become easier. This approach was first employed when sequencing *Dibaeis baeomyces* (McDonald et al. 2013), and has been used more recently to sequence the

Table 7.1 Available genome assemblies for lichenized fungi

Year	Classification	Species name	Accession number[a]	References
2013	Lecanoromycetes, Lecanorales	*Cladonia macilenta*	GCA_000444155.1	Park et al. (2013a)
2013	Lecanoromycetes, Teloschistales	*Gyalolechia flavorubescens*	GCA_000442125.1	Park et al. (2013b)
2014	Lecanoromycetes, Lecanorales	*Cladonia metacorallifera*	GCA_000482085.2	Park et al. (2014a)
2014	Lecanoromycetes, Umbilicariales	*Umbilicaria muehlenbergii*	GCA_000611775.1	Park et al. (2014b)
2014	Eurotiomycetes, Verrucariales	*Endocarpon pusillum*	GCA_000464535.1 GCA_000611755.1 GCA_014607435.1	Wang et al. (2014) Park et al. (2014c) Mead and Gueidan (2020)
2015	Lecanoromycetes, Umbilicariales	*Umbilicaria pustulata*	GCA_000938525.1 GCA_900169345.1 GCA_008636195.1 (m)	Unpublished Dal Grande et al. (2017) Greshake Tzovaras et al. (2020)
2017	Lecanoromycetes, Lecanorales	*Ramalina peruviana*	GCA_001956345.1	Unpublished
2018	Lecanoromycetes, Lecanorales	*Cetradonia linearis*	GCA_003521265.1	Allen et al. (2018)
2018	Lecanoromycetes, Lecanorales	*Cladonia uncialis*	GCA_002927785.1	Bertrand et al. (2018a)
2018	Lecanoromycetes, Lecanorales	*Evernia prunastri*	GCA_003184365.1	Meiser et al. (2017)
2018	Lecanoromycetes, Umbilicariales	*Umbilicaria hispanica*	GCA_003254425.1	Paul et al. (2018)
2018	Lecanoromycetes, Lecanorales	*Pseudevernia furfuracea*	GCA_003184345.1	Meiser et al. (2017)
2018	Lecanoromycetes, Lecanorales	*Ramalina intermedia*	GCA_003073195.1	Wang et al. (2018b)
2018	Arthoniomycetes, Arthoniales	*Arthonia radiata*	GCA_002989075.1	Armstrong et al. (2018)
2019	Lecanoromycetes, Lecanorales	*Alectoria sarmentosa*	GCA_009733775.1	Liu et al. (2019)
2019	Lecanoromycetes, Lecanorales	*Cladonia rangiferina*	GCA_006146055.1	Unpublished
2020	Lecanoromycetes, Lecanorales	*Letharia columbiana*	GCF_014066305.1 (m)	McKenzie et al. (2020)
2020	Lecanoromycetes, Lecanorales	*Letharia lupina*	GCF_014066315.1 (m)	McKenzie et al. (2020)
2020	Lecanoromycetes, Teloschistales	*Xanthoria elegans*	GCA_011316305.1	Unpublished
2020	Lecanoromycetes, Lecanorales	*Usnea hakonensis*	GCA_013423325.1	Kono et al. (2020)
2020	Dothideomycetes, Trypetheliales	*Viridothelium virens*	GCA_010094025.1	Haridas et al. (2020)
2020	Dothideomycetes, Racodiales	*Racodium therryanum*	GCA_014905255.1	Unpublished
2021	Lecanoromycetes, Lecanorales	*Bacidia gigantensis*	GCF_019456465.1 (m)	Allen et al. (2021)
2021	Lecanoromycetes, Lecanorales	*Cladonia borealis*	GCA_018257855.1	Unpublished
2021	Lecanoromycetes, Lecanorales	*Niebla homalea*	GCA_019925105.1	Duong et al. (2021)
2021	Lecanoromycetes, Lecanorales	*Parmelia sp. 050094*	GCA_018257885.1	Unpublished

(continued)

Table 7.1 (continued)

Year	Classification	Species name	Accession number[a]	References
2021	Lecanoromycetes, Caliciales	*Physcia stellaris*	GCA_018902385.1	Wilken et al. (2020)
2021	Lecanoromycetes, Lecanorales	*Stereocaulon alpinum*	GCA_018257865.1	Kim et al. (2021b)
2011	Lecanoromycetes, Lecanorales	*Cladonia grayi*	JGI Clagr3	Armaleo et al. (2019)
2011	Lecanoromycetes, Teloschistales	*Xanthoria parietina*	JGI Xanpar2	Unpublished
2015	Lecanoromycetes, Lecanorales	*Usnea florida*	JGI Usnflo1	Unpublished
2016	Lecanoromycetes, Acarosporales	*Acarospora strigata*	JGI Acastr1	McDonald et al. (2013)
2016	Lecanoromycetes, Pertusariales	*Dibaeis baeomyces*	JGI Dibbae1 (m)	McDonald et al. (2013)
2016	Eurotiomycetes, Verrucariales	*Endocarpon pallidulum*	JGI Endpal1	McDonald et al. 2013
2016	Lecanoromycetes, Ostropales	*Graphis scripta*	JGI Grascr1	McDonald et al. (2013)
2017	Lecanoromycetes, Peltigerales	*Lobaria pulmonaria*	JGI Lobpul1 JGI LobpulAf1 (m) JGI LobpulSc1 (m) JGI LobpulSp1 (m) JGI LobpulSw1 (m)	Unpublished

[a]GCA numbers are from the NCBI genome repository and JGI numbers are from the Joint Genome Institute MycoCosm repository (Grigoriev et al. 2014); (m) assembly obtained from metagenome sequencing

Table 7.2 Status of the genome sequencing effort for Ascomycota lichenized fungi

	Number of species[a]		Number of genomes	
Fungal class	Non-lichenized	Lichenized	Non-lichenized	Lichenized
Dothideomycetes	20,495	316	274	2
Eurotiomycetes	10,336	616	274	1
Lecanoromycetes	31	8414	1	24
Leotiomycetes	6711	2	138	0
Arthoniomycetes	0	464	0	1
Lichinomycetes	0	90	0	0
Candelariomycetes	14	46	0	0
Collemopsidiomycetes	45	18	0	0
Coniocybomycetes	4	28	0	0
Sareomycetes	13	2	0	0
Sordariomycetes	34,075	0	658	0
Pezizomycetes	3095	0	26	0
Orbiliomycetes	548	0	9	0
Laboulbeniomycetes	143	0	1	0
Geoglossomycetes	77	0	2	0
Xylonomycetes	16	0	3	0
Xylobotryomycetes	3	0	0	0
Total	75,605	9997	1386	27

[a]Number of species in fungal lineages and available non-redundant fungal genomes deposited at NCBI (13/12/2021). Lichenized species were retrieved according to the LIAS light database for rapid identification of lichens (http://liaslight.lias.net; 13/12/2021). Numbers from NCBI taxonomy were retrieved using TaxonKit (Shen and Ren 2021). Numbers of species in these databases are an underestimation of the numbers of lichenized species (Lücking et al. 2017) and should be considered as orders of magnitude

perfume lichens *E. prunastri* and *P. furfuracea* (Meiser et al. 2017; Singh et al. 2021), *Letharia* lichens (McKenzie et al. 2020), *Umbilicaria pustulata* (Greshake Tzovaras et al. 2020), and *Bacidia gigantensis* (Allen et al. 2021). Of particular importance, these studies showed that the quality and completeness of the obtained mycobiont assemblies are nearly similar to those obtained from the mycobiont alone, which in the case of perfume lichens could be explained by the fact that over 70% of the metagenome reads were of mycobiont origin (Meiser et al. 2017). However, the gene content encoding SM biosynthetic pathways appears to differ slightly because of the more fragmented metagenome assemblies and contaminants by lichenicolous fungi present in the thallus (Meiser et al. 2017). Another advantage of this metagenomics approach is that high-quality sequencing also allows the generation of assemblies for the photobiont and the microbiome all at once. Although no sequencing data is available at NCBI or JGI yet, good quality mycobiont genomes obtained from metagenome sequencing have been reported for *Alectoria sarmentosa* (Tagirdzhanova et al. 2021) and for 39 additional Lecanorales species (Pizarro et al. 2020). Thus, we are entering a new era of lichen sequencing that will provide a more complete view of the SM biosynthetic potential of lichenized fungi, as well as common biological processes involved in establishing the lichen communities.

7.3.2 An Abundance of Polyketide Biosynthetic Pathways

In fungi, SM biosynthetic pathways are controlled by genes that are often located at the same genomic locus and are co-regulated, defining a BGC organization (Keller 2019). These BGCs are organized around the so-called core gene that encodes the enzyme responsible for the first raw stable intermediate. These core enzymes are classified into four major groups as polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), terpene cyclases (TCs), and dimethylallyl tryptophan synthetases (DMATs). Analysis of mycobiont genomes has revealed that Lecanoromycetes are particularly enriched in polyketide pathways (Mosunova et al. 2020). Thus, the genomic information is consistent with the observation that the vast majority of SMs identified from lichens are polyketides. However, most of the reported compounds are aromatic, which are typically synthesized by non-reducing PKSs, while reducing PKSs usually produce linear polyketides. Comparative genomics of Lecanoromycetes and lichenized fungi show that their genomes are as rich in non-reducing as in reducing PKSs (Fig. 7.5; Calchera et al. 2019). Such an abundance in reducing PKSs suggests that many more lichen polyketides await to be discovered. Considering that the few available mycobiont genomes have already revealed a very high number of BGCs, especially for polyketides, obtaining more genome sequences will reveal many more biosynthetic pathways and will provide access to the complete chemical diversity of lichenized fungi, which is expected to be much larger than currently reported.

7.3.3 Linking Biosynthetic Gene Clusters to Known Lichen Compounds

Phylogenetic analyses of fungal PKSs have revealed that enzymes belonging to the same monophyletic clade tend to share the same enzymatic activities and produce the same polyketide backbone. Phylogenetic analyses of core enzymes is thus a very useful approach to investigate the chemical diversity of fungal polyketides. In addition, the large diversity of chemical structures and biological activities is further enhanced by tailoring genes located next to the PKS gene. Phylogenetic analyses of PKSs, comparative genomics and transcriptomics studies have identified candidate BGCs for a number of well-known lichen polyketides.

Grayanic acid was the first compound linked to a BGC in the genome of *C. grayi* thanks to the correlation between grayanic acid production and expression of the *CgrPKS16* gene (Armaleo et al. 2011). In addition, the predicted BGC comprises

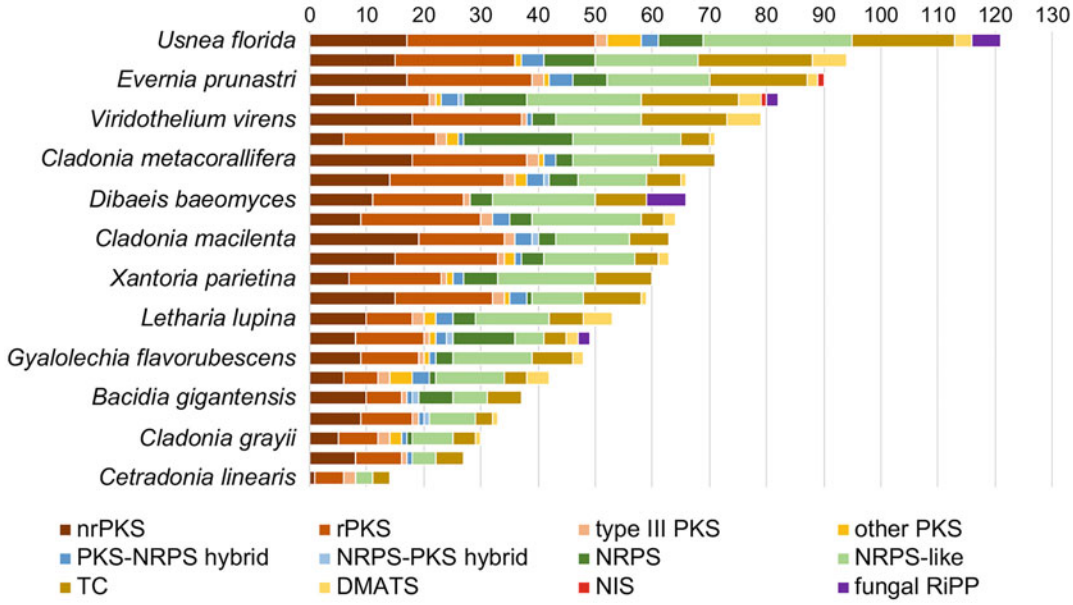

Fig. 7.5 Number of predicted biosynthetic pathways in lichen mycobiont genomes. Biosynthetic gene clusters (BGCs) as predicted by fungiSMASH (Blin et al. 2021) were classified according to conserved domains present in the core enzyme. *nrPKS* non-reducing polyketide synthase, *rPKS* reducing polyketide synthase, *NRPS* non-ribosomal peptide synthetase, *TC* terpene cyclase, *DMATS* dimethylallyl tryptophan synthetase, *NIS* NRPS-independent siderophore, *RiPP* ribosomally synthesized and post-translationally modified peptide

genes encoding an *O*-methyltransferase and a cytochrome P450 which exhibit enzymatic activities consistent with the grayanic acid structure. This BGC was also found conserved in *C. uncialis* (Bertrand et al. 2018b), and in *E. prunastri* and *P. furfuracea* (Calchera et al. 2019). Resequencing of two different chemotypes of *P. furfuracea* from metagenomes indicated that the homolog of the PKS assigned to grayanic acid is likely responsible for the production of olivetoric and physoric acids in this lichen (Singh et al. 2021).

Another important lichen polyketide due to its potential applications is usnic acid. The genome of *C. uncialis* was sequenced with the specific aim to identify the BGC involved in the production of usnic acid (Abdel-Hameed et al. 2016a; Bertrand et al. 2018a). Chemical studies indicated that usnic acid is produced by a non-reducing PKS with a carbon methylation domain and a single tailoring enzyme that is required for oxidative dimerization (Abdel-Hameed et al. 2016a). The genome of *C. uncialis* contains a single candidate BGC consistent with the predicted requirements, which was found to be expressed in the lichen thallus where usnic acid is detected (Abdel-Hameed et al. 2016a). This candidate BGC was also found in *E. prunastri* and *P. furfuracea* (Calchera et al. 2019). A more recent comparative genomic analysis of lichenized fungi found a correlation between the presence of this BGC and usnic acid production (Pizarro et al. 2020). The correlation between transcriptomics and usnic acid production in *Nephromopsis pallescens* suggested that the Nppks7 PKS is responsible for the production of this SM (Wang et al. 2018a).

Similarly, the mycobiont *Cladonia macilenta* was sequenced in order to identify the BGC involved in the production of biruloquinone, an acetylcholinesterase inhibitor that is a promising candidate to prevent Alzheimer's disease (Park et al. 2013a). A recent transcriptomics approach enabled the assignment of this compound to a

BGC which comprises *PKS21*, a close homolog of the cercosporin *CTB1* gene, and five other genes (Kim et al. 2021a). This BGC is fully conserved in *Cladonia borealis* and *Cladonia metacorallifera*. While the genome of *C. metacorallifera* was released in 2014 (Park et al. 2014a), it has only recently been used to search for the BGC involved in the production of the red pigment cristazarin (Jeong et al. 2021). Unfortunately, 27 out of 30 PKS genes were differentially regulated when cristazarin is produced and it was not possible to assign any of them to this pigment. The *Stereocaulon alpinum* genome was compared to *Cladonia* genomes in order to identify a candidate BGC for atranorin biosynthesis (Kim et al. 2021b). A comparative genomics and BGC clustering approach determined the set of PKSs common to these mycobionts and the ones most likely involved in the production of depsides and depsidones. This approach identified the *PKS23* BGC in *S. alpinum* and *C. rangiferina* as candidate for the production of atranorin because this SM was reported in these two lichens only (Kim et al. 2021b). Gene composition of the predicted BGC was also consistent with the chemical structure of atranorin, which has an unusual methoxycarbonyl group. This BGC is also present in the genomes of 5 other mycobionts, which are also able to produce atranorin. A phylogenetic analysis of the PKS showed that Pks23 belongs to a new group of non-reducing PKSs. This BGC was fully validated with functional analyses (see Sect. 7.4.2).

In addition to linking BGCs to compounds isolated from lichen thalli, genome analyses allow one to predict the production of other SMs. For example, the combination of *C. uncialis* genome and expression data suggested that one BGC encoding a PKS homologous to the terrein synthase produces halogenated derivatives of 6-hydoxymellein (Abdel-Hameed et al. 2016b). Other BGCs appear highly conserved with the betaenone BGC in *Phoma betae*, patulin BGC in *Aspergillus clavatus*, azaphilone BGC in *Monascus pilosus*, and emodin-derived anthraquinone BGCs, suggesting that *C. uncialis* can also produce these kinds of SMs, although they have not been reported yet (Bertrand et al. 2018b).

The different genome analyses that focused on lichen SM pathways not only linked candidate BGCs to known lichen molecules, they also have revealed a much larger and diverse biosynthetic production potential than previously known, which may lead to the identification of new chemical backbones and new biological roles in lichen communities. Functional analyses of these candidate BGCs will be key to understanding further their biological roles.

7.4 Heterologous Expression as a Strategy to Elucidate Lichen Biosynthetic Pathways

The difficulty in reconstituting lichen communities in the laboratory and their slow growth, which is also true for each isolated partner, make functional studies very difficult. Another challenge is that laboratory conditions are often non-conducive for the production of SMs produced by the isolated mycobiont, as found for many other fungi. Indeed, it is well-known that SMs are produced under very specific conditions that are difficult to determine and reproduce in the laboratory, resulting in cryptic biosynthetic pathways or silent BGCs (Keller 2019). Therefore, it is important to find a reliable strategy to link genes to compounds. One approach that is widely implemented to elucidate fungal biosynthetic pathways is heterologous expression in a fungal host where gene expression can be controlled. Surrogate hosts like *Aspergillus oryzae* (NSAR1), *Aspergillus nidulans*, or *Saccharomyces cerevisiae* are routinely used to characterize BGCs from non-lichenized fungi and have been used for a few lichen pathways (Table 7.3). However, successful heterologous expression of PKSs from lichenized fungi has been reported for only a very limited number of cases (Table 7.3), and each of these achievements represents a significant breakthrough in lichen research.

Table 7.3 Heterologous expression of polyketide synthases (PKSs) from lichen mycobionts

PKS	Native host	Surrogate host	Molecule	References
PFUR17_02294	*Pseudevernia furfuracea*	*Saccharomyces cerevisiae*	Lecanoric acid	Kealey et al. (2021)
Pks23	*Endocarpon pusillum*	*Saccharomyces cerevisiae*	Naphthalene pyrone	Harvey et al. (2018)
Atr1	*Stereocaulon alpinum*	*Ascochyta rabiei*	Atranorin	Kim et al. (2021b)
MPAS[a]	*Cladonia uncialis*	*Aspergillus oryzae*	No production	Bertrand and Sorensen (2019)
6-MSAS[b]	*Cladonia uncialis*	*Aspergillus oryzae*	No production	Bertrand and Sorensen 2019
OAS[c]	*Cladonia uncialis*	*Aspergillus oryzae*	No production	Bertrand and Sorensen (2019)
UlPks6	*Usnea longissima*	*Aspergillus oryzae*	No production	Yi et al. (2016)
CgrPks2	*Cladonia grayi*	*Aspergillus oryzae*	No production	Armaleo et al. (2011)
ScPks1	*Solorina crocea*	*Aspergillus oryzae*	No production	Gagunashvili et al. (2009)
XsePks1	*Xanthoparmelia semiviridis*	*Aspergillus nidulans*	No production	Chooi et al. (2008)

[a]Methyl-phloroacetophenone synthase
[b]6-methylsalicylic acid synthase
[c]Orsellinic acid synthase

7.4.1 Successful Expression of Lichen Polyketide Synthases in *Saccharomyces cerevisiae*

Heterologous expression of a lichen depside PKS has been recently reported in yeast (Kealey et al. 2021). Expression of codon-optimized *PFUR17_02294* from the perfume lichen *P. furfuracea* was performed in a yeast strain that was engineered to contain a copy of the *Bacillus subtilis sfp* gene which encodes a phosphopantetheinyl transferase needed to activate the acyl carrier protein domain of the PKS. Transformants produced an orsellinic acid dimer that was identified as a depside, lecanoric acid. It is unusual that a PKS is able to synthesize lecanoric acid which is a depside formed from the dimerization of two orsellinic acid precursors. The presence of orsellinic acid was also observed after prolonged culture fermentation, resulting from the hydrolysis of the ester linkage (Kealey et al. 2021). How this dimerization is performed by the PKS requires an in-depth investigation at the mechanistic level, and may suggest that lichen mycobionts exhibit more diverse enzymatic capabilities.

Harvey and colleagues (Harvey et al. 2018) have also developed a *S. cerevisiae* strain for heterologous expression of various fungal BGCs. In this work, 41 cryptic BGCs (28 PKSs and 13 TCs) were selected from diverse Ascomycota and Basidiomycota species, and 22 of them yielded detectable SMs. One of the successful expression trials involved the Pks23 from the lichenized fungus *Endocarpon pusillum*. Pks23 produced two related naphthalene pyrones, which are known precursors of phenalenone in *Penicillium herquei* in which the biosynthetic pathway was elucidated through gene deletions and heterologous expression in yeast (Gao et al. 2016). However, the BGC does not seem to be fully conserved and *E. pusillum* most likely produces related but different compounds.

7.4.2 Successful Expression of a Lichen Gene Cluster in *Ascochyta rabiei*

Kim and coworkers (Kim et al. 2021b) have recently published an article demonstrating the first successful functional heterologous expression of a novel lichen polyketide BGC from the lichen mycobiont *S. alpinium*. The presence of methoxycarbonyl within the 3-methyl orsellinic acid makes it a unique molecule among other

depsidones and depsides. The BGC is comprised of genes encoding three enzymes (Atr1: PKS; Atr2: P450 monooxygenase; Atr3: O-methyltransferase) and a transporter (Atr4). They chose to express Atr1, Atr2, and Atr3 in a strain of the plant pathogenic fungus, *Ascochyta rabiei*, in which the BGC for solanapyrone production was removed (Kim et al. 2021b). Genes were cloned from the genomic DNA of *S. alpinum*. 4-O-demethylbarbatic acid was observed after the successful expression of Atr1, consistent with the atranorin structure. Similarly to lecanoric acid, 4-O-demethylbarbatic acid is a dimer produced by the PKS, indicating that lichen PKSs commonly produce dimeric SMs. Transformants co-expressing Atr1 with either Atr2 or Atr3 yielded different intermediates (proatranorins) and a shunt product (baeomycesic acid), while co-expression of all three genes resulted in the production of atranorin. This study represents a major breakthrough in the study of lichen SMs because not only did it demonstrate the efficiency of comparative genomics and phylogeny to select candidate BGCs, it also resulted in the first full elucidation of a lichen biosynthetic pathway.

7.4.3 Difficulties in Heterologously Expressing Lichen Polyketide Synthases in *Aspergillus oryzae*

Although *A. oryzae* has a proven record as an amenable host for the functional analysis of fungal PKSs, successful expression in this host of PKSs from lichenized fungi is still a daunting task. To date, various heterologous expression attempts in *A. oryzae* have been reported with non-reducing PKSs (Table 7.3). Unfortunately, in all the reported cases, no corresponding metabolite could be detected in fermentation culture extracts. Transcription of the PKS genes was observed in each case, and the transcripts were consistent with the expected gene structure, meaning that introns were accurately spliced in *A. oryzae* (Bertrand and Sorensen 2019). The ketosynthase domain of *C. uncialis* PKSs were further investigated for functionality and they did not show any particular features compared to non-lichenized PKSs (Abdel-Hameed et al. 2018). Moreover, they were successfully produced in *Escherichia coli*, suggesting that at least partial lichen PKSs can be translated accurately (Abdel-Hameed et al. 2018). Thus, the reasons for the absence of metabolite production remain unclear although it may be linked to the translation of the full-length PKS. For example, expressing the lecanoric acid or atranorin PKSs in *A. oryzae* would inform about whether the PKS or the host is the limiting factor. Conversely, expressing in yeast or *A. rabiei* the PKS genes that failed to produce compounds in *A. oryzae* could represent an interesting alternative. Although the KS domain was successfully produced in *E. coli*, one hypothesis could be that *A. oryzae* degrades lichen PKSs after translation. An *A. oryzae* strain lacking the PepE and tripeptidyl peptidase genes produced high-titers of recombinant human lysozyme and bovine chymosin proteins (Jin et al. 2007). Similarly, autophagy and vacuolar protein sorting (VPS) degrade accumulated, misfolded, or unfolded proteins. *A. oryzae* autophagy-deficient mutants showed increased bovine chymosin production levels up to threefold (Yoon et al. 2013), while deletion mutants of VPS10 gene resulted in 2.2- and 3-fold increase of the production of human lysozyme and chymosin, respectively (Yoon et al. 2010). Such modifications could be added in the *A. oryzae* NSAR1 strain used for SM production as it may help in successful heterologous expression. Finally, other issues could be related to missing precursors for the PKS, or missing tailoring enzyme to release the polyketide chain. While it remains unclear why strains of *A. oryzae* that express the lichen PKS genes do not produce any detectable SMs, addressing the PKS stability and engineering a dedicated strain devoid of proteases could unlock the use of this host.

7.4.4 Indirect Studies Using Orthologous Pathways Provide Information About Potential Lichen Compounds

BGCs are often conserved in distant fungal lineages, yet show few differences in their gene component due to single gene gains or losses. These variations refer to BGC families that produce different derivatives of the same chemical backbones. Although heterologous expression of lichen PKSs remains a complex task, an alternative approach is to make use of BGC families. Because PKS phylogenetic studies indicate that orthologous PKSs produce similar backbones, it is possible to express orthologs of lichen PKSs from non-lichenized fungi. While the *C. uncialis* candidate 6-methylsalicylic acid and orsellinic acid PKSs did not produce any compound in *A. oryzae*, their orthologs from *Penicillium* sp. and *Fusarium* sp., respectively, did yield the expected compounds (Bertrand and Sorensen 2019).

Recently, a combined phylogenetic and comparative genomics approach was used to identify novel polyketide BGCs in seven mycobiont genomes (Mosunova et al. 2022). This approach identified one new phylogenetic clade that comprised enzymes from *L. pulmonaria*, *U. pustulata*, and *C. grayi* and corresponds to the new group XI of non-reducing PKSs. This clade actually corresponds to a conserved BGC that was found in distant fungal lineages, from Lecanoromycetes to Dothideomycetes. Expression of the homologous BGC found in *Aspergillus parvulus* was correlated with the production of naphthalenone compounds, 6-methyl asparvenone, and 1-ethyl parvulenone. Heterologous expression in *A. oryzae* of the PKS gene revealed that the PKS from the group XI clade produced acetyl tetrahydroxynaphthalene, an intermediate consistent with the production of naphthalenones. While the PKS gene was found to be inactivated by disruptive mutations in *C. grayi*, the pathway is predicted to be functional in *L. pulmonaria* and *U. pustulata*. Based on the BGC content that slightly differs from *A. parvulus*, it is predicted that *L. pulmonaria* and *U. pustulata* produce non-methylated and non-acetylated derivatives of asparvenone. This class of SMs has not been reported for both lichens and it will be interesting to search for derivatives in the lichen thallus. The only lichen compound that resembles naphthalenones is the red pigment cristazarin from *Cladonia cristatella* and *C. metacorallifera*. However, the genome of *C. metacorallifera* does not encode any group XI PKS and future research is needed to determine the cristazarin biosynthetic pathway. These examples show that studying orthologous pathways in other fungi provides hints about SMs produced by lichens and thus, it is an interesting strategy to uncover the complete chemical diversity of lichen mycobionts.

7.5 Toward Understanding the Role of Mycobiont Secondary Metabolites in Lichen Ecosystems

SMs isolated from lichens have been extensively used as natural dyes and have been screened for biological activities relevant to medical and industrial applications. The biological role of these SMs for the lichen itself is mostly based on assumptions, covering protection from UV-light and desiccation, and defense against other microbes and herbivores. So far, genomic and transcriptomic analyses have not provided insights into the role of mycobiont SMs in the lichen ecosystem, but mostly identified nutrient exchange, cell wall reorganization, and lipid metabolism as major biological processes (McDonald et al. 2013; Wang et al. 2014; Armaleo et al. 2019; Kono et al. 2020). Understanding the biological functions of SMs in their interactions with other organisms and environment is extremely challenging. It is a major research question for fungal plant pathogens and remains an open question for lichens. Here, we would like to provide some complementary research directions toward understanding the role of SMs in the establishment and survival of lichen ecosystems.

7.5.1 *In vitro* Reconstitution of Lichen Ecosystems

It is possible to study the functional genomics of the whole thallus by collecting long-lived thalli from nature, but the slow growth restricts harvesting large biomass. Therefore, *in vitro* culturing of lichen communities is a fascinating alternative tool for identifying specifically induced BGCs and investigating linkage with the possible novel SMs. Such a reconstitution is an incredibly difficult task, but was achieved for a few lichens since the early days of lichenology (Stocker-Wörgötter 2001). In the genomic era, investing again in reconstituting lichens under controlled conditions is important for functional analyses as it allows comparing gene expression and metabolite production when partners are grown isolated or together. The lichen *Usnea hakonensis* was successfully reconstituted in the laboratory after co-culturing *U. hakonensis* mycobiont and *Trebouxia* photobiont (Kono et al. 2020). This experiment allowed a comparison of the transcriptomes between isolated partners, and the reconstituted community and lichen from the field. Only one PKS gene appeared to be up-regulated in the natural lichen, which requires further characterization to identify the kind of polyketide that is produced. Because this PKS belongs to the reducing group, it is expected that the corresponding SM is different from all the aromatic polyketides identified in lichens. Comparison of the metabolic profiles of *Xanthoria elegans* grown aposymbiotically or with its photobiont did not reveal major qualitative differences (Brunauer et al. 2007). In light of the complex microbiome hosted in lichens, investigating the impact of bacteria and lichenicolous fungi on co-cultures of the mycobiont and photobiont could provide new insights into how lichen ecosystems function. Considering that the recent reconstitution of *C. grayi* and *U. hakonensis* lichens did not reveal significant induction of mycobiont BGCs (Armaleo et al. 2019; Kono et al. 2020), the microbiome might be the missing partner for SM production.

7.5.2 Spatial Distribution of Mycobiont Secondary Metabolites

Some biological functions can be deduced from biological activities like antibiotic or antifungal properties. SMs with such bioactivity are most likely involved in colonizing or protecting an ecological niche from other competing microbes. However, hints about biological functions can also be obtained by determining where in the thallus specific SMs are produced. Here again, reconstituted communities will be very useful, but methods like mass spectrometry imaging (MSI) allow SM localization to be studied in natural ecosystems too. The spatial organization of SMs in a lichen using MSI was first reported in *O. ventosa* (Le Pogam et al. 2016). SMs show clear distinct localization within the thallus, with the polyketides hemoventosin located on the surface of the apotheciate thallus (red pigment found in the fruiting bodies), thamnolic acid found in the upper cortex and medulla, and divaricatic and miriquidic acids accumulating in the lower medulla and at the lichen/rock interface. Usnic acid appears to be vertically distributed equally across the thallus, but is detected on the edges of the lichen and accumulates above the photobiont layer. SM spatial organization has also been investigated in the lichen *Peltigera* (Garg et al. 2016). This study employed an untargeted metabolomics approach relying on molecular networking. As expected, comparison of the community to isolated partners (mycobiont, phytobiont, and bacteria) showed little overlap in the metabolic profile, confirming that studying the whole community is particularly important. While this study used the Global Natural Product Social (GNPS) database to rapidly identify known molecules, very few structures could be assigned to the novel metabolites that were detected in these extracts. Nevertheless, MSI of three different layers of *Peltigera*, sun-exposed top layer, middle layer, and bottom layer, revealed different metabolite distribution. For example, the fungal pyridone alkaloid PF1140 was found to be most abundant in the bottom

layer, while asperphenamate and sesquiterpene lactones were mostly found in the middle layer. All these molecules exhibit diverse biological activities, including antimicrobial activities, and are expected to be produced by different lichenicolous fungi rather than the mycobiont. Although a promising approach, MSI remains difficult to employ for unknown molecules, and the strategy to use heterologous expression to identify SMs produced by mycobionts will provide the necessary information to perform targeted MSI.

7.5.3 Genetic Manipulation of Mycobionts

A common limitation to functional analyses in fungi is their amenability to molecular biology tools, and lichenized fungi are particularly difficult to work with. The development of tools to delete, disrupt, or over-express genes is a traditional strategy that would help assigning BGCs to molecules and study the biological functions of these SMs. *Agrobacterium tumefaciens*-mediated transformation protocols have been reported for *Umbilicaria muehlenbergii* (Park et al. 2013c) and *C. macilenta* (Liu et al. 2021), which have been employed for random mutagenesis only as a proof-of-concept. Although both genetic manipulation tools and genome information are available for *U. muehlenbergii*, so far no functional study has been reported. CRISPR-Cas9 has been developed for many model and non-model non-lichenized fungi, but so far, no report has mentioned its use in mycobionts. While transformation procedures and low homologous recombination rates are key limiting factors in functional analyses and are not solved with this this genome editing tool, it would still be interesting to develop this method for lichenized fungi because the dramatic increased frequency of true mutants will facilitate obtaining the desired mutations.

7.6 Conclusion

The last decade has seen great strides in the advancement of linking genes to molecules in lichen fungi. The inexpensive next-generation sequencing technology had led to sequencing the genomes of a significant number of lichenized fungi, and readily available annotation tools have revealed the BGCs involved in the production of lichen SMs. This progress has allowed for the linking of BGCs to chemical structures for a number of lichen SMs. Consistent with earlier observations on non-lichenized fungi, these studies revealed that the number and diversity of BGCs far exceed the observed SM production. This hidden biosynthetic potential has yet to be fully realized, in part because of the challenge described above with successful heterologous expression. The reasons for the failure of metabolite production in these expression systems remain unclear, but there remains a high degree of optimism that solutions will be found. The development of new expression platforms, especially using yeast systems where some signs of success, such as with lecanoric acid, have been observed, leave some room for optimism. The advent of a general expression platform for lichen genes that allows for the successful expression of complete BGCs will represent a significant advance in our understanding of SM biosynthesis. The conclusive linking of genes-to-molecules across a broad array of lichen species will surely follow this advance. More significantly, the discovery of new chemical structures and classes of natural products will surely be the result from decoding the cryptic BGCs in the genomes. Last, but not least, lichen communities have been found to be more complex than originally imagined with the presence of a diverse microbiome, whose impacts on the lichen ecosystem remains to be determined. The communication between all of the partners in this symbiotic association is almost certain to involve small

molecule signaling. With the advances in genomics, DNA synthesis, heterologous expression, mass spectrometry imaging, and reconstituted lichen communities, we are now entering a new era in which we will not only discover the full and unique chemical diversity of these ubiquitous organisms, but also understand the true biological functions of all these lichen metabolites.

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Part III

Sensing and Signalling in Fungus–Plant Interactions

Regulation of Plant Infection Processes by MAP Kinase Pathways in Ascomycetous Pathogens

8

Zeyi Wang, Xue Zhang, Cong Jiang, and Jin-Rong Xu

Abstract

Plant pathogenic fungi use the well-conserved MAP kinase (MAPK) pathways to mediate responses to external stimuli and regulate various infection and developmental processes. Most ascomycetous fungal pathogens have three MAPK cascades. In general, the Pmk1/Kss1 invasive growth (IG) pathway is essential for pathogenesis by regulating infection-related morphogenesis, such as formation of appressoria or hyphopodia, penetration, and invasive growth in infected plant tissues. The cell wall integrity (CWI) MAPK pathway is also normally important for plant infection by regulating species-specific infection processes, cell wall integrity, infectious growth, and responses to cell wall stress. Unlike the IG and CWI pathways, the HOG (high osmolarity glycerol) pathway is dispensable for virulence in some fungal pathogens such as *Magnaporthe oryzae* but plays a critical role in pathogenesis in many others. Besides its conserved role in osmoregulation, the HOG pathway is usually important for responses to oxidative and other environmental stresses. Overall, both conserved and species-specific functions have been identified for individual MAP kinase cascades in plant pathogenic fungi, likely due to variations in upstream signaling and downstream transcriptional regulation. Limited studies in a few fungal pathogens have also shown that there is crosstalk among three MAPK pathways to regulate various infection processes and responses to biotic and abiotic stresses, indicating the complex regulatory networks associated with these MAP kinase pathways.

Keywords

Signal transduction · Pathogenesis · *PMK1* · Appressorium formation · Plant penetration · Cell wall integrity

8.1 Introduction

In fungi and other eukaryotic organisms, mitogen-activated protein (MAP) kinase pathways play critical roles in regulating responses to various extracellular cues. A typical MAP kinase cascade consists of a MAP kinase (MAPK), a MAPK kinase (MEK), and a MEK kinase (MEKK). The sequential activation of these protein kinases results in the dual phosphorylation of MAPKs at the T-X-Y activation motif,

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which then phosphorylates downstream targets to regulate transcriptional changes and cellular responses. The model organism *Saccharomyces cerevisiae* has five MAPK genes due to its whole genome duplication event. Fus3 and Kss1 are two paralogous MAPKs that have overlapping functions in pheromone response but only Kss1 is involved in regulating filamentation and invasive growth into agar (Schwartz and Madhani 2004; Chen et al. 2012). From Ste2 and Ste3 pheromone receptors to transcription factors such as Ste12 and Dig1, the pheromone response pathway is the best characterized MAPK pathway in eukaryotic organisms. Slt2 and Hog1 MAPKs mainly regulate cell wall integrity and osmoregulation, respectively, although they are also involved in responses to other stresses. Smk1 is a meiosis-specific MAPK regulating ascospore wall assembly. Unlike other yeast MAPKs, Smk1 lacks upstream MEK or MEKK and it is activated by autophosphorylation and phosphorylation by Cak1 (Schwartz and Madhani 2004; Chen et al. 2012).

Whereas orthologs of Fus3/Kss1, Slt2, and Hog1 are well-conserved in plant pathogenic ascomycetes, Smk1 appears to be unique to *S. cerevisiae*. In fact, most plant pathogenic ascomycetes have only three MAPKs, three MEKs, and three MEKKs that are orthologous to the key components of the yeast Fus3/Kss1, Slt2, and Hog1 MAP kinase cascades, with a few exceptions such as two MAPKs homologous to yeast Hog1 in *Verticillium dahliae* and two MEKKs functioning upstream from the cell wall integrity MAPK in *Fusarium oxysporum*. Various components of these three well-conserved MAPK pathways have been characterized in different plant pathogenic ascomycetes for their functions in pathogenesis, sexual and asexual reproduction, mycotoxin production, and stress responses (Jiang et al. 2018). To date, all three MAPK cascades have been characterized in the rice blast fungus *Magnaporthe oryzae*, wheat scab fungus *Fusarium graminearum*, and several other plant pathogenic fungi. Whereas only two MAPKs are important for pathogenesis in *M. oryzae*, a model for studying fungal–plant interactions, all three MAPKs play critical roles in pathogenesis of *F. graminearum*, indicating variations in the functions of individual MAPKs among different fungal pathogens.

8.2 The Pmk1/Kss1 Invasive Growth (IG) Pathway

In general, plant pathogenic ascomycetes have only one MAPK that is orthologous to Fus3 and Kss1, which are activated by upstream MEK Ste7 and MEKK Ste11 in yeast. Studies in a number of fungal pathogens have showed that this MAPK pathway is important for regulating infection-related morphogenesis and invasive growth in plant tissues (Li et al. 2012; Turrà et al. 2014). Although they share similar amino acid sequence identity with yeast Fus3 and Kss1, the IG MAPKs from plant pathogens are considered to be more closely related to the latter because of the role of Kss1 in agar invasion in *S. cerevisiae*.

8.2.1 Regulation of Appressorium Formation by the PMK1 Pathway in M. oryzae

Like many other foliar pathogens, *M. oryzae* forms melanized, dome-shaped appressoria for plant penetration. As the first MAPK gene characterized in plant pathogens, *PMK1* (pathogenicity MAP kinase 1) is essential for appressorium formation and pathogenesis in the rice blast fungus. Germ tubes of the *pmk1* deletion mutant form subapical swollen bodies instead of appressoria on artificial hydrophobic surfaces and rice leaves. Deletion of *PMK1* blocks the formation of appressoria but not surface recognition, which is regulated by the cAMP-PKA pathway in *M. oryzae* (Xu and Hamer 1996). Pmk1 is activated by the Mst7 MEK, which is in turn activated by Mst11 MEKK (Fig. 8.1). The *mst7* and *mst11* mutants have the same defects in appressorium formation and plant infection as the *pmk1* mutant. Although the Mst11-Mst7-Pmk1 MAPK cascade lacks a scaffold protein, Mst7 directly interacts with Pmk1 via its MAPK docking site and both Mst11 and Mst7 interact

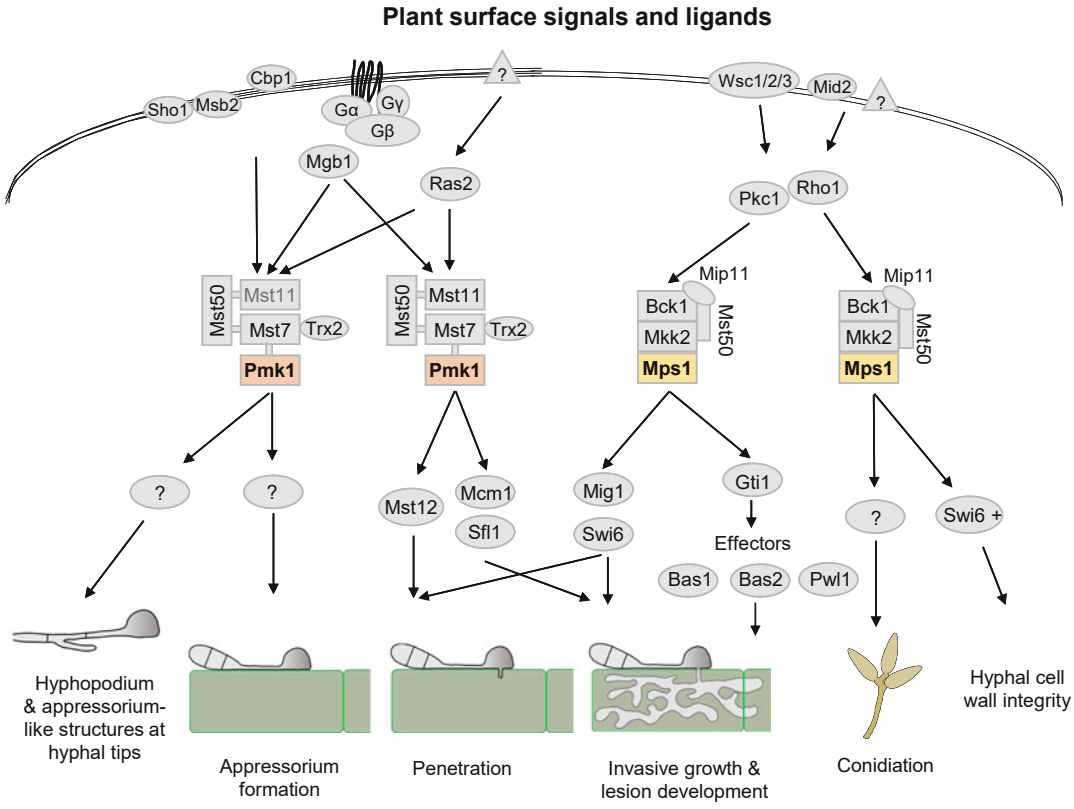

Fig. 8.1 Distinct and overlapping functions of the Pmk1 and Mps1 MAPK pathways in *Magnaporthe oryzae*. The Mst11-Mst7-Pmk1 MAPK cascade is involved in regulating appressorium formation, penetration, and invasive growth (moving from cell to cell) in infected plant tissues. Both trimeric G-proteins and small GTPase Ras2 have been implicated in activating the Pmk1 pathway via Msb2 and Cbp1 mucins, Sho1, and possibly GPCRs as the receptors for physical and chemical cues such as surface hydrophobicity and hardness, cutin monomers, and primary alcohols. Thioredoxin Trx2 affects the activation of Mst7 but the role of Mst20 and Chm1 PAK kinases in Mst11 activation is not clear. Known downstream transcription factors of Pmk1 include Mst12, Mcm1, and Sfl1 but none of them is essential for appressorium formation, indicating the existence of other Pmk1 targets. The Bck1-Mkk2-Mps1 cascade is involved in regulating appressorium penetration, invasive growth, disease development, and conidiation. *M. oryzae* has orthologs of Wsc1–3 and Mid1 that may function as the cell wall stress sensors. Based on its conserved functions, this CWI MAPK pathway likely functions downstream from PKC and Rho1. Transcription factors known to function downstream from Mps1 MAPK include Gti1, Swi1, and Mig1. Adaptor protein Mst50 is involved in both Pmk1 MAPK and Mps1 MAPK pathways. Mip11 functions as a RACK protein that interacts with both Mst50 and Mck1

with the adaptor protein Mst50 (Zhao and Xu 2007; Park et al. 2006). The Trx2 thioredoxin is involved in the activation of Pmk1 by affecting the folding or intra−/inter-molecular interaction of Mst7 (Zhang et al. 2016). One of the downstream targets of the Pmk1 pathway is Mst12, a Ste12 ortholog that is essential for appressorium penetration and pathogenicity. *MST12* is dispensable for appressorium formation but regulates septin-mediated cytoskeleton reorganizations in mature appressoria (Park et al. 2002, 2004; Dagdas et al. 2012). MoMcm1 and MoSfl1 are the other two transcription factors that likely function downstream from the Pmk1 MAPK cascade for appressorium penetration and invasive growth (Li et al. 2011; Zhou et al. 2011). MoSfl1 is identified as one of the proteins phosphorylated by Pmk1 in vitro (Li et al. 2011). Deletion of

MoSFL1 rescues the defect of the *cpk1 cpk2* mutant in vegetative growth by relieving transcriptional suppression of the Cyc8-Tup1 co-suppressor (Li et al. 2017b), suggesting that it may be functionally related to both Pmk1 and cAMP-PKA pathways.

In yeast, the PAK kinase *STE20* functions upstream from the pheromone response pathway. In *M. oryzae*, deletion of the *STE20* ortholog does not block appressorium formation or plant infection. Deletion of the only other PAK kinase gene, *CHM1*, results in pleiotropic defects in growth, conidiation, and plant infection but the *chm1* mutant still forms melanized appressoria (Li et al. 2004). Both Mst50 and Mst11 have the Ras-associating domain and Mst50 physically interacts with Ras1 and Ras2 in yeast two-hybrid assays (Park et al. 2006). In *M. oryzae*, *RAS2* is an essential gene and functions upstream of both the cAMP-PKA and MAPK pathways. Expressing the dominant $RAS2^{DA}$ allele in the wild type, but not in the *mst50* mutant, results in the formation of melanized appressoria in liquid droplets, indicating the bypass of the requirement of surface attachment and recognition (Zhou et al. 2012; Qi et al. 2015). Besides Ras2, trimeric G-proteins also are involved in regulating appressorium formation and pathogenesis in *M. oryzae* and Mst50 interacts with Mgb1 Gβ subunit (Nishimura et al. 2003; Park et al. 2006). For upstream receptors, the *M. oryzae* genome has over 40 putative G protein-coupled receptor (GPCR) genes, including two pheromone receptors. However, deletion of *MoSTE2* and/or *MoSTE3* has no effect on appressorium formation. Although the CFEM (conserved fungal-specific extracellular membrane-spanning)-domain containing GPCR encoded by *PTH11* is important for surface recognition, and plant infection, treatments with cAMP suppress the defects of *pth11* mutant in plant infection, indicating that *PTH11* mainly functions via cAMP signaling in *M. oryzae* (DeZwaan et al. 1999; Nishimura et al. 2003). In contrast, the orthologs of yeast Sho1 and Msb2 mucin have overlapping roles in acting as the sensors for plant surface chemicals such as primary alcohols to activate the Pmk1 pathway for regulating appressorium formation (Liu et al. 2011). In addition, the *CBP1* gene encoding a putative extracellular chitin-binding protein appears to be involved in sensing hydrophobic surfaces in *M. oryzae* (Kamakura et al. 2002).

8.2.2 Regulating the Formation of Various Infection Structures in Fungal Pathogens

The Pmk1/Kss1 MAPK pathway also has been functionally characterized in several other plant pathogenic fungi that form appressoria for plant penetration, including *Bipolaris sorokiniana, Cochliobolus heterostrophus*, *Colletotrichum gloeosporioides, Colletotrichum fructicola*, and *Colletotrichum lagenarium.* In all of them, this MAPK pathway is required for appressorium formation (Leng and Zhong 2015; Li et al. 2012; Liang et al. 2019). Furthermore, transforming the *CMK1* gene of *C. lagenarium* into the *pmk1* mutant rescues its defect in appressorium formation. Expression of *CPMK1* from *Claviceps purpurea*, a non-appressorium-forming ascomycete, or *PsMAPK1* from the wheat stripe rust *Puccinia striiformis* f. sp. *tritici*, a basidiomycete, also complements the *pmk1* mutant for appressorium formation and pathogenesis (Mey et al. 2002; Guo et al. 2011a), indicating that this MAPK is well-conserved in sequence and function among different fungal pathogens.

Similar to appressoria formed by foliar pathogens, hyphopodia are formed by root pathogens for plant invasion. Under laboratory conditions, *M. oryzae* also forms hyphopodia for infection of rice roots. The Pmk1 MAPK cascade, but not the cAMP-PKA pathway, is essential for hyphopodium formation in *M. oryzae* and likely other root pathogens (Kong et al. 2013; Sesma and Osbourn 2004). On rice leaves or artificial hydrophobic surfaces, hyphal tips of *M. oryzae* also form melanized, swollen apical structures that are morphologically similar to appressoria formed by germ tubes. *PMK1* is also essential for the formation of appressorium-like structures at hyphal tips (Kong et al. 2013). In the gray mold

fungus *Botrytis cinerea*, the formation of infection cushions or compound appressoria by hyphae attached to plant surface is blocked in the *msb2* and *bmk1* mutants (Leroch et al. 2015). In *F. graminearum*, the *GIV1* GPCR gene that appears to function upstream of Gpmk1 is important for infection cushion formation (Jiang et al. 2019). In *Sclerotinia sclerotiorum*, *SMK1* is characterized for its function in regulating sclerotium formation but has not been examined for its role in infection cushion formation (Chen et al. 2004). Nevertheless, the *smk1* mutant is reduced in the expression of the *RGB1* type 2A protein phosphatase gene and silencing of *RGB1* results in a significant reduction in infection cushion formation (Erental et al. 2007). Although the functions of two *PMK1* orthologs in the rice sheath blight fungus *Rhizoctonia solani*, a basidiomycetous pathogen, have not be directly characterized, expression of the RNA interference (RNAi) construct targeting both *RPMK*1–1 and *RPMK*1–2 in transgenic rice plants significantly reduces infection cushion formation and disease severity (Tiwari et al. 2017). Therefore, it is likely that the Pmk1/Kss1 IG MAPK pathway has a conserved role in regulating infection structure formation in plant pathogenic fungi.

8.2.3 Invasive Growth After Penetration

In *M. oryzae*, Pmk1 is important for invasive growth after penetration as well, and the *pmk1* mutant fails to infect rice leaves through wound sites (Fig. 8.1). As a hemibiotrophic pathogen, invasive hyphae of *M. oryzae* spread from the initial colonized cell to neighboring compartments before killing plant cells. Pmk1 plays a critical role in cell-to-cell spread of invasive hyphae in infected rice tissues (Sakulkoo et al. 2018). Its orthologs have a conserved role in invasive growth after penetration in other appressorium-forming plant pathogens (Jiang et al. 2018).

PMK1 orthologs also are important for plant infection in various plant pathogenic fungi that do not form appressoria, including the biotrophic pathogen, *Claviceps purpurea*, vascular wilt pathogens, *F. oxysporum* and *V. dahliae*, canker pathogens, *Cryphonectria parasítica* and *Valsa mali*, corn stalk and ear rot pathogen, *Fusarium verticillioides*, wheat pathogens, *Zymoseptoria tritici* and *Parastagonospora nodorum*, and the banana pathogen, *Mycosphaerella fijiensis* (Hamel et al. 2012; Jiang et al. 2018; Li et al. 2012). In *Z. tritici* and *P. nodorum,* the Pmk1 ortholog is important for infectious growth in mesophyll tissues after invasion through stomata (Solomon et al. 2005; Cousin et al. 2006). This IG MAPK pathway regulates the expression of various cell wall-degrading enzyme (CWDE) genes in *F. oxysporum*, *F. graminearum*, *V. mali*, and *C. parasitica* (Jiang et al. 2018). In *F. graminearum*, the *gpmk1* (*fmk1*) mutant is non-pathogenic and fails to cause disease symptoms on drop-inoculated wheat kernels. Deletion of its upstream MEK and MEKK genes results in the same defects in plant infection and all the mutants disrupted in this MAPK cascade are defective in the production of deoxynivalenol (DON), a potent inhibitor of eukaryotic protein synthesis (Wang et al. 2011). DON is an important virulence factor required for the spread of invasive hyphae from inoculated sites to neighboring spikelets through rachis tissues in *F. graminearum*. In *F. verticillioides*, *FvMK1* regulates the biosynthesis of fumonisins that are also toxic to plant cells (Zhang et al. 2011).

In summary, the Pmk1/Kss1 IG MAPK pathway is conserved for regulating penetration-related morphogenesis and invasive growth in fungal pathogens. It may regulate the expression of various stage-specific genes during disease development, likely in response to plant signals recognized at different infection stages. In *M. oryzae*, genes of diverse functions are regulated by the IG MAPK pathway, including *PTH11* GPCR, *GAS2/GAS2* hypothetical proteins, and *MoHOX7* homeobox transcription factor (Jiang et al. 2018; Zhang et al. 2021). For plant signals, ethylene, wheat floral tissue extract, and secreted class III peroxidases are known to activate the IG MAPK cascade in *C. gloeosporioides, F. graminearum*, and

F. oxysporum, respectively (Jiang et al. 2019; Turra et al. 2015; Kim et al. 2000).

8.2.4 Sexual Reproduction

Sexual reproduction is important to increase genetic variation in plant pathogenic fungi. In *S. cerevisiae* that forms naked asci, mating occurs between two regular yeast cells of compatible mating types. Fus3 and Kss1 have overlapping functions in pheromone response and the *fus3 kss1* double mutant is sterile. In contrast, most ascomycetous crop pathogens form asci and ascospores inside ascocarps such as perithecia and pseudothecia and often involve the development of female-specific mating structures know as ascogonia. In *M. oryzae*, a heterothallic fungus, the *pmk1* mutant is fertile when mated as the male but sterile when mated as the female. The *PMK1* ortholog is also essential for female fertility but dispensable for male fertility in *C. heterostrophus*, *F. verticillioides,* and *F. graminearum* (Jenczmionka et al. 2003; Zhang et al. 2011; Takano et al. 2000).

However, many other genes are known to be essential for female fertility in *M. oryzae* and other fungal pathogens. In fact, deletion of the CWI MPAK *MPS1* also results in the loss of female fertility in *M. oryzae*. In *F. graminearum,* a homothallic fungus that can be forced to outcross, mutants deleted of the other two MAPKs also are female sterile in outcrosses. In *C. heterostrophus*, the *mps1* and *hog1* MAPK deletion mutants are female fertile although pseudothecia are not developed in the *mps1* × *mps1* cross (Igbaria et al. 2008). Nevertheless, all these MAPK mutants retain male fertility. Therefore, it appears that none of the MAPKs is essential for male fertility in ascomycetous fungal pathogens but the Pmk1/Kss1 IG pathway has a conserved role in female fertility. The IG MAPK may also play a role in ascus development and ascospore formation because expressing a dominant active *FST7* MEK allele rescues the defect of a mutant blocked in ascus/ascospore formation but not perithecium development (Jiang and Xu, unpublished). Overall, in comparison with *S. cerevisiae*, the regulation of sexual reproduction is much more complex in filamentous ascomycetes that form sexual fruiting bodies. Unlike in yeast, deletion of the individual pheromone or pheromone receptor genes does not block perithecium formation in *F. graminearum* (Lee et al. 2008).

8.3 The Cell Wall Integrity (CWI) MAPK Pathway

In the budding yeast, the CWI pathway consisting of the Bck1-Mkk1/Mkk22-Slt2 MAPK cascade is activated by Rho1 and Pkc1 to regulate gene expression changes via transcription factors Rlm1 and Swi6 (Jiménez-Gutiérrez et al. 2020). It is required for remodeling of the fungal cell wall during growth, development, and for responding to environmental stimuli. The key components of this CWI MAPK pathway are conserved in ascomycetous phytopathogens and have been shown to play important roles in regulating various infection and developmental processes besides responses to cell wall stress.

8.3.1 Penetration and Infectious Growth

In *M. oryzae*, the *MPS1* MAP kinase gene is important for appressorial penetration and infectious growth. The *mps1* deletion mutant forms melanized appressoria, but its appressoria are defective in penetration and it fails to infect through wounds (Xu et al. 1998). Deletion of the *MoMCK1* MEKK gene results in similar defects with the *mps1* mutant in plant infection (Jeon et al. 2008). Interestingly, Mst50 also interacts with MoMck1 and MoMkk2, and both Mst50 and MoMck1 interact with RACK1 protein Mip1 (Li et al. 2017a). Deletion of *MST50* or *MIP1* reduces the phosphorylation level of Mps1 under stress conditions and results in cell wall integrity defects, indicating the involvement of Mst50 and Mip11 for tethering the CWI MAPK cascade together in *M. oryzae* (Fig. 8.1). In *S. cerevisiae*, cell wall stressors or damages are

recognized by sensor proteins Mid2, Wsc1-Wsc3, Sho1, and Hkr1. Their orthologs are conserved in *M. oryzae* and other fungal pathogens, and some of them may function as the sensors for the CWI MAPK pathway (Carbó and Pérez-Martín 2010; Xu et al. 2019). For downstream targets, *MIG1* and *MoSWI6* encode transcription factors orthologous to yeast Rlm1 and Swi6. Like the *mps1* mutant, the *mig1* mutant still forms appressoria, but is defective in the differentiation and growth of invasive hyphae, likely due to defects in overcoming plant defense responses (Mehrabi et al. 2008). Whereas the *mps1* and *mig1* mutants are non-pathogenic, the *Moswi6* mutant causes small specks but not typical blast lesions on infected rice leaves (Qi et al. 2012). Appressoria formed by the *Moswi6* mutant are defective in appressorium turgor generation. Another likely downstream target of the Mps1 pathway is the MoGti1 transcription factor that is important for penetration peg formation and invasive growth in *M. oryzae* (Li et al. 2016). Although it forms melanized appressoria with normal turgor pressure, the *Mogti1* deletion mutant is non-pathogenic because MoGti1 regulates the expression of many effector genes, including *BAS1*, *BAS2*, and *PWL1* (Li et al. 2016). Interestingly, expression of the bacterial effector *HopAI* with the infection-specific *MIR1* promoter (Li et al. 2007) significantly reduces the phosphorylation of Mps1 and results in defects in invasive growth and lesion development (Zhang et al. 2017).

The CWI MAPK pathway also is important for plant infection in other fungal pathogens with different tissue specificity or infection mechanisms, such as *B. cinerea, C. parasitica, C. purpurea, F. graminearum, Z. tritici, M. fijiensis,* and *S. sclerotiorum* (Sanz et al. 2017; Jiang et al. 2018). However, although it has a conserved role in pathogenesis, this MAPK pathway varies in the actual infection processes under its regulation among different plant pathogenic fungi. Whereas Mps1 is dispensable for appressorium formation in *M. oryzae*, its ortholog is important for appressorium development in *C. lagenarium* and *C. gloeosporioides* (Yong et al. 2013; Kojima et al. 2002). In *S. sclerotiorum*, *SMK3* is important for infection cushion formation and initial infection but it is not essential for lesion expansion (Bashi et al. 2016). In *F. graminearum*, the Mgv1 and Gpmk1 MAPKs are involved in regulating basal resistance to plant defensin MsDef1 (Ramamoorthy et al. 2007). Similarly, both CWI and HOG pathways are important for responding to cell wall stresses caused by the phytoalexins camalexin and brassinin in *Alternaria brassicicola* (Joubert et al. 2011). In *Z. tritici*, the *MgSlt2* mutant is normal in stomata penetration but defective in developing invasive hyphae in wheat leaves (Mehrabi et al. 2006). In *Aspergillus flavus* and *F. verticillioides*, deletion of the *BCK1* MEKK gene results in a significant reduction in virulence. However, the *Afbck1* deletion mutant is increased in aflatoxin production but the *Fvbck1* mutant is increased in fumonisin production (Zhang et al. 2020). In *F. graminearum*, mutants deleted of any component of the CWI MAPK cascade are significantly reduced in DON production (Wang et al. 2011). In *A. alternata*, deletion of *AaSLT2* results in failure to produce host-selective toxins and loss of pathogenicity (Yago et al. 2011). In *B. sorokiniana*, the *Bsslt2* mutant is normal in appressorium formation and root infection but has a reduced virulence on leaves (Leng and Zhong 2015). These observations show that the CWI MAPK pathway has species-specific roles in fungal pathogenesis and secondary metabolism.

8.3.2 Cell Wall Integrity and Hyphal Growth

Like in *S. cerevisiae*, in all the plant pathogenic fungi that have been studied, mutants disrupted in the CWI MAPK pathway by targeted deletion of its key components are hypersensitive to cell wall lytic enzymes and cell wall stressors such as Congo Red (CR) or Calcofluor White (CFW) (Jiang et al. 2018; Hamel et al. 2012). In *M. oryzae*, the *mps1* mutant is normal in growth rate on oatmeal agar but produces only limited aerial hyphae, conidiophores, and conidia. In cultures older than 1 week, autolysis of aerial

hyphae can be observed in the center of *mps1* and *Mobck1* colonies (Xu et al. 1998; Jeon et al. 2008). Autolysis of aerial hyphae in aging cultures also has been observed in mutants deleted of key components of the CWI MAPK pathway in other fungi, including *Sordaria macrospora* and *Coniothyrium minitans* (Zhang et al. 2020).

Whereas *MPS1* orthologs also are dispensable for normal growth rate in *Colletotrichum* species, mutants disrupted in the CWI MAPK pathway have severe growth defects in other fungal pathogens, including *A. flavus, B. cinerea, F. graminearum,* and *F. verticillioides* (So et al. 2017; Hou et al. 2002; Rui and Hahn 2007; Zhang et al. 2020). In *F. graminearum*, *C. parasitica*, and *B. cinerea*, mutants deleted of the CWI MAPK form compact colonies with limited whitish aerial hyphae. In *S. sclerotiorum*, the *smk3* mutant is reduced in growth rate, blocked in sclerotium formation, but increased in aerial hyphal growth (Bashi et al. 2016). In fungal pathogens, reduced growth rate and increased sensitivity to cell wall stresses may directly contribute the defects of CWI mutants in plant infection. Nevertheless, a functional CWI MAPK pathway may be necessary for masking cell wall components to avoid being degraded or recognized by the host to trigger immunity response.

In *C. parasitica*, the *Cpslt2* and *Cpbck1* deletion mutants often produce spontaneous suppressors with faster growth rate although these suppressor strains still grow slower than the wild type and are similar to the original mutants in virulence (So et al. 2017). Therefore, only the defects of the CWI mutants in growth, but not their defects in plant infection, are partially rescued by spontaneous mutations that remain to be identified in these suppressor strains. In *F. graminearum*, the *mgv1* mutant also is unstable and produces spontaneous suppressors with faster growth rate that have nonsense or frameshift mutations in *FgHOG1*, an ortholog of yeast *HOG1* MAPK (Ren et al. 2019). Deletion of *FgHOG1* is confirmed to partially rescue the growth defect of the *mgv1* mutant but not its defect in pathogenesis. One possible explanation is that deletion of *MGV1* results in the overstimulation of the HOG pathway, which is detrimental to hyphal growth (see below) but can be suppressed by nonsense or frameshift mutations in the *FgHOG1* ortholog (Ren et al. 2019). Similar suppressor mutations may occur in the suppressor strains of *Cpslt2* and *Cpbck1* mutants in *C. parasitica* and other fungi.

8.3.3 Hyphal Fusion and Parasexual Reproduction

Hyphal fusion between hyphae of different strains can lead to heterokaryon formation and parasexual reproduction that are unique to fungi and contribute to genetic variations in many asexual fungal pathogens (Clutterbuck 1996; Daskalov et al. 2017). The first fungal MAPK gene found to be essential for hyphal fusion and heterokaryon formation is *MGV1* of *F. graminearum* (Hou et al. 2002). Anastomosis is not observed in the *mgv1* mutant and the *mgv1 nit1* mutant fails to form heterokaryons with a *nitM* mutant (Hou et al. 2002). In the model filamentous fungus *Neurospora crassa*, Mak-1 (Slt2) and Mak-2 (Kss1) MAPKs interact with Cot-1 to regulate hyphal fusion. Further studies showed that the Mak-1 and Mak-2 MAPK pathways crosstalk to regulate hyphal fusion together with the striatin-interacting protein phosphatase and kinase (STRIPAK) complex (Dettmann et al. 2014; Fischer and Glass 2019). In *N. crassa*, the So protein functions as a scaffold for the upstream components of the CWI MAPK pathway. Interestingly, So is one of the proteins phosphorylated by Mak-2. However, unlike the *N. crassa so* and *mak-2* mutants, hyphal fusion still occurs in the *Fgso* (*Fgsoft*) and *Gpmk1* deletion mutants in *F. graminearum* (Zheng et al. 2013). Therefore, the functions of So and other components of STRIPAK in hyphal fusion may be not conserved in all the phytopathogenic ascomycetous species. Furthermore, the roles of MAPKs in steps of parasexual reproduction after hyphal fusion and heterokaryon formation, such as fate and stability of heterokaryons, diploidization, and somatic recombination, remain to be characterized.

8.4 The High-Osmolarity Glycerol (HOG) Pathway

Whereas the other two fungal MAPKs have the TEY dual phosphorylation site, Hog1 and its orthologs have the TGY motif, which is similar to p38 stress activated MAP kinases (SAPKs) in animals. In yeast, the Ssk2/Ssk22-Pbs2-Hog1 MAPK cascade mainly regulates responses to hyperosmotic stress. In plant pathogenic fungi, besides its conserved role in osmoregulation, the HOG pathway in general is important for regulating responses to other environmental stresses, including antifungal chemicals, reactive oxygen species (ROS), and plant defense compounds (Dunayevich et al. 2018; Lee et al. 2017; Yang et al. 2020a, b).

8.4.1 Species-Specific Roles in Pathogenesis

In *M. oryzae*, the *osm1* deletion mutant is normal in appressorium formation and plant infection (Dixon et al. 1999). Although deletion of *OSM1* affects glycerol accumulation in vegetative hyphae under hyperosmotic conditions, the *osm1* mutant has no defects in appressorium turgor generation (Fig. 8.2), indicating that glycerol accumulation in appressoria is not regulated by *OSM1*. Its upstream sensor histidine kinases MoSln1 and MoHik1, phosphotransfer protein MoYpd1p, and MoSsk1 MEKK also are important for osmoregulation during vegetative growth but dispensable for pathogenesis (Jacob et al. 2016).

Like in *M. oryzae*, the Hog1 ortholog is dispensable for plant infection in some fungal pathogens, such as *Cochliobolus orbiculare* and *Bipolaris oryzae* (Jiang et al. 2018; Moriwaki et al. 2006). However, the HOG MAPK pathway is important for plant infection in many other plant pathogenic fungi. For example, Sak1 is important for appressorium development and penetration of epidermal cells in *B. cinerea* (Liu et al. 2008). Silencing of Hog1 and Pbs2 reduces infectious growth and virulence in *F. oxysporum* (Pareek and Rajam 2017). In *F. graminearum* (Fig. 8.2), the *Fghog1* mutant is defective in DON production and fails to spread through rachis tissues in wheat heads after the initial infection (Zheng et al. 2012). In *Ustilaginoidea virens*, UvHog1 regulates the production of secondary metabolites that are toxic to plant cells (Zheng et al. 2016). Therefore, the HOG pathway likely has species-specific roles during plant infection in fungal pathogens.

The best characterized downstream target of the HOG MAPK in plant pathogenic fungi is the Atf1 bZIP transcription factor. In *F. graminearum*, Atf1 interacts with FgOs2 (FgHog1) in the nucleus under osmotic stress and constitutive expression of *FgATF1* suppresses the defects of *Fgos2* mutant in osmoregulation and pathogenesis (Van Nguyen et al. 2013). Atf1 orthologs also are important for virulence in *M. oryzae, F. verticillioides, V. dahliae*, and other fungal pathogens (Jiang et al. 2018; Szabó et al. 2020; Tang et al. 2020). However, the *ATF1* ortholog mainly regulates responses to oxidative stress instead of osmoregulation in these plant pathogenic fungi. For orthologs of yeast Skn7, a response regulator of the HOG pathway, they are important for plant infection in a number of fungi, but *MoSKN7* is dispensable for virulence in *M. oryzae* (Motoyama et al. 2008), further indicating the differences among various plant pathogenic fungi in the roles of HOG pathway during plant infection.

8.4.2 Osmoregulation and Survival

Although the importance of the HOG pathway for plant infection varies, its function in regulating adaptive responses to hyperosmotic stress is well-conserved in fungal pathogens. Deletion of the *HOG1* ortholog results in increased sensitivity to hyperosmotic stress in all the plant pathogenic fungi studied, including *Z. tritici* and *F. graminearum*. Like in yeast, Hog1 orthologs are rapidly phosphorylated in response to hyperosmotic stress in fungal pathogens such as *C. heterostrophus* (Yoshimi et al. 2005). In *M. oryzae*, the *osm1* deletion mutant is

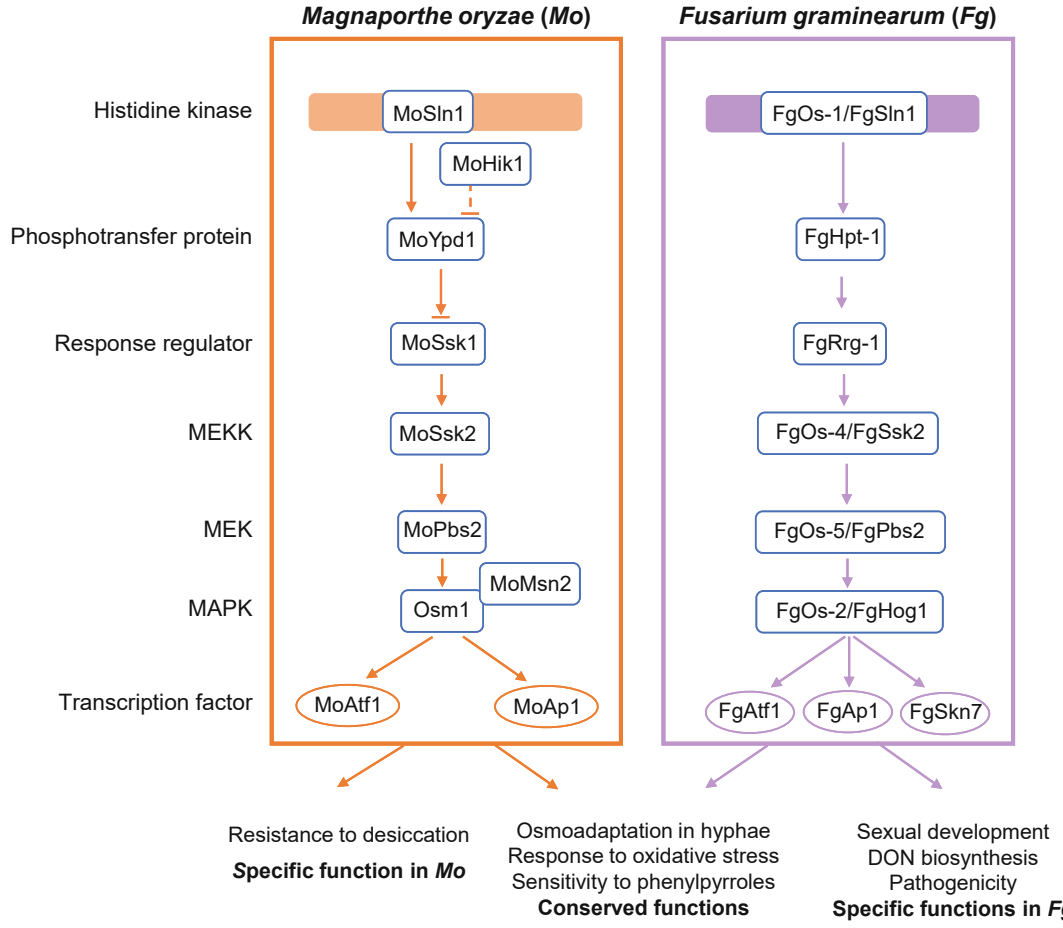

Fig. 8.2 The HOG MAPK pathway in *Magnaporthe oryzae* and *Fusarium graminearum*. All of the key components of HOG pathway, including the three-tiered protein kinase cascade and upstream phosphorelay and sensor proteins, are conserved in *M. oryzae* (*Mo*) and *F. Graminearum* (*Fg*). Besides its conserved function in osmoadaptation, the Hog1 MAPK pathway also has species-specific roles in these two important plant pathogens. Although this pathway is dispensable for pathogenesis in *M. oryzae*, it is important for plant infection in *F. graminearum*. In *F. graminearum*, the FgHog1 MAPK is also important for sexual development and secondary metabolism

hypersensitive to osmotic stress and desiccation (Dixon et al. 1999). Although it is normal in plant infection under laboratory conditions, the *osm1* mutant will face problems to survive in desiccated plant tissues in the field. In fact, the HOG pathway may be important for survival in nature in many other plant pathogens because of its essential role in adaptive responses to hyperosmotic stress associated with desiccation.

Interestingly, phenylpyrrole fungicides, fludioxonil and fenpiclonil, overstimulate the HOG pathway and result in the accumulation of intracellular glycerol and cell burst. Mutants deleted for key components of the HOG MAPK pathway are resistant to these fungicides in *N. crassa*, *C. lagenarium*, and other fungi (Brandhorst et al. 2019; Jiang et al. 2018). Remarkably, fludioxonil and fenpiclonil have been applied to control foliar pathogens for over 30 years, but field isolates with complete resistance against these phenylpyrrole fungicides have not emerged and spread widely in crop fields

(Kilani and Fillinger 2016), which may be related to the defects of HOG mutants in stress response and survival in nature. Resistance against dicarboximide fungicides also has been observed in HOG pathway mutants in *N. crassa* and fungal pathogens (Zhang et al. 2002; Fujimura et al. 2003). For example, the *hog1* mutant has increased tolerance to vinclozolin in *A. alternata* (Yu et al. 2016). However, the direct targets of these fungicides are not key components of the HOG pathway and remain to be identified in plant pathogenic fungi.

8.4.3 Oxidative Stress

In fungal pathogens, the HOG pathway plays a critical role in regulating responses to oxidative stress caused by oxidants produced by plant cells or present in the environment. Mutants deleted of the Hog1 MAPK or other key components of this pathway have increased sensitivity to oxidative stress, which may be related to defects in plant infection observed in some fungal HOG mutants as described above. In pathogens where the HOG MAPK is dispensable for virulence, they may use effector proteins to effectively suppress the oxidative burst in infected plant tissues. In general, the Atf1 ortholog is one major transcription factor functioning downstream from the HOG MAPK to regulate the expression of genes important for oxidative responses in fungal pathogens (Guo et al. 2011b; Tang et al. 2020). In contrast, the role of the Skn7 ortholog in oxidative stress response differs significantly among different pathogens, such as being dispensable in *M. oryzae* but critical in *A. alternata* (Motoyama et al. 2008; Chen et al. 2012). AP1 is another transcription factor known to be involved in regulating oxidative stress-related genes in fungi but its relationship with the HOG MAPK pathway is not clear. In yeast, *YAP1* is not known to be related to the HOG pathway. In *M. oryzae*, whereas the *osm1* mutant is normal in pathogenesis, the *MoAP1* deletion mutant is defective in plant infection (Guo et al. 2011b).

In some fungal pathogens, the Hog1 MAPK pathway also has been implicated in regulating responses to other environmental stresses, such as UV irradiation, hypoxia-inducing $NaNO_2$-treatment, and heavy metals (for reviews, see Zhang et al. 2021). However, although Hog1 MAPK plays a major role, the other two MAPK pathways often are involved in stress responses by directly regulating downstream targets or crosstalk with the HOG pathway. For example, mutants deleted of key components of the CWI MAPK pathway have increased sensitivities to oxidative stress in *B. cinerea* (Yin et al. 2018) and *F. verticillioides* (Zhang et al. 2015). In *F. graminearum*, the *Gpmk1 mgv1 Fghog1* mutant, the only triple MAPK mutant that has been reported in plant pathogenic fungi, is viable but hypersensitive to various environmental stresses (Ren et al. 2022).

8.5 Concluding Remarks

The well-conserved MAPK pathways regulate various plant infection and developmental processes in ascomycetous plant pathogens. Most of them have three linear MAPK cascades without redundancy at the MAPK, MEK, or MEKK level. In different plant pathogenic ascomycetes, individual MAPK pathways have both conserved and species-specific functions, such as the regulation of invasive growth and DON biosynthesis by the IG MAPK pathway in *F. graminearum* (Hamel et al. 2012; Jiang et al. 2018). MAPK signaling also has been characterized in basidiomycetous plant pathogens but mainly limited to *U. maydis*, in which two Kss1-like MAPKs, Kpp2 and Kpp6, have overlapping functions in plant infection (Brachmann et al. 2003; Di Stasio et al. 2009). For the diverse roles of MAPKs that have been observed, fungal pathogens must be able to recognize various plant and environmental signals with upstream sensors or receptors. Among the predicted sensor or receptor genes, only GPCRs are significantly expanded in fungal pathogens in comparison with the budding yeast. For example, *M. oryzae* and *F. graminearum* have over 40 and 100 putative GPCRs, respectively, which is more than 10 times the three GPCRs found in yeast. Some of these GPCRs may be responsible for sensing host and

environmental signals to regulate plant infection processes, such as *PTH11* in *M. oryzae* and *GIV1* in *F. graminearum* (Jiang et al. 2019; Kulkarni et al. 2005).

Unlike their roles in pathogenesis and stress response, the functions of fungal MAPK pathways in defense against mycoviruses, bacteria, and other fungi have not been well characterized although limited studies indicate their involvement in fungal–fungal/bacterial/viral interactions. Ascomycetous fungal pathogens lack receptor kinases or receptor-like kinases but have putative nucleotide-binding and leucine-rich repeat domain-containing (NLR) immune receptors (Uehling et al. 2017). Like in plants and animals, these NLRs may recognize certain microbe-associated molecular patterns (MAMPs) and function upstream from MAPK cascades in fungal pathogens to regulate the expression of genes related to defense or antagonistic interactions with bacteria or other fungi. Plant pathogenic fungi are known to produce various anti-microbial/fungal compounds and secrete various hydrolytic enzymes such as chitinases and glucanases. Therefore, it is not only interesting to characterize the possible functional relationships between MAMP recognition by NLRs and MAPK signaling to regulate anti-microbial/fungal activities but also helpful to improve biocontrol agents.

In yeast, MAPKs are hubs of protein–protein interaction networks and they influence each other as part of the interconnected signaling networks to ensure appropriate cellular responses to external cues (Saito 2010; Van Drogen et al. 2020). Plant pathogenic fungi have much more complex developmental and infection processes and they likely use these three MAPK pathways to coordinately regulate responses to host and environmental signals. To date, most of the MAPK studies in plant pathogenic fungi deal with individual MAPKs or MAPK pathways. There are only a few reports on mutants disrupted in two MAPK pathways, such as the *mps1 hog1* mutant of C. *heterostrophus* and *mgv1 Fghog1* mutant of *F. graminearum* (Ren et al. 2019; Igbaria et al. 2008). To better understand the crosstalk among these MAPKs, systematic transcript profiling with mutants disrupted in multiple MAPK pathways and different components of MAPK pathways is needed to establish the regulatory networks involving these MAPKs in fungal pathogens. Similarly, systematic proteomics analysis is needed to establish protein–protein interaction networks and determine the positions and links of individual MAPKs in *M. oryzae* or other plant pathogenic fungi.

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Role of pH in the Control of Fungal MAPK Signalling and Pathogenicity

9

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Abstract

Like all organisms, fungi have evolved sophisticated mechanisms for ambient pH sensing and adaptation, as well as an exquisitely tuned system for regulating cytosolic pH (pH_c). Moreover, fungal plant and animal pathogens can sense and modify the pH of the surrounding host tissue to promote infection. Here we discuss the current understanding of the cellular mechanisms mediating fungal responses and modulation of ambient pH as well as homeostasis of pH_c. Furthermore, we review recent findings revealing the role of pH in the regulation of mitogen-activated protein kinases (MAPKs), which act as crucial modulators of fungal growth, development and pathogenicity. The emerging link between pH and MAPK signalling may have significant implications for the development of new antifungal control strategies.

Keywords

Fungus · MAPK · Pathogen · pH · Signalling

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9.1 Ambient pH Sensing and Adaptation in Fungi

9.1.1 Sensing and Adaptation to Alkaline Ambient pH: the Pal/Rim Pathway

For survival, growth and reproduction, fungi must be able to sense and respond to changes in the ambient pH (Obara and Kamura 2021; Selvig and Alspaugh 2011). The best studied pH response mechanism in fungi is the Pal/Rim alkalinization response pathway (Peñalva et al. 2008). Studies conducted primarily in the model organisms *Saccharomyces cerevisiae* and *Aspergillus nidulans* have cemented our understanding on this conserved fungal pH response pathway (Peñalva et al. 2014). Alkalinization is sensed through the concerted action of two cell surface proteins, PalH/Rim21and PalI/Rim9, which carry seven and four transmembrane domains, respectively (Calcagno-Pizarelli et al. 2007; Lucena-Agell et al. 2016). The mechanisms underlying alkaline pH sensing by PalH/Rim21 remain poorly understood. Recent studies in *S. cerevisiae* demonstrated that the C-terminal cytosolic domain of PalH/Rim21 detects altered lipid asymmetry in the plasma membrane resulting from alkaline-induced depolarization (Obara and Kamura 2021). This leads to dissociation of PalH/Rim21 from the plasma membrane and to ubiquitination and phosphorylation of its associated α-arrestin PalF/Rim8 (Herranz et al.

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2005; Hervás-Aguilar et al. 2010; Obara and Kamura 2021). Endocytosis of the PalH–PalF complex triggers recruitment of the components Vps20 and Snf7 of the endosomal sorting complexes required for transport (ESCRT), and subsequently of the interacting proteins PalA/Rim20 and the papain-like protease PalB/Rim13 to the endomembranes (Henne et al. 2012). PalA/Rim20 binds to the C-terminal inhibitory domain of the inactive full-length version of the zinc finger transcription factor PacC/Rim101 resulting in its proteolytic cleavage and activation by PalB/Rim13. Processed PacC/Rim101 protein can function both as an activator of alkaline-expressed and as a repressor of acidic-expressed genes, thereby orchestrating the cellular response to alkaline ambient pH (Peñalva et al. 2014).

Besides its role in pH adaptation, PacC/Rim101 is also involved in other key processes of fungi such as sporulation and host infection (Fernandes et al. 2017; Wasserstrom and Wendland 2021). Loss of PacC/Rim101 leads to reduced virulence in the fungal pathogens of humans *Candida albicans* and *Aspergillus fumigatus* (Bertuzzi et al. 2014; Davis 2009; Landraud et al. 2013; Rascle et al. 2018) or the insect pathogen *Metarhizium robertsii* (Huang et al. 2015). PacC also contributes to virulence in certain plant pathogens such as *Magnaporthe oryzae*, *Botrytis cinerea*, *Penicillium expansum* or *Trichothecium roseum* (Bertuzzi et al. 2014; Chen et al. 2018; Davis 2009; Landraud et al. 2013; Rascle et al. 2018; Wang et al. 2022) but is dispensable for virulence in others such as *Fusarium graminearum* (Caracuel et al. 2003; Merhej et al. 2011) or the biotroph *Ustilago maydis* (Cervantes-Chávez et al. 2010). Interestingly, in the cross-kingdom pathogen *Fusarium oxysporum* f. sp. *lycopersici*, PacC was dispensable for infection of tomato plants but required for full virulence on immunodepressed mice (Caracuel et al. 2003; Merhej et al. 2011). Moreover, an expansion in the number of *pacC* genes in accessory genome regions was recently reported for *F. oxysporum* strains isolated from infected humans suggesting a possible role of PacC in fungal adaptation to the environment encountered in the mammalian host (Zhang et al. 2020).

9.1.2 Sensing and Adaptation to Acid Ambient pH

Acid pH sensing and adaptation has been mainly studied in S. cerevisiae. Genome-wide expression analysis identified a number of genes showing an opposite response to extracellular acidification or alkalinization, indicative of a specific function in pH adaptation (Causton et al. 2001). Among these, PDR12 encoding an ABC transporter involved in weak acid resistance and TRK2 encoding a potassium transporter responsible for K^+ currents at low pH, were induced under acidic and repressed under alkaline conditions (Causton et al. 2001). By contrast, Pho89 encoding a sodium phosphatase symporter, which mediates sodium-dependent phosphate uptake at alkaline pH (Serra-Cardona et al. 2014), was upregulated at high pH and downregulated at low pH (Causton et al. 2001). This pH-mediated transcriptional control required the calcineurin-dependent transcription factor Crz1p and several repressors coordinately regulated by the protein kinase Snf1 and the transcription factor Rim101 (Serra-Cardona et al. 2014).

In yeast, several genomic screens for sensitivity to different weak acids have been conducted (Mira et al. 2010). These studies were mainly motivated by the commercial importance of these acids as fungal growth inhibitors (Mollapour et al. 2004). Fungi themselves can secrete weak organic acids such as gluconic, oxalic and citric acid (Kawahata et al. 2006; Park et al. 2017), which are known virulence factors in necrotrophic plant pathogens (Jiao et al. 2022). Weak acids are usually uncharged at low extracellular pH, but after crossing the plasma membrane will dissociate at the high pH of the cytosol leading to proton accumulation and cytosolic acidification (Stratford et al. 2013). A decrease in cytosolic pH leads to activation of the major plasma membrane H^+-ATPase Pma1 (see also Sect. 9.2.1) in a futile cycle, where protonated weak acids can re-enter the cell immediately after proton export, leading to depletion of the cellular energy stores and proton accumulation to levels toxic for the cell (Ullah et al. 2012). Genome-wide studies addressing the mechanisms

of adaptation to different weak acids revealed both general and specific responses, the latter being correlated to the structure and hydrophobicity of the anion (Mira et al. 2010). Thus, the transcriptional regulators Msn2 and Msn4 coordinate the general environmental stress response to weak acids (Gasch et al. 2000; Schüller et al. 2004) including upregulation of chaperones and proteins involved in energy metabolism and ergosterol biosynthesis. On the other hand, the transcription factor War1 responds specifically to sorbic acid, triggering upregulation of Pdr12, which encodes a plasma membrane ABC transporter that mediates sorbate efflux (Schüller et al. 2004).

Screens for yeast mutants hypersensitive to weak acids have been useful for identifying proteins involved in the general and specific response to weak acids. For example, multiple studies indicate that the plasma membrane H^+-ATPase Pma1, the vacuolar H^+-ATPase (V-ATPase) and the ergosterol biosynthesis pathway play central roles in weak acid stress resistance (Mira et al. 2010; Ullah et al. 2012). However, mutants exhibiting hypersensitivity to closely related weak acids such as acetic and propionic acid may differ significantly in the genes affected (Mira et al. 2010; Ullah et al. 2012). Overall, these findings suggest that the fungal cell detects and responds to both common features of weak acid stress as well as to others specifically associated with individual organic acids.

9.1.3 Modulation of Ambient pH and Its Role in Plant Pathogenicity

Besides developing efficient mechanisms for adapting to ambient pH changes, fungal pathogens can efficiently modulate the pH of the host tissue by secreting acids or alkali. While host acidification has been mostly described for necrotrophic fungal pathogens, alkalinization has been reported in hemibiotrophs, particularly during early biotrophic stages of infection (Alkan et al. 2013).

Acidification by secretion of organic acids is used by several plant pathogens as a strategy to damage the host tissue (Jiao et al. 2022). Necrotrophs such as *Sclerotinia sclerotiorum* or *Botrytis* spp. decrease the host pH by secreting significant amounts of oxalic acid (Manteau et al. 2003; Rollins and Dickman 2001), whereas other fungi release gluconic acid, either alone as in *Phomopsis mangiferae* (Davidzon et al. 2010), or in combination with citric acid such as in *Penicillium* and *Aspergillus* spp. (Prusky et al. 2004; Ruijter et al. 1999).

Alkalinization of the host environment as a means to promote fungal infection is mainly achieved through the release of ammonia (Miyara et al. 2012). For instance, in *Colletotrichum gloeosporioides* the release of ammonia and the consequent alkaninization leads to activation of infection-related mechanisms such as appressorium formation or the secretion of cell wall-degrading enzymes (Shnaiderman et al. 2013). Similarly, alkalinization induces asexual sporulation and secretion of lytic enzymes in *M. oryzae* (Landraud et al. 2013). In the human pathogens *Cryptococcus neoformans* and *C. albicans,* alkalinization triggers melanin formation and capsule production (Vecchiarelli et al. 2013) or filamentation, adhesion and invasion, respectively (Nobile et al. 2008; Sun et al. 2015). Although the precise mechanism leading to the release and accumulation of extracellular ammonia during host infection remains to be elucidated, this process appears to require the regulated uptake of amino acids via amino acid permeases or their mobilization from vacuolar stores, followed by catabolism through different routes involving steps of deamination (Bi et al. 2016; Miyara et al. 2012; Shnaiderman et al. 2013; Vylkova et al. 2011). The subsequent release of ammonia may primarily serve to protect the cell from the toxic effects of ammonium accumulation and has been suggested to occur either through passive diffusion or by means of transporters such as the members of the Ato protein family (Danhof and Lorenz 2015; Vylkova et al. 2011).

In many fungi, the ability to acidify or alkalinize the environment is largely dictated by the availability and nature of the carbon source. It

has been suggested that excess carbon is metabolized by glucose oxidase to gluconic acid whereas non-preferred carbon sources, including certain amino acids such as glutamate, are deaminated resulting in the accumulation and release of ammonia (Bi et al. 2016; Vylkova et al. 2011). These findings are of biological relevance, because fungal pathogens are likely to encounter different types and levels of carbon sources depending on the given host niche and stage of infection (e.g. biotrophic *versus* necrotrophic). Thus, carbon availability during host infection may act as a master regulator to determine whether a pathogen follows either an acidic necrotrophic or an alkaline biotrophic infection strategy.

An alternative strategy of host tissue alkalinization during early stages of infection was reported in the hemibiotrophic phytopathogen *F. oxysporum* and involves the secretion of a functional homologue of plant rapid alkalinizing factor (RALF) peptides (Masachis et al. 2016). RALFs are extracellular peptides present in all land plants, which were first identified for their ability to trigger a rapid increase in ambient pH when added to plant cell cultures (Pearce et al. 2001). Interestingly, the genome of *F. oxysporum* encodes a functional homologue of plant RALFs (Fo-RALF), which carries a fungal secretion signal and triggers rapid extracellular alkalinization in plant cells. Isogenic *F. oxysporum* mutants lacking Fo-RALF failed to induce root alkalinization and displayed reduced virulence on tomato plants (Masachis et al. 2016). A likely host target of Fo-RALF is the receptor-like kinase FERONIA, which mediates the plant responses to endogenous RALF peptides, including the inactivation of the plasma membrane H^+-ATPase AHA2 that results in rapid extracellular alkalinization (Haruta et al. 2014). This process regulates a series of complex developmental and physiological responses including modulation of the plant immune response (Blackburn et al. 2020; Stegmann et al. 2017). Intriguingly, an *Arabidopsis* mutant defective in FERONIA displayed enhanced resistance against *F. oxysporum* (Masachis et al. 2016). By contrast, in the flower-infecting wheat pathogen *F. graminearum* RALF was found to be dispensable for virulence, and transient silencing of two *FERONIA* homologues in wheat prior to *F. graminearum* inoculation did not affect the outcome of the interaction (Wood et al. 2020). Overall, these studies illustrate the diversity of mechanisms employed by fungal pathogens to manipulate the host pH and increase the efficiency of infection.

9.2 Role of Cytosolic pH in Fungal Signalling and Pathogenicity

In contrast to ambient pH, cytosolic pH (pH_c) tends to be constant and is generally tightly regulated (Kane 2016; Zhou et al. 2021). Tight regulation of pHcyt is of utmost importance for processes in eukaryotic cells. For instance, changes in pH_c can interfere with protein folding and enzyme activity, vesicle trafficking or even impact the function and integrity of organelles. In fungi, pH_c homeostasis regulates fundamental processes such as growth, development, ageing or virulence (Deprez et al. 2018; Dolz-Edo et al. 2019). That said, rapid fluctuations in pH_c have been observed in response to different stimuli (Orij et al. 2011). Among these, changes in ambient pH have been shown to affect pH_c dynamics in filamentous fungi (Bagar et al. 2009).

9.2.1 Cytosolic pH Homeostasis

The best studied determinant of pH_c homeostasis in fungi is the H^+-ATPase Pma1, which is essential for survival and represents the most abundant plasma membrane protein (Kane 2016). Pma1 mediates ATP-dependent proton extrusion, thereby establishing an electrochemical gradient across the plasma membrane that drives the active uptake of nutrients and inorganic ions by secondary transporters. At the stuctural level, Pma1 consists of a single large catalytic subunit of around 100 kDa, which is embedded in the membrane lipid bilayer by 10 hydrophobic α-helices with both termini exposed to the cytosol. Due to the high abundance of Pma1 in the plasma

membrane and its relative long half-life, regulation occurs mainly at the post-translational level (Kane 2016). Under glucose-limiting conditions, the C-terminal regulatory domain inhibits hydrolysis of ATP, whereas under glucose availability phosphorylation of this domain releases its inhibitory effect, leading to strongly increased affinity for ATP (Portillo et al. 1991). Mass spectrometry studies of *S. cerevisiae* Pma1 identified key phosphorylation sites in the C-terminal domain mediating Pma1 activation in response to glucose, including S899, S911 and Thr912 (Lecchi et al. 2007). Moreover, truncation of the Pma1 C-terminal domain in *C. albicans* led to drastic cytosolic acidification which strongly affected fungal growth and filamentation (Rane et al. 2019).

Besides Pma1, the vacuolar H^+-ATPase (V-ATPase) also plays a key role in fungal pH_c regulation by maintaining the vacuole more acidic than the surrounding cytosol (Martínez-Muñoz and Kane 2017). In contrast to the single-subunit Pma1, fungal V-ATPase consists of fourteen subunits arranged in two subcomplexes. The peripheral subcomplex V1 harbours the site for ATP hydrolysis, while the V0 subcomplex is embedded in the organellar membrane and contains the proton pore. Similar to Pma1, V-ATPase activity is tightly regulated by glucose, with glucose-depletion leading to reversible disassembly of the peripheral V1 subunits from the membrane-bound V0 sector (Kane 2016).

Besides glucose availability, ambient pH acts as a major regulator of Pma1 and V-ATPase activity. Pma1 activity was reported to be increased by external acidification (Carmelo et al. 1997; Orij et al. 2012), while V-ATPase assembly was higher at alkaline ambient pH (Diakov and Kane 2010). Furthermore, the activity of both Pma1 and V-ATPase is affected by fluctuations in pH_c. For instance, at low pHcyt Pma1 activity was enhanced (Holyoak et al. 1996; Orij et al. 2011; Ullah et al. 2012) and the Km of the enzyme for ATP decreased without a corresponding increase in Vmax (Eraso and Gancedo 1987). On the contrary, V-ATPase assembly was negatively regulated by a decrease in pH_c (Dechant et al. 2010). Meanwhile, an alkaline pHcyt led to higher levels of the V1 subunit and an enhanced V-ATPase activity suggesting that regulation in response to pH occurs in part via V1-V0 assembly (Diakov and Kane 2010).

The fact that Pma1 and V-ATPase activity is differentially regulated by ambient pH may have implications for the balanced activity of these two proton pumps. In fact, cells of yeast *vma*Δ mutants showing reduced V-ATPase activity displayed increased internalization of Pma1 (Smardon and Kane 2014). Similarly, acute or chronic loss of V-ATPase activity triggered internalization of approximately half of the cell surface Pma1, suggesting that internalization of Pma1 may compensate for the loss of V-ATPase activity (Velivela and Kane 2018). Pma1 endocytosis required its ubiquitination by Rsp5 and by the α-arrestin Rim8 via a mechanism independent of the Pal/Rim pathway (Velivela and Kane 2018). Taken together, these findings reveal a complex interplay between the two major ATPases Pma1 and V-ATPase in the maintenance of fungal pHcyt homeostasis.

9.2.2 Role of pH_c in Signalling and Cell Growth

Studies in yeast have established a functional link between pH_c and cell growth. As a general rule, acidification of the cytosol correlates with slow growth whereas alkaline pHcyt is associated with fast growth. Young et al. (2010) described a mechanism of membrane biosynthesis by pH_c, whereby cytosolic acidification triggers the release of Opi1, a transcriptional repressor, from its binding to phosphatidic acid in the ER membrane. This results in translocation of Opi1 into the nucleus where it inhibits transcription of multiple phospholipid biosynthetic genes, thereby limiting membrane biosynthesis and cell growth (Young et al. 2010). These findings uncovered a new role of pH_c in coordinating synthesis of membrane precursors with nutrient availability and cell growth rate.

Dechant et al. (2010) reported a mechanism by which growth rate and cell size are regulated by pH_c in response to glucose availability (Dechant et al. 2010). Glucose limitation induces a decrease in pH_c and depletion of the H^+-ATPase Pma1 from the daughter cell, triggering cell cycle arrest in early G1. Importantly this G1 arrest could be reversed by increasing ambient pH, suggesting that it was caused by acidification of pH_c. Furthermore, a decrease of pH_c caused inactivation of the Ras signalling pathway, consistent with pH_c acting as a signal for nutrient status (Dechant et al. 2010; Diakov and Kane 2010). In addition, the V-ATPase plays a central role in pH_c signalling in response to nutrient status, as its correct assembly was required for transmission of the pH_c signal to its downstream effectors such as protein kinase A (PKA), Ras1 or Target of Rapamycin Complex 1 (TORC1) (Dechant et al. 2010, 2014). This mechanism involves the interaction of the small GTPase Arf1 with the Stv1-containing (Golgi/endosome) form of V-ATPase, while TORC1 activation required the interaction of the Vph1-containing V-ATPase with the small GTPase Gtr1p at the vacuole (Dechant et al. 2010, 2014). TORC1 activation and phosphorylation of the Sch9 kinase (Novarina et al. 2021) then trigger the disassembly of V-ATPase, representing a negative feedback loop (Wilms et al. 2017). This interplay between Sch9 and V-ATPase allows for proper control of pH_c and vacuolar pH (pH_v) and integrates them with nutrient sensing (Deprez et al. 2018; Wilms et al. 2017). More studies are required to fully understand the molecular mechanisms of pH_c in cell growth control, but work done so far points towards V-ATPase activity acting as the key signalling intermediate between nutrient status, pH_c and critical nutrient sensors.

9.2.3 Cytosolic pH As a Regulator of Fungal Pathogenicity

While the link between ambient pH and fungal pathogenicity has been recognized for a long time, recent findings suggest that at least part of this role may be mediated by pH_c control of mitogen-activated protein kinase (MAPK) activity. MAPK cascades are broadly conserved eukaryotic signalling pathways that channel a huge number of external cues into appropriate cellular responses, thereby regulating a wide variety of processes such as cell growth, differentiation, survival and pathogenicity (Chen and Thorner 2007). In most fungal pathogens, three MAPK signalling pathways have been reported (Turrà et al. 2014). The orthologues of the Fus3/Kss1 MAPKs from *S. cerevisiae* are crucial for invasive growth and appressorium formation in plant pathogens including the rice blast fungus *M. oryzae* (Lev et al. 1999; Ruiz-Roldán et al. 2001; Takano et al. 2000; Xu and Hamer 1996; Zhao et al. 2005). In the root-infecting vascular wilt fungus *F. oxysporum*, the Fus3/Kss1 homologue Fmk1 controls a number of infection-related functions such as invasive growth, vegetative hyphal fusion, root adhesion, chemotropic sensing of nutrients and virulence on tomato plants (Di Pietro et al. 2001; Prados Rosales and Di Pietro 2008; Turrà et al. 2015). On the other hand, the orthologues of the *S. cerevisiae* cell wall integrity (CWI) MAPK Mpk1/Slt2 have been widely shown to be involved in fungal infection (Turrà et al. 2014). *F. oxysporum mpk1*Δ mutants have severe defects in host sensing, penetration and colonization, leading to a decrease in fungal pathogenicity (Segorbe et al. 2017; Turrà et al. 2014). Finally, the orthologues of the *S. cerevisiae* high osmolarity glycerol MAPK Hog1, which mediates response to hyperosmotic stress, were also shown to contribute to virulence in a number of fungal pathogens although their exact role during infection remains to be determined (Day et al. 2017; Day et al. 2018; Igbaria et al. 2008; Turrà et al. 2014).

Although both pH and MAPKs have long been known as master regulators of fungal pathogenicity, a possible link between these two mechanisms has not been considered until recently (Fernandes et al. 2017). In *F. oxysporum,* extracellular alkalinization was found to trigger rapid phosphorylation of the invasive growth MAPK Fmk1 promoting penetration and virulence on tomato plants, while low

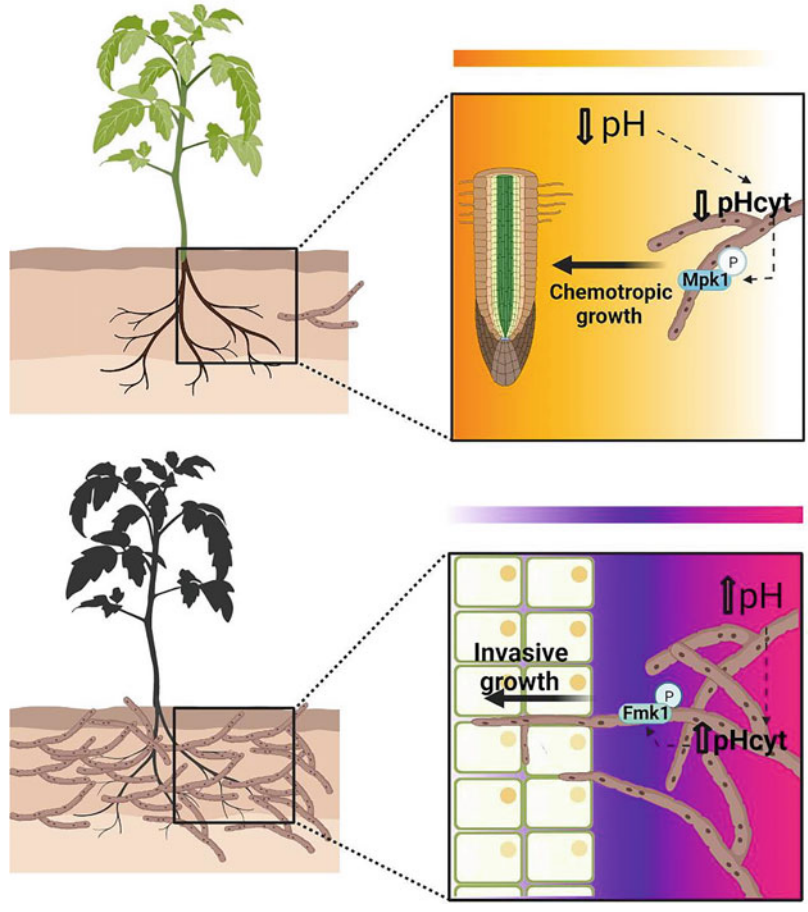

Fig. 9.1 pH-mediated MAPK regulation acts as a master switch in the control of fungal pathogenicity on plants. (**a**) Signal molecules released by plant roots trigger chemotropic attraction of root-colonizing fungi. Chemotropic growth of *F. oxysporum* towards tomato root exudates was previously shown to require the MAPK Mpk1 (Turrà et al. 2015). (**b**) Fungal phytopathogens such as *F. oxysporum* induce alkalinization of the surrounding host tissue to increase virulence (Masachis et al. 2016; Fernandes et al. 2017). Recent evidence suggests that the effect of ambient pH on virulence-related functions is largely mediated by changes in pH_c (Fernandes et al. unpublished data)

pH induced a decrease in Fmk1 phosphorylation, invasive growth and virulence (Masachis et al. 2016). Interestingly the other two MAPKs, Mpk1 and Hog1, are also regulated by ambient pH, although in a manner opposite to Fmk1 with an increase in phosphorylation upon extracellular acidification and a decrease upon alkalinization. Moreover, a recent study revealed that control of fungal MAPK signalling by ambient pH is mediated by rapid and transitory changes in pH_c (Fernandes et al. unpublished data). This finding suggests that pH_c acts as a master switch of MAPK signalling and pathogenicity (Fig. 9.1).

9.3 Conclusions

Many fungal plant and animal pathogens can sense and modify the pH of the surrounding host tissue to increase virulence, using a variety of mechanisms. Changes in ambient pH have been shown to affect pH_c dynamics, suggesting that pH_c acts a key player in fungal virulence. Moreover, pH_c controls fundamental processes such as cell growth, development or ageing. The H^+-ATPase Pma1 and V-ATPase are major regulators of pH_c homeostasis, which are required for transmission of the pH signal to its downstream effector pathways, including MAPKs, TORC1 and PKA. The emerging link between

pH_c and different signalling pathways has significant implications for the development of new approaches for the control of fungal disease.

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Role of Volatile Organic Compounds in Establishment of the *Trichoderma*–Plant Interaction

10

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Abstract

Trichoderma species are multifaceted fungi that proliferate in the soil around roots or live inside plants as endophytes. The emission of volatile organic compounds (VOCs), including sesquiterpenes, 6-n-pentyl-2H-pyran-2-one (6-PP), and ß-caryophyllene by *Trichoderma* appears to be integral for recognition as beneficial organisms and enables not only plant colonization, but also promotes root branching and reinforces immunity. Genes encoding proteins for the biosynthesis of VOCs in fungi and their signaling targets in plants have just begun to be identified, which unveil an intimate relationship that promotes fungal growth on one side and shapes plant physiology and metabolism for improved survival and adaptation on the other.

Keywords

Trichoderma · Volatile organic compounds · Root development · Plant defense · Sugar exudation · Gene expression

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10.1 *Trichoderma*: Multifaceted Plant Symbionts

Plants are resilient organisms that adapt to the ever changing environmental conditions through interactions with their fungal partners in the rhizosphere. Fungi strongly depend on root exudates as nutritional cues and so their mutualistic relationships with plants are at the center of symbiosis events in natural and agricultural ecosystems (Werner et al. 2016). Fossil evidence of mycelia inside roots dates from the early Devonian period, around 400 million years ago, and it is thought that migration of plants from the aquatic environment to land required fungal colonization and spread within plant tissues (Remy et al. 1994; Rich et al. 2021).

Free-living and endophytic fungi of the genus *Trichoderma* establish highly dynamic interactions with crop and horticultural species and are increasingly important for farmers due to their promising plant growth-promoting properties (Lamdan et al. 2015; López-Bucio et al. 2015; Xu et al. 2018; Mayo-Prieto et al. 2019; Carro-Huerga et al. 2020). Major traits modulated by *Trichoderma* include higher biomass accumulation through an enhanced capability of colonized roots to solubilize and take up mineral nutrients, an improved root growth and branching, and the activation of induced systemic defenses that extend throughout the shoot system (López-Bucio et al. 2015; La Spada et al. 2020;

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Lombardi et al. 2020; de Sousa et al. 2021; Li et al. 2021).

Currently, at least 200 *Trichoderma* species have been investigated by both phenotypical and molecular analyses and every day more species are discovered from rhizosphere soil, aboveground tissues of plants, and decaying organic matter (Saravanakumar and Wang 2020; Xue et al. 2021). Studies into the evolution and comparative genomics of these fungi from phylogenetically distant clades including *Trichoderma reesei*, *Trichoderma virens*, and *Trichoderma atroviride* suggest mycoparasitism as an ancestral feature, from which novel relationships with microorganisms, animals, and plants further evolved (Kubicek et al. 2019; Rodríguez et al. 2021; Mukherjee et al. 2022). Every recognition stage during plant colonization involves a highly sophisticated network of genes and proteins ultimately responsible for adjusting the balance between growth and defense (Contreras-Cornejo et al. 2009; Nieto-Jacobo et al. 2017; Pelagio-Flores et al. 2017; Bader et al. 2020; Bean et al. 2021).

Volatile organic compound (VOC) emissions are integral to the communication of *Trichoderma* with plants. VOCs have a low molecular weight and high vapor pressure and are emitted as gases from the producing organisms, and thus represent an affordable and highly efficient mechanism for inter-kingdom recognition (Werner et al. 2016; Inamdar et al. 2020). VOC blends are responsible for major odors perceived by the human nose, and they are well-known for the sweet and delicate smells emitted by flowers to attract pollinators. They also serve as alarm cues by which damaged plants attract predators of their enemies (Schulz-Bohm et al. 2017; Fincheira and Quiroz 2018; Inamdar et al. 2020).

This chapter aims to describe the VOCs produced by *Trichoderma* species and their role in interactions with plants. The fungal blends are highly specific in their chemical composition and abundance of compounds produced by each strain and either VOC mixtures or single compounds may enhance growth and plant biomass, change root morphogenesis, and induce plant defense mechanisms, which depend on fungal and plant proteins for molecular recognition.

10.2 Volatile Organic Compound Profiling of *Trichoderma*

The composition of VOCs from *Trichoderma* is highly variable depending upon the fungal species, developmental stage, growth conditions, and ecological interactions (Lee et al. 2015, 2016, 2019; Guo et al. 2019, 2020). More than 470 *Trichoderma* VOCs have been identified, including alcohols, hydrocarbons, aldehydes, alkaloids, ketones, sesquiterpenes, monoterpenes, alkanes, ethers, heterocyclic compounds, phenol, and benzene (Stoppacher et al. 2010; Hung et al. 2013; Estrada-Rivera et al. 2019; Guo et al. 2019). The use of pure analytical standards confirmed the identity of 2-heptanone, 3-octanone, 2-nonanone, 2-undecanone, 1-octen-3-ol, 3-octanol, phenylethyl alcohol, alpha-phellandrene, alpha-terpinene, 6-n-pentyl-2H-pyran-2-one (6-PP), and nerolidol as part of the VOCs produced by *T. atroviride* (Stoppacher et al. 2010). Noteworthy, 6-PP appears to be a major VOC from *T. atroviride* frequently reported in different studies regardless of the growth conditions, purification procedures, and/or detection systems (Reithner et al. 2005; Vinale et al. 2008; Stoppacher et al. 2010; Garnica-Vergara et al. 2016; Moreno-Ruiz et al. 2020).

Headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography–mass spectrometry (GC-MS) analysis indicated that *T. virens* Gv29–8 produces three major categories of VOCs in potato dextrose agar (PDA) medium: 24 sesquiterpenes, five monoterpenes, and five C8 alkane compounds (Crutcher et al. 2013). During the interaction with *Arabidopsis* seedlings, *T. virens* emitted mainly sesquiterpenes, including β-caryophyllene, (−)-β-elemene, germacrene D, τ-cadinene, α-amorphene, δ-cadinene, and τ-selinene, which account for more than 95% of total VOCs (Contreras-Cornejo et al. 2014). Interestingly, δ-cadinene has been classified as a

phytoalexin (Wu et al. 2005), compound with antimicrobial properties, which helps to restrict the dispersion of pathogens and antioxidant activities (Jeandet 2015). The terpenoid biosynthesis machinery is ubiquitous to *Trichoderma* and families of genes encoding for terpene cyclases have been identified in the genomes of *T. virens*, *T. atroviride*, and *T. reesei* (Bansal and Mukherjee 2016), which indicates their possible roles in VOC biosynthesis.

The VOC blends of plant-endophytic species such as *Trichoderma asperelloides* T1 and *Trichoderma asperellum* PSU-P1 comprised 22 compounds including ethanol, 2-methyl-1-butanol, 2-ethylhexanol, 1-nonanol, 2-pentylfuran, acetic acid, and 6-PP (Phoka et al. 2020; Wonglom et al. 2020). Comparison of VOC emissions from *Trichoderma harzianum* WM24a1, *Trichoderma hamatum* QL15d1, *T. reesei* QM6a, and *Trichoderma velutinum* GL1561 identified alcohols, aldehydes, and ketones from all three species (Guo et al. 2020). In addition, sesquiterpenes were abundant in cultures of *T. hamatum* and *T. reesei*, but their production was negligible in *T. harzianum* and *T. velutinum*. The VOC emission patterns also differed between species with ß-elemene, α-selinene, and trans-γ-bisabolene being the major VOCs of *T. hamatum*, whereas α-cedrene, β-cedrene, and β-curcumene were more abundant in *T. reesei* (Guo et al. 2020).

A recent study by Lazazzara et al. (2021) characterized the VOCs produced by *T. asperellum* T34, *T. harzianum* T39, and *T. atroviride* SC1, and confirmed the presence of 6-PP as well as alkenes such as 1,3-octadiene, the furanes 2-pentylfuran and 2-n-heptylfuran, the ketones 3-octanone and 2-undecanone, and many monoterpenes (α-phellandrene, α-terpinene, limonene, γ-terpinene and β-phellandrene) and sesquiterpenes ([Z, E]-α-farnesene, γ-cadinene, γ-muurolene, α-curcumene, α-zingiberene, trans-β-farnesene, germacrene A, β sesquiphellandrene, β-himachalene, β-bisabolene, and δ-cadinene). Thus, although the heterogeneity and chemical composition of *Trichoderma* VOCs may change spatially and temporally, a frequently found metabolite is 6-PP and it may explain, in a significant part, the biological properties of fungal isolates already described.

10.3 *Trichoderma* Genes for Volatile Organic Compound Emission

Genomic analyses and disruption or overexpression strategies in different *Trichoderma* species enabled the identification of single genes or gene clusters encoding for diverse enzymes involved in the VOC biosynthesis-related pathways, such as non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), or terpene synthases, as well as regulatory proteins and transcription factors. Many of these genes are tightly linked to developmental and adaptive processes such as conidiation and regeneration (Zeilinger et al. 2016, Table 10.1).

The G-protein A subunit TGA1 of *T. atroviride* is involved in the production of 6-PP and sesquiterpenes. When compared to the wild-type fungus, the Δ*tga1* mutant secreted reduced amounts of 6-PP into the growth medium (Reithner et al. 2005). The production of 6-PP by *T. atroviride*, *Trichoderma gamsii*, and *T. harzianum* depends on the oxidation of linoleic acid to 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13-HPOD) by lipoxygenase 1 (LOX1), and consistently, their genomes encode a *LOX* gene that is absent in non-producing species such as *T. virens* and *T. reesei* (Atanasova et al. 2013; Zeilinger et al. 2016). In *T. atroviride*, a single gene encodes LOX1, and its deletion inhibits biosynthesis of 6-PP, oxylipins, 1-octen-3-ol, 2-heptanone, and 3-octanone, which affects conidiation and the induction of plant systemic resistance against *Botrytis cinerea* (Speckbacher et al. 2020).

The transcriptional regulation of 6-PP biosynthesis in *T. atroviride* and *T. harzianum* was critically influenced by the histone deacetylase-encoding gene *HDA-2* and the transcription factor *THCTF1*, respectively (Rubio et al. 2009; Estrada-Rivera et al. 2019). Besides, the emission patterns of 6-PP and its analog 6-pent-1-enyl-2H-pyran-2-one are affected by the absence of a functional *NOX2* gene, which determines the

Table 10.1 Volatile organic compounds (VOCs) from *Trichoderma* and their effects in plants

Volatile compounds	Fungal species	Host plant	Effect on plants	References
6-PP	*T. atroviride*	*Arabidopsis* Tomato Grapevine Strawberry Olive tree	Promotes plant growth, regulates root architecture and sucrose transport, induces defense mechanisms against pathogens, improves fruit quality	Vinale et al. (2008), Garnica-Vergara et al. (2016), Carillo et al. (2020), Lombardi et al. (2020), Esparza-Reynoso et al. (2021), Dini et al. (2021), Lazazzara et al. (2021)
Blend of VOCs including 1-octen-3-ol, nonanal, and 6-PP	*T. asperellum* IsmT5	*Arabidopsis*	Reduces fresh weight, root length, and leaf area, and increases the accumulation of defense-related compounds and expression of defense-related genes	Kottb et al. (2015)
6-PP 1-octen-3-ol, 3-octanone β-elemene and ε-amorphene 1,3-octadiene, limonene and β-eudesmol + valerianol	*T. atroviride* LU132 *T. atroviride* IMI206040 *T. virens* Gv29.8, 29 *T. asperellum* LU1370	*Arabidopsis*	Increases shoot, root, and total biomass, and chlorophyll content	Nieto-Jacobo et al. (2017)
2-heptanone and 3-octanone	*T. atroviride* wild-type (WT)	*Arabidopsis*	Promotes plant growth	Estrada-Rivera et al. (2019)
6-PP	Mutant strain Δ*hda-2*			
3-methyl-1-butanol, 1-decene, and 2-heptylfuran	*Trichoderma* spp.	*Arabidopsis*	Increases fresh weight and total chlorophyll content	Lee et al. (2019)
Blend of VOCs with 2-ethyl-1-hexanol, 1-nonanol, ethanol, succinic acid, and 6-PP	*T. asperellum* T1	Lettuce	Increases the development of leaves and roots, plant biomass and total chlorophyll content, and improves the accumulation of cell-wall degrading enzymes	Wonglom et al. (2020)
Blend of VOCs with high presence of ketones	*T. azevedoi* CEN1241	Lettuce	Increases plant growth, the content of chlorophyll and carotenoids	da Silva et al. (2021)
Blend of VOCs with high presence of 2-methyl-1-butanol, 2-pentylfuran, acetic acid, and 6-PP	*T. asperelloides* PSU-P1	*Arabidopsis*	Increases fresh weight, root length, and chlorophyll content	Phoka et al. (2020)
Heptane, 2,5-dimethylethylbenzene, 4-hydroxy-2-butenoic acid (methyl ester), acetylcarbonyl, benzylamine and carbolic acid, α-farnesene, caryophyllene oxide, 2 methyl-1-butanol, ß-bisabolene, and pyridine	*T. harzianum* recombinant strains (T13 and T15)	Bean	Enhances plant disease resistance and improves plant growth	Eslahi et al. (2021)

(continued)

Table 10.1 (continued)

Volatile compounds	Fungal species	Host plant	Effect on plants	References
γ-cadinene and δ-cadinene, 6α-cadina-4,9-diene, α-elemene and valencene	*T. virens*	*Arabidopsis*	Increases fresh weight, main root length, and lateral root number	González-Pérez et al. (2018)
Isocembrene, 6-PP, 4(10)-thujene, and cyclopenten-2-one	*T. atroviride*			
VOC mixtures	*Trichoderma* spp.	*Arabidopsis*	Improves salt tolerance and increases plant growth	Jalali et al. (2017)
Blend of VOCs enriched in sesquiterpenes	*T. viride* *T. virens*		Elicits development and defense programs	Hung et al. (2013, 2014), Contreras-Cornejo et al. (2014)
VOC mixtures	*Trichoderma* spp.	*Arabidopsis* Tomato	Increases fresh weight, chlorophyll, and root growth	Lee et al. (2015, 2016)
Blend of VOCs	*T. atroviride*	*Arabidopsis*	Improves sucrose metabolism and transport, and the root exudation of sugars	Esparza-Reynoso et al. (2021)
Blend of VOCs with high content of 2-heptanone, 6-PP, and (E)-6-pentyl-pyran-2-one	*T. atroviride* knock-out strains Δ*nox1*, Δ*nox2*, and Δ*noxR*	*Arabidopsis*	Increases root and shoot biomass	Cruz-Magalhães et al. (2019)

fungistatic effect of *T. atroviride* against *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Cruz-Magalhães et al. 2019).

The emission of terpenes by *T. virens*, including β-caryophyllene is critical for plant growth and immunity (Crutcher et al. 2013; Contreras-Cornejo et al. 2014). Noteworthy, in the *4-phosphopantetheinyl transferase 1 mutant* (Δ*ppt1*), which fails to promote growth and activate the defense reaction in *Arabidopsis* seedlings, the amount of terpenes strongly decreased, indicating an important role for these VOCs in the fungal–plant dialog that reinforces the symbiosis (Contreras-Cornejo et al. 2014).

10.4 Plant Responses to *Trichoderma* Volatile Organic Compounds

The specific contribution of *Trichoderma* VOCs to plants has just begun to be clarified. Either the blended VOCs from growing *Trichoderma* colonies or direct application of purified compounds orchestrate the plant cellular responses depending on the time of exposure, type of strains, culture media, and the age of the fungal culture (Chen et al. 2016; Jalali et al. 2017; González-Pérez et al. 2018; Esparza-Reynoso et al. 2021). Examples on how the *Trichoderma* VOCs modulate plant traits are indicated in Table 10.2.

10.4.1 Growth and Morphogenesis

The release of specific VOCs is integral to the initial events of recognition between the plant and *Trichoderma* for root developmental reprogramming and increases the colonization of surfaces through inducing the formation and growth of lateral roots and root hairs for more efficient soil exploration and nutrient acquisition, which often leads to further improvement of whole plant biomass (Contreras-Cornejo et al. 2014; Moisan et al. 2019).

Arabidopsis seedlings exposed for 6 days to VOCs emitted by *T. virens* showed a two-fold

Table 10.2 Genes involved in the biosynthesis of volatile organic compounds (VOCs) from *Trichoderma*

Gene	Fungal species	VOC	References
TGA1 *GPR1* *TMK1* *LAE1*	*T. atroviride*	6-PP	Reithner et al. (2005), Karimi Aghcheh et al. (2013)
Gene cluster	*T. reesei* *T. atroviride* *T. virens*	Several sesquiterpenes	Bansal and Mukherjee (2016)
TRI4, *TRI5* *ERG1*	*T. arundinaceum* *T. brevicompactum* *T. harzianum*	Trichodiene	Tijerino et al. (2011) Cardoza et al. (2015) Malmierca et al. (2015a, b)
THCTF1	*T. harzianum*	6-PP	Rubio et al. (2009)
PPT1	*T. virens*	β-phellandrene, p-menth-3-ene, and β-cedrene	Contreras-Cornejo et al. (2014)
VIR4	*T. virens*	Volatile terpenes	Crutcher et al. (2013) Bansal et al. (2021)
NOX1 *NOX2* *NOXR*	*T. atroviride*	6-PP and 6-pent-1-enyl-2H-pyran-2-one	Cruz-Magalhães et al. (2019)
LOX1	*T. atroviride*	1-octen-3-ol, 2-heptanone, and 3-octanone	Speckbacher et al. (2020)

increase in fresh weight when compared to axenically-grown seedlings. The main VOCs apparently responsible for enhanced root branching were sesquiterpenes and well-known cellular messengers potentially involved in the plant response, including jasmonic acid and hydrogen peroxide (Contreras-Cornejo et al. 2011). *Arabidopsis* and tomato plants exposed to VOC blends emitted by cultures of 11 *Trichoderma* species showed roughly a 40% plant biomass and 80% chlorophyll content increase. *Trichoderma pseudokoningii* (CBS 130756) had the greatest biostimulant effect in *Arabidopsis*, whereas *T. viride* (BBA 70239) improved by 90% the biomass and size of tomato plants. Diterpenes and sesquiterpenes such as β-elemene and ε-amorphene, γ-cadinene, δ-cadinene, and α-farnesene, among others, may be directly linked to the growth and development of *Arabidopsis* and bean plants through their antioxidant properties (Nieto-Jacobo et al. 2017; González-Pérez et al. 2018; Eslahi et al. 2021).

6-PP is one of the most interesting and highly bioactive VOCs due to its auxin-like effect on plants manifested upon exposure to *Trichoderma* cultures. Application of the purified and/or synthetic 6-PP promotes growth and biomass in tomato, canola, bean, and *Arabidopsis* (Vinale et al. 2008; Garnica-Vergara et al. 2016; Estrada-Rivera et al. 2019). Sprays of 6-PP onto tomato plants grown in pots under greenhouse conditions increased root biomass and branching, while supplementation of this VOC to agar-solidified growth medium of tomato and canola seedlings led to better germination and more vigorous individuals (Vinale et al. 2008). 6-PP inhibited the growth of roots at 100–300 μM concentrations. Nevertheless, more root branches were formed that further extended the absorptive capacity and interaction area. In *Arabidopsis* seedlings, irrespective of the mode by which 6-PP was applied, the shoot area increased and leaves remained healthy with a dark green color indicative of high levels of chlorophyll and leaf pigments (Garnica-Vergara et al. 2016; Esparza-Reynoso et al. 2021).

1-octen-3-ol and its analog 3-octanone, also called "mushroom alcohols", are VOCs emitted from *T. atroviride* IMI206040 and *T. asperellum* IsmT5, which have plant growth-promoting activity at low concentrations (Hung et al. 2014; Kottb et al. 2015; Nieto-Jacobo et al. 2017). In addition, some alcohols emitted by *T. asperellum* T1 such as 1-nonanol, 2-ethyl-1-hexanol, and ethanol increased leaf number, lateral root density, plant biomass, and total chlorophyll content

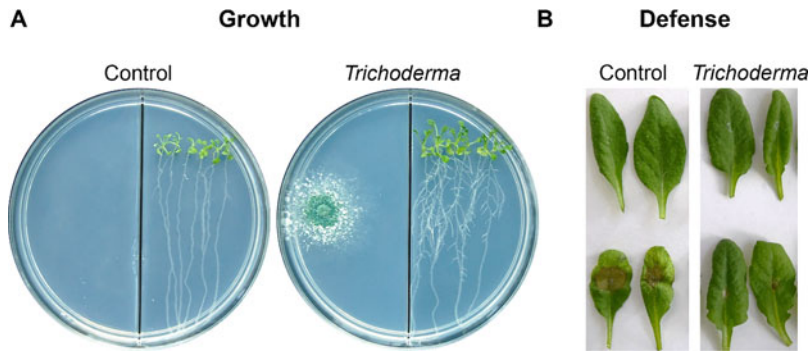

Fig. 10.1 *Trichoderma* volatile organic compounds (VOCs) promote plant growth and immunity. (**a**) Images of divided Petri plates showing the growth of *Arabidopsis* seedlings exposed or not exposed to VOCs emitted by a colony of *Trichoderma atroviride* from the opposite side of the plate. The VOC blend promotes root growth, lateral root formation, and overall plant biomass. (**b**) Leaves of healthy *Arabidopsis* plants (upper) or challenged with the phytopathogen *Botrytis cinerea* (lower) are protected by previous exposure to *T. atroviride* VOCs

in lettuce (Wonglom et al. 2020). Comparable effects have been attributed to 3-methyl-1-butanol, 1-decene, and 2-heptylfuran, which at a low concentrations (10 ng/l) increased the biomass and chlorophyll content of *Arabidopsis* seedlings (Lee et al. 2019; Phoka et al. 2020). Besides, ketones like 2-heptanone released by *T. atroviride* enhanced the content of chlorophyll and carotenoids in leaves of *Arabidopsis* and lettuce (Cruz-Magalhães et al. 2019; Estrada-Rivera et al. 2019; da Silva et al. 2021). Figure 10.1 shows the effects of *T. atroviride* VOCs on *Arabidopsis* development and immunity. Thus, although the VOC profiles may vary among *Trichoderma* species, a common feature is their overall plant growth-promoting properties.

10.4.2 Reinforcement of Immunity

Trichoderma VOCs reinforce the defense system of their plant hosts, ensuring resistance against pathogenic fungi and bacteria, and may confer protection from insect pests. The defensive properties rely not only on the overproduction of the canonical plant defense hormone jasmonic acid, but also on the production of the oxylipins 12-oxo-phytodienoic acid (12-OPDA) and α-ketol of octadecadienoic acid (KODA), which are distributed over long distances within tissues of the plant and act as alarm signals (Martínez-Medina et al. 2017a; Wang et al. 2020).

In the research by Vinale et al. (2008), tomato and oilseed rape seedlings were sprayed with 6-PP and then inoculated with spores of *B. cinerea* or *Leptosphaeria maculans*. This compound reduced disease symptoms in the plants caused by the pathogens, which could be correlated with overexpression of pathogenesis-related (PR) proteins. *T. virens, T. atroviride*, and *T. asperellum* strongly induced defense responses related to the activity of the phytohormones salicylic acid, jasmonic acid, ethylene, and abscisic acid (Contreras-Cornejo et al. 2011, 2014, 2015; Velázquez-Robledo et al. 2011; Kottb et al. 2015). Plants exposed to *Trichoderma* VOC blends overproduce substances that protect from pathogen attack such as camalexin and phytoalexins as well as PR proteins (Contreras-Cornejo et al. 2011; Kottb et al. 2015). The VOCs from *T. asperelloides* PSU-P1 and *T. asperellum* T1 induced the expression of chitinase (*CHI*) and β-1,3-glucanase (*GLU*) genes and both *Arabidopsis* and lettuce plants had higher peroxidase, chitinase, and β-1,3-glucanase enzyme activities (Phoka et al. 2020; Wonglom et al. 2020).

Application of 6-PP was sufficient to enhance the expression of defense-related genes and to confer protection against *B. cinerea* as well as to

induce the formation of trichomes on leaves that may protect from feeding insects (Kottb et al. 2015). Indeed, 6-PP and 1-octen-3-ol produced by *T. atroviride* could be responsible for modulating the production of plant terpenes and the accumulation of jasmonic acid in maize plants, which led to greater resistance to *Spodoptera frugiperda*, an herbivore of agronomical importance (Contreras-Cornejo et al. 2016).

The sesquiterpene ß-caryophyllene could orchestrate the resistance of *Arabidopsis* to *Pseudomonas syringae* through binding to the transcriptional co-repressor TOPLESS (TPL) complex, and in this manner influenced jasmonic acid signaling (Nagashima et al. 2019; Frank et al. 2021). Some of the other already detected VOCs such as ethanol and 1-octen-3-ol, α-farnesene, cadinene, 1,3-octadiene, 2-pentylfuran, and 6-PP harbor antibacterial and antifungal activity (Xiong et al. 2017; Lee et al. 2019). In particular, 6-PP and 2-pentylfuran strongly inhibited downy mildew (*Plasmopara viticola*) in leaf disks of grapevine when applied as a VOC blend (Lazazzara et al. 2021). Thus, the VOCs emitted by *Trichoderma* may protect plants from pathogens via their antimicrobial properties or eliciting the biosynthesis of hormones that induce defense gene expression.

10.4.3 Metabolic Reprogramming

An emerging trend of *Trichoderma* VOCs has been related to the nutritional content and production of regulatory metabolites in their plant hosts. Metabolomics analysis from tomato leaves whose seeds were treated with 6-PP unveiled gamma-aminobutyric acid (GABA) and acetylcholine, molecules typically associated with plant stress resistance and carbon and nitrogen metabolism, as part of the metabolites being overproduced and with beneficial effects in plants (Mazzei et al. 2016).

In soybean seeds harvested from plants treated with 6-PP, accumulation of stearic acid and 11-eicosanoic acid was reported (Marra et al. 2019), whereas tomato plants inoculated with *T. harzianum* T22 or supplemented with 6-PP had a roughly 52% increase in the content of the antioxidant lycopene (Carillo et al. 2020), thus increasing their nutritional value. While investigating whether *T. atroviride* VOCs could influence carbon metabolism and root exudation patterns in *Arabidopsis*, Esparza-Reynoso et al. (2021) found that 6-PP critically influences sucrose transport from the shoot to the root by modulating the expression of the major sucrose transporter AtSUC2 as well as increasing the amount of sucrose in root exudates. In a recent study, 6-PP applied in olive trees enhanced the emission of monoterpenes and hydrocarbon aldehyde (Dini et al. 2021). Thus, 6-PP is one of the factors that plants sense to emit their own VOCs, many of them with highly relevant adaptive functions.

10.4.4 Genetic Responses

Recent advances have increased our understanding into how plants decode the chemical message of *Trichoderma* VOCs 6-PP and ß-caryophyllene (Fig. 10.2). In *T. atroviride–Arabidopsis* interactions, the production of 6-PP increased in the presence of plants when compared to the levels emitted by the fungal colony alone (Garnica-Vergara et al. 2016). The repressing effect of this VOC in primary root growth and inducing root branching was strongly dependent on ETHYLENE INSENSITIVE 2 (EIN2) as demonstrated by the resistance of the *Arabidopsis ein2–1* mutant to application of 6-PP regarding the growth of the main root axis.

An auxin–ethylene relationship for sensing 6-PP was demonstrated by the selective regulation of PIN auxin-transport proteins in primary root tips and developing lateral root primordia as well as the dependence of TIR1, AFB2, and AFB3 auxin receptors and ARF7 and ARF19 transcription factors for 6-PP-mediated regulation of lateral root development. Consistently, genes from the auxin and ethylene pathways including *TIR1*, *AUX1*, *PIN3*, *PIN7* and *ACO2*, *ETR1*, *ERS1*, *EIN2* were induced in response to VOCs from *T. atroviride* (Estrada-Rivera et al. 2019).

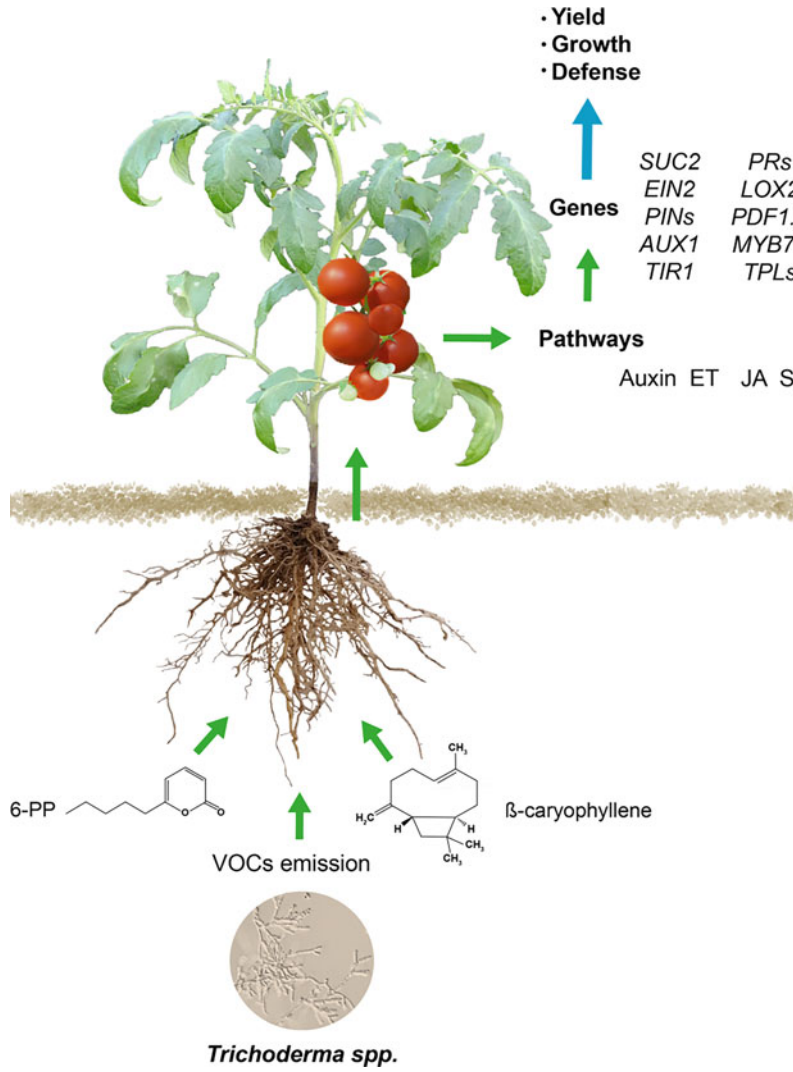

Fig. 10.2 Genes involved in the hormonal signaling network in response to volatile organic compounds (VOCs) emitted by *Trichoderma*. *Trichoderma* spp. release VOCs including 6-PP and ß-caryophyllene, which act as info-chemicals for modulation of the plant hormonal status, development, and defense. The perception of fungal VOCs may affect auxin, ethylene (ET), jasmonic acid (JA), and salicylic acid (SA)-dependent processes for regulation of gene expression, and in this manner, tunes the balance between growth and defense

Noteworthy, although the phenotypes of plants exposed to VOCs from bacteria, fungal pathogens, and fungal symbionts may be somewhat comparable (Camarena-Pozos et al. 2019; García-Gómez et al. 2020; Esparza-Reynoso et al. 2021), the molecular mechanisms required to decode the informational cues present in the corresponding microbial VOCs depend on specific target proteins in plant tissues (Table 10.3).

T. virens colonies emit ß-caryophyllene as a major VOC involved in plant growth and defense (Contreras-Cornejo et al. 2014). In an effort to identify the protein targets by which bioactive VOCs modulate plant signal transduction pathways, Nagashima et al. (2019) tested a series of monoterpenes and sesquiterpenes as well as the canonical defense regulators methyl jasmonate and salicylic acid to induce the expression of the tobacco *OSMOTIN* gene (*NtOSMOTIN*), which encodes a PR protein involved in tolerance to salt stress and infection by fungal pathogens. ß-caryophyllene and its structural analogs α-caryophyllene, or caryophyllene oxide induced at low concentrations, and in a dose-dependent manner, NtOSMOTIN expression in tobacco BY-2 cells and in leaves of tobacco seedlings. Using labeled ß-caryophyllene derivatives, six TOPLESS (TPL)-like proteins that act as

Table 10.3 Plant genes mediating responses to *Trichoderma* volatile organic compounds (VOCs)

Individual VOC or blends	Genes	Plant species	Associated response	References
1-octen-3-ol	*AOS*, *HPL*, *PDF1.2*, and *PR-3*	*Arabidopsis*	Defense	Kishimoto et al. (2007)
6-PP	*PR1*	Oilseed rape (*Brassica napus*)	Defense	Vinale et al. (2008)
VOC blends from *T. virens*	*LOX2*	*Arabidopsis*	Defense	Contreras-Cornejo et al. (2014)
VOC blends from *T. asperellum* IsmT5	*PR-1*, *GL3*, *VSP2*, *PDF1.2*, *MYB51*, *YUC8*	*Arabidopsis*	Growth and defense	Kottb et al. (2015)
6-PP	*EIN2, PIN1, PIN2, PIN3, PIN7, TIR1, AFB2, AFB3, ARF7, ARF19, AXR1–3, AUX1–7.*	*Arabidopsis*	Growth	Garnica-Vergara et al. (2016)
VOC blends from *T. asperellum and T. harzianum*	*MYB72, FRO2, IRT1, bHLH38, bHLH39, PDF1.2*, *VSP2.*	*Arabidopsis* and tomato (*Solanum lycopersicum*)	Growth, Fe uptake, and defense	Martínez-Medina et al. (2017b)
VOC blends from *T. virens* and *T. atroviride*	*ERD14*	*Arabidopsis*	Cold stress	González-Pérez et al. (2018)
VOC blends from *T. atroviride*	*TIR1, AUX1, PIN3, PIN7, ACO2, ETR1, ERS1, EIN2.*	*Arabidopsis*	Growth	Estrada-Rivera et al. (2019)
VOC blends from *T. asperelloides* PSU-P1	*CHI*, *GLU*, *POD.*	*Arabidopsis*	Defense	Phoka et al. (2020)
6-PP	*SUC2*	*Arabidopsis*	Sucrose transport and exudation	Esparza-Reynoso et al. (2021)
6-PP	*PR2*, *OSM1*, *OSM2*, *CHIT3,* HSR.	Grapevine (*Vitis vinifera*)	Defense	Lazazzara et al. (2021)
VOC blends from *T. harzianum* T-78 or *T. asperellum* T-34	*MYB72, FRO2, IRT1, PR1, PDF1.2, NIA1, NIA2, PHYTOGB1.*	*Arabidopsis*	Defense and Fe uptake	Pescador et al. (2021)

transcriptional co-repressors were identified and found to bind ß-caryophyllene. The interaction of NtTPL3 and caryophyllene oxide was demonstrated *in vitro*, thus ß-caryophyllene diffuses into plant cells, binds NtTPLs in nuclei, and releases them from the transcriptional complexes that repress defense-related gene expression.

10.5 Concluding Remarks

The chemical communication among organisms from different kingdoms involves VOC emissions as specific footprints that elicit a response in the detecting cells and tissues (Giordano et al. 2021). The recent identification of TOPLESS (TPL)-like proteins as targets of ß-caryophyllene and the finding that 6-PP perception by roots involves the ethylene-response gene *EIN2*, help to explain how *Trichoderma* VOCs affect plant development and defense, via fine-tuning hormonal responses (Garnica-Vergara et al. 2016; Nagashima et al. 2019; Navarrete et al. 2022). TOPLESS proteins belong to a family of transcriptional co-repressors involved in both auxin and jasmonate signaling, whereas EIN2 plays a critical role in sensing ethylene, the bacterial phytotoxin pyocyanin and jasmonic acid (Ortiz-Castro et al. 2014; Barrera-Ortiz et al. 2018). Thus, it is tempting to speculate that as part of *Trichoderma* VOCs, ß-caryophyllene and

6-PP play a role in the molecular recognition among fungi and their plant hosts.

Trichoderma species are at the forefront of the myriad of microorganism that colonize plants with potential biotechnological applications not only because of their effective antagonism to plant pathogens, but also because these fungi produce molecules with very interesting properties. The recent finding that 6-PP drives sucrose mobilization within the plant and increases its exudation by roots (Esparza-Reynoso et al. 2021), and that sugar availability changes the metabolism of *T. atroviride* (Villalobos-Escobedo et al. 2020), may open up new avenues to manage the symbiosis in the field. The knowledge into how plants recognize and respond to *Trichoderma* VOCs is at the center of the initiative to develop natural and effective bio-stimulants for their use in agriculture.

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Part IV

Regulation of Fungal Gene Expression and Development

Epigenetic Regulation of Fungal Genes Involved in Plant Colonization

11

Jessica L. Soyer and Isabelle Fudal

Abstract

Plant-pathogenic fungi have a major impact on agriculture and human health, as well as a remarkable ability to adapt to new conditions. This adaptability relies, at least in part, on the fine-tuned expression of genes involved in plant colonization. The large-scale sequencing of fungal genomes, together with the wealth of associated transcriptomic data collected during plant infection, highlights that genes involved in plant colonization exhibit sophisticated waves of expression and are often enriched in particular regions of the fungal genome (e.g., in repeat-rich heterochromatic or sub-telomeric regions, or on accessory chromosomes). These specific locations suggest that an epigenetic control mechanism might be involved in the regulation of their expression. In this chapter, we provide a historical perspective on the tremendous amount of work accumulated on chromatin organization and remodeling, and on recent data on genomes and epigenomes of plant-interacting fungi, which have refined our understanding of the impact of chromatin structure on the regulation of genes involved in host colonization. We also highlight how functional analyses support the hypothesis of a chromatin-based control of gene expression in plant-interacting fungi, associated with the action of specific transcription factor(s). We conclude by outlining the next challenges to be addressed concerning the epigenetic regulation of fungal genes involved in plant colonization.

Keywords

Plant-interacting fungi · Effectors · Chromatin · Epigenetics · Fungal epigenome · Gene regulation · Transcription factors

11.1 Introduction

Plant-pathogenic fungi have a major impact on agriculture and human health and show a remarkable ability to adapt rapidly to new environmental conditions (Fisher et al. 2018). They occupy most ecological niches and present a great diversity of lifestyles, nutritional strategies, and interactions with their hosts. Their success in infecting a host plant and adapting to new environmental conditions or to new hosts depends, at least in part, on their ability to fine-tune the expression of pathogenicity genes and to dynamically regulate their transcriptional profiles. During plant colonization, plant-associated fungi secrete molecules, commonly called effectors, that can modulate plant immunity and promote colonization (Sánchez-Vallet et al. 2018; Rocafort et al. 2020). In plants carrying major resistance (*R*)

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genes, some of these effectors can be specifically recognized and are called avirulence (AVR) proteins. Effectors were initially referred to as (small) secreted proteins ((S)SPs), but some secondary metabolites and small RNAs (sRNAs) were also found to play a role as effectors (Weiberg et al. 2013; Wang et al. 2015; Sánchez-Vallet et al. 2018; Collemare et al. 2019). The large-scale increase in the sequencing of fungal genomes, together with advances in the quality of genome assemblies and the acquisition of associated high-quality transcriptomic data during plant infection, have highlighted the importance of effector genes: they exhibit complex waves of expression during host colonization and are enriched in particular regions of the fungal genome (e.g., repeat-rich regions, accessory chromosomes, sub-telomeric regions). The location of effector genes in particular genomic regions suggests that an epigenetic control mechanism, through chromatin remodeling, might be involved in the regulation of their expression. Use of the term "epigenetic"—literally meaning "above genetic"—is still controversial and represents a "semantic morass" (Lederberg 2001). This term was initially proposed by Waddington (1942, 1957) to describe interactions between the environment and the genes leading to the development of a new phenotype, with no involvement of the underlying DNA sequence.

In this chapter, we provide a historical overview of the deciphering of chromatin organization and the accumulation of data regarding genomes and epigenomes of plant-associated fungi. Pioneering analyses performed in the model fungus, *Neurospora crassa*, helped scientists working on plant-related fungi to build hypotheses on the epigenetic control of plant colonization. Here, our ambition is to reconcile data gathered on *N. crassa* with the latest knowledge obtained from -omic (genomic, transcriptomic, epigenomic) analyses on plant-associated fungi to establish a framework on how plant-related genes are regulated. Then, we report on the functional analyses that support the hypothesis of an epigenetic control mechanism of plant-associated genes in fungi and highlight the next challenges to be tackled concerning our understanding of this regulation.

11.2 Current Knowledge on Fungal Chromatin Organization and Key Elements Involved in Chromatin Remodeling

11.2.1 Historical Overview of Chromosome Organization

Within the eukaryotic nucleus, chromatin folding from the DNA fiber to the chromosome territories represents intertwined levels of compactions of which each level is regulated and each scale is an important regulator of physiological processes. The two centuries between Brown's discovery of the nucleus in 1831 (cited in Jost et al. 2012) and the decoding of the human genome in 2001 (International Human Genome Consortium 2001) have led to an accumulation of knowledge about the composition of the nucleus and its role. Nucleic acids constituting the substrate of the genetic code were first isolated by Friedrich Miescher in 1869 (cited in Jost et al. 2012). With advances in staining and cytological techniques, Walther Flemming was able to visualize a highly organized fibrous structure within eukaryotic nuclei in 1879, which he referred to as chromatin. The DNA molecule itself is static, whereas the chromatin fiber is a highly dynamic structure. Beyond its role in maintaining the physical compaction of the DNA molecule, chromatin forms a platform where the main cellular functions are regulated: replication, transcription, chromosome segregation, or DNA repair. Chromatin consists of a complex made up of DNA and proteins. The nucleosome is the fundamental unit of chromatin, composed of histones, described by Kossel in 1884 (cited by Jost et al. 2012), and represents the structural component supporting the information carried by DNA. It was Kornberg, in Kornberg 1974, who first postulated that chromatin was composed of a portion of DNA of about 200 bp wrapped around the "core" nucleosome, composed of an octamer of four histones (two H3–H4 and two H2A–H2B), giving it the appearance of a "pearl necklace." In the nucleus, the chromatin fiber undergoes several levels of compaction and folding until it forms the

chromosome (according to the term proposed by Waldeyer in 1888).

On the basis of all this accumulated knowledge, it is undeniable that the regulation of gene expression is a multifactorial process and relies on intertwined factors. Gene transcription relies on the binding of one (or more) transcription factor(s) (TFs) to a *cis*-regulatory element in a given chromatin context. The first layer governing gene expression relies on the accessibility of TFs to promoter sequences. This accessibility is influenced by post-translational modifications of histone proteins of the chromatin. These modifications define at least two distinct condensation states of the chromatin, firstly identified based on cytological studies: euchromatin, a relaxed structure, permissive for gene expression, and heterochromatin, highly condensed and repressive for gene expression (Heitz 1928; Jenuwein and Allis 2001; Huisinga et al. 2006; Grewal and Jia 2007). Heterochromatin constitutes the highly condensed fraction of the genome and, in contrast to euchromatin, is gene-poor, has a low rate of recombination, and is usually Transposable Element (TE)-rich (Richards and Elgin 2002; Huisinga et al. 2006; Grewal and Jia 2007). Two forms of heterochromatin can be distinguished: constitutive and facultative heterochromatin (Craig 2005). The former is a permanent structure, mainly found in centromeres and telomeres, to ensure chromosome segregation and genome stability by preventing the transposition of repetitive sequences, whereas facultative heterochromatin is a dynamic and regulated structure, involved in the regulation of gene expression. These two types of chromatin states are also distinguished by molecular "signatures," conserved from *Schizosaccharomyces pombe* to humans, which are the presence of proteins and post-translational modifications targeting the DNA or histone tails that protrude from nucleosomes. These modifications alter chromatin folding and thus accessibility of the genetic information stored therein (Luger and Richmond 1998). Since the work of Allfrey et al. (1964), who first described histone modifications and their association with the regulation of gene expression, many different types of histone modifications have been described. These modifications are referred to as the "histone code" because the state of chromatin condensation is not conditioned by an isolated type of modification, but rather by a density and local combination of modifications (Jenuwein and Allis 2001). According to Jenuwein and Allis (2001), the histone code "extends the information potential of the genetic code" and determines the two opposite states of chromatin.

At least eight post-translational modifications can affect histone tails, i.e., acetylation, phosphorylation, methylation, de-imination, ADP ribosylation, ubiquitylation, sumoylation, isomerization (for reviews, see (Kouzarides 2007; Bannister and Kouzarides 2011)), and thus influence gene expression. These modifications were initially highlighted by the use of specific antibodies, by a mass spectrometry approach and, more recently, the development of the Chromatin ImmunoPrecipitation (ChIP) technique that has made it possible to map and characterize more precisely these modifications, their specificity, and associated function (Solomon et al. 1988; Ren et al. 2000; Johnson et al. 2007; Park 2009).

The location of nucleosomes along the chromatin also influences gene expression by altering the accessibility of promoters or regulatory sequences to TFs or histone-modifying enzymes (HMEs; Radman-Livaja and Rando 2010; Struhl and Segal 2013). Nucleosome positioning and occupancy are determined by a combination of DNA sequence features, TFs, chromatin remodelers, and histones modifiers (Singh and Mueller-Planitz 2021). Genome-wide maps of nucleosome occupancy and positioning have only been developed in a few Hemiascomycota yeast species, including *Saccharomyces cerevisiae* (Yuan et al. 2005; Struhl and Segal 2013), in the ascomycete *Aspergillus fumigatus* (Nishida et al. 2009), the basidiomycete *Mixia osmundae* (Nishida et al. 2012), and recently in a few plant-pathogenic fungi (*Leptosphaeria maculans* 'brassicae', *Leptosphaeria maculans* 'lepidii', *Botrytis cinerea*, *Fusarium graminearum,* and *Verticillium verticilloides*; Cook et al. 2020; Clairet et al. 2021a). These results showed that nucleosomes are generally

depleted in promoters, enhancers, and terminators of transcriptionally active genes, indicating nucleosomal control of gene expression and that the nucleosomal DNA length distribution was similar in *M. osmundae*, *A. fumigatus*, *B. cinerea*, and *F. graminearum*, but differed from that of hemiascomycetous yeasts. *L. maculans* 'brassicae' and *L. maculans* 'lepidii' distinguished themselves by the presence of a shorter nucleosomal DNA length distribution, suggesting a narrower chromatin fiber structure.

Within the nucleus, chromosomes are not randomly organized but display specific spatial localization in interphase as first observed in animal cells by Rabl in 1885. This territorial organization was referred to as "chromosome territories" (CTs) by Theodor Boveri (1909). While histone modifications have long been known to influence chromatin compaction and thereby gene expression, our understanding of the role of the 3D organization of the chromosomes has dramatically changed over the last 10 years. Long distance contacts occur in chromatin loops bringing together the *cis*-regulators and the target genes. A central role of chromatin loops and long-distance chromosomal interactions has been recognized as important for several physiological processes (replication, DNA repair, transcription, etc.; for a review, see (Bonev and Cavalli 2016)). In terms of gene regulation, CTs are also dynamic and genes expressed together can be closely located within these territories although located on different chromosomes (Cremer and Cremer 2010). Hence, CTs represent an important template for gene regulation (Duan et al. 2010).

11.2.2 Key Players Ensuring the Equilibrium Between Heterochromatin and Euchromatin

Although many interconnected levels are involved in chromatin organization and regulation of cellular processes, the focus so far in plant-pathogenic fungi has been the analysis of DNA methylation and post-translational modifications (PTMs) of histones. Indeed, chromatin state has a direct and quantifiable impact on gene expression and can be monitored in different cell types or growth conditions. HMEs catalyze the deposition or removal of any covalent histone modifications (Kouzarides 2007). Different "actors" are involved in remodeling the chromatin structure including the so-called "writer" proteins, which include histone acetyltransferases (HATs), methyltransferases (HMT), deacetylases (HDACs), and demethylases. These enzymes are directly responsible for the deposition or removal of histone or DNA modifications. They act together with the so-called "reader" proteins (such as heterochromatin protein 1, HP1, or the Polycomb proteins), which recognize specific modifications after their deposition and subsequently recruit other enzymes to define the local chromatin state (for reviews, see (Musselman et al. 2012; Lalonde et al. 2014)). Among the HMTs, the SET-domain (Su(var)3–9, Enhancer of zeste, Trithorax) family is specific for methylation of lysine residues (histone methyltransferases, KMTs). Apart from DNA methylation, associated with heterochromatin, different histone modifications serve as markers of the chromatin state. Heterochromatin is characterized by the presence of histone hypoacetylation, methylation at lysine 9 of histone H3 (H3K9me) or lysine 27 of histone H3 (H3K27me) and the presence of HP1. In filamentous fungi, such as in *N. crassa*, heterochromatin assembly and maintenance are relatively well worked out (Tamaru and Selker 2001; Kouzminova and Selker 2001; Tamaru et al. 2003; Freitag et al. 2004a, b; Honda and Selker 2008; Lewis et al. 2009; Honda et al. 2010; Jamieson et al. 2013; Jamieson et al. 2016). In this fungus, KMT1 (also called DIM5 or ClrD) is responsible for tri-methylation of H3K9 (Tamaru and Selker 2001; Tamaru et al. 2003) at domains associated with constitutive heterochromatin. This modification is then specifically recognized by the chromodomain of HP1 which in turn recruits the DNA-methyltransferase DIM-2 via its chromo-shadow domain (Honda and Selker 2008). Recruitment of DIM-2 catalyzes DNA methylation, which promotes and stabilizes heterochromatin. Although involved in the maintenance of constitutive heterochromatin, HP1 is also important for H3K27me3 maintenance in

domains of facultative heterochromatin (Basenko et al. 2015; Jamieson et al. 2016). The histone lysine methyltransferase KMT6 (also called EzhB) is part of the Polycomb Repressive Complex 2 (PRC2) and catalyzes H3K27 methylation (Jamieson et al. 2013) at facultative, gene-rich heterochromatin domains. In *N. crassa*, DNA methylation and DIM-2 are not essential for heterochromatin assembly in contrast to the need for KMT1 and HP1 (Foss et al. 1993; Kouzminova and Selker 2001; Tamaru and Selker 2001; Honda et al. 2012). This might explain why DNA methylation has not been found to be widely conserved in filamentous fungi (Bewick et al. 2019). In *N. crassa*, the action of HDACs is also paramount to this process, as inactivation of *had-1* induces a loss of DNA methylation but is also associated with an increase of H3K9ac which compromises methylation of this lysine (Selker 1998). Hence, de-acetylation of H3K9 seems to be a pre-requisite to DNA methylation, H3K9 methylation, and heterochromatin assembly (Smith et al. 2010). In contrast to histone acetylation, histone methylations were originally considered to be irreversible for the following reasons: (i) the C–N bond is more stable than the amine bond established during acetylation, (ii) the half-life of the methyl mark is equal to that of the histones (Byvoet et al. 1972). However, recent biochemical analyses in model organisms have identified histone demethylases, that have specificity for particular residues and are now considered to be key "actors" of transcription regulation (for a review, see (Kooistra and Helin 2012)).

11.3 Contribution of Omics Data to Our Understanding of the Genomic, Epigenomic, and Transcriptomic Context of Fungal Genes Involved in Plant Colonization

11.3.1 Fungal Genomes Are as Puzzling as the Fungal Kingdom

Fungal species are important for human activities including research and industrial applications. They are in the kingdom for which there is the largest number of genomes sequenced, therefore providing a rich source of information and data to answer many key biological questions. The genome of the yeast *S. cerevisiae* was the first genome of a eukaryotic organism to be sequenced (Goffeau et al. 1996), followed 6 years later by that of another yeast, *S. pombe* (Wood et al. 2002). These assemblies paved the way for the first genome-level functional analysis of the complete set of genes within a eukaryote. This was followed by the publication of the first genomes of Ascomycete fungi (*N. crassa* and *Aspergillus* sp.; Galagan et al. 2003, 2005; Machida et al. 2005) and of *Ustilago maydis*, a plant-pathogenic basidiomycete (Kämper et al. 2006). The sequencing of fungal genomes has since accelerated following the advent of high-throughput sequencing technologies, leading to an exponential accumulation of genomic data from plant-associated fungi, of many different lifestyles. To date, more than 10,000 fungal genomes are available on NCBI, including sequences of several strains within the same species and re-sequence data using different technologies. While the first genome analyses focused on one genome, the increasing data now available has opened up the possibility for comparative genomic studies within closely related species (e.g., for the *Leptosphaeria* species complex, *Zymoseptoria* sp., *Epichloë* sp. or *Ustilago* sp.; Stukenbrock et al. 2011; Laurie et al. 2012; Grandaubert et al. 2014; Treindl et al. 2021) to outstanding comparative analyses of fungal genomes from multiple lineages and host-interaction modes (e.g., Miyauchi et al. 2020). The size of fungal genomes ranges from less than 10 Mb (for the Basidiomycete *Wallemia sebi*, 9.82 Mb; Padamsee et al. 2012) to several hundred Mb (e.g., for the mycorrhizal fungus *Gigaspora rosea*, 567 Mb or for *Phakopsora pachyrhizi* (strain 1057), 43 Mb; https://mycocosm.jgi.doe.gov/Phakopsora/Phakopsora.info.html; Miyauchi et al. 2020). Finally, complete genome assemblies were recently obtained with the advent of third-generation sequencing strategies, e.g., for *F. graminearum*, *Verticillium dahliae*, *Colletotrichum higginsianum, B. cinerea*, *Epichloë festucae*. (Faino et al. 2015;

King et al. 2015; Dallery et al. 2017; Van Kan et al. 2017; Winter et al. 2018; Treindl et al. 2021). This re-sequencing allowed complete genome assemblies from telomere to telomere, shedding light on previously unidentified TE sequences, which were under-estimated in previous sequencing strategies (e.g., in the *C. higginsianum* genome, 7% is made up of TEs in the new assembly vs. 1.2% in the first assembly; or for *L. biglobosa* 'brassicae', with 17.8% of TEs in the new assembly vs. 2% in the first assembly; Dallery et al. 2017; Dutreux et al. 2018)-, or TE-rich accessory chromosomes. Besides representing a major driver of rapid evolution of effector genes (for reviews, see (Rouxel and Balesdent 2017; Möller and Stukenbrock 2017)), TEs have also been shown to play a crucial role in genome architecture and expansion of genome size. TEs are often found to be organized in clusters, compartmentalizing the genome into gene-rich regions and gene-poor TE-rich regions (e.g., in *L. maculans*, *Pseudocercospora fijiensis*, and *Epichloë festucae*; Rouxel et al. 2011; Ohm et al. 2012; Grandaubert et al. 2014; Winter et al. 2018). In contrast, in some of the larger genomes, although characterized by an expansion of TEs (e.g., *Melampsora larici-populina*, having 45% TEs, or *Blumeria graminis*, having 75% TEs), the TEs are not confined to specific locations but rather are homogeneously scattered throughout the genome (e.g., in Spanu et al. 2010; Duplessis et al. 2011a). An extreme feature of fungi, although not limited to this kingdom, is the prevalence of accessory chromosomes (also called B chromosomes or dispensable chromosomes) in their genomes (for reviews, see (Galazka and Freitag 2014; Soyer et al. 2018)). Some species harbor TE-rich accessory chromosome(s) (e.g., *L. maculans, Zymoseptoria tritici*, *Fusarium oxysporum*, *Fusarium poae*, *Magnaporthe oryzae*; Ma et al. 2010; Rouxel et al. 2011; Balesdent et al. 2013; Dhillon et al. 2014; Grandaubert et al. 2015; Vanheule et al. 2016; Peng et al. 2019). However, there is a cost associated with the maintenance of TE-rich regions and eukaryotes have different strategies to limit the spread of TEs within their genomes, including packaging of them into heterochromatin regions (Hollister and Gaut 2009).

11.3.2 Organization of the Epigenomic Landscape in Plant-Interacting Fungi

So far, most genome-wide descriptions of the epigenomic landscapes in plant-interacting fungi have been performed using Chromatin Immunoprecipitation followed by high-throughput sequencing (ChIP-seq). This technique allows precise location, throughout the genome, of histone modifications associated with euchromatin or heterochromatin (Johnson et al. 2007; Park 2009; Soyer et al. 2015a). The establishment of this technique has proven challenging, even using mycelia from axenic cultures, explaining that genome-wide studies of the chromatin structure in fungi are still sparse. ChIP-seq analyses have so far focused mostly on the location of the euchromatin modification H3K4me2/3 and of the heterochromatin modifications H3K9me3 and H3K27me3, as exemplified for the two phytopathogenic fungi, *Z. tritici* and *L. maculans* (Schotanus et al. 2015; Soyer et al. 2021). These analyses, together with numerous others, have allowed us to determine generic characteristics of the organization of the epigenome in fungi and complemented the knowledge previously gathered for the model ascomycete, *N. crassa*. However, these studies also highlight the fact that each fungus can display different specificities in its epigenomic organization. The most consistent feature regarding the distribution of these histone marks is that domains enriched in H3K4me2/3 and domains enriched in H3K9me3 are mutually exclusive in the genomes. This was shown for instance in *N. crassa*, *Fusarium fujikuroi, Z. tritici*, and *L. maculans* (Smith et al. 2008; Jamieson et al. 2013; Wiemann et al. 2013; Schotanus et al. 2015; Soyer et al. 2021). So far, and as in *N. crassa*, TE-rich regions have always been found to be associated with heterochromatin (e.g., in *Z. tritici*, *F. fujikuroi*, *L. maculans*; Wiemann et al. 2013; Schotanus et al. 2015; Soyer et al. 2021). Centromeres are enriched

with H3K9me2/3 for *N. crassa*, *F. graminearum*, *V. dahliae*, *F. fujikuroi*, and *Z. tritici* (Smith et al. 2011; Wiemann et al. 2013; Schotanus et al. 2015; Seidl et al. 2020), and are usually devoid of coding sequences, except for *Z. tritici* (Schotanus et al. 2015). Sub-telomeric regions are often found associated with both H3K9me3 and H3K27me3, overlapping with repetitive sequences, which is a classic feature that is also observed in *N. crassa*, *Z. tritici*, and *L. maculans* (Smith et al. 2008; Jamieson et al. 2013; Schotanus et al. 2015; Soyer et al. 2021). Only H3K27me3 has been shown to be located in sub-telomeric areas in *F. graminearum* and *F. fujikuroi* (Connolly et al. 2013; Niehaus et al. 2016a; Studt et al. 2016).

Along with the increased amount of ChIP-seq data, specific features of plant-associated fungi epigenomes have been brought to light. Although TE-rich regions are typically associated with heterochromatin, in *Z. tritici*, TE-rich regions are enriched in both H3K9me3 and H3K27me3 modifications (Schotanus et al. 2015). TEs of *V. dahliae* are enriched in DNA methylation, H3K9me3 or H3K27me3, although association depends on the genomic context and on the TE families considered (Cook et al. 2020). Accessory chromosomes of *F. oxysporum*, *F. fujikuroi*, *F. graminearum*, or *Fusarium asiaticum* are enriched in H3K27me3 (Connolly et al. 2013; Galazka and Freitag 2014; Niehaus et al. 2016a; Studt et al. 2016; Fokkens et al. 2018). Adaptive genomic regions of *V. dahliae* are also enriched in H3K27me3 (Cook et al. 2020). In *Z. tritici*, accessory chromosomes are associated with both H3K9me3 and H3K27me3, while in *L. maculans* they are associated only with H3K9me3 (Schotanus et al. 2015; Soyer et al. 2021). An epigenomic analysis was set up in *B. cinerea*, allowing detection of H3K9me3 and H3K27me3 in this species (Schumacher et al. 2019), but a precise location of these two histone modifications is lacking so far. As for H3K9me3 or H3K27me3, which show considerable variation among different fungi, DNA methylation is not always conserved either. For instance, it is found in *N. crassa*, *P. fijiensis*, *Ascobolus immersus*, *Coprinus cinereus*, *M. oryzae*, and *V. dahliae* (Selker and Stevens 1985; Zolan and Pukkila 1986; Goyon et al. 1996; Dhillon et al. 2010; Jeon et al. 2015; Cook et al. 2020), but is absent from some other species (e.g., *S. pombe* and *L. maculans*; Antequera et al. 1984; Bewick et al. 2019). In *Z. tritici*, DNA methylation was initially not detected (Dhillon et al. 2010), but a recent analysis has pointed out that DNA methylation was observed on TEs in isolates having a functional DIM-2 protein, and that presence/absence of DNA methylation has an impact on genome evolution of this species (Möller et al. 2021).

As for genomic analyses, the next step forward for epigenomic analyses is for comparative epigenomic analyses. This has been done in the closely related species *L. maculans* 'brassicae' and *L. maculans* 'lepidii' (Soyer et al. 2021) through ChIP-seq to map H3K4me2, H3K9me3, and H3K27me3. The comparative epigenomic analysis corroborated a previous comparative genomic analysis and previous epigenetic studies led in this species complex (Grandaubert et al. 2014; Soyer et al. 2014). Difference in terms of genome organization between *L. maculans* 'brassicae' and *L. maculans* 'lepidii' (i.e., alternation of large stretches of TEs and large gene-rich compartments in the former vs. little TE content, no bipartite organization of the genome, in the latter) is consistent with the underlying organization of the epigenomic landscape in both species. Location of H3K4me3 was analyzed and compared among different subspecies of the *Zymoseptoria* genus (i.e., *Z. tritici*, *Zymoseptoria ardabiliae*, *Zymoseptoria pseudotritici*) showing that accessory chromosomes are consistently depleted in H3K4me2 (Feurtey et al. 2020). Recent groundbreaking comparative epigenomic analyses were performed in several species of the *Fusarium* genus showing that genes conserved among the genus are not associated with H3K27me3 and that two-thirds of the genes associated with this modification are consistently found associated in the other species analyzed (Moser Tralamazza et al. 2022), as it was shown in *Z. tritici* (Soyer et al. 2019). On this basis, comparative epigenomics appear essential to understand the impact of the epigenomic

landscape on adaptation toward rapid environmental changes, modulation of interactions with the holobiont, host adaptation and specialization. This type of analysis provides an opportunity to understand the evolutionary significance of epigenetic modifications and will undoubtedly be extended to other model fungal species in the near future.

In filamentous fungi, 3D organization of the chromosomes remains poorly investigated, although development of new techniques, such as Hi-C, allows us to decipher chromosome organization in the nucleus and its impact on genes expression. Hi-C allows the mapping of the chromosome interactions within the nucleus, at a genome-wide scale (Lieberman-Aiden et al. 2009). As often is the case, this new approach was first applied to the model filamentous fungus, *N. crassa* (Galazka et al. 2016; Klocko et al. 2016) and showed that H3K9me3-rich regions physically interact. Functional analyses of the role of KMT1 and KMT6 showed that KMT1 is not involved in the 3D organization of the nucleus. In contrast, H3K27me3 domains bind to the nuclear membrane and are essential for the integrity of the 3D genome organization (Klocko et al. 2016). In *N. crassa*, inactivation of *KMT1* and loss of H3K9me3 marks result in relocation of H3K27me3 marks, which, once relocated to sites of H3K9me3, do not participate in the 3D genome organization. These results indicate a robustness of 3D genome structure that is not undermined by inactivation of key factors involved in establishing chromatin structure (Basenko et al. 2015; Galazka et al. 2016; Klocko et al. 2016). The 3D genome organization of *E. festucae*, an endophytic fungus with an isochore genomic structure, was recently described (Winter et al. 2018). Winter et al. (2018) found physical contacts between different AT-isochores and GC-isochores but very few interactions between the two types of domains. Furthermore, contacts between AT-rich regions overwhelmingly dominate (50% of all inter-chromosomal contacts), indicating that AT-isochores play a fundamental role in topologically structuring the chromosomes (Winter et al. 2018). Epigenome analyses of plant-associated fungi have defined both common features and specificities in the content and distribution of euchromatin and heterochromatin throughout the genomes. These distribution variations have consequences for genome stability, heritability of associated regions and genes, and gene expression.

11.3.3 Complex Expression Patterns of Genes Involved in Host Interactions

Accumulation of transcriptomic data from different stages of plant infection highlights how waves of expression of subsets of genes, including effector genes, occur over the course of infection, how they vary depending on the host-plant infected, and between different species or strains infecting the same host. The first transcriptomic analyses revealed that the infection cycle was underpinned by specific temporal patterns of expression of effector genes (e.g., in *M. larici-populina*, *L. maculans*, *C. higginsianum*, *M. oryzae*, *Z. tritici*; Duplessis et al. 2011b; Rouxel et al. 2011; O'Connell et al. 2012; Hacquard et al. 2012; Petre et al. 2012; Dong et al. 2015; Mirzadi Gohari et al. 2015; Rudd et al. 2015). In *C. higginsianum*, different sets of effector genes are expressed in pre-penetrating appressoria, during the early biotrophic phase and during the transition to necrotrophy (O'Connell et al. 2012), with effectors expressed during the first stages of infection favoring cell viability, while effectors expressed during necrotrophy being involved in cell death induction (Kleemann et al. 2012). Expression of specific sets of (effector) genes was also found to represent a signature of the infection structures (e.g., in *M. oryzae*, *M. larici-populina*, and *C. higginsianum*; Soanes et al. 2012; Hacquard et al. 2012; Kleemann et al. 2012). Transcriptomic analyses were also performed on fungi showing a complex lifecycle on their host. The fungus *L. maculans* exhibits a very complex lifecycle on oilseed rape, lasting several months in the field during which the fungus alternates different lifestyles on different plant organs (Rouxel and Balesdent 2005). Gene

expression analysis throughout the life cycle of *L. maculans* -under controlled conditions or in the field- on different oilseed rape organs revealed a very complex regulation of the genes involved in pathogenesis, which is far more sophisticated than initially postulated from analyses carried out during early infection of cotyledons or petioles under controlled conditions (Rouxel et al. 2011; Gervais et al. 2017; Gay et al. 2021). Eight specific clusters of genes, all enriched in effector genes, are expressed during interaction with oilseed rape and are associated with a given lifestyle and/or an infected tissue. Based on previous analyses, all *AVR* effector genes were thought to be expressed only during the early infection stages on cotyledons (Rouxel et al. 2011; Gervais et al. 2017), but Gay et al. (2021) showed that expression of these genes was finely regulated to be activated exclusively during the asymptomatic colonization stages.

Such specific expression of subsets of candidate effector genes, associated with a specific infection stage, is also observed in *Z. tritici* (Mirzadi Gohari et al. 2015; Haueisen et al. 2019). Besides expressing specific subsets of effector genes at different stages of infection, plant-associated fungi also express different sets of genes, including effectors, on different hosts (e.g., Hacquard et al. 2013; Kellner et al. 2014; Plett et al. 2015; Lorrain et al. 2018; for a review, see (Petre et al. 2020)). When comparing infection of wheat (compatible host) and *Brachypodium distachyon* (non-compatible host) by *Z. tritici*, Kellner et al. (2014) identified genes (including putative effector genes) specifically expressed during wheat infection. Due to their exceptionally elaborate life cycle (Kolmer 2013; Leonard and Szabo 2005; Chen et al. 2014), rust fungi represent a perfect framework to decipher gene expression associated with infection of taxonomically distant hosts, associated with a specific stage of the infection. A transcriptomic analysis was performed in *M. larici-populina* to highlight genes underlying specific types of spores and or hosts (Lorrain et al. 2018). Genes differentially expressed between the three stages of infection on poplar are enriched in effector genes and a comparison between poplar and larch, the alternate host on which sexual reproduction occurs, highlights that subsets of effector genes might be involved in host specialization. Species/strain-specific effector gene expression patterns were also found through a comparative transcriptomic analysis (e.g., in *L. maculans* and *L. biglobosa*, *Z. tritici*, *M. oryzae*; Lowe et al. 2014; Dong et al. 2015; Palma-Guerrero et al. 2016, 2017; Haueisen et al. 2019) or even on different host genotypes (Schurack et al. 2021). Expression of genes associated with early stages of oilseed rape infection was compared between two closely related species, the hemibiotroph *L. maculans* 'brassicae' and the necrotroph *L. biglobosa* '*canadiensis*'. Although infecting the same host and same organs, *L. maculans* 'brassicae' expresses a large number of genes with no identifiable domains, many of them encoding (putative) effectors, while *L. biglobosa* 'canadensis' expresses cell wall-degrading Carbohydrate-Active enZymes (CAZmes) (Lowe et al. 2014). Comparative transcriptomic analyses of wheat infection by several *Z. tritici* strains differing in virulence revealed strain-specific regulation of putative effectors, proteases and cell wall-degrading enzymes, suggesting that differences in gene expression could be a major determinant of virulence variation among *Z. tritici* strains (Palma-Guerrero et al. 2016, 2017; Haueisen et al. 2019). Sophisticated spatio-temporal or host-specific expression of effector genes indicates that the fine-tuned regulation of these genes is crucial for the successful outcome of plant infection. A combined analysis of epigenomic and transcriptomic data will help decipher the impact of the fungal epigenome on the regulation of gene expression.

11.3.4 Lessons from Combined Analysis of Genomic, Transcriptomic, and Epigenomic Data

Heterochromatin is subdivided into two states: constitutive and facultative. Constitutive heterochromatin is a permanent structure, found in all cell types and tissues, at centromeres, telomeres,

and TEs and, as such, is involved in genome integrity and stability and ensures proper chromosome segregation. In contrast to constitutive heterochromatin, facultative heterochromatin defines genomic regions in the nucleus of a eukaryotic cell that have the opportunity to adopt either an open or a compact conformation depending on the temporal and spatial context (Trojer and Reinberg 2007). However, accepted dogma regarding conventional definitions of facultative or constitutive heterochromatin seems not to be valid in all plant-associated fungi. While histone modifications H3K9me3 and H3K27me3 are both a signature of heterochromatin, H3K9me3 is considered to be a typical mark for constitutive heterochromatin, as it is associated with repeats and involved in genome stability, whereas H3K27me3 is associated with facultative heterochromatin but can easily be reversed toward a euchromatin state under some stresses from the environment. As H3K9me3 is found associated with TE-rich regions, the proportion of H3K9me3 in a genome generally reflects the TE content, as described for *Z. tritici* (Schotanus et al. 2015). This is also the case for *L. maculans* 'brassicae' and *L. maculans* 'lepidii'. In *L. maculans* 'brassicae', which have >30% TEs, 33% of the genome is associated with H3K9me3, while the genome of *L. maculans* 'lepidii', having a low TE content, has a low enrichment in H3K9me3 (4% of H3K9me3 in its genome). The unique dispensable chromosome of *L. maculans* 'brassicae' is mostly comprised of repetitive DNA and 90% of this chromosome is associated with H3K9me3 (Rouxel et al. 2011; Balesdent et al. 2013; Soyer et al. 2021). Co-location of H3K9me3 with TEs supports the "constitutive" nature of this modification, together with the fact that H3K9me3-domains encompass a small number of genes (as in *L. maculans* 'brassicae' or *L. maculans* 'lepidii', with 70 and 104 genes, respectively, or in *Z. tritici* with 86 genes located within H3K9me3-domains; Schotanus et al. 2015; Soyer et al. 2019; Soyer et al. 2021). Genes associated with H3K9me3 are almost all located in the middle of repetitive elements, in sub-telomeric areas, or very close to the edge of regions enriched in repetitive elements. This observation is in accordance with the fact that TEs are targeted by H3K9me3 which might leak to neighboring genes. Nevertheless, some of these genes are heavily transcribed upon host interaction (Chujo and Scott 2014; de Jonge et al. 2013; Schotanus et al. 2015; Soyer et al. 2014, 2019, 2021; Winter et al. 2018; Gay et al. 2021). This does not match the "constitutive" nature of H3K9me3 modification. In contrast to H3K9me3 domains, H3K27me3 domains encompass a high proportion of predicted genes, with nearly 20% of the genes in both *L. maculans* subspecies and in *Z. tritici* and 30% of the genome for *F. graminearum* or *F. fujikuroi* (Connolly et al. 2013; Wiemann et al. 2013; Schotanus et al. 2015; Niehaus et al. 2016a; Studt et al. 2016; Soyer et al. 2019; Soyer et al. 2021). This is consistent with the "facultative" nature of this histone modification. Nevertheless, some evidence supports an important role for this modification in genome organization and stability. For instance, dispensable chromosomes of *Z. tritici* are twice as rich in TEs as core chromosomes, but no significant enrichment of these chromosomes in H3K9me3 has been identified, and they are entirely covered by H3K27me3 (Schotanus et al. 2015). Besides, the loss of H3K27me3 in *Z. tritici* increases the stability of some accessory chromosomes (Möller et al. 2019). These features seem to indicate that although H3K27me3 is an important regulator of gene expression involved in the development or response to various stresses, it might also play a role in the stability of some parts of fungal genomes.

An additional effect of genes located in TE-rich regions or accessory chromosomes is their poor expression during axenic growth (Rouxel et al. 2011; Dallery et al. 2017; Haueisen et al. 2019; Gay et al. 2021). Interestingly, in many cases, genes associated with TE-rich regions can be upregulated upon host infection (e.g., Rouxel et al. 2011; de Jonge et al. 2013; Chujo and Scott 2014; Dallery et al. 2017; Kombrink et al. 2017; Winter et al. 2018; Soyer et al. 2021; Gay et al. 2021). The upregulated TE-associated genes include pathogenicity-related genes such as effector genes or secondary

metabolite gene clusters. Heterochromatin is often found outside of TE-rich regions but is frequently associated with genes involved in interaction with the host, including genes encoding effectors or secondary metabolites (Connolly et al. 2013; Wiemann et al. 2013; Chujo and Scott 2014; Schotanus et al. 2015; Studt et al. 2016; Niehaus et al. 2016a; Fokkens et al. 2018; Soyer et al. 2019; Gay et al. 2021; Soyer et al. 2021; Zhang et al. 2021). In *L. maculans*, a combined analysis of ChIP-seq data together with RNA-seq data generated from samples taken throughout the lifecycle of *L. maculans* on its host showed that H3K9me3 and H3K27me3 domains are significantly enriched in (predicted) effector genes and in genes upregulated *in planta* (Gay et al. 2021). Importantly, all characterized *AVR* genes of *L. maculans* are associated with H3K9me3 domains during axenic growth (Gay et al. 2021). Likewise, in *M. oryzae* and *F. graminearum*, genes located in H3K27me3 domains are enriched in genes upregulated upon infection (Zhang et al. 2021; Moser Tralamazza et al. 2022). Moreover, in both species, genes not associated with H3K27me3 or associated with H3K4me2 during axenic growth show the same expression pattern between *in vitro* and *in planta* conditions (Zhang et al. 2021; Moser Tralamazza et al. 2022). Similarly, for *L. maculans*, the well-characterized *AVR* genes of *M. oryzae* are enriched in heterochromatin, although this is the H3K27me3-heterochromatin type (Zhang et al. 2021). In *V. dahliae*, subsets of genes differentially expressed between *in planta* and axenic growth conditions were associated with H3K27me3. The authors hypothesized that specific expression of genes during host infection might be under epigenetic control (Kramer et al. 2022). Additional genome-wide histone maps together with transcriptomic studies *in vitro* and *in planta* will allow us to determine whether an association of effector genes not only with TEs, but also with different histone modifications, is a general feature of plant-associated fungi and could potentially be involved in their regulation. Taken together, the combined analysis of transcriptomic and epigenomic data supports the hypothesis that genes involved in plant interactions are in distinct genomic regions that share common epigenomic characteristics and therefore represent an efficient way to concertedly regulate their expression (Soyer et al. 2015b).

11.4 Chromatin-based Regulation of Effector Gene Expression Combined or not to the Action of Specific Transcription Factors

The localization of effector genes in dynamic regions of fungal genomes, associated with heterochromatin domains, as well as the fine control of their expression during infection, and a global repression of expression during axenic growth, suggest that the expression of these effector genes may be under chromatin control and may or may not be associated with the action of specific TFs. Thus, several pioneering studies have been carried out in recent years to address this question using complementary strategies: (i) by moving effector genes from heterochromatin to euchromatin regions, (ii) by inactivating key genes that encode proteins involved in chromatin remodeling, and (iii) by inactivating or overexpressing TF encoding genes.

11.4.1 Effect of a Change in Genomic Context on the Expression of Effector Genes

The effect of genomic and epigenomic context on gene expression has, historically, been particularly well documented in *Drosophila melanogaster*. This mechanism, called Position-effect variegation (PEV), leads to the silencing of genes normally located in a euchromatin region that are found in the proximity of a heterochromatin region after rearrangement or transposition. This mechanism is notably dependent on HP1 and KMT1 (for a review, see (Elgin and Reuter 2013)). In fungi, the effect of a change in genomic/epigenomic context on effector gene expression has been studied in a reverse manner, i.e., by

moving effector genes from a heterochromatin to a euchromatin domain, in the plant-pathogenic fungi *L. maculans* and *Z. tritici* (Soyer et al. 2014; Meile et al. 2020).

As previously mentioned, *L. maculans* exhibits a compartmentalized genome structure, with alternating GC- and AT- repeat-rich regions (Rouxel et al. 2011). The latter are enriched in H3K9me3 heterochromatin domains (Soyer et al. 2021). While gene-poor (only 5% of the predicted genes), AT-rich regions are significantly enriched in putative effector genes (20% of the genes in this genomic environment against 4.2% of the genes in gene-rich regions), which are mainly expressed during plant infection and repressed during axenic growth (Rouxel et al. 2011; Gay et al. 2021). These observations led to the hypothesis that the chromatin structure in AT-rich regions could influence the expression of the associated genes. Soyer et al. (2014) developed a strategy aimed at determining the influence of the AT-rich regions on effector gene expression. Four effector genes were moved from AT- to GC-rich regions by *Agrobacterium tumefaciens*-mediated transformation, and expression of these genes was analyzed by qRT-PCR. The transfer of these effector genes from AT- to GC-rich regions strongly increased their expression in axenic culture, demonstrating that AT-rich regions repressed the expression of these genes. In contrast, these effector genes were upregulated during plant infection whatever their genomic context, suggesting that at that developmental stage, repression due to the AT-rich regions was released.

In *Z. tritici*, Meile et al. (2020) proposed an elegant strategy to study the effect of both the genomic context and the presence of a native promoter on the expression level of effector genes and on the spatio-temporal pattern of expression. They studied effector genes located in a heterochromatin context during axenic growth and moved these effector genes under the control of their native promoter or of a constitutive promoter into a euchromatin genomic environment, fusing them either with fluorochromes or with a selection marker. These experiments showed that changing the genomic environment from heterochromatin to euchromatin allowed a higher expression of these genes under axenic growth conditions, and thus, as in the study by Soyer et al. (2014), showed that the heterochromatin environment repressed the expression of these effector genes during axenic growth. Also, this repression was shown to be independent of the promoter (the same effect was observed in the presence of a native or a constitutive promoter). In contrast, *in planta*, the expression of effector genes was derepressed regardless of the genomic context, although the change in genomic context modified the spatio-temporal pattern of expression of these effector genes. Indeed, in the case of localization of effector genes in their native context, Meile et al. (2020) observed an induction of expression only in fungal cells near the host penetration sites and inside the leaf, and not in cells located on the leaf surface. In contrast, when the genomic environment was changed, a global overexpression of effector genes in all the fungal hyphae was observed.

Taken together, these data show that the genomic environment in which effector genes are located controls their expression during axenic growth, but that control is released during plant infection. Nevertheless, the *in planta* expression pattern of effector genes is not dependent on the genomic context. This suggests that although chromatin-based repression needs to be released to allow for the expression of effector genes, other factors might be involved to ensure their concerted expression *in planta*.

11.4.2 Role of Proteins Involved in Chromatin Remodeling on the Control of Effector Gene Expression

The role of chromatin remodeling on the control of gene expression in plant-pathogenic fungi has been mainly investigated through the inactivation of key players involved in the establishment of heterochromatin, notably KMT1 and KMT6. For example, in *F. graminearum*, secondary metabolism gene clusters were found to be enriched in the H3K27me3 mark and inactivation of *KMT6*

led to overexpression of a large number of gene clusters in axenic culture (Connolly et al. 2013). Similarly, in *F. fujikuroi*, inactivation of *KMT6* led to a decrease of the H3K27me3 modification that was associated with the induction of several secondary metabolism gene clusters and the production of secondary metabolites (Studt et al. 2016; Niehaus et al. 2016b). In *E. festucae*, an endophyte that grows in the apoplast of *Lolium perenne* to establish a mutually beneficial association, an epigenetic mechanism regulates the expression of at least two biosynthetic SM clusters, i.e., those for lolitrems (*ltm*) and ergot alkaloids (*eas*). These two gene clusters are located close to AT- and repeat-rich regions, and are upregulated during host colonization. To decipher the role of the chromatin context on their regulation, Chujo and Scott (2014) generated knock-out mutants for *clrD* (*KMT1*) and *ezhB* (*KMT6*), and subsequently analyzed the expression of the *ltm* and *eas* genes together with the presence of H3K9me3 and H3K27me3 at these loci both *in vitro* and *in planta*, via ChIP-qPCR. Deletion of *clrD* and *ezhB* resulted in the induction, at least in part, of the genes located in these two clusters. In addition, both mutants had altered symbiotic interaction phenotypes with *L. perenne*. Moreover, derepression *in planta* of *ltm* and *eas* was found to be associated with a decrease of both H3K9me3 and H3K27me3 marks in the promoters of these cluster genes.

The first demonstration of a chromatin-based control of proteinaceous effector gene expression was done in *L. maculans* by Soyer et al. (2014). Expression of two genes encoding proteins involved in heterochromatin establishment was silenced using RNAi and a global transcriptomic analysis was performed on the silenced mutants using oligoarrays. RNAi silencing of *LmDIM5* (*KMT1*) and *LmHP1* allowed derepression, during axenic growth, of 3% of the genes located in GC-rich regions compared with more than 30% of the genes located in AT-rich regions, and specifically effector genes. Moreover, genes derepressed in both transformants were those that were naturally upregulated, in the wild-type (WT) strain, during primary infection of oilseed rape. ChIP-qPCR analyses showed that overexpression of at least two effector genes was associated with a decrease of the repressive histone modification H3K9me3 in the genomic environment of these genes (Soyer et al. 2014). However, the level of derepression of effector genes obtained during axenic growth in the *LmDIM5* and *LmHP1* mutants did not reach the level of *in planta* expression. This suggests that other components, such as specific TF(s), could be involved in effector gene induction *in planta*. Moreover, the silenced transformants obtained by Soyer et al. (2014) still expressed ~20% of *KMT1* which did not allow the authors to fully decipher the effects of a complete lack of H3K9me3 on the chromatin structure and gene expression. CRISPR-Cas9 technology was recently established for *L. maculans* to generate knock-out mutants (Idnurm et al. 2017), and this technology used to inactivate *KMT1* (Clairet et al. 2021b). Mutants inactivated for *KMT1* displayed reduced virulence on oilseed rape but normal growth and conidiation in culture. Clairet et al. (2021b) found that inactivation of *KMT1* had a significant impact on effector gene expression, but that only one *AVR* gene and three genes located in H3K9me3 domains were upregulated during axenic growth. In contrast, inactivation of *KMT1* had a significant effect on genes located in H3K27me3 domains. Clairet et al. (2021b) hypothesized that complete inactivation of *KMT1* led to H3K27me3 relocation at native H3K9me3 domains, as previously reported for *N. crassa* and *Z. tritici* (Basenko et al. 2015; Möller et al. 2019), thereby explaining why complete removal of H3K9me3 did not induce a global overexpression of genes located in repeat-rich regions.

Zhang et al. (2021) addressed the role of H3K27me3 in *M. oryzae* effector regulation both *in vitro* and *in planta*. Using ChIP-qPCR during plant infection, they found the H3K27me3 marks were replaced by H3K27ac, notably in regions containing genes overexpressed *in planta*, resulting in increased transcription. H3K27me3 covered half of the annotated TEs, while H3K27ac was almost exclusively associated with coding regions. They generated a *kmt6* mutant and found that most of

the genes overexpressed in the mutant (~88%) were covered in the WT by H3K27me3. They also inactivated *GCN5*, which encodes the acetyltransferase responsible for H3K27 acetylation and made a *kmt6/gcn5* double mutant. Unlike the two single mutants, the double mutant was affected in growth, conidia morphology and infection of rice. In addition, the H3K27me3 and H3K27Ac modifications were completely absent and genes that were upregulated in the *kmt6* mutant were not upregulated in the double mutant due to the absence of H3K27Ac. Kramer et al. (2022) found that the inactivation of *KMT6* in *V. dahliae* preferentially induced the expression of genes located in H3K27me3 regions during axenic growth. However, while H3K27me3 is associated with gene repression, activation of genes located in an H3K27me3 context is not always associated with the removal of that heterochromatin mark.

In a reverse and complementary approach, several analyses have studied the effect of inactivation of proteins involved in the establishment of euchromatin marks (H3K4me2/3, H3K36me2/3). Janevska et al. (2018) investigated the role of two H3K36me3 histone methyltransferases, SET2 and ASH1, in *F. fujikuroi*. SET2 is responsible for H3K36me3 deposition in euchromatin regions of the genome, whereas ASH1 is responsible for H3K36 methylation in sub-telomeric regions. Inactivation of *ASH1* led to an increased presence of H3K27me3 in sub-telomeric regions and increased chromosomal instability. Furthermore, loss of the H3K36me3 marks after inactivation of *SET2* or *ASH1* led to growth and sporulation defects, induction of secondary metabolite synthesis and reduced pathogenicity. Zhou et al. (2021) investigated the role of the H3K4me3 euchromatin mark through the functional analysis of several members of the COMPASS-like complex in *M. oryzae*. Inactivation of these components leads to defects in fungal development and pathogenicity. Similarly, in *C. higginsianum*, inactivation of CCLA, a sub-unit of the COMPASS complex involved in H3K4 trimethylation, led to reduced mycelial growth, sporulation, and pathogenicity, but also to an enriched production of secondary metabolites, including several terpenoid compounds (Dallery et al. 2019). Lukito et al. (2019) also investigated the role of CclA on the control of the sub-telomeric gene clusters *IDT* and *EAS* in *E. festucae*. Inactivation of *cclA* led to the activation of *IDT* and *EAS* gene clusters in axenic culture. Inactivation of *kdmB*, the demethylase responsible for H3K4me3 removal, decreased *in planta* expression of *IDT* and *EAS* gene clusters. However, both *cclA* and *kdmB* mutants were still able to establish a symbiotic interaction with their host plant. In contrast, the inactivation of *setB* (*set2*) led to hyphal growth defects similar to the ones observed for the *clrD* (*kmt1)* mutant, with both mutants being unable to infect *L. perenne* (Lukito et al. 2021). Transcriptomic analysis at an early stage of plant colonization showed that many effector genes, overexpressed *in planta* in the WT strain, are down-regulated both in *setB* and *clrD* mutants.

Thus, while various key players of chromatin remodeling are involved in controlling the expression of effector genes and secondary metabolism gene clusters, much remains to be discovered regarding the way this control can be released and identification of the "actors" (chromatin readers, specific TFs, etc.) that could be involved.

11.4.3 First Evidence of Dual Control for the Expression of Effector Genes by Specific Transcription Factors and Chromatin Remodelers

Changes in the genomic context and the manipulation of proteins involved in chromatin remodeling have revealed a repressive role during axenic growth of the heterochromatin regions in which some of the fungal effector genes are located. This repression is lifted *in planta* allowing for a specific expression pattern of effector genes throughout infection. However, these experiments also showed that the release of the chromatin-based control of expression was not sufficient to reach the level of induction of effector gene expression *in planta*, suggesting the

involvement of other partners, such as specific TF (s).

Only a few TFs affecting effector gene expression have been identified in fungi so far (for reviews, see (Tan and Oliver 2017; John et al. 2021)). Most is known about the role of Sge1/Ros1 orthologues in the regulation of effector gene expression. Sge1 was first described in *F. oxysporum* f. sp. *lycopersici* as a positive regulator of effector gene expression (Michielse et al. 2009). Since then, Sge1 has been found to function as a master regulator of effector genes in *V. dahliae, Z. tritici*, *B. cinerea*, and *Fulvia fulva* (Michielse et al. 2011; Santhanam and Thomma 2013; Mirzadi Gohari et al. 2013; Okmen et al. 2014). In *U. maydis*, Sge1 negatively regulates the expression of effector genes associated with biotrophic development and positively regulates effector gene expression associated with late infection, indicating that Sge1 is a master regulator of the infection process in *U. maydis* (Tollot et al. 2016). In *F. oxysporum* f. sp. *lycopersici*, Sge1 was able to regulate the expression of effector genes located on an accessory chromosome independently of chromatin-remodeling (van der Does et al. 2016). In *Alternaria brassicicola*, a zinc cluster- TF specific to Pleosporales, AbPf2, regulates the expression of 33 genes encoding secreted proteins, including eight putative effectors (Cho et al. 2013). In *Parastagonospora nodorum*, the AbPf2 orthologue PnPf2 positively regulates two genes encoding necrotrophic effectors, *SnToxA* and *SnTox3*, and the orthologue of *SnToxA*, *ToxA*, is regulated by PtrPf2 in *Pyrenophora tritici-repentis* (Rybak et al. 2017). In *P. nodorum*, a recent transcriptomic analysis comparing the WT and the *PnPf2* mutant during axenic culture and infection of wheat revealed involvement of *PnPf2* in the regulation of twelve effector genes and of genes associated with plant cell wall degradation and nutrient assimilation (Jones et al. 2019). In *Z. tritici*, the Pf2 orthologue was found to be essential for virulence, but also regulates dimorphic switching, axenic growth, fungal cell wall composition, and carbon-sensing pathways (Habig et al. 2020). Although Pf2 was demonstrated to be a positive regulator of effector gene expression, no investigation of a link between the action of a histone-modifying enzyme and Pf2 was performed.

In order to test the hypothesis that effector genes would be under multiple level control of chromatin-based regulation and of specific TF(s), Clairet et al. (2021b) combined functional analyses on *KMT1* and the TF *Pf2* in *L. maculans*. *LmPf2* has an expression profile similar to that of the *L. maculans AVR* genes and effector genes expressed during the asymptomatic phases of infection, while expression of *KMT1* is inversely correlated to their expression during axenic growth, cotyledon and petiole infection. To investigate further CRISPR-Cas9 inactivated mutants of *KMT1* and *LmPf2*, together with strains overexpressing *LmPf2* were generated, either in a WT background or in a *kmt1* mutant background. No major defect in conidia production, growth rate or morphology was associated with the inactivation of *LmPf2*. In contrast, inactivation of *LmPf2* had a major effect on pathogenicity since the mutants were unable to invade the cotyledon further than the inoculation site, did not induce any visible symptoms on the host, and were highly impaired in effector gene expression *in planta*. In contrast, overexpression of *LmPf2* in a *kmt1* mutant background significantly induced the expression of effector genes, including eight *AVR* genes (the upregulation being much higher than when *LmPf2* was overexpressed in a WT background) and of genes associated with heterochromatin. In conclusion, in *L. maculans*, there is a major effect of the chromatin-context on the ability of LmPf2 to regulate effector gene expression. Whether that model of double control of effector gene expression involving a specific TF and a histone-modifying protein could be generalized to other plant-pathogenic fungi or if it is specific to *L. maculans* needs to be further investigated.

11.5 Future Challenges Concerning the Chromatin-Based Control of Plant-Associated Genes

The discovery of the central role of chromatin structure as a regulator of plant-associated genes in fungi has deepened our understanding of how plant colonization takes place. Identifying the molecular determinants underlying this epigenetic regulation may eventually provide us with generic means to sustainably control fungal pathogens. However, much remains to be discovered, notably regarding the environmental/plant signals allowing targeted chromatin remodeling at the onset of plant colonization and chromatin dynamics during colonization, as well as the possible interplay between epigenetic mechanisms and the action of TFs.

Location of subsets of effector genes specifically expressed during infection in TE-rich heterochromatin domains, or gene-rich H3K27me3 domains, together with accumulation of functional analyses support the hypothesis that expression of effector genes is governed by an epigenetic control that ensures repression of these genes during axenic growth and expression during host infection. The environmental or host signals that induce the release of the epigenetic control and the expression of effector genes at the early stages of infection remain unknown. To our knowledge, the signals emitted by the host plants that control these pathogenicity-related mechanisms have only been studied in root-colonizing oomycetes and fungi and no study has been reported for leaf-colonizing fungi (Morris et al. 1998; Akiyama et al. 2005; Turrà et al. 2015). Morris et al. (1998) reported that the oomycete *Phytophthora sojae* grows toward daidzein and genistein, two isoflavones secreted from the soybean roots, while Akiyama et al. (2005) showed that symbiotic arbuscular-mycorrhizal fungi were attracted by strigolactones, plant hormones that are secreted from *Lonicera japonica* roots. Turrà et al. (2015) identified peroxidases secreted from tomato roots as the compounds that trigger attraction in *F. oxsyporum*. Identification of the host signals that mediate the interaction between plant and leaf-colonizing fungi has been hampered by the lack of reliable quantitative assays measuring a pathogenesis-related trait/aspect such as directed hyphal growth for root-colonizing fungi. Meyer et al. 2017) investigated environmental stimuli triggering effector gene expression in *L. maculans*. Among different biotic and abiotic factors tested, antibiotics such as cycloheximide, an antifungal compound that inhibits protein synthesis, increased the expression of several effector genes when used at concentrations ten times lower than that needed to block the ribosome machinery. Physical and chemical stimuli, such as pH, osmolarity, carbon and ammonium sources, and temperature also influenced, but to a lesser extent, the expression of effector genes. In contrast, no plant leaf signals influencing effector gene expression have so far been identified. The identification of host signals that trigger changes in chromatin status and the induction of gene expression is of major scientific and applied importance since it would provide us with new, non-chemical strategies to control leaf-colonizing fungi, e.g., by allowing the breeding of plant genotypes that would be less favorable to the expression of effectors genes.

While the importance of histone modifications in the regulation of the expression of a few effectors or secondary metabolite gene clusters has been demonstrated by ChIP-qPCR *in planta* (Chujo and Scott 2014; Soyer et al. 2019; Meile et al. 2020; Zhang et al. 2021), a complete view of the *in planta* chromatin dynamics of a plant-associated fungus has not been generated yet, mainly because of the technical difficulty. The first *in planta* demonstration of a decrease of histone modifications associated with heterochromatin and underlying expression of genes involved in host colonization was performed in the endophytic fungus *E. festucae* (Chujo and Scott 2014). Using ChIP-qPCR, they assessed enrichment of H3K9me3 and H3K27me3 for a few genomic loci of secondary metabolite gene clusters and compared this level between axenic culture and host colonization. This analysis showed that local decrease of H3K9me3 and/or H3K27me3 was associated with the expression of

genes during host infection (Chujo and Scott 2014). The same strategy was developed for three putative effector genes in *Z. tritici* during infection of wheat leaves, showing that their high expression at 13 dpi (corresponding to the switch from asymptomatic growth to necrotrophy for *Z. tritici*) compared to axenic culture was associated with a decrease in the level of H3K9me3 and/or H3K27me3 in their genomic environment (Soyer et al. 2019), as also observed for other effector genes of *Z. tritici* by Meile et al. (2020). Similar observations were made in *M. oryzae* (Zhang et al. 2021). Altogether, these analyses of distantly related fungi, displaying various interaction modes with their host confirmed that, during host interaction, specific induction of secondary metabolites/effector genes, located within heterochromatin domains during axenic growth, is associated with a dynamic re-organization of the histone modifications at their loci. The next challenge to be tackled now is to assess the chromatin dynamics *in planta* to fully decipher the influence of histone modifications in the regulation of (effector) gene expression. So far, and as discussed before, genome-wide scale epigenomic analyses of plant-interacting fungi have only been performed during axenic growth. This lack of a global comprehensive view of the chromatin dynamics of the fungal genome during plant interaction is currently due to technical limitations. Indeed, the success of performing such epigenomic analyses is hampered by the low amount of fungal biomass during plant infection combined with the fact that fungal genomes are usually small compared to the large size of the host genomes. The next step to move forward would be to analyze the fungal epigenomic dynamics during host interaction through deep-sequencing or by developing biochemical approaches to isolate the fungus from infected tissues and conduct the necessary epigenomic analysis approaches.

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12 Toward Understanding the Role of Chromatin in Secondary Metabolite Gene Regulation in the Rice Pathogen *Fusarium fujikuroi*

Lena Studt

Abstract

Fusarium fujikuroi (teleomorph *Gibberella fujikuroi*) is a notorious rice pathogen and the causative agent of the *bakanae* (foolish seedling) disease. The symptoms of this disease, i.e., thin, chlorotic, and hyper-elongated rice internodes and often sterile grains, are due to the ability of this fungus to produce and secrete the phytohormones gibberellins (GAs). In addition to the GAs, *F. fujikuroi* produces a plethora of other secondary metabolites (SMs), including pigments but also potent mycotoxins. SMs are not essential for growth but may pose a selective advantage under certain environmental conditions, and as such are regulated by a complex gene regulatory network. The genes involved in the biosynthesis of individual SMs are physically linked in the fungal genome, thereby facilitating a coordinated and timely response when needed. Several of these regulatory components have been studied in *F. fujikuroi*. These include signal transduction pathways as well as narrow-domain and wide-domain transcriptional regulators. Another regulatory layer that has emerged as a key player in fungal SM gene regulation is chromatin structure. This chapter summarizes the current knowledge on SM biosynthesis in *F. fujikuroi* and the regulatory mechanisms that control their expression with a focus on chromatin structure.

Keywords

Fusarium fujikuroi · Secondary metabolite · Mycotoxin · Chromatin · Gene regulation · Histone modification

12.1 Introduction

Fusarium is a species-rich group of mycotoxigenic plant pathogens and ranks as one of the most economically important fungal genera in the world (Aoki et al. 2014). This genus comprises at least 300 phylogenetically distinct species (O'Donnell et al. 2015) that collectively cause agriculturally significant diseases on virtually all crop plants (Leslie and Summerell 2006). Next to infection, fusaria are well-known to produce a broad range of low molecular-weight compounds, or secondary metabolites (SMs), including potent toxins that may accumulate during infection (O'Donnell et al. 2018), thereby contaminating food and feed. *Fusarium*-induced crop diseases and mycotoxin contamination cost the global agricultural economy multi-billion euro losses each year (O'Donnell et al. 2015).

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The *Fusarium fujikuroi* species complex (FFSC) comprises important members of these notorious plant pathogens (Niehaus et al. 2016a). *F. fujikuroi* Nirenberg (teleomorph *Gibberella fujikuroi*), the founding member of the FFSC, is a heterothallic ascomycete (Nirenberg and O'Donnell 1998; Leslie and Summerell 2006). *F. fujikuroi* is a rice pathogen and was first isolated as the causal agent of the so-called *bakanae* (foolish seedling) disease (Hori 1890). Since then, the fungus has gained considerable attention as *bakanae* is one of the most important seed-borne diseases and constitutes a major threat to rice-growing countries worldwide. Its occurrence has increased in recent years due to environmentally-friendly rice cultivation (Jeon et al. 2013). Though the preferred host plant of *F. fujikuroi* is rice, it has also been isolated from other crop plants with some frequency (Leslie et al. 2004; Carter et al. 2008; Chiara et al. 2015; Pedrozo et al. 2015; Bolton et al. 2016). The most characteristic symptoms of *bakanae* are thin, chlorotic, and hyper-elongated rice internodes, as well as sterile or empty grains of fully-grown plants (Sun and Snyder 1981; Bömke and Tudzynski 2009). These symptoms are caused by the ability of the fungus to produce gibberellins (GAs), bioactive phytohormones that are secreted by the fungus during host infection (Yabuta and Hayashi 1939). This biochemical characteristic stimulated the exploitation of *F. fujikuroi* for commercial GA production worldwide. In addition to GAs, *F. fujikuroi* produces several other SMs, including the pigments neurosporaxanthin, bikaverin and fusarubins but also harmful mycotoxins such as fumonisins, fusarins, gibepyrones and fusaric acid (Wiemann et al. 2013; Niehaus et al. 2017a). Generally, the genes involved in the biosynthesis of single SMs are physically linked in the genome (Keller and Hohn 1997), thereby facilitating coordinated expression. Several of the *F. fujikuroi* biosynthetic gene clusters (BGCs) have been functionally characterized. This knowledge has not only allowed us to link known or novel SMs to their respective BGCs but also enabled us to study their regulation at the transcriptional level.

Generally, SMs are not essential for fungal growth, but often display biologic activities that may provide a selective advantage for the producing organism under certain environmental conditions, and as such may very well become essential for fungal survival (e.g., Pusztahelyi et al. 2015; Macheleidt et al. 2016; Keller 2019). Due to these specialized functions, the genes involved in their biosynthesis are often silent under standard laboratory conditions and only induced upon specific stimuli mimicking natural conditions in which biosynthesis of the respective SM becomes beneficial for the producing organism. This observation implies that the expression of these genes is controlled by complex regulatory circuits that ensure a coordinated and timely response to these stimuli. Several components of these gene networks have been studied in *F. fujikuroi*. These include signaling cascades as well as narrow-domain and broad-domain transcription factors (TFs). In addition, another level of regulation based on chromatin structure has emerged as a key player in fungal secondary metabolism. This chapter summarizes our current knowledge on SM biosynthesis in *F. fujikuroi* and provides an overview of the regulatory network with a focus on chromatin structure.

12.2 Secondary Metabolism in *F. fujikuroi*: The Road So Far

12.2.1 Gene Architecture and Cluster Organization

Fungal SMs comprise a vast collection of low molecular-weight compounds with an enormous chemical diversity. SMs are synthesized from only a few building blocks such as acetyl-CoA and/or amino acids originating from primary metabolism, and usually belong to one of the four chemical families: polyketides, non-ribosomal peptides, terpenoids, and indole alkaloids, named according to the key enzyme involved in their synthesis. The biosynthesis starts with the assembly of the structural backbone by polymerization of a precise number of

building blocks typically orchestrated by the so-called key signature enzyme, i.e., polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS), terpene cyclase (TC), or dimethylallyltryptophan synthase (DMATS) (Mosunova et al. 2021). Hybrids of two different chemical classes also exist. In this case, the two enzymes involved are either encoded within one gene (hybrid) or appear as two distinct key enzyme-encoding genes. Two prominent examples are Fus1 (PKS-NRPS10) or Fub1 and Fub8 (PKS6 and NRPS34) involved in fusarin and fusaric acid biosynthesis, respectively (Niehaus et al. 2013, 2014b; Studt et al. 2016a). Further modifications of the carbon backbone are carried out by additional enzymes that attach functional chemical groups to the SM backbone. The genes involved in the biosynthesis of a single SM are often arranged in a contiguous BGC and may encode additional accessory enzymes such as a pathway-specific TF that positively regulates genes within the BGC, or enzymes involved in the transport or the detoxification of the SM generated (Keller 2019).

Advances in genome sequencing combined with algorithms involved in mining SM genes have shown that fungi have the genetic capacity to produce a much higher number of SMs than previously anticipated (Keller 2019). This also holds true for *F. fujikuroi*. Before the genome sequence of the *F. fujikuroi* reference strain IMI58289 had been published (Wiemann et al. 2013), only a handful of SMs were known to be produced by this fungus and even fewer had been connected to their respective BGCs. Those characterized included the seven-gene cluster involved in the biosynthesis of GAs (Bömke and Tudzynski 2009), the six-gene cluster (*BIK1-BIK6*) involved in the formation of the pigment bikaverin (Linnemannstöns et al. 2002b; Wiemann et al. 2009), and the four genes involved in neurosporaxanthin biosynthesis (Linnemannstöns et al. 2002a). The *F. fujikuroi* genome sequence not only allowed researchers to link several additional known SMs to their respective BGCs but also revealed the great potential of *F. fujikuroi* to produce many more SMs, many of which were cryptic (Wiemann et al. 2013). Overall, 48 key signature enzyme-encoding genes have been identified in *F. fujikuroi* IMI58289 comprising 16 NRPSs, 14 PKSs, 4 PKS-NRPS hybrids, 12 TCs and 2 DMATSs. Recently, the genomes of eight additional naturally occurring *F. fujikuroi* isolates from distant geographic regions have been published (Niehaus et al. 2017a). Interestingly, they all differ in the size of their chromosomes, the type of asexual spores they produce (macro and/or microconidia), and the number of BGCs present in their genomes, ranging from 41 to 53 (Niehaus et al. 2017a). Some BGCs are conserved among the different *F. fujikuroi* isolates and also in most fusaria *i.e.*, genes involved in the biosynthesis of fusarubins (Studt et al. 2012), fusahexin (Westphal et al. 2021) or the two siderophores ferricrocin and ferrichrome (Varga et al. 2005; Tobiasen et al. 2006), while others appear to be unique to just some fungal isolates. One marked example is the *F. fujikuroi* isolate B14 which differs from the other isolates by the presence of three additional PKS-encoding genes, i.e., *PKS40*, *PKS43*, *PKS51*, while the BGC involved in the formation of apicidin F, *APF1-APF12* (*APF10* is missing in *F. fujikuroi*), is absent (von Bargen et al. 2013; Niehaus et al. 2014a, 2017a). Notably, the BGC involved in apicidin F biosynthesis is also absent from the genome of *F. fujikuroi* KSU X-10626 (Chiara et al. 2015). It is intriguing to speculate that gain or loss of certain BGCs is connected with the lifestyle and the specific requirements of the different *F. fujikuroi* isolates. Overall, 56 key enzyme-encoding genes were identified in the *F. fujikuroi* isolates, giving rise to potentially 55 distinct SMs (two separate key enzymes are involved in fusaric acid biosynthesis) (Niehaus et al. 2017a). For 25 of them, the corresponding SM is known—either identified from *F. fujikuroi* cultures directly or predicted based on comparative genomics approaches (Fig. 12.1).

Knowledge on the BGCs present in *F. fujikuroi* has paved the way for new strategies to decrypt the secondary metabolome of this important plant pathogen. Bioinformatics combined with transcriptional and chemical analyses has now allowed for the linkage of several already

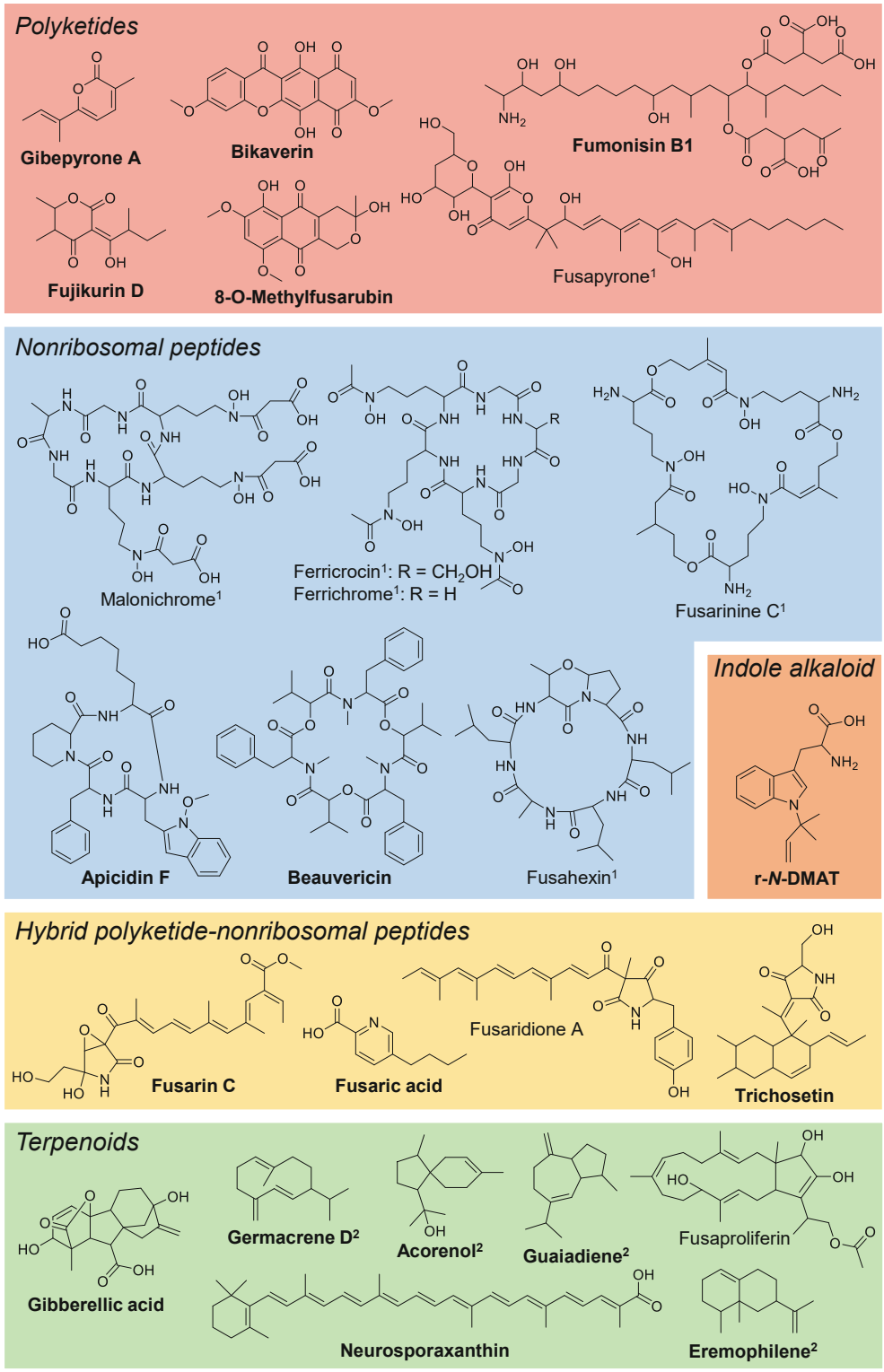

Fig. 12.1 Overview of *F. fujikuroi* SMs with known chemical origin. SMs depicted in bold letters have been determined in *F. fujikuroi* experimentally, while the others are predicted based on comparative genomics. [1]BGC is absent from the reference strain IMI58289 but present in other natural *F. fujikuroi* isolates; [2]the depicted compound is derived from the key enzyme only. Depending on additional decorating enzymes, the final product may differ

known SMs with their respective BGCs. Examples are the identification of the BGC involved in the biosynthesis of the perithecial pigments fusarubins, *FSR1-FSR6* (Studt et al. 2012), or the BGCs involved in the biosynthesis of fusarins, *FUS1-FUS9* (Niehaus et al. 2013), and fusaric acid, *FUB1-FUB12* (Niehaus et al. 2014b; Studt et al. 2016a). In the latter, two separate key enzyme-encoding genes (*FUB1* and *FUB8*) are present and essential for the biosynthesis of this mycotoxin (Niehaus et al. 2014b; Studt et al. 2016a). Notably, most of the BGCs identified *in silico* constituted orphan clusters for which the corresponding product was unknown, and functional characterization was hindered by the fact that most of the genes are not expressed or expressed only at very low levels under standard laboratory conditions (Wiemann et al. 2013; Niehaus et al. 2017a). So knowledge of the BGCs present allowed for a more targeted approach, i.e., the overexpression of genes encoding the key enzyme and/or the pathway-specific TF. For example, overexpression of the DMATS1-encoding gene, *FFUJ_09179*, resulted in the identification of the reverse *N*-prenylated tryptophan r-*N*-DMAT (Arndt et al. 2017). Overexpression of the pathway-specific TFs encoded within the PKS-NRPS1 and the NRPS31 (later referred to as APF) BGCs, *FFUJ_02222* and *FFUJ_00012*, allowed for the structural elucidation of the corresponding products, trichosetin and apicidin F, respectively (Wiemann et al. 2013; Niehaus et al. 2014a; Janevska et al. 2017). In the case of the polyketides fujikurins derived from the PKS19 BGC, only simultaneous overexpression of both the key enzyme- and the pathway-specific TF-encoding genes, *FFUJ_12239* and *FFUJ_12243*, respectively, resulted in sufficient levels of metabolite for structural elucidation (Wiemann et al. 2013; von Bargen et al. 2015). It is noteworthy, that overexpression of a putative pathway-specific TF-encoding gene did not always result in elevated biosynthesis of the respective SM. This is exemplified by the TFs encoded within the beauvericin and r-N-DMAT BGCs, *BEA4* and *FFUJ_09177*, respectively (Studt et al. 2016a; Arndt et al. 2017).

Subsequent molecular and chemical characterization of additional cluster genes revealed their function and/or contribution to the final product. An overview of the cluster organization of BGCs functionally characterized in *F. fujikuroi* over the past years is shown in Fig. 12.2. In some cases, genes within a predicted BGC, though co-regulated, appear dispensable for the biosynthesis of the corresponding SM. Examples are *FUS3-FUS7* of the BGC involved in fusarin biosynthesis (Niehaus et al. 2013), and *FFUJ_09176-FFUJ_09178* encoded within the predicted DMATS1 BGC (Arndt et al. 2017).

Also, SMs have been assigned to three additional BGCs not present in the *F. fujikuroi* reference strain IMI58289, but present in other *F. fujikuroi* isolates as determined by comparative genomics (Niehaus et al. 2017a). Those include the polyketide fusapyrone (Atanasoff-Kardjalieff et al. 2021), the hybrid polyketide-nonribosomal peptide fusaridione A (Kakule et al. 2013), and the sesterterpene fusaproliferin (Ćeranić et al. 2021).

12.2.2 Distinct SM Profiles Determine the Pathotype in *F. fujikuroi*

Host-pathogen interactions constitute fine-tuned interplays where both participating organisms have evolved efficient strategies to interact with the other. The virulence of *F. fujikuroi* is intimately connected with the production of GAs, although their biological role during infection is not yet fully understood (Hedden and Sponsel 2015). *Bakanae* symptoms are abolished in rice plants infected with strains that have lost either the biosynthetic genes or regulatory components known to be fundamental for GA biosynthesis such as AreA or Vel1 (Mihlan et al. 2003; Wiemann et al. 2010, 2013), thereby resembling MOCK-infected control samples. Interestingly, rice seedlings infected with the *F. fujikuroi* isolate B14 did not show *bakanae* symptoms but instead stunting and advanced crown and root rot (Niehaus et al. 2017a). This phenotype was attributable to the biosynthesis of fusaric acid and fumonisins, and simultaneous absence of GAs in

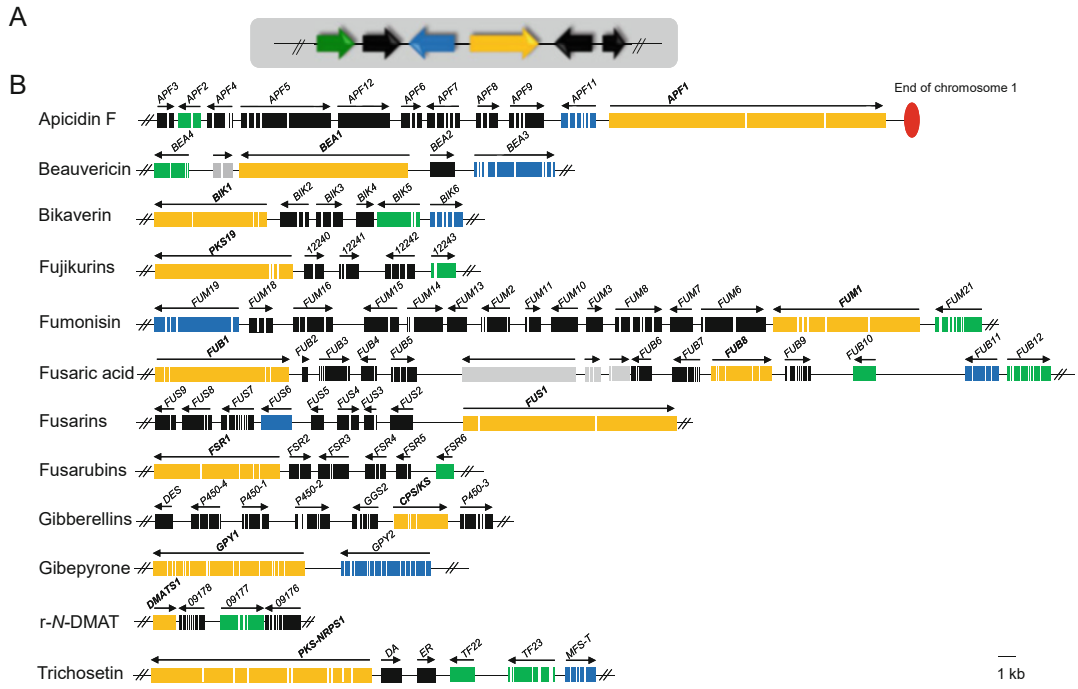

Fig. 12.2 Organization of selected BGCs in *F. fujikuroi*. (**a**) Schematic overview of the common composition of a fungal biosynthetic gene cluster (BGC). (**b**) Schematic organization of BGCs that have been functionally characterized in *F. fujikuroi*. The key enzyme-encoding gene is shown in yellow, putative transporters and pathway-specific transcription factors encoded within the BGC are shown in blue and green, respectively. Other cluster genes are depicted in black, while genes that do not belong to the cluster based on coregulation are shown in grey. Gene identifiers are as published. White bars represent introns and arrows show translation direction. References: Linnemannstöns et al. (2002b), Wiemann et al. (2009), Bömke and Tudzynski (2009), Studt et al. (2012), von Bargen et al. (2013), Wiemann et al. (2013), Niehaus et al. (2013, 2014a, b, 2016b), von Bargen et al. (2015), Studt et al. (2016a), Rösler et al. (2016b), Arndt et al. (2017), Janevska et al. (2016, 2017)

this particular strain. Strains deleted for both fusaric acid and fumonisin biosynthesis (ΔΔ*fub1*/*fum1*) closely resembled MOCK-infected rice plants, and exogenous addition of gibberellic acid (GA_3) to ΔΔ*fub1*/*fum1*-infected samples induced the *bakanae* symptoms (Niehaus et al. 2017a). Characterization of 15 additional *F. fujikuroi* field isolates revealed that there are two subclades (phylogenetically distinct groups) of *F. fujikuroi* strains according to their pathotype and SM profile (Niehaus et al. 2017a). It is noteworthy that irrespective of the pathotype, all harbor the BGCs involved in GA, fusaric acid, and fumonisin biosynthesis. However, while fusaric acid production was similar in all strains, the biosynthesis of GAs and fumonisins differed markedly. GA biosynthesis was highly abundant in strains causing the *bakanae* phenotype, whereas fumonisin was not detected, while no GAs but high amounts of fumonisin were detected in strains causing the stunting phenotype (Niehaus et al. 2017a). This suggests that some *F. fujikuroi* strains have evolved different strategies to infect and establish themselves within the plant. Further research awaits to understand the drivers for these distinct expression patterns even though they have a common genetic SM repertoire.

Another unique aspect of the *F. fujikuroi* isolates exhibiting the stunting phenotype is the presence of the PKS51 BGC, putatively comprising *FFB14_06367-FFB14_06375* (Niehaus et al. 2017a), that is specifically expressed during *in planta* growth, and absent from all *bakanae*

strains. Deletion of *PKS51* did not abolish but intensified the disease symptoms (Niehaus et al. 2017a), suggesting a distinct role of *PKS51* and/or its product during the *F. fujikuroi*-rice interaction. In *F. fujikuroi* strain B14, there are two additional BGCs, i.e., PKS40 and PKS43 next to PKS51. While PKS43 appears to be non-functional in this strain, the PKS40 BGC comprises seven genes, *FFB14_11407-FFB14_11413*, and was recently shown to be involved in the biosynthesis of fusapyrone in the closely related *F. mangiferae* (Atanasoff-Kardjalieff et al. 2021). Absence of *PKS40* transcript during *in planta* growth, however, suggests that this γ-pyrone does not play a role during the infection, but instead may provide a selective advantage under distinct environmental conditions.

12.3 Regulation of SM Gene Expression in *F. fujikuroi*

SM biosynthesis is energy consuming and, as such only initiated upon the receipt of appropriate environmental signals. These may include changes in carbon and nitrogen sources, iron availability, temperature, pH, reactive oxygen species, or stimuli derived from other (competing) organisms (Macheleidt et al. 2016). To respond to these stimuli, the fungus has to interact with its environment. This is generally orchestrated via signal transduction pathways that transmit signals that lead to (de)activation of downstream targets such as pathway-specific ("narrow"-domain) or globally acting ("wide"--domain) TFs. A timely response to an external stimulus is often vital for fungal survival. Thus, the transcription of these regulatory circuits is tightly controlled. Several of these components have been studied in *F. fujikuroi*. These include the G protein signaling cascade that perceives and processes external environmental stimuli as well as the main down-stream target, the adenylate cyclase (Li et al. 2007). This pathway plays a key role in wild type-like expression of several BGCs in *F. fujikuroi* (Studt et al. 2013a). Well-known global regulators affecting the expression of several SM-encoding genes in *F. fujikuroi* are components of the VELVET complex that coordinates light, fungal development, and secondary metabolism (Wiemann et al. 2010; Niehaus et al. 2018), the wide-domain TF Sge1 originally identified as the master regulator of morphological switching in yeast (Michielse et al. 2015), and the two GATA TFs AreA and Csm1, connecting nitrogen availability and asexual development, respectively, to secondary metabolism (Pfannmüller et al. 2017; Niehaus et al. 2017b). Though the relevance of environmental signals and the contribution of several global regulators for SM gene expression have been analyzed, the full complexity of the regulation of fungal secondary metabolism is still puzzling. Adding to this complexity, another regulatory level has emerged as crucially important for SM gene regulation i.e., chromatin structure. In the following section, our knowledge on chromatin-dependent SM gene regulation in *F. fujikuroi* is summarized and discussed.

12.3.1 Chromatin Structure: A Natural Obstacle for Transcription

Chromatin is the fundamental packaging form of DNA. In eukaryotes, genomic DNA wraps around histone protein octamers to form nucleosome chains, each consisting of two of the core histone proteins H2A, H2B, H3, and H4 wrapped by 147 base pairs of DNA (Kornberg 1974). The assembly of DNA with histones and other proteins is called "chromatin." Distinct types of chromatin impact the spatial and temporal regulation of gene expression. Heterochromatin corresponds to genomic regions condensed even during interphase and is differentiated from euchromatin which is more accessible. As such, the chromatin structure generates a natural obstacle to the transcription machinery and thus must be highly dynamic and well-coordinated upon the receipt of external stimuli. Alterations in the chromatin structure involve ATP-dependent chromatin-modifying enzyme complexes that mobilize the histones within the chromatin (Becker and Workman 2013), histone chaperones that

facilitate the exchange of core histones with their respective variants (Almouzni and Cedar 2016; Chen and Ponts 2020), and posttranslational modifying enzymes that act on DNA or histones by adding or removing chemical groups (Bannister and Kouzarides 2011). Little information is still available on the role of ATP-dependent chromatin-modifying enzyme complexes or histone variants in filamentous fungi including *F. fujikuroi*. Notably, attempts to delete the gene encoding the histone variant H2A.Z from the genome of *F. fujikuroi* failed thus far, suggesting that H2A.Z is essential in this fungus (Chen et al. 2020). Future research is required to determine the role of H2A.Z for gene transcription and SM biosynthesis in *F. fujikuroi*.

Since the discovery that histones may become acetylated and methylated by Allfrey et al. (1964), many more types of posttranslational modifications (PTMs or histone marks) have been identified and characterized. In general, the core histone proteins that make up the nucleosome are composed of a globular domain and a flexible histone tail that protrudes from the histone-DNA surface giving rise to a flexible platform for PTMs. Each histone mark is associated with a specific chromatin state. Thus, the incidence and/or change in histone marks present can serve as markers for changes in chromatin structure associated with either gene expression (euchromatin) or silencing (heterochromatin). These histone marks are established by histone-modifying enzymes ("writers"), recognized by proteins containing domains that harbor an affinity for a specific histone mark ("readers"), and removed by antagonizing activities ("erasers"). Among all known histone marks to date, acetylation and methylation of histone 3 have been studied the most, and their relevance for transcription has been assessed in many organisms, including *F. fujikuroi*. For several euchromatic and heterochromatic histone marks, the genome-wide distribution has been determined by chromatin immunoprecipitation-coupled sequencing (ChIP-seq) in *F. fujikuroi* (Studt et al. 2013b, 2016b; Wiemann et al. 2013; Janevska et al. 2018a). Distribution of the euchromatic histone marks H3K9ac and H3K4me2 co-occurred primarily at the centers or arms of most chromosomes, and as such were found to largely associate with gene expression (Wiemann et al. 2013). H3K9ac was found to be highly abundant at several actively transcribed BGCs but absent under non-inducing conditions. Examples are the BGCs involved in fusarin, apicidin F, GA and bikaverin biosynthesis (Studt et al. 2013b; Wiemann et al. 2013; Niehaus et al. 2013). In contrast, H2K4me2 is largely absent from BGCs in *F. fujikuroi*. One exception is the BGC involved in GA biosynthesis: two of the seven cluster genes are enriched for H3K4me2 under inducing conditions only (Wiemann et al. 2013; Studt et al. 2017). Heterochromatin is further divided into facultative and constitutive heterochromatin, and both types are established and maintained by distinct histone PTM pathways to silence transposons, regulate cell type-specific gene expression, and organize chromosomes inside the nucleus (Lewis 2017). Constitutive heterochromatin is typically associated with H3K9me3 and found in centromeric and gene-poor regions. Genes decorated with this histone mark are never or hardly ever expressed (Freitag 2017). In *F. fujikuroi*, H3K9me3 is mostly limited to pericentric and centromeric regions. All such, regions are associated with AT- and transposon-rich DNA with few, if any, annotated genes. None of the BGCs are enriched for H3K9me3 (Wiemann et al. 2013). One marked exception is the PKS19 BGC involved in the biosynthesis of fujikurins (von Bargen et al. 2015). Here, the AT-rich flanking regions are enriched for H3K9me3, although this BGC is not associated with telomeric or centromeric regions but in fact is located in the center of chromosome eight (Wiemann et al. 2013). It is noteworthy, that the BGC involved in fujikurin biosynthesis is missing from some of the additionally sequenced *F. fujikuroi* isolates though adjacent genes at the left and the right borders show collinearity suggesting that this BGC is the result of a horizontal gene transfer or cluster duplication and loss (birth and death) (Niehaus et al. 2017a). Overall, the PKS19 cluster genes exhibit low expression levels during axenic growth but are specifically induced upon

infection of rice seedlings, suggesting that the product may contribute to the virulence of *F. fujikuroi* in some way (Wiemann et al. 2013). This suggestion, however, awaits further proof. In contrast to constitutive heterochromatin, facultative heterochromatin is located in gene-rich chromosomal regions that are subject to dynamic regulation and are typically decorated with H3K27me2/3. These regions may switch from a silent (H3K27me2/me3-decorated) to a transcriptionally active state (and vice versa) when expression of the underlying genes is favored (Ridenour et al. 2020). In *F. fujikuroi*, many BGCs fall within regions of facultative heterochromatin (Studt et al. 2016b). Contrary to the discrete distributions of these euchromatic and heterochromatic marks, H3K36me3 is present at nearly every gene in *F. fujikuroi* regardless of the transcriptional state (Janevska et al. 2018a). Figure 12.3 summarizes the current knowledge on the general function (euchromatin or heterochromatin) and distribution of histone marks in *F. fujikuroi*.

In the following sections, selected histone marks and their relevance for SM gene regulation in *F. fujikuroi* are discussed in more detail.

12.3.2 Prominent Histone Marks: Of "Writers, Readers and Erasers"

12.3.2.1 Histone Acetylation and Its Role for SM Gene Regulation in *F. fujikuroi*

Histone acetylation is thought to alter the chromatin structure in *cis* by changing the physical properties of modified histone tails. The addition of acetyl groups neutralizes the positively charged lysine residues of the highly basic histone tails thereby generating a localized expansion of the chromatin fiber and thus enabling a better access to the DNA for other regulators, DNA-binding TFs, and the RNA polymerase II transcription machinery. As such, histone acetylation is generally associated with active chromatin or regions that are generally permissive for transcription. Histone acetylation is established by histone acetyltransferase (HATs), also referred to as lysine acetyltransferases (KATs), that form a superfamily of enzymes, further divided into nuclear A-type or cytoplasmic B-type acetyltransferases, depending on their localization and targets within the cell. Five well-studied KAT subfamilies include HAT1 (KAT1), GNAT (general control non-derepressible 5 (Gcn5)-related KAT, or KAT2), MYST (MOZ, Ybf2/Sas3, Sas2, and TIP60, or KAT5), as well as the structurally related metazoan-specific p300/CBP (KAT3) and fungal-specific Rtt109 (regulator of Ty1 transposition gene product 109, or KAT11) (Marmorstein and Zhou 2014).

Overall, the genome of *F. fujikuroi* IMI58289 harbors 68 predicted KATs, of which the GNAT subfamily constitutes the largest group with 63 members (Atanasoff-Kardjalieff and Studt 2022). To date, only the GNAT member Gcn5 (KAT2) has been characterized in great detail in *F. fujikuroi* (Rösler et al. 2016a). Gcn5 is the catalytic subunit of the SAGA (**S**pt-**A**da-**G**cn5-**A**cetyltransferase) complex, which is responsible for the acetylation of several histone lysine residues in *F. fujikuroi*. These include H3K4, H3K9, H3K18, H3K27, and H3K36, and it is likely that more target sites will be discovered in the future. Not surprisingly, loss of Gcn5 (or of the two transcriptional adaptors Ada2 and Ada3) resulted in the de-regulation of a large gene set. Overall, 27 SM key enzyme-encoding genes were found to be affected by the loss of Gcn5, with the majority of them being downregulated (Rösler et al. 2016a; Table 12.1), a phenotype that is in agreement with the activating role of histone acetylation. However, for some SMs loss of Gcn5 led to an increase in SM biosynthesis, e.g., in the case of bikaverin. This is particularly interesting as the bikaverin BGC is enriched for H3K9ac specifically under inducing conditions (Studt et al. 2013b; Wiemann et al. 2013), thereby implying a role of Gcn5 in regulating *BIK* gene expression in the wild type.

In addition to Gcn5, another unrelated KAT of the class B-type, HAT1, has been functionally characterized in *F. fujikuroi* (Niehaus et al. 2018). In contrast to Gcn5 and most of the known KATs, HAT1 is not part of a multimodule enzyme complex but associated with

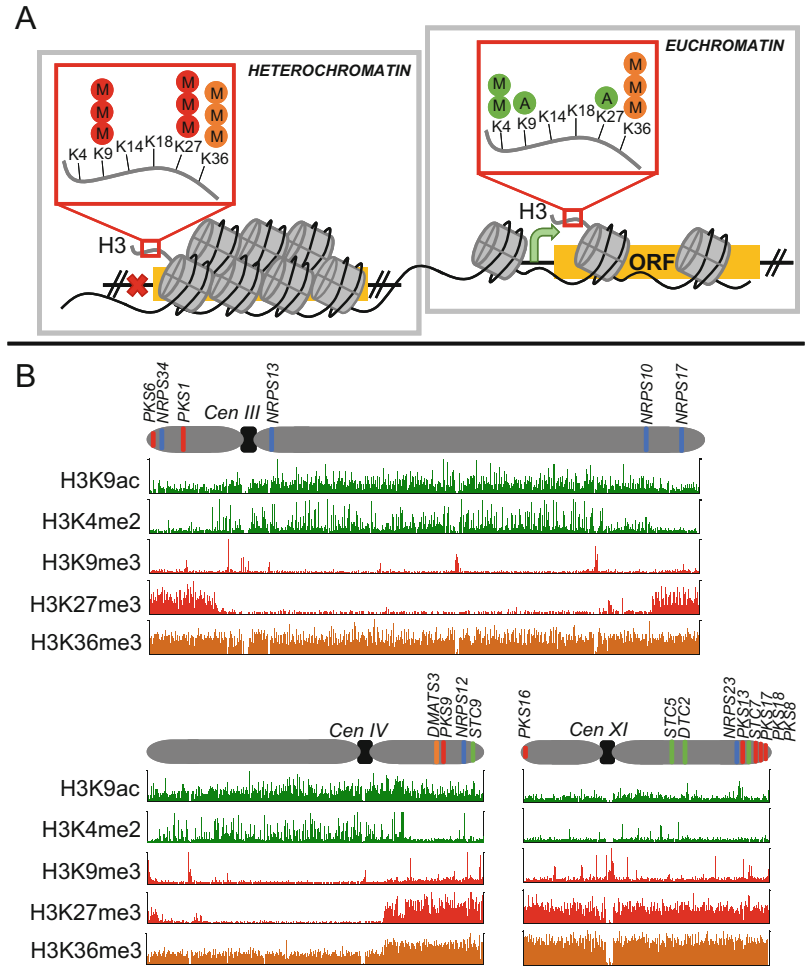

Fig. 12.3 Well-known histone marks, their association with gene expression and distribution in *F. fujikuroi*. (**a**) Histone proteins are depicted in grey; genomic DNA wrapped around histones in black. N-terminal tails and histone marks either associated with heterochromatin or euchromatin are indicated in the respective box and exemplars shown for one histone protein each. Active and repressive marks are depicted in green and red, respectively, while H3K36me3 present at genes regardless of their transcriptional state is shown in orange; M, methylation; A, acetylation. Genomic region, non-accessible in heterochromatic and accessible in euchromatic region is depicted in yellow. Both chromatin states can merge with the other thus losing prior and gaining novel histone marks. (**b**) Chromatin marks for chromosomes III, IV, and XI are shown as examples of the twelve *F. fujikuroi* chromosomes. For each chromosome (gray), the position of the centromere (black) and the locations of BGCs are shown at the top; below this in descending order are histone 3 lysine 9 acetylation (H3K9ac), H3K4me2, H3K9me3, H3K27me3, and H3K36me3. The key enzyme-encoding gene is depicted for each BGC according to the following color code: polyketide synthase (PKS), red; non-ribosomal peptide synthetase (NRPS), blue; terpene cyclase (DTC means diterpene cyclase, STC means sesquiterpene cyclase), green; dimethylallyl tryptophan synthase (DMATS), orange (modified after Wiemann et al. 2013; Studt et al. 2016b; Janevska et al. 2018a)

another single factor, HAT2, which is involved in the stabilization of HAT1 function. HAT1 was initially proposed to be a cytoplasmic HAT but later shown to also harbor nuclear functions (Ruiz-García et al. 1998; Poveda et al. 2004; Ai and Parthun 2004). In line with these findings, HAT1 localizes mainly to the nucleus in *F. fujikuroi*, suggesting that HAT1 is involved

Table 12.1 Involvement of histone-modifying enzymes in SM gene expression in *F. fujikuroi* IMI58289

Signature gene	Gene ID	Product	Histone modifier
Non-ribosomal peptide synthetases (NRPSs)			
NRPS2	FFUJ_04614	Ferricrocin	Gcn5, Hda1
NRPS3	FFUJ_06929		
NRPS4	FFUJ_08113	Fusahexin	Gcn5, Set1, Kdm5, Set2, Ash1, Kmt6
NRPS6	FFUJ_10736	Fusarinine	Set1, Set2
NRPS10	FFUJ_03506		Set2
NRPS11	FFUJ_10934		Gcn5, Set1, Set2, Ash1
NRPS12	FFUJ_14790		Set1, Ash1
NRPS13	FFUJ_02440		Kmt6
NRPS17	FFUJ_03641	Ferrichrome	Gcn5
NRPS20	FFUJ_06720		
NRPS21	FFUJ_02022		Ash1
NRPS22	FFUJ_09296	Beauvericin	Gcn5, **Hda1**, Ash1, **Kmt6**
NRPS23	FFUJ_12008		Gcn5, Set1, Set2
NRPS25	FFUJ_05347		
NRPS31	FFUJ_00003	Apicidin F	Gcn5, Set1, Kdm5, Set2, Ash1, Kmt6
NRPS34	FFUJ_02115	Fusaric acid	**Gcn5**, **Set1**, Kdm5, **Hda1**, **Hda2**, **Set2**, **Ash1**. Kmt6, **HAT1**
Polyketide synthases (PKSs)			
PKS-NRPS1	FFUJ_02219	Trichosetin	Gcn5, Set1, **Kmt6**
PKS2	FFUJ_00118		Gcn5, Kdm5, Set2, Kmt6
PKS3	FFUJ_03984	Fusarubins	**Gcn5**, **Set1**, **Kdm5**, **Hda1**, **Hda2**, **Ash1**, Kmt6
PKS4	FFUJ_06742	Bikaverin	**Gcn5**, **Set1**, **Kdm5**, **Hda1**, **Hda2**, **Set2**, **Ash1**, **Kmt5**
PKS6	FFUJ_02105	Fusaric acid	**Gcn5**, **Set1**, Kdm5, **Hda1**, **Hda2**, **Set2**, **Ash1**, Kmt6, **HAT1**
PKS7	FFUJ_06260		Set2, Ash1, Kmt6
PKS8	FFUJ_12090		Hda1, Set2, Kmt6
PKS-NRPS9	FFUJ_14695		Gcn5, Set1, Set2, Ash1
PKS-NRPS10	FFUJ_10058	Fusarins	Gcn5, **Set1**, **Kdm5**, Hda1, **Hda2**, **Set2**, **Ash1**, **Kmt5**
PKS11	FFUJ_09241	Fumonisins	Gcn5, Set1, Kdm5, Hda1, Set2, Ash1, Kmt6
PKS12	FFUJ_10347		
PKS13	FFUJ_12020	Gibepyrones	Gcn5, Set1, Hda1, Set2, Ash1, Kmt6
PKS14	FFUJ_11034		
PKS16	FFUJ_11199		Gcn5, Kmt6
PKS17	FFUJ_12066		
PKS18	FFUJ_12074		
PKS19	FFUJ_12239	Fujikurins	Kmt6
PKS-NRPS20	FFUJ_12707		Gcn5
PKS type III	FFUJ_05866		Gcn5, Set1
Terpene cyclases (TCs)			
DTC1–1	FFUJ_14336	Gibberellins	**Gcn5**, **Set1**, **Kdm5**, **Hda1**, **Hda2**, **Set2**, **Ash1**, Kmt6, **Kmt5**, **HAT1**
TrTC1	FFUJ_13140		
TeTC1	FFUJ_11802	Carotenoids	Gcn5, Set1, Hda1, Set2, Ash1, Kmt6
STC1	FFUJ_00036	Germacrene D	Gcn5, Set1, Kdm5, Set2, Ash1, Kmt6, Hda1
STC2	FFUJ_00969		
STC3	FFUJ_04067	Eremophilene	Ash1
STC4	FFUJ_10353	Koraiol	Gcn5, Set1, Ash1
STC5	FFUJ_11739	Guaiadiene	Gcn5, **Kmt6**
STC6	FFUJ_12585	Acorenol	Kmt6

(continued)

Table 12.1 (continued)

Signature gene	Gene ID	Product	Histone modifier
STC7	FFUJ_12026		Gcn5
STC8	FFUJ_09423		Kmt6
STC9	FFUJ_14833		Gcn5
Dimethylallyltryptophane synthases (DMATSs)			
DMATS1	FFUJ_09179	r-*N*-DMAT	Gcn5
DMATS3	FFUJ_14683		Set1, Set2, Ash1, Kmt6

Histone modifiers known to affect the transcription of specific SMs are listed and highlighted in bold if transcriptional deregulation was verified by chemical analysis. References: Niehaus et al. (2016b, 2017a, 2018), Studt et al. (2013, 2016b), Rösler et al. (2016a), Janevska et al. (2018a, b), Bachleitner et al. (2021)

in the PTM of histones (Niehaus et al. 2018). Though the target of HAT1 is unknown, deletion and overexpression of *HAT1* clearly impacted SM biosynthesis in *F. fujikuroi* i.e., HAT1 positively and negatively affects GA and fusaric acid biosynthesis, respectively (Niehaus et al. 2018; Table 12.1). Intriguingly, overexpression of *HAT1* in a *LAE1* deletion background was able to partially complement the Δ*lae1* phenotype with regard to GA and fusarubin biosynthesis, both being nearly abolished in Δ*lae1* (Wiemann et al. 2010; Niehaus et al. 2018). This result points toward a dynamic interplay on different regulatory levels. Further work is required to connect the different components of gene regulation with one another.

Histone acetylation is commonly, though not exclusively, read by bromodomain-containing proteins, also referred to as "readers." This motif is often part of a KAT assembled in a larger chromatin-remodeling complex (Marmorstein and Zhou 2014). The *F. fujikuroi* genome harbors 13 predicted bromodomain-containing proteins (Atanasoff-Kardjalieff and Studt 2022), including Gcn5 which also exhibits histone acetyltransferase activity (Rösler et al. 2016a). No additional bromodomain-containing proteins have been studied thus far in *F. fujikuroi*. Histone acetylation is opposed by the action of histone deacetylases (HDACs) that actively remove the acetyl groups ("erasers"), thereby generating a hypoacetylated environment resulting in a condensed chromatin structure not accessible for the transcriptional machinery (Park and Kim 2020). Thus, while hyperacetylation of histones is strongly associated with gene transcription, hypoacetylation is associated with gene repression. HDACs are categorized, depending on their mode-of-action, into Class I and II Zn^{2+}-dependent HDACs as well as Class III NAD^{+}-dependent silent information regulator 2 (Sir2) ("sirtuins") (Seto and Yoshida 2014). Overall, four and eight Zn^{2+}-dependent HDACs and sirtuins, respectively, are predicted in the genome of *F. fujikuroi* (Atanasoff-Kardjalieff and Studt 2022). While little information is available in filamentous fungi in general for the latter, Zn^{2+}-dependent HDACs have been characterized in more detail in several fungi, including *F. fujikuroi* (Studt et al. 2013b).

Four Zn^{2+}-dependent HDACs have been identified in the genome of *F. fujikuroi* designated FfHda1 through FfHda4. While attempts to generate homokaryotic strains lacking the RpdA homolog, FfHda3, failed in *F. fujikuroi*, suggesting an essential function of this protein, the impact of the other three HDACs on SM gene regulation has been studied by overexpression and/or deletion of the respective genes, i.e., *FfHDA1*, *FfHDA2*, and *FfHDA4*. While deletion of *FfHDA4* did not affect SM biosynthesis, loss of either FfHda1 or FfHda2 strongly affected SM biosynthesis in *F. fujikuroi* (Studt et al. 2013b; Table 12.1). Strains lacking FfHda1 resulted in reduced concentrations of almost every SM tested: biosynthesis of GAs, fusarubins, fusaric acid and bikaverin was significantly decreased under standard inducing conditions. Production of bikaverin is known to be repressed by nitrogen or alkaline conditions (Wiemann et al. 2009) but

intriguingly, this metabolite was produced in high nitrogen conditions in the Δ*ffhda1* strain (Studt et al. 2013b), suggesting that the nitrogen repression is overruled in the absence of FfHda1, possibly due to the role of FfHda1 in removing the active H3K9ac marks present at this BGC (Studt et al. 2013b; Wiemann et al. 2013). Surprisingly, overexpression of *FfHDA1* did not oppose the phenotype of Δ*ffhda1*, but instead gave quite similar results, with one exception: the de-repression of *BIK* genes under repressing (high nitrogen) conditions was not observed (Studt et al. 2013b). It is possible that FfHda1 is indeed involved in the removal of H3K9ac at *BIK* genes under unfavorable conditions, thereby explaining a similar phenotype under inducing but an opposing phenotype under repressing conditions in Δ*ffhda1* and OE::*FfHDA1* with regard to bikaverin biosynthesis (Studt et al. 2013b). It is noteworthy, that deletion of *FfHDA1* accompanied by slightly increased H3K9ac levels at the bikaverin BGC also resulted in reduced amounts of the red pigment, suggesting that H3K9ac levels are not crucial for gene transcription. In addition to already known SMs, deletion of *FfHDA1* also induced the expression of an, at that time cryptic, BGC involved in the biosynthesis of the depsipeptide, beauvericin (Niehaus et al. 2016b). Increase in beauvericin biosynthesis was accompanied by an increase in H3K27ac at the respective BGC (Niehaus et al. 2016b), suggesting that FfHda1 may also be involved in removing H2K27ac in this fungus. Loss of the second class I HDAC, FfHda2, had a similar though distinct impact on secondary metabolism in *F. fujikuroi*. Four out of five tested SMs were de-regulated: while the amount of GAs, bikaverin, fusaric acid, and fusarins was significantly decreased, fusarubin biosynthesis remained unaffected under inducing conditions but was observed under repressing, acidic pH conditions in Δ*ffhda2* (Studt et al. 2013b). Nothing is yet known about the target sites of FfHda2. In accordance with the decrease in GA biosynthesis, the major virulence factor in *F. fujikuroi*, strains lacking FfHda1 and FfHda2 were hypovirulent on rice, and *bakanae* symptoms were abolished in a strain deleted for both *FfHDA1* and *FfHDA2* (Studt et al. 2013b). This result suggests that balanced histone acetylation is crucial for successful infection by *F. fujikuroi*. Whether this phenotype is merely associated with the reduced GA biosynthesis is currently unclear, but it seems plausible that histone acetylation coordinates the expression of various pathogenesis-related genes during the infection process. HDACs are generally seen to have a rather broad target range and this is underlined by the relatively low number of HDACs predicted in the *F. fujikuroi* genome compared to KATs (Atanasoff-Kardjalieff and Studt 2022). Further efforts are required to determine the precise role of HDACs in SM gene regulation.

12.3.2.2 Histone Methylation and Its Role in SM Gene Regulation in *F. fujikuroi*

While histone acetylation is connected to euchromatin and thus gene transcription, the role of histone methylation is much more variable. Two general classes of histone methyltransferases are known, i.e., the histone lysine methyltransferases (KMTs) and the protein arginine methyltransferases, also referred to as "writers." So far, only KMTs have been investigated in *F. fujikuroi*. They generally contain a catalytically conserved SET (**S**u(var)3–9, **E**nhancer of Zeste, and **T**rithorax) domain (Jones and Gelbart 1993; Tschiersch et al. 1994; Stassen et al. 1995). The SET domain recognizes lysine residues at distinct positions on the N-termini thereby facilitating the relocation of a methyl group from S-adenosyl-L-methionine (AdoMet) to a lysine residue. Thus far, there is only one KMT known that does not harbor the SET domain, i.e., DOT1 involved in methylation of H3K79 (Freitag 2017). In addition to the putative DOT1 homolog, *F. fujikuroi* contains 26 predicted SET domain-containing proteins (Atanasoff-Kardjalieff and Studt 2022), and several of them have been studied in considerable detail in recent years. These include Set1 (Kmt2) involved in H3K4me (Janevska et al. 2018b), Set2 (Kmt3) and Ash1 in methylating H3K36 (Janevska et al. 2018a), Kmt5 required for H4K20 mono-, di- and trimethylation

(Bachleitner et al. 2021), as well as Kmt6 in methylating H3K27 (Studt et al. 2016b). The histone lysine residue(s) may be mono-, di-, or trimethylated and, depending on the modified residue(s), this methylation is associated with heterochromatin formation, transcriptional silencing of euchromatic genes, or transcriptional activation and elongation by RNA polymerase II (Dillon et al. 2005). Histone lysine methylation is read by chromodomain domain-containing proteins ("readers") (Musselman et al. 2014), and antagonized by histone lysine demethylases (KDMs), also referred to as "erasers" (Black et al. 2012). The genome of *F. fujikuroi* encodes 15 predicted histone-"reader" protein-encoding genes, but none of them has been characterized thus far, leaving their contribution to SM gene regulation in this fungus yet to be determined. More information is available with regard to lysine demethylation. There are two types of KDMs: the amino oxidases with the lysine-specific histone demethylase 1 (Lsd1) that uses FAD and oxygen as cofactors for the demethylation of H3K4 and H3K9, and hydroxylases all harboring the catalytically active Jumonji C (JmjC) domain, that is involved in demethylation using 2-oxoglutarate and iron as cofactors (Black et al. 2012). Overall, eleven KDMs are predicted in the genome of *F. fujikuroi* (Atanasoff-Kardjalieff and Studt 2022). So far, only two of them, FfKdm4 and FfKdm5 involved in demethylating H3K36 and H3K4, respectively, have been characterized in detail in *F. fujikuroi* (Janevska et al. 2018a, b). The contribution of characterized histone methylation marks for SM gene expression in *F. fujikuroi* is summarized below.

12.3.2.2.1 H3K4 Methylation

Methylation of H3K4 orchestrated by Set1, also referred to as Kmt2 (Allis et al. 2007), is generally accepted as a hallmark of transcription, but several studies also imply a role for H3K4 methylation in gene silencing in yeast (Briggs et al. 2001; Krogan et al. 2002; Bryk et al. 2002; Fingerman et al. 2005; Weiner et al. 2012). Kmt2 is the catalytic component of the COMPASS (**Com**plex of **P**roteins **As**sociated with **S**et1) complex and as such is required for mono-, di-, and trimethylation of H3K4 (Takahashi and Shilatifard 2010; Takahashi et al. 2011). In addition to Set1, seven additional subunits are assembled in the COMPASS complex, and each subunit is important to establish the distinct global H3K4 methylation pattern (Shilatifard 2012). FfSet1 is essential for mono-, di-, and trimethylation of H3K4me in *F. fujikuroi*, and its loss greatly impacted SM biosynthesis in this fungus. Strains lacking FfSet1 showed elevated biosynthesis of bikaverin, fusarubins, and fusarins, while GA biosynthesis was abolished in axenic culture (Janevska et al. 2018b; Table 12.1). This phenotype is largely phenocopied in a second COMPASS member, FfCcl1, which is crucial for trimethylation of H3K4 (Studt et al. 2017). BGCs are largely devoid of H3K4 methylation in *F. fujikuroi*. One prominent exception is the BGC involved in GA biosynthesis. Here, two out of the seven cluster genes are enriched for H3K4me2 under inducing conditions only (Wiemann et al. 2013). This observation is in line with the results of strains deleted for either *FfSET1* or *FfCCL1* as both show an impaired GA biosynthesis, though to a different extent (Studt et al. 2017; Janevska et al. 2018b; Table 12.1). This suggests that the COMPASS complex directly targets this BGC, while expression of the other BGCs (not enriched for H3K4 methylation) is regulated further downstream. However, H3K4me2 is enriched at both the *GA* and *BIK* genes in Δ*ffccl1*, yet gene expression and biosynthesis are only increased in the latter (Studt et al. 2017). Thus, H3K4me2 alone does not determine the gene fate.

The function of FfSet1 is antagonized by the H3K4-specific histone demethylase FfKdm5 in *F. fujikuroi*. Deletion and overexpression of *FfKDM5* results in increased and decreased H3K4me3 levels, respectively, which was opposed by H3K4me2 levels (Janevska et al. 2018b). It is likely that the Lsd1 homolog, FfKdm1 also contributes to H3K4 demethylation, as has been shown for other organisms (Shi et al. 2004). Strains deleted for *FfLSD1* did not deviate from the wild type significantly (Tudzynski, unpublished data); however, this might be

attributable to absence of the substrate, i.e., H3K4me1/me2, without the simultaneous overexpression of *FfKDM5*. Thus, the role of FfLsd1 in H3K4 demethylation and secondary metabolism in *F. fujikuroi* remains enigmatic at this point. Similar to FfSet1, deletion as well as overexpression of *FfKDM5* significantly affected SM biosynthesis in *F. fujikuroi* (Table 12.1). In agreement with their opposing functions, deletion and overexpression of *FfKDM5* mostly antagonized and phenocopied the effect of Δ*ffset1*, respectively. Deletion of *FfSET1* as well as constitutive overexpression of *FfKMD5* resulted in elevated biosynthesis of the two red pigments (fusarubins and bikaverin), and fusarins, whereas the phenotype of Δ*ffkdm5* was the opposite (Janevska et al. 2018b). Interestingly, a histone demethylase-independent role in regulating SM gene expression has been shown for the FfKdm5 homologs, FgKdm5 and AnKdmB in *Fusarium graminearum* and *Aspergillus nidulans*, respectively (Bachleitner et al. 2019). Whether this also holds true for *F. fujikuroi* is yet to be determined.

12.3.2.2.2 H3K36 Methylation

Another histone mark that has long been associated with euchromatin is methylation of H3K36 (Ho et al. 2014), which was shown to facilitate transcription *via* associating with RNA polymerase II (Li et al. 2003; Kizer et al. 2005; Morris et al. 2005). In *S. cerevisiae* mono-, di-, and trimethylation of H3K36 is established by a single KMT designated ScSet2 (Strahl et al. 2002), or Kmt3 (Allis et al. 2007). Notably, H3K36me3 was found to be ubiquitous in the genome of *F. fujikuroi* regardless of the transcriptional state of the underlying genes (Janevska et al. 2018a). The same is true for *A. nidulans*, *Neurospora crassa*, and *F. graminearum* (Connolly et al. 2013; Gacek-Matthews et al. 2016; Bicocca et al. 2018). This observation is attributable to the presence of two H3K36-specific KMTs in *F. fujikuroi,* i.e., FfSet2 and FfAsh1, that establish H3K36me3 at distinct genomic locations: while FfSet2-mediated H3K36me3 contributes to regions associated with euchromatin and largely overlaps with H3K4me2, FfAsh1-mediated H3K36me3 is mainly associated with subtelomeric regions of facultative heterochromatin and coexists with H3K27me3 (Janevska et al. 2018a). No significant correlation between H3K36me3 and active transcription was found in this fungus. Upon loss of FfSet2 or FfAsh1, several key SM enzyme-encoding genes were de-regulated. While some BGCs appear to be selectively regulated by only one of the KMTs, the SM profile of both mutant strains was similar for others, e.g., elevated fusarin and fusaric acid levels, but a decrease in GA biosynthesis (Janevska et al. 2018a; Table 12.1). This contradicts the distinct functions of both KMTs but may be attributable to secondary effects. Also, no correlation was found between H3K27me3 and/or H3K36me3 levels with regard to SM gene expression in this fungus (Janevska et al. 2018a), leaving the mode-of-action unclear at the moment. Notably, GA biosynthesis was decreased upon loss of both FfAsh1 and FfSet2, a phenotype that was in line with an attenuated pathogenicity (Janevska et al. 2018a). Future research is required to dissect the unique functions of FfSet2 and FfAsh1 in *F. fujikuroi* in general and for secondary metabolism in particular.

H3K36me3 is antagonized by the JmjC domain-containing protein Kdm4 (Tu et al. 2007; Klose et al. 2007; Gacek-Matthews et al. 2015; Janevska et al. 2018a; Schumacher et al. 2019). Deletion of *FfKDM4* did not result in an overall increase in H3K36me3, which, however, is not surprising since H3K36me3 is already omnipresent in the wild-type strain, but overexpression of *FfKDM4* resulted in globally decreased H3K36me3 levels. Interestingly, overexpression of *FfKDM4* appears to be detrimental to the fungus (Janevska et al. 2018a). Whether this phenomenon is related to H3K36me3 or another histone mark, e.g., H3K9me3 or H3K27me3, remains to be elucidated, but it is noteworthy that attempts to delete either of the KMT-encoding genes, i.e., *FfKMT1* or *FfKMT6*, respectively, failed in *F. fujikuroi* thus far (Studt et al. 2016b; Studt

and Tudzynski, unpublished data), suggesting that both histone marks exert pivotal functions in this fungus.

12.3.2.2.3 H3K9 Methylation

H3K9me3 is a hallmark for formation of constitutive heterochromatin, and as such is tightly associated with gene silencing. Constitutive heterochromatin is typically associated with H3K9me3 and found in telomeric and centromeric gene-poor regions (Craig 2005; Smith et al. 2008; Becker et al. 2016). Genes decorated with H3K9me3 are hardly ever expressed (Freitag 2017). H3K9me3 is established by the H3K9-methyltransferase Kmt1, also known as SpClr4 in the fission yeast *Schizosaccharomyces pombe* (Nakayama et al. 2001) or NcDim5 in *N. crassa* (Tamaru and Selker 2001). Kmt1 (Dim-5) is the catalytic subunit of the DCDC (**D**IM-5/-7/-9/**C**UL4/**D**DB1 **C**omplex), involved in guiding Kmt1 and facilitating H3K9me3 (Freitag 2017). H3K9me3 is recognized and bound by heterochromatin protein 1 (HP1) through its chromodomain (Bannister et al. 2001; Lachner et al. 2001; Freitag et al. 2004), and is thought to inhibit euchromatin spread. As such, HP1 is often a component of condensed heterochromatin. Homologs of both are present in *F. fujikuroi*, i.e., FfKmt1 and FfHP1, but so far attempts to generate homokaryotic deletion mutants failed for either of them thereby impeding their functional characterization (Studt and Tudzynski, unpublished data). This stands in marked contrast to other filamentous fungi, e.g., *N. crassa*, *Aspergillus* spp., *Zymoseptoria tritici*, *Epichloë festucae*, or *Botrytis cinerea* (Tamaru and Selker 2001; Palmer et al. 2008; Reyes-Dominguez et al. 2010; Chujo and Scott 2014; Zhang et al. 2016). Notably, Kmt1 is also dispensable in at least two other closely related *Fusarium* spp., i.e., *F. verticillioides* and *F. mangiferae* (Gu et al. 2017; Atanasoff-Kardjalieff et al. 2021). In the latter, the KMT1 homolog, FmKmt1, appears essential for the wild type-like expression of several BGCs, while fungal growth remained largely unaffected (Atanasoff-Kardjalieff et al. 2021). Thus, H3K9me3 seems to play a role in SM gene expression in some way. Unfortunately, the lack of a suitable H3K9me3-specific antibody prohibited genome-wide association studies in this fungus (Atanasoff-Kardjalieff et al. 2021). Notably, expression of two PKS-encoding BGCs depends on FmKmt1, i.e., FmPKS8 and FmPKS40. These rather contradictory findings may very well reflect an indirect role of H3K9me3 in regulating SM gene expression, consistent with the fact that BGCs lack H3K9me3 marks in the closely related *F. fujikuroi* (Wiemann et al. 2013). FmPKS40 has only recently been shown to be involved in fusapyrone biosynthesis in *F. mangiferae* (Atanasoff-Kardjalieff et al. 2021). Here, fusapyrone biosynthesis is facilitated by a seven-gene cluster, designated *FPY1-FPY7*, in *F. mangiferae* (Atanasoff-Kardjalieff et al. 2021). Notably, the FPY BGC is present also in the *F. fujikuroi* isolate B14 (Niehaus et al. 2017a). However, fusapyrone was not detected in liquid cultures likely due to the very low expression of the *FPY* genes in this isolate (Atanasoff-Kardjalieff et al. 2021), thereby suggesting distinct regulation mechanisms in both fusaria.

H3K9me3 is tightly associated with DNA methylation in filamentous fungi. Here, HP1 acts as a scaffold for the recruitment of the DNA methyltransferase DIM-2 *via* interacting with the chromoshadow domain as described for *N. crassa*. DNA methylation is connected with the deactivation of invasive transposable elements and as such with gene silencing as shown for *N. crassa* (Freitag and Selker 2005). Two DNA methyltransferases have recently been identified and functionally characterized in *F. graminearum*, i.e., *FgRID* and *FgDIM-2* (Bonner et al. 2021). Deletion of each of them had an impact on SM biosynthesis in this fungus. Homologs are also present in *F. fujikuroi*, but their biological role remains to be elucidated.

To sum up, from our current knowledge it seems that BGCs are not targeted directly by Kmt1 or the DCDC complex in *F. fujikuroi*. However, data from other filamentous fungi suggest that H3K9me3 plays a pivotal role in wild type-like secondary metabolism in some way. Future research will determine the biological

role of constitutive heterochromatin in *F. fujikuroi*.

12.3.2.2.4 H3K27 Methylation

H3K27me3 is a repressive histone mark and is typically associated with chromosomal regions subject to a dynamic regulation, also called facultative heterochromatin (Ridenour et al. 2020). H3K27me3 is established by the **P**olycomb **R**epressive **C**omplex 2 (PRC2) with the lysine methyltransferase Kmt6 as the catalytic unit (Allis et al. 2007). Notably, distribution of H3K27 methylation but also presence of the PRC2 complex varies between organisms, and even between different fungi. While components of PRC2 and H3K27me3 occur in *N. crassa* (Jamieson et al. 2013), *Fusarium* spp. (Connolly et al. 2013; Studt et al. 2016b), and many other taxa, they are absent from *S. cerevisiae*, *S. pombe*, *Aspergillus* spp. or *Ustilago maydis* (Freitag 2017). In addition to Kmt6, two additional core components, FgEed and FgSuz12, are present and required for H3K27 methylation in *F. graminearum*. Deletion of either of them resulted in a complete loss of H3K27me3 and closely resembled the strain deleted for *FgKMT6* in *F. graminearum* (Tang et al. 2021). One marked difference to published data is that FfKmt6 and H3K27me3 are vital in *F. fujikuroi* (Studt et al. 2016b), but not in other published fungi (Connolly et al. 2013; Jamieson et al. 2013; Chujo and Scott 2014; Pham et al. 2015; Möller et al. 2019). Though the reason for this still remains unclear, this result suggests species-specific functions of Kmt6 and H3K27me3 even in closely related fungi.

Long stretches of H3K27me3 are present in *F. fujikuroi* specifically localized to subtelomeric regions (Studt et al. 2016b). Exceptions are chromosomes ten and eleven that appear nearly completely covered with H3K27me3, a result consistent with the fact that most of the genes located on these chromosomes are silent (Wiemann et al. 2013). Notably, the majority of BGCs are localized to these facultative regions in *F. fujikuroi*, suggesting that H3K27me3 plays a pivotal role in SM gene regulation in this fungus, potentially by orchestrating the expression of genes under distinct environmental conditions. Consequently, knock-down of *FfKMT6* using RNA interference resulted in reduced global H3K27me3, which was accompanied by the expression of several previously silenced, mostly cryptic BGCs. The overall expression of 20 of the 48 key enzyme-encoding genes present in *F. fujikuroi* was affected by reduced H3K27me3 levels (Studt et al. 2016b; Table 12.1). Thus, H3K27me3 is an important regulatory layer for SM gene expression in this fungus.

While the establishment of H3K27me3 by the PRC2 complex has now been studied in several organisms, it has long remained a mystery how facultative heterochromatin is "read" or reversed when the expression of the underlying genes becomes favorable. PRC1 components that "read" H3K27me3 and promote chromatin compaction and maintenance of facultative heterochromatin in higher organisms are absent from filamentous fungi (Ridenour et al. 2020). Only recently, a BAH domain-containing protein was identified that recognizes H3K27me3 in *N. crassa* and *F. graminearum*, i.e., EPR-1 and FgBP1, respectively (Wiles et al. 2020; Tang et al. 2021). Consistent with the predicted function, loss of FgBP1 has a similar phenotype as Δ*fgkmt6* with regard to SM biosynthesis. A homolog of FgBP1 is also present in *F. fujikuroi*, and future research will determine its contribution to facultative heterochromatin and (SM) gene silencing.

At present, it is unclear how and if Kmt6 is actively removed to allow for gene expression. Homologs of known H3K27me3-specific JmjC domain-containing KDMs are absent from fungal genomes (Freitag 2017). This suggests that either distinct JmjC or novel proteins are involved, or that H3K27me3 is reversed by a different mechanism. Overall, ten JmjC domain-containing proteins are predicted in the *F. fujikuroi* genome (Atanasoff-Kardjalieff and Studt 2022), and only two have been functionally characterized, thus far, including FfKdm4 and FfKdm5, which are involved in H3K36 and H3K4 demethylation, respectively (Janevska et al. 2018a, b). Future molecular characterization of the remaining KDMs will determine their role in the regulation

of gene expression in *F. fujikuroi* in general and for SM genes in particular.

12.3.2.2.5 H4K20 Methylation

Generally, trimethylation of H4K20 is considered a repressive histone mark and as such is associated with gene silencing in higher eukaryotes (Kourmouli et al. 2004; Schotta et al. 2004), while H4K20me3 does not seem to regulate gene expression in the fission yeast *S. pombe* (Sanders et al. 2004). In *S. pombe* only one KMT, i.e., SET9/Kmt5 (Allis et al. 2007), establishes mono-, di-, and trimethylation of H4K20 (Sanders et al. 2004), while KMT5A, KMT5B, and KMT5C establish mono-, di-, and trimethylation in higher eukaryotes (Nishioka et al. 2002; Fang et al. 2002; Schotta et al. 2004). Very little is currently known about the role of H4K20me3 and/or Kmt5 homologs in filamentous fungi. Only recently, the relevance of H4K20 methylation for fungal development in general and for SM gene expression in particular was analyzed in *F. fujikuroi* (Bachleitner et al. 2021; Table 12.1). Here, FfKmt5 establishes mono-, di-, and trimethylation of H4K20. Deletion as well as overexpression of *FfKMT5* have distinct effects on SM biosynthesis in this fungus. The most significant finding was increased fusarin biosynthesis upon loss of FfKmt5, a phenotype that was reversed to wild-type levels in the complemented strain. Conversely, overexpression of *FfKMT5* resulted in reduced biosynthesis of bikaverin and GAs. Deregulated GA levels, however, did not have an impact on virulence (Bachleitner et al. 2021). Notably, deletion and overexpression often did not result in opposing phenotypes, leaving the role of H4K20me3 in regulating SM gene expression unresolved. Future research is required to record the genome-wide deposition of H4K20me3 in this (or any other filamentous fungus) and to determine the relevance of this histone mark for SM gene regulation.

12.4 Concluding Remarks and Perspectives

Fungi are well-known for their ability to produce a plethora of SMs, often comprising diverse bioactivities. In fact, advances in genome sequencing combined with algorithms involved in mining BGCs have revealed that fungi have the genetic capacity to produce a much higher number of SMs than previously anticipated (Keller 2019). While much progress has been made in decoding the cryptic secondary metabolome, the regulatory circuits that govern SM gene expression are still not well understood. Over the past years, the chromatin structure (as determined by changes in histone marks) has received a lot of attention, and evolved as a key component in SM gene regulation. Several prominent histone marks have been characterized in *F. fujikuroi*, and the function of most of these appears to be crucial for wild type-like secondary metabolism in this fungus. However, evidence for a direct association with a given BGC by genome-wide mapping techniques is still scarce. A breakthrough towards understanding chromatin-level gene regulation of SMs in *F. fujikuroi* was the discovery that a large portion of histones associated with silent BGCs is trimethylated at H3K27, but again, the understanding of the exact regulatory mechanisms remains unknown. This may be attributable to the fact that histone marks do not function in isolation. The establishment of one histone mark often requires the presence or absence of others, and recognition of such is orchestrated by other factors. While the interdependence of selected histone marks has been studied in other fungi, e.g., *N. crassa* (Jamieson et al. 2016; Bicocca et al. 2018) or *Z. tritici* (Schotanus et al. 2015), nothing is yet known in the genus *Fusarium*. Future research will reveal whether a similar histone crosstalk is also present in *F. fujikuroi*, and if this at least to some extent provides an explanation of phenotypes related to histone marks not directly associated with a deregulated BGC. Moreover, though it is evident that other global regulators "interact" with histone-modifying enzymes in some way, their interplay at the molecular level remains largely elusive. Analyzing the hierarchical order of events will be vital to further our understanding of SM gene regulation in the future. Next to known chromatin components, *F. fujikuroi* harbors a series of predicted yet cryptic histone-modifying proteins,

many types of histone marks (apart from acetylation and methylation) are now known, and many more certainly await description in the future. Their characterization will almost inevitably reveal novel players in SM gene regulation. Thus, although several components affecting the expression of single BGCs are known now, the complexity of their regulation is still puzzling, as often one-and-the-same regulator exerts distinct functions on selected BGCs. Though this may not come as a surprise, as each SM likely serves a distinct purpose, time will tell whether a broad scheme for SM gene regulation can be drawn up, and how chromatin is connected to other factors regulating SM gene expression.

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The Rice Blast Fungus *Magnaporthe oryzae* Uses a Turgor-Dependent, Septin-Mediated Mechanism to Invade Rice

13

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Abstract

Rice blast disease is a major threat to global food security and remains challenging to control in all rice-growing regions of the world. The rice blast fungus *Magnaporthe oryzae* infects plants with a specialised single-celled infection structure called an appressorium, which develops enormous intracellular turgor to forcibly drive a rigid penetration peg through the rice leaf cuticle. Appressoria form in response to physical and chemical cues from the hydrophobic rice leaf cuticle, coupled with starvation stress. Here, we review the signalling pathways involved in the perception of surface signals and the mechanisms responsible for appressorium-mediated turgor-driven plant infection. We discuss how appressorium-mediated plant infection requires the assembly of a higher-order septin ring structure at the base of the appressorium, to spatially orchestrate and facilitate appressorium repolarisation. Septin ring organisation requires the turgor-dependent kinase Sln1, which is necessary for modulation of turgor once a threshold has been reached and is a prerequisite for repolarisation of the appressorium, penetration-peg emergence and plant infection.

Keywords

Appressorium · Fungus · *Pyricularia* · Septins · MAP kinase · NADPH oxidases · Melanin · Turgor · Virulence · Plasmodesmata · Rice · Wheat · Pathogenesis

13.1 Introduction

13.1.1 Rice Blast Disease

At least 20–40% of the world's food crop production is lost to plant pests and pathogens each year and highlights the urgency of scientific research in this area (Douglas 2018; Strange and Scott 2005; Vurro et al. 2010). It has been estimated that more than 800 million people are not adequately fed (Strange and Scott 2005), and in developing countries, rice is often a key dietary component. Indeed more than 50% of the global population rely on rice for their major calorific intake (Khush 2005). By 2050, the human world population is predicted to increase from 7.6 to 9.8 billion people (Elert 2014), and a recent analysis by the International Food Policy Research Institute indicates that to keep up with future rice demand, global production must increase by 38% before 2030. Rice production currently faces several challenges that negatively impact

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production. Pathogens including fungi, bacteria, viruses and nematodes cause substantial losses to yield (Asibi et al. 2019). However, the most devastating and economically important disease that affects cultivated rice is caused by a heterothallic ascomycete fungus *Magnaporthe oryzae* [synonym of *Pyricularia oryzae*] (Zhang et al. 2016a). The blast fungus, *M. oryzae* has multiple pathotypes and can infect more than 50 different grass species, including economically important staple crops such as rice (*Oryza sativa*), finger millet (*Eleusine coracana*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*) (Langner et al. 2018). The rice blast fungus is generally considered a foliar pathogen but can also attack stems, nodes and panicles of rice plants, accounting for approximately 6% loss of the global rice harvest annually, (Savary et al. 2019), but epidemics account for up to 30% yield losses (Fisher et al. 2012; Wilson and Talbot 2009). Outbreaks of rice blast disease are a serious and recurrent problem in the 85 countries where rice is grown (Skamnioti and Gurr 2007) including Southeast Asia (China, Sri Lanka, Indonesia, Bangladesh, India) (Kumar and Kalita 2017; Suprapta 2012; Wilson and Talbot 2009) as well as South America, Australia, Korea and the Philippines (Greer and Webster 2001; Shahriar et al. 2020).

M. oryzae also has the capacity to jump from one host to another which can have devastating consequences. In 1985, for example, the *Magnaporthe oryzae Triticum* pathotype (MoT), the causal agent of wheat blast disease, first appeared in Brazil following a likely jump from a grass-infecting isolate of the fungus (Inoue et al. 2017). Growth in global trade and agriculture in that time has enabled the spread of wheat blast, first identified in 2016 in Bangladesh, where it now threatens wheat production (Islam et al. 2016), with the capacity to spread to India, the world's second-largest wheat producer (Islam et al. 2019), and most recently to Zambia (Tembo et al. 2020). When considered together, *M. oryzae* poses a significant threat to global food security, and so understanding the basic biology of blast disease is important for the development of new disease control strategies (Eseola et al. 2021).

13.2 Life Cycle of *M. oryzae*

Rice blast disease is initiated when a three-celled conidium forms under humid conditions, from characteristic ellipsoid disease lesions, on the surface of rice leaves (Ou 1985). Asexual spores are transferred to neighbouring plants *via* wind and dew drop dispersal (Balhadère et al. 1999; Talbot 1995), rapidly adhering to the waxy, hydrophobic host leaf surface (Uchiyama and Okuyama 1990) with the aid of a proteinaceous spore tip mucilage released from the apex of the cell (Hamer et al. 1988). To cause plant disease *M. oryzae* forms a specialised infection structure called an appressorium. This specialised infection cell generates enormous turgor pressure of up to 8.0 MPa, by accumulating high concentrations of glycerol. The appressorium has a differentiated cell wall rich in melanin, which is essential for turgor generation and acts as a structural barrier to the efflux of solutes from the appressorium (Talbot 2003; Wilson and Talbot 2009). Cellular turgor is rapidly translated into mechanical force and a narrow penetration hypha emerges from the base of the appressorium, forcibly rupturing the rice leaf cuticle and allowing the fungus to invade rice tissue and cause disease. *M. oryzae* has emerged as a model experimental organism for understanding plant infection processes by pathogenic fungi, including appressorium morphogenesis (Talbot 2019; Xu et al. 2007), appressorium function (Fernandez and Orth 2018; Wilson and Talbot 2009), secretory processes involving effector proteins (Giraldo et al. 2013) and structural biology, and pathogen recognition by host immune receptors (Bentham et al. 2020; Dean et al. 2005; Langner et al. 2021).

13.3 Cell Signalling and Fungal Pathogenicity in *M. oryzae*

There are several important signal transduction pathways involved in host surface recognition

and subsequent fungal development and pathogenicity. The early stages of infection-related development are initiated when a three-celled conidium lands and attaches itself to the hydrophobic cuticle of the host leaf surface, triggering the cyclic AMP response pathway. The adenylate cyclase mutant Δ*mac1*, for example, is unable to elaborate appressoria to cause disease (Choi and Dean 1997). However, application of exogenous cAMP restores appressorium formation and pathogenicity, suggesting that *MAC1* is required for cAMP production (Adachi and Hamer 1998; Choi and Dean 1997). It has also been shown that appressorium formation on hydrophilic surfaces can be induced with exogenous cAMP (Gilbert et al. 1996). Upon germination, the spore sends out a polarised germ tube via the action of hydrophobin proteins. In addition, two proteins have been identified as having a potential role in recognition of the host surface during appressorium formation. Mpg1, for example, is a class I fungal hydrophobin, and disruption of the *MPG1* gene leads to a reduction in virulence at the point of appressorium formation (Kershaw and Talbot 1998; Talbot et al. 1996). When hydrophobins are secreted, they form an amphipathic layer at the host-pathogen interface where the pathogen can attach (Wessels 1996). Addition of exogenous cAMP remediates the Δ*mpg1* mutant phenotype, restoring pathogenicity (Talbot et al. 1993). Surface attachment is a prerequisite for triggering signalling cascades responsible for appressorium development. The class II hydrophobin *MHP1* gene shares 20% sequence similarity with *MPG1,* and disruption of *MHP1* was found to result in a reduction in pathogenicity (Kim et al. 2005). In contrast, recent studies have shown in the wheat blast isolate Br24, *MPG1* directly contributes to adhesion while *MHP1* was found to be dispensable for pathogenicity (Inoue et al. 2016). A combination of protein crystallisation and atomic force microscopy has revealed that Mpg1 self assembles at the fungus-host interface into an amyloid-like, rodlet structure (Pham et al. 2016). This is similar to the rodlet layers observed on the spore surface of *M. oryzae*, which are absent in Δ*mpg1* mutants (Talbot et al. 1996). Hydrophobins have also been implicated in direct interactions with cutinases and methyl esterase, secreted by *M. oryzae* on the host surface to aid tight adhesion, necessary for appressorium development (Skamnioti and Gurr 2007).

A second protein thought to be involved in host surface recognition is Pth11. Pth11 has previously been identified as a G-protein-coupled receptor necessary for appressorium formation (Fig. 13.1). The Δ*pth11* mutant phenotype could be reversed upon application of exogenous cAMP or diacylglycerol (DAG), suggesting a connection with downstream cAMP-dependent signalling (DeZwaan et al. 1999). Further studies have shown that Pth11 contains seven transmembrane regions and a CFEM (*C*ommon in several *F*ungal *E*xtracellular *M*embrane proteins) domain, comprised of eight cysteine residues (Kulkarni et al. 2005). Furthermore, the CFEM domain has been shown to be required for appressorium development and pathogenicity, suggesting that the CFEM domain is necessary for the putative surface-sensing function of Pth11 (Kou et al. 2017). In addition, highly regulated reactive oxygen species (ROS) homeostasis was also found to be important for Pth11-mediated appressorium formation in *M. oryzae* (Kou et al. 2017).

The *M. oryzae* genome contains sixty-one uncharacterised membrane-bound proteins, all thought to be G-proteins (Dean et al. 2012), suggesting that *M. oryzae* needs to respond to a considerable number of environmental and physical cues. G-proteins are involved in the transduction of signals from activated cell surface receptors to intracellular target effectors such as adenylate cyclase, phospholipases, kinases and ion channels (Gilman 1987; Simon et al. 1991; Strathmann et al. 1989; Stryer and Bourne 1986). Heterotrimeric G-proteins are well characterised, and comprised of three subunits: α, β and γ which are anchored to the plasma membrane through hydrophobic regions located on the β and γ subunits (Liu and Dean 1997; Malbon 2005). Once bound to GTP, the Gα subunits dissociate from the βγ subunit and are then free to interact with effector proteins in the cytoplasm, and in *M. oryzae*, three groups of subunits, MagA, MagB and MagC have been characterised (Liu

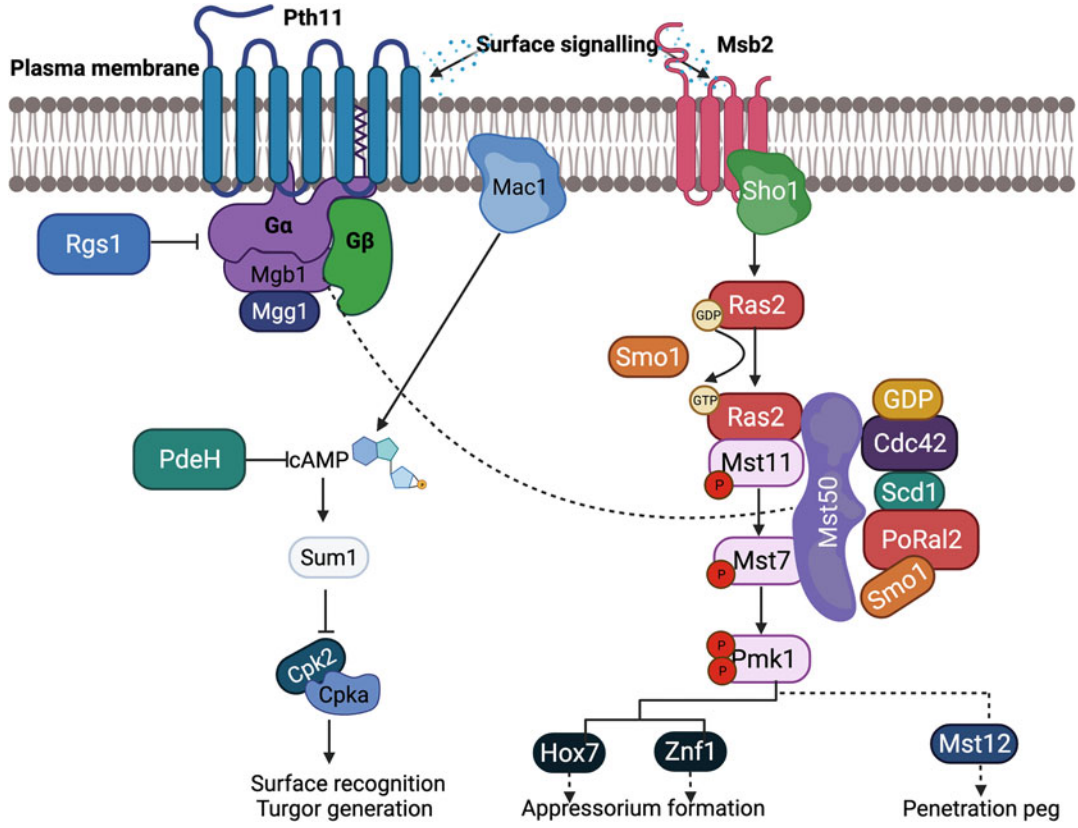

Fig. 13.1 Surface signalling transduction and appressorium formation in *M. oryzae.* Cross talk with the cAMP response pathway may occur through the G-subunit protein MagB. The G proteins MagA and MagB interact with the G-protein coupled receptor Pth11 to regulate the cAMP-response pathway, which is negatively regulated by Rgs1. The adenylate cyclase Mac1 is responsible for the accumulation of cAMP, which in turn binds to the regulatory protein kinase A subunit Sum1, resulting in detachment of the catalytic subunit CpkA and phosphorylation of downstream target proteins such as triacylglycerol lipases. The Mst50 scaffold protein tethers the Pmk1 MAPK module to produce a phospho-relay, where Pmk1 is phosphorylated and thereby activates transcription factors such as Mst12, Hox7 and Znf1. Mst50 interacts with proteins to regulate Pmk1 MAPK pathway including Gef1, Cdc42, Scd1 Ral2 and Smo1. Msb2 and Sho1 activate the Pmk1 MAPK pathway by recognising surface hydrophobicity and plant-derived compounds, such as cutin monomers and leaf waxes. Ras proteins Cdc42 and the Gβ subunit protein Mgb1 are also involved in activating the Pmk1 MAPK pathway. Figure modified from (Qu et al. 2021). Created with BioRender.com

and Dean 1997). Targeted gene deletion of *MAGA, MAGB* and *MAGC* showed *MAGB* to exhibit the strongest phenotype, displaying significantly reduced vegetative growth, conidiation and appressorium formation (Liu and Dean 1997). Once again, application of exogenous cAMP restored the Δ*magB* phenotype back to wild type, suggesting that MagB is involved in appressorium development upstream of the cAMP signal (Deising et al. 2000; Lengeler et al. 2000). In the absence of the Gα subunit, it is thought that the remaining Gβγ subunit prevents appressorium formation by constitutive repression of adenylate cyclase (Talbot 2003). *R*egulators of *G*-protein *S*ignalling (RGS) proteins function as GTPase-activating proteins (GAPs) and act to negatively regulate heterotrimeric G-protein cascades (Siderovski and Willard 2005). Disruption of the *RGS1* gene, for instance, results in precocious

appressoria which can form on both inductive and non-inductive surfaces, suggesting Rgs1 has a role to play in negatively regulating appressorium development (Liu et al. 2007). Considering that Δ*rgs1* mutants are unable to elaborate appressoria on soft surfaces even in the presence of exogenous cAMP, this highlights the importance of a thigmotropic cue for appressorium formation and cAMP accumulation. Moreover, a recent study revealed that the transcription factor Cos1, previously shown to be required for appressorium formation, displays elevated levels of *RGS1* transcription. A double Δ*cos1*Δ*rgs1* mutant displayed a delay in appressorium formation, and reduced melanisation and pathogenicity, suggesting that synergy between *RGS1* and *COS1* may be important for pathogenicity of *M. oryzae* (Na et al. 2019).

13.4 Pmk1 MAPK Signalling Pathway

Mitogen-Activated Protein Kinases (MAPKs) have important roles in signalling pathways and are highly conserved in a diverse range of eukaryotes ranging from yeast to mammals (Herskowitz 1995; Waskiewicz and Cooper 1995; Xu and Malave 2000). The development of appressoria in the absence of exogenous nutrients, on a hard hydrophobic surface requires the *P*athogenicity *M*AP *K*inase *1* (Pmk1) signalling pathway, critical for both appressorium formation and invasive hyphal growth (Sakulkoo et al. 2018; Zhao and Xu 2007). In fact, this signalling pathway has been reported to be essential for plant infection in more than 20 different phytopathogens (Jiang et al. 2018; Turrà et al. 2014). Pmk1 is orthologous to the Fus3/Kss1 MAPKs found in the budding yeast *Saccharomyces cerevisiae*. The Fus3 kinase is involved in pheromone signalling (Xu and Hamer 1996) while Pmk1 is necessary for to surface perception and appressorium morphogenesis (Turrà et al. 2014; Xu and Malave 2000). Disruption of *PMK1* through either homologous recombination, or replacement with an analogue-sensitive allele ($pmk1^{AS}$), renders the mutant unable to elaborate appressoria, and unable to grow invasively and cause disease, even after wound inoculation (Sakulkoo et al. 2018; Xu and Hamer 1996). Addition of exogenous cAMP initiates appressorium formation, but it is insufficient to complete appressorium formation and maturation, suggesting that Pmk1 operates downstream of the cAMP response pathway. MAPK signalling cascades are activated through a set of sequential phosphorylation events, which ultimately result in the activation of the MAPK by dual phosphorylation on a conserved Thr-Xxx-Tyr motif (Raman and Cobb 2003). Pmk1 appears to be activated by the MAPKK, Mst7, which in turn is activated by the MAPKKK Mst11 (Zhao et al. 2005). The adaptor protein, Mst50, was found to interact with both Mst11 and Mst7 and has an important role in the Pmk1 MAPK signalling cascade (Park et al. 2002). In fact, it has been suggested that Mst50 regulates activation of the Pmk1 MAPK pathway through interaction with Gef1, Cdc42, Scd1, Smo1 and Ral2 (Qu et al. 2021) (Fig. 13.1). Mst50 has a sterile α motif (SAM) and a Ras association domain (RAD), and physically interacts with two Ras proteins in *M. oryzae*, Ras1 and Ras2. Ras2 has been reported to have an important role in both cAMP-signalling and the Pmk1 MAPK pathway (Park et al. 2006). More recently, PoRal2 was identified in *M. oryzae* and shown to have an important role in sporulation, appressorium formation, plant penetration and virulence. Δ*PoRal2* mutants, for instance, were found to generate appressoria with elongated germ tubes on hydrophobic surfaces and exhibited a defective response to exogenous cAMP and activated $RAS2^{G18V}$ on a hydrophilic surface, indicating impairment in the cAMP-PKA or Pmk1-MAPK signalling pathways (Qu et al. 2021). Furthermore, compared to the wild type, Δ*PoRal2* mutants exhibited a lower level of Pmk1 phosphorylation, and PoRal2 was found to interact with Scd1, Smo1, and Mst50, which are all thought to be involved in Pmk1 activation (Qu et al. 2021) (Fig. 13.1). Additionally, two thioredoxin-encoding genes, *TRX1* and *TRX2*, have an important role in pathogenesis. Trx2 was shown to interact with Mst7 resulting in the

regulation of the Pmk1 MAPK pathway (Zhang et al. 2016b).

A recent study using a combination of transcriptomics, discovery phosphoproteomics and molecular biology has provided evidence that Pmk1 acts as a central regulator of appressorium morphogenesis (Oses-Ruiz et al. 2021). Pmk1 directly phosphorylates a suite of transcription factors including Mst12, required for appressorium repolarisation, and Hox7 which is required for appressorium formation (Cao et al. 2016; Kim et al. 2009; Oses-Ruiz et al. 2021; Park et al. 2002; Sweigard et al. 1998; Yue et al. 2016) (Fig. 13.1). However, understanding how Pmk1 and its target proteins regulate such a diverse range of genes involved in appressorium morphogenesis, plant penetration and host colonisation remains to be elucidated.

13.5 How Is Turgor Sensed Within the Appressorium

Appressorium morphogenesis takes place inside dew drops on the rice leaf surface, where free water acts as an essential prerequisite for appressorium formation. Appressorium turgor pressure is generated by an osmotic mechanism, whereby water flows into these cells against a concentration gradient, generated by the synthesis of compatible solutes such as glycerol to molar concentrations. A study by de Jong and co-workers identified glycerol as the main solute to accumulate in the *M. oryzae* appressorium (de Jong et al. 1997). Considering that appressoria form in free water on the host leaf surface in the absence of any external nutrients, this suggests that glycerol synthesis originates solely from storage products present in the conidium of the fungus. The likely precursors for glycerol production found in conidia are lipids, glycogen, mannitol and trehalose (Foster et al. 2003; Talbot 1995; Thines et al. 2000). During spore germination, degradation, and/or transport of trehalose, glycogen and lipids to the germ tube apex occurs (Foster et al. 2003, 2017; Thines et al. 2000).

Melanin synthesis and glycerol production collectively drive turgor-mediated plant infection. In *M. oryzae*, the melanin layer located on the inner side of the appressorium cell wall creates a semi-permeable barrier to retain glycerol and draws water into the cell by osmosis (Chumley and Valent 1990). *M. oryzae* melanin biosynthetic mutants, for instance, are non-pathogenic and unable to generate turgor (Chumley and Valent 1990). Upon germination, glycogen reserves are rapidly utilised (Bourett and Howard 1990; Thines et al. 2000). The amyloglucosidase-encoding gene *AGL1*, and glycogen phosphorylase-encoding gene *GPH1*, for example, were both found to be dispensable for turgor generation and rice penetration. However, *in planta* proliferation was disrupted, suggesting that glycogen metabolism is likely to be required for completion of the fungal life cycle (Badaruddin et al. 2013).

Trehalose, a carbohydrate common to algae and fungi, is thought to be involved in the conversion of glycogen to triacylglycerides for energy. Trehalose is synthesised by a multienzyme complex, the catalytic subunit of which is trehalose-6-phosphate synthase 1 (Tps1) required to produce the non-reducing dissacharide trehalose from G6P and uridine-disphosphate (UDP)-glucose (Fernandez and Orth 2018; Fernandez and Wilson 2012; Wilson et al. 2007, 2010). Tps1 has an important role in glucose-6-phosphate (G6P) sensing, possibly allowing the fungus to adapt to nutrient oscillations during plant colonisation. Δ*tps1* mutants, for instance, are non-pathogenic due to a role in G6P-binding, regulation of NADPH levels and the control of sugar sensing and nitrogen source utilisation, independent of its trehalose-6-phosphate production (Foster et al. 2003; Wilson et al. 2007). Furthermore, screening of a chemical database alongside structural modelling of the Tps1 active site, identified a potential inhibitor of Tps1, chemical Lead 25, which displayed a high affinity for Tps1, potentially providing a means by which rice blast disease could be controlled in the field (Xue et al. 2014).

Conidia also contain lipid droplets, and lipid degradation has been proposed as a major route to

glycerol accumulation in appressoria (Thines et al. 2000). Lipid bodies are transported to the developing appressorium in a Pmk1-dependent manner, but their degradation and subsequent fatty acid and glycerol synthesis requires the cAMP/PKA pathway (Wang et al. 2007). Protein kinase A is a cAMP-dependent tetrameric holoenzyme present in eukaryotic cells involved in cAMP signalling. PKA becomes activated when cAMP binds to two regulatory subunits allowing two remaining catalytic subunits to be released, allowing phosphorylation of downstream target proteins (Kronstad et al. 1998). Gene replacement of *CPKA*, a gene encoding the catalytic subunit of the cAMP-dependent protein kinase A, failed to block appressorium formation and the response to exogenous cAMP, but did, however, generate smaller appressoria with less turgor that are unable to penetrate rice. A second gene encoding the catalytic subunit *CPK2* was also identified and is dispensable for pathogenicity. However, surprisingly, a double Δ*cpkA*Δ*cpk2* mutant displayed a significant reduction in growth rate and conidiation. In addition, the mutant exhibited morphogenetic defects in germ tubes on hydrophobic surfaces, and complete failure to elaborate appressoria, suggesting an important role for Cpk2-mediated cAMP-PKA in surface sensing and response (Selvaraj et al. 2017). Moreover, spontaneous suppressors of Δ*cpkA*Δ*cpk2* restore hyphal growth and appressorium formation on hydrophobic surfaces, but not pathogenicity. Intriguingly, loss of function mutations in the transcription factor MoSfl1 can bypass the requirement for PKA activity and restore growth and appressorium formation to the Δ*cpkA*Δ*cpk2* double mutant (Li et al. 2017). Phosphorylation of Sfl1 by PKA disrupts the MoSfl1-Cyc8-Tup1 cascade to regulate the expression of downstream genes involved in growth and pathogenicity (Li et al. 2017). High levels of triacylglycerol lipase activity have been found in germinating conidia and enzyme activity increases upon the onset of turgor generation, concomitant with the breakdown of lipid bodies (Thines et al. 2000). Deletion of any of the eight predicted intracellular triacylglycerol lipase-encoding genes had no effect on pathogenicity, including enzymes predicted to be lipid droplet-associated lipases, patatin-like phospholipases, and orthologues of hormone-dependent lipases, as well as the major predicted cytoplasmic triacylglycerol lipases. A high level of functional redundancy amongst triacylglycerol lipases makes them a suitable target for CRISR-Cas9-based gene editing, where a counterselection strategy could be used to precisely edit each triacylglycerol lipase-encoding gene without insertion of foreign DNA, allowing for multiple triacylglycerol lipase-encoding gene disruptions in one isogenic wild type background (Foster et al. 2018). Such advances in technology and successful deployment in *M. oryzae* would help determine, for example, the importance of these enzymes in lipid breakdown and subsequent glycerol production and turgor generation. Mutation of the multifunctional-β-oxidation gene *MFP1,* caused a reduction in virulence, predominantly linked to melanisation highlighting the requirement for lipid metabolism in fuelling polyketide synthase biosynthesis (Wang et al. 2007).

13.6 Melanin Biosynthesis and Regulation

There are two major pathways responsible for melanin production in the fungal kingdom. The first includes polymerisation of phenolic compounds derived from the environment or metabolic pathways, for example, L-3-4-dihydroxyphenylalanine (LDOPA) melanin and pyomelanin, produced by the human fungal pathogen *Aspergillus fumigatus* (Eisenman and Casadevall 2012) and (Schmaler-Ripcke et al. 2009). The second pathway includes *de novo* synthesis of the phenolic compound 1,8-dihydroxynaphthalene (DHN) by a polyketide synthase (PKS) pathway and its subsequent polymerisation leading to DHN-melanin (Bell and Wheeler 1986; Butler and Day 1998; Henson et al. 1999; Wheeler 1983). Acetate is a precursor for melanin biosynthesis, entering a series of sequential enzymatic steps via the enzymes Alb1->4Hnr->Rsy1->Buf1. The first intermediate in the pathway is 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-HN; 4HN).

Alternating reduction and dehydration reactions lead to scytalone, 1,3,8-trihydroxynaphthalene (1,3,8-HN; 3HN), vermelone, and 1,8-dihydroxynaphthalene (1,8-HN; DHN) (Chumley and Valent 1990). DHN monomers are subsequently polymerised to yield melanin around the appressorial cell wall through oxidative activity of laccases. Laccases are multi-copper oxidases that participate in pigment biosynthesis. In *Colletotrichum orbiculare*, for example, *LAC2* was shown to be important for polymerisation of appressorium-specific melanin (Lin et al. 2012). In *Magnaporthe*, however, 18 putative laccase-encoding genes are present with different expression profiles during both fungal mycelium growth and appressorium-mediated plant infection, so their function during DHN melanin biosynthesis remains to be determined. In addition to melanin biosynthetic enzymes, several genes have been implicated in regulation of melanin biosynthesis in *M. oryzae*. Melanin biosynthesis in appressoria is developmentally regulated and likely regulated by both transcriptional and post-translational means (Takano et al. 1995; Tsuji et al. 2000). Mutation of the *ALB1*, *RSY1* and *BUF1* genes causes a deficiency in both appressorial and hyphal melanisation (Chumley and Valent 1990). The transcription factor, *PIG1* (*pig*ment of *M. oryzae*) is required for hyphal melanisation but dispensable for appressorium-specific melanin production. The transcription factors Cnf1 and Cos1 have also been implicated in regulating melanin production in hyphae (Li et al. 2013; Lu et al. 2014; Tsuji et al. 2000). In appressoria, disruption of the Cys_2-His_2 zinc *M. oryzae* transcription factor *VRF1* (*V*irulence *R*egulatory *F*actor 1), caused abnormal growth of appressoria and a significant reduction in melanin production. In a Δ*vrf1* mutant (also named *ZNF1*, (Yue et al. 2016), RNA-Seq analysis from 5 h appressoria revealed a significant reduction in expression of all the core melanin biosynthetic enzymes. Interestingly, in contrast, RNA-Seq data from mycelium showed disruption of *VRF1* had no effect on the expression of melanin biosynthetic enzyme-encoding genes, suggesting that Vrf1 is required for appressorium-specific melanisation (Cao et al. 2016; Yue et al. 2016). There are many diverse strains of *M. oryzae* in the field, and it is evident that some are lighter in colonial colour than others, which are darker due to excess melanin content. A comparison between Guy11, a strain isolated from rice originating from French Guyana (Leung et al. 1988) and the reference genome strain 70–15 (Dean et al. 2005), for example, revealed differences in mycelial melanisation. 70–15 was obtained by a series of genetic crosses, initially between the rice-infecting strain 104–3 and a weeping lovegrass (*Eragrostis curvula*)-pathogenic strain AR4 (Kolmer and Ellingboe 1988) and then cross/backcrossed with Guy11 (Chao and Ellingboe 1991). Guy11 was found to display a much higher level of melanin biosynthetic gene transcripts when compared to 70–15. Furthermore, paired culture experiments between deletion mutants or melanin overexpression strains suggest that the reaction catalysed by Buf1, but not Alb1 and Rsy1, is the likely rate-limiting step in melanin biosynthesis in 70–15 (Zhu et al. 2021). In addition to melanin regulation, it is also important to consider how melanin is trafficked and deposited extracellularly on the outside of the appressorium cell wall. Melanosomes, for instance, are organelles that produce and store melanin. A forward genetic screen in *Aspergillus fumigatus* identified mutations in an endosomal-sorting nexin *MVP1*, which abolished melanin cell wall deposition. Furthermore, fungal melanin biosynthesis was initiated in endosomes with exocytosis leading to melanin extracellular deposition, analogous to the synthesis and trafficking of mammalian melanin (Upadhyay et al. 2016). Determining the exact mechanism for melanin delivery and secretion in *M. oryzae* would expand our understanding of the synthesis and delivery of melanin. This could potentially be an important pathway to define for future design of effective antifungal agents, like tricyclazole, pyroquilon and phthalide, all of which are known to cause accumulation of 3HN by-products in fungal

cultures and inhibit rice blast disease (Howard and Valent 1996).

13.7 Turgor Sensing

The internal hydrostatic turgor of filamentous fungi is an important feature of growing hyphae and appressorium development, requiring precise and coordinated synthesis of both the plasma membrane and the cell wall for controlled cellular expansion (Wessels 1993). In the oomycete, *Saprolegnia ferax,* the rate of growth through solid medium decreases as turgor pressure is lowered, and is more pronounced at higher agar concentrations, suggesting that turgor provides force for invasive hyphal growth (Money 1995). *M. oryzae* can breach the surface of rice leaves and a variety of synthetic membranes. In fact, the famous 'gold leaf' experiment performed by Brown and Harvey in 1927, in which plant leaves were wrapped in a thin gold layer and inoculated with fungal spores, elegantly demonstrated that fungi can puncture an inert surface, requiring force generation rather than enzymatic activity (Brown and Harvey 1927; Talbot 2019). It has been noted that there is variation in the amount of force being exerted by appressorium-forming species. In *M. oryzae*, the appressorium generates up to 8.0 MPa (80 atmospheres) of turgor (Talbot 2003). In the related fungal species, *Colletotrichum graminicola*, the force measured at the base of the appressorium has been measured using an optical waveguide to be 17 μN (Bechinger et al. 1999). Determining how the appressorium perceives a signal to repolarise and cause disease has remained a long-standing question in the field of *M. oryzae* research.

In budding yeast *Saccharomyces cerevisiae,* a branched multistep phospho-relay signalling pathway regulates cellular adaptation to osmotic stress (Xu et al. 2003). Two-component signalling pathways are well conserved, ranging from prokaryotes to lower eukaryotes including plants and fungi (Reiser et al. 2003), and undergo a sequential histidine to aspartate phosphoryl transfer between a membrane-bound sensor histidine aspartate kinase, a histidine-containing phosphotransfer (HPt) protein and a cytoplasmic response regulator protein. In *S. cerevisiae* the sensor kinase Sln1 has a periplasmic region, flanked by two transmembrane (TM) helices, followed by a cytoplasmic region containing the kinase and receiver domains (Mascher et al. 2006). Under normal growth conditions, Sln1 is autophosphorylated, but upon exposure to hyperosmotic stress, when for example, osmolarity rises, it becomes dephosphorylated. Dephosphorylated Sln1–Ypd1–Ssk1 causes the unphosphorylated form of Ssk1 to interact and stimulate the activity of a redundant pair of (MAPKKK) protein kinases Ssk2 and Ssk22 (Horie et al. 2008). These in turn phosphorylate the Pbs2 MAPKK and the *H*igh *O*smolarity *G*lycerol response signalling pathway (*HOG1* pathway) (Saito and Posas 2012). The Hog1 MAPK is responsible for sensing hyperosmotic stress and for transmitting these signals to the nucleus to modulate gene expression. Phosphorylated Hog1 induces expression of genes encoding enzymes involved in glycerol production and uptake (Babazadeh et al. 2014; Brewster and Gustin 2014; Gomar-Alba and Alepuz 2013; Lee et al. 2013; O'Rourke et al. 2002; Saito and Posas 2012). Increased expression of glycerol-3-phosphate dehydrogenase 1 (*GPD1*) for instance, enhances glycerol production under hyperosmotic stress (Albertyn et al. 1994; Rep et al. 1999). Hog1 has also been shown to regulate the aquaglyceroporin Fps1, responsible for glycerol transport in response to changes in extracellular osmolarity. Upon exposure to hyperosmotic shock, Rgc2 (*R*egulator of *G*lycerol *C*hannel) rapidly dissociates from Fps1 consequently causing channel closure (Lee et al. 2013).

Sln1 histidine aspartate kinase is therefore an osmosensory kinase and may act in a similar way in *M. oryzae* during hyperosmotic stress adaptation. However, Sln1 signalling during appressorium turgor generation is different, and it operates independently of the Hog pathway. The *HOG1* homologue *OSM1* is dispensable for glycerol production and pathogenicity (Dixon et al. 1999). A recent study has suggested an additional role for Osm1 in detoxifying plant-derived ROS, through phosphorylation of the transcription factor Aftf1,

causing a dissociation of the Aft1-Tup1 complex, releasing Tup1-mediated transcriptional repression of genes involved in oxidoreduction pathways such as thioredoxins (Liu et al. 2020). Furthermore, *M. oryzae* Ypd1 is required for sporulation (Jacob et al. 2015). Silencing of *YPD1* by RNA interference disrupts appressorium-mediated plant infection (Mohanan et al. 2017), and Δ*ssk1* mutants display reduced pathogenicity (Motoyama et al. 2008).

In *M. oryzae* recent findings have suggested an important role for Sln1 in modulating appressorium-specific turgor, enabling the appressorium to sense when a critical threshold of turgor has been reached to enable host penetration (Ryder et al. 2019). Δ*sln1* mutants in *M. oryzae* generate runaway turgor pressure, have hyper-melanised appressoria, and are significantly reduced in pathogenicity (Ryder et al. 2019; Zhang et al. 2010). A combination of mathematical modelling, based on reactive diffusion differential equations to identify prerequisites for appressorium repolarisation, RNA-Seq analysis and discovery phosphoproteomics showed that the Sln1 sensor kinase interacts with mechanosensitive ion channel proteins (Mic1, Mic2 and Mic3) to maintain intracellular osmotic homeostasis. Once a sufficient turgor threshold has been achieved, it is maintained through action of Sln1 which negatively regulates melanin biosynthesis and the cAMP/Protein kinase A pathway. It is likely Sln1 works in parallel with the protein kinase C (Pkc1) cell integrity pathway, phosphorylating the phosphodiesterase enzyme PdeH to modulate cAMP levels, which in turn controls lipolysis and glycerol production through the action of Sum1/CpkA dissociation (Thines et al. 2000). Pkc1 may also target the NADPH oxidase R regulatory subunit (NoxR), the homologue to mammalian $p67^{phox}$ which controls the regulated synthesis of ROS, necessary for septin-mediated cytoskeletal reorganisation (Ryder et al. 2013, 2019) (Fig. 13.2). Determining a detailed molecular mechanism responsible for turgor sensing and how it is transmitted downstream of Sln1 remains to be determined.

13.8 Septin-Dependent Plant Infection

Septins are a family of small morphogenetic filament-forming guanosine triphosphatases (GTPases), first discovered by Hartwell in *S. cerevisiae* (Hartwell 1971). Septins are highly conserved from yeast to humans (Gladfelter et al. 2005; Lindsey and Momany 2006), and are implicated in a variety of biological processes such as cytokinesis, polarity determination, secretion, as well as membrane remodelling and scaffolding capabilities (Douglas et al. 2005; Robertin and Mostowy 2020; Spiliotis and Nelson 2006; Szuba et al. 2021). Septins are thought to interact with other cytoskeletal elements, for example, in the nucleation and branching of actin filaments (Hu et al. 2012; Mavrakis et al. 2014). However, it is not fully understood how these interactions occur, and whether they depend on polymerisation of septins (Cavini et al. 2021; Mavrakis et al. 2014; Spiliotis and Nakos 2021). Recently, it was shown in budding yeast that septin hetero-octamers were able to reshape membranes (Vial et al. 2021). How septins assemble into filaments and other more sophisticated structures such as collars and gauzes remains an important biological question. Studies have already revealed post-translational modifications including phosphorylation, acetylation, ubiquitination and sumoylation modulate septin dynamics (Hernández-Rodríguez and Momany 2012; Johnson and Blobel 1999; Ribet et al. 2017; Takahashi et al. 1999; Zhang et al. 2000). A total of five septin genes have been characterised in *M. oryzae,* four of which share similarity to core septins identified in yeast (Cdc3, Cdc10, Cdc11 and Cdc12) (Hartwell 1971) namely Sep3, Sep4, Sep5 and Sep6, respectively. Septins are important for *M. oryzae* pathogenicity and are independently required for correct F-actin localisation. Expression and localisation of septins using GFP (green fluorescent protein) revealed how appressorium-mediated plant infection requires the assembly of a dynamic higher-order septin ring structure at the base of the appressorium, to facilitate and

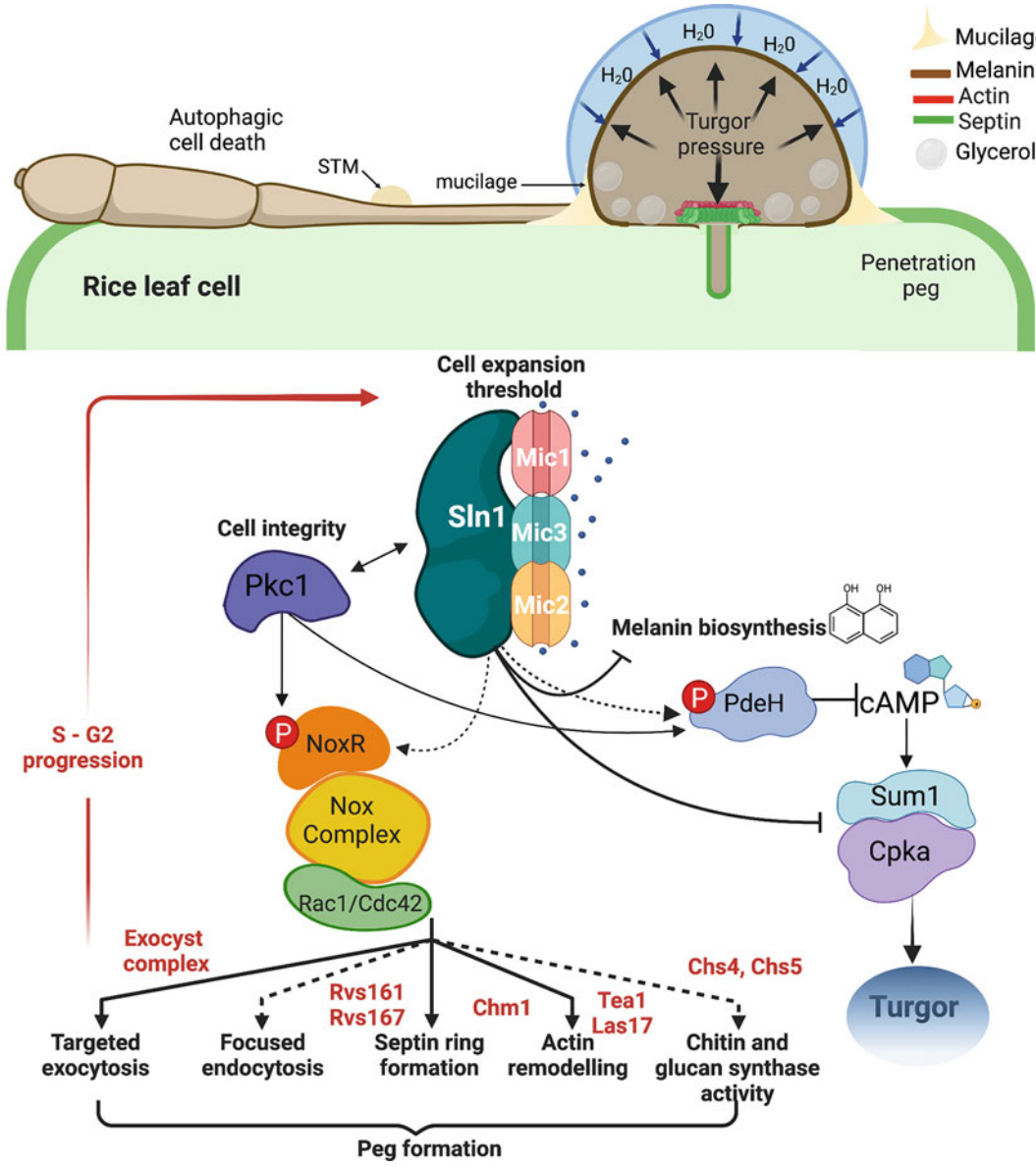

Fig. 13.2 Model of turgor-driven plant cell invasion by *M. oryzae*. The Sln1 sensor kinase responds to appressorium turgor by interaction with a set of upstream monitors of cell expansion, including stretch-activated ion channel proteins, Mic1, Mic2 and Mic3. Once a turgor threshold is reached, Sln1 negatively regulates melanin biosynthesis and the cAMP/PKA pathway controlling likely routes to glycerol production. Sln1 can interact with Pkc1 (a central regulator of cell integrity), which may phosphorylate PdeH phosphodiesterase to modulate cAMP levels, as well as lipolysis and glycerol production. Sln1 acts to directly recruit septins to the appressorium pore, requiring Pkc1 and NADPH oxidase Nox2. Septins and F-actin together help to maintain cortical rigidity inside the appressorium, as well as organising the exocyst complex and a large family of endocytic proteins at the appressorium pore. Septins act as a lateral diffusion barrier to ensure the switch from isotropic expansion to polarised cell growth, requiring focused polymerisation of F-actin as well as chitin and glucan synthases. A pressure-dependent S-phase checkpoint is also activated and is required for Sln1 activity. Collectively, these processes lead to host leaf cuticle penetration. Created with BioRender.com

spatially orchestrate appressorium repolarisation (Dagdas et al. 2012; Dulal et al. 2020) (Fig. 13.3). A recent study using two-colour 4D fluorescence imaging elegantly showed the dynamic recruitment of F-actin to the surface of the *M. oryzae* septin ring, confirming that formation of an actin ring precedes septin remodelling and is necessary for contraction and remodelling of the septin ring (Dulal et al. 2020, 2021). Furthermore, both F-actin ring and septin ring recruitment occur in a pressure-dependent manner (Dulal et al. 2021; Ryder et al. 2019). The melanin-deficient mutants *alb1*$^{-}$, *rsy1*$^{-}$ and *buf1*$^{-}$, for example, cannot generate high pressure (Chumley and Valent 1990). In a Δ*buf1* mutant, septins and F-actin are recruited in a disordered fashion, leaving them unable to contract and reorganise (Dulal et al. 2020, 2021). Similar outcomes occurred when melanin biosynthesis was chemically disrupted by addition of tricyclazole, or when intracellular turgor production was perturbed upon application of exogenous glycerol to developing appressoria (Dulal et al. 2020, 2021).

In addition to maintaining cortical rigidification inside the appressorium, septins also provide a lateral diffusion barrier for correct localisation of numerous proteins including the actin-associated protein Las17, required for F-actin polymerisation via the arp2/3 complex, and Tea1, an ERM protein (ezrin, radixin, moesin) required to tether actin to the plasma membrane (Dagdas et al. 2012; Dulal et al. 2020; Van Ngo and Mostowy 2019). More recently, very long chain fatty acid (VLCFA) biosynthesis was shown to regulate phosphatidylinositol phosphate (PIP)-mediated septin ring formation by recruiting septins to curved plasma membranes, initiating septin ring formation and subsequent penetration peg emergence (He et al. 2020). Targeted gene disruption of the conserved fatty acid elongase gene *ELO1* necessary for VLCFA production, compromised both VLCFA and PIP-containing VLCFAs, causing disruption in septin ring formation and pathogenicity. Intriguingly, compounds specifically targeting VLCFA biosynthesis effectively inhibited rice blast disease by preventing septin-dependent penetration, but remarkably, these chemicals were also found

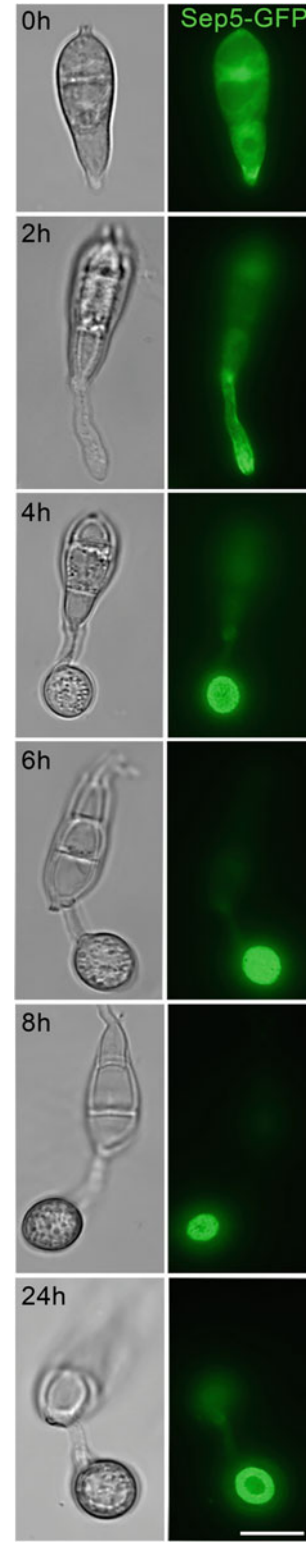

Fig. 13.3 Septin-mediated cytoskeletal reorganisation in *M. oryzae* appressoria. Time course of septin ring

to be effective at inhibiting other fungal pathogens, including the corn leaf blight fungus *Bipolaris maydis*, wheat powdery mildew fungus *Blumeria graminis* and the locust pathogen *Metarhizium acridum* (He et al. 2020).

ROS generated by membrane-bound NADPH oxidases (Nox) have important roles in defence, cell signalling and act as second messengers in animals and plants. Noxs are a family of flavocytochromes that appear highly conserved from algae to humans. Nox proteins are able to transfer electrons across biological membranes onto molecular oxygen to generate superoxide anions ($O_2^{\cdot-}$). Superoxide can be released unmodified or protonated and reduced to form H_2O_2. In fungal systems, specific isoforms of Nox have been shown to be important for various physiological processes involving cellular differentiation including the development of sexual fruiting bodies, ascospore germination, hyphal defence, and hyphal growth in both mutualistic and antagonistic plant-fungal interactions (Takemoto et al. 2007; Tanaka et al. 2008). In *M. oryzae,* a regulated burst of ROS is required for septin-mediated cytoskeletal reorganisation and appressorium repolarisation. In the absence of *NOX2* and *NOXR*, septins are recruited as a dense patch at the appressorium pore and do not form the toroidal network essential for facilitating plant infection (Egan et al. 2007; Ryder et al. 2013). A second Nox-encoding enzyme, Nox1, was found to be necessary for the maintenance of polarity and organisation of the F-actin network at the base of the appressorium (Egan et al. 2007; Ryder et al. 2013). Furthermore, the discovery and disruption of the *NOXD*-encoding gene, a homologue of mammalian $p22^{phox}$, was shown to be required for septin ring assembly at the appressorium pore. This study also identified the transcription factor Tpc1 to be required for regulation of *NOXD* expression via an interaction with Mst12 (Galhano et al. 2017). Appressorium-specific ROS is likely to stimulate actin cytoskeletal reorganisation directly by modifying the uncapping ability of actin-binding proteins, such as gelsolin (Ryder et al. 2013), or indirectly via signalling components likely to act downstream of the turgor-sensing kinase Sln1. Components likely to be involved in this process include the protein kinase Chm1, implicated in septin ring phosphorylation, and Cdc42, a member of the Rho family controlling actin cytoskeleton organisation and cell polarity (Dagdas et al. 2012; Takemoto et al. 2007; Zheng et al. 2009). In the biotrophic fungus *Epichloë festucae*, a mutualistic association is established as it colonises the intracellular spaces of aerial tissues to form an endophytic hyphal network in *Lolium perenne*. Although this fungus does not require appressoria to breach its host leaf cuticle, it does, however, generate appressorium-like structures called 'expressoria' to exit the plant. These structures require components from the NoxA and NoxB NADPH oxidase complexes. Moreover, septin ring formation was observed at the point of cuticle exit, requiring NoxB for correct localisation and function (Becker et al. 2016).

Intriguingly, Nox enzymes have been implicated in the chemiosmotic generation of turgor pressure (Segal 2016). In mammalian systems, for example, it is known that the passage of electrons across the membrane causes membrane depolarisation (Henderson et al. 1987), which in turn generates a charge that requires compensating protons to allow further movement of electrons. The electrons, not the charge-compensating proteins, influence the osmotic status of the cell. However, other charge-compensating ions do. In a phagocytic vacuole, for instance, an influx of cations such as K^+ ions causes swelling (Levine et al. 2015). Most of the charge, however, is compensated by protons, meaning there is little osmotic effect, compared to a scenario where instead, the charge-compensating protons come from cations, which could be the case if Nox was functioning to increase tonicity (Segal 2016). When we consider

Fig. 13.3 (continued) formation during appressorium morphogenesis. Micrographs of septin ring organisation imaged by expressing Sep5-GFP driven by Sep5 native promotor in wild type strain of *M. oryzae*, Guy11. Conidial suspensions of $5x10^4$ spores/mL were inoculated onto hydrophobic glass coverslips and images captured at different time intervals during infection-related development (0–24 h). Scale bar = 10 μm

M. oryzae appressoria, the accumulation of glycerol and other polyols essential for turgor generation could therefore act as a source for Nox-compensating cations. Future experiments to measure ion fluxes directly linked to Nox would be interesting to explore, in order to characterise the role of Nox in possible cellular turgor generation.

Finally, Spermine synthase (Ssp1) has been shown to act as an important antioxidant against Nox-derived reactive oxygen species in the endoplasmic reticulum, leading to correct spatial production and secretion of spore tip mucilage (Bechinger et al. 1999; Rocha et al. 2020). In a Δ*ssp1* mutant, for instance, the seal is lost between the host surface and the appressorium pore, resulting in solute leakage and the appressorium being unable to generate sufficient turgor for plant infection.

13.9 Conclusions

There is considerable diversity in the ways in which appressorium turgor is generated in different fungal pathogens (Chang et al. 2014; Howard et al. 1991; Loehrer et al. 2014; Ludwig et al. 2014; Ryder and Talbot 2015). In contrast, similarities in the regulation of appressorium morphogenesis in diverse organisms are much more apparent, including cell cycle control and operation of a conserved MAP kinase pathway for appressorium differentiation. During the last decade, key advances have begun to elucidate how isotropic expansion of the appressorium is translated into generation of invasive force necessary to breach the host cuticle (Dagdas et al. 2012; Gupta et al. 2015; Ryder et al. 2013). Moreover, recent work has demonstrated a working model for turgor-driven plant infection in *M. oryzae*, in which the Sln1 histidine aspartate kinase is required for the modulation of turgor, acting as a regulator of downstream processes to ensure the optimal timing of repolarisation of the appressorium (Ryder et al. 2019) (Fig. 13.2). Future experiments will need to define precisely how turgor is sensed within a *M. oryzae* appressorium and how the cell uses this information to repolarise and invade plant tissue. Recent advances in *M. oryzae* discovery phosphoproteomics, proximity labelling, and Selective Reaction Monitoring (SRM) mass spectrometry, will help define exactly how Sln1 operates during turgor-driven plant infection, and elucidate its interplay with the cell integrity, osmo-sensory, cell cycle, NADPH oxidase, autophagy, and cAMP-dependent signalling pathways, necessary for appressorium function (Dulal et al. 2021; Kadota et al. 2014; Ryder et al. 2019).

How pathogens move from cell to cell in host tissue remains to be resolved in both animal and plant-pathogenic fungi (Cruz-Mireles et al. 2021). It has been observed, for instance, that severe hyphal constriction occurs at pit field sites between rice cells where plasmodesmata accumulate (Faulkner et al. 2008). In a *M. oryzae* compatible interaction, invasive hyphae swell at crossing points before undergoing severe constriction to 0.6–0.8 μm to enter neighbouring cells, which is highly reminiscent of appressorium-dependent penetration. These hyphal swellings have been coined 'transpressoria' and are specific *in planta* infection structures formed by fungi to move between host cells (Liese 1970; Liese and Schmid 1964; Emmett and Parbery 1975). It has been noted, for instance, that penetration through pit fields outlining host rice epidermal cells requires isotropic expansion followed by severe hyphal constriction, which is Pmk1 MAPK-dependent (Sakulkoo et al. 2018). Intriguingly, it is yet to be determined whether transpressoria requires a pressure-dependent mechanism to forcibly invade neighbouring cells and whether this mechanism requires the Sln1 histidine aspartate kinase to modulate this pressure, and if so, is this achieved in the same way as in an appressorium. Do transpressoria, for instance, ever melanise, or are there other cell wall modifications that enable the transpressorium to achieve sufficient pressure to breach neighbouring cells? Is this turgor pressure solute-dependent, for instance, and do transpressoria synthesise glycerol to molar concentrations in the same way as an appressorium?

Direct measurement of appressorium turgor pressure has so far been challenging, largely because *M. oryzae* appressoria generate a substantial amount of pressure, making it extremely difficult to reliably quantify using physical techniques such as pressure probes. Appressorium turgor measurements have therefore relied on proxy measures with the use of the incipient cytorrhysis assay, where appressoria are incubated in hyperosmotic concentrations of glycerol or PEG, and the subsequent cell collapse rate is recorded, providing an indirect measure of appressorium turgor (de Jong et al. 1997; Foster et al. 2017). Now, with huge advances in mechanobiology, a subset of chemically modified molecular rotors that yield complete microviscosity maps of cell membranes has been developed (Michels et al. 2020). These borondipyrromethene (BODIPY)-based molecular rotors are rigidochromic by means of coupling the rate of intramolecular rotation, which depends on the mechanics of their direct surroundings, with their fluorescence lifetime. The plasma membrane-associated probe N^+-BDP was shown to measure the viscosity of membranes, and in plant root cells, for instance, various properties of different tissue types, which are essentially a function of the viscosity of their membranes, could be quantified. Future experiments employing the use of such molecular rotors in *M. oryzae* appressoria, for example, may provide new quantifiable insights into spatial variations in microviscosity and appressorium-mediated turgor-driven plant cell infection.

Finally, it is important that fundamental knowledge of appressorium biology is translated into new methods for disease control. A recent example in which a fundamental analysis of septin recruitment to the appressorium pore resulted in the discovery of new broad-spectrum fungicides (He et al. 2020) shows how this is possible. More studies of this kind could therefore provide durable strategies for controlling devastating crop diseases such as rice blasts.

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Role of Light in the Life Cycle of *Botrytis cinerea*

14

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Abstract

The fungus *Botrytis cinerea (Botryotinia fuckeliana)* infects more than 500 plant species and causes a wide range of symptoms: soft rots, accompanied by collapse and water-soaking of tissues followed by the appearance of gray masses of conidia on leaves and soft fruits (gray mold), and spots that may turn brown to full-scale soft rotting on flower petals (Botrytis blight). In general, *B. cinerea* is responsible for severe economic losses that are either due to the damage of growing plants in the field or the rot of harvested fruits, flowers, and vegetables during storage under cold and humid conditions. *B. cinerea* has adapted to the plant host and its environment by evolving strategies to use plant tissues for proliferation in terms of a necrotrophic lifestyle and to survive biotic (host responses) as well as abiotic stresses such as sunlight. *B. cinerea* maintains a complex regulatory network of light-sensitive proteins and signal transduction pathways to use light for coordinating stress responses, virulence, and reproduction. Different light-controlled reproduction cycles enable *B. cinerea* to live in moderate climate zones by infecting and propagating in summer and remaining dormant in winter when green host tissues are unavailable as a nutrient source.

Keywords

Gray mold fungus · Plant pathogen · Light · Photoreceptors · Development

14.1 Adaptations to the Plant Host

The interaction between *B. cinerea* and its host plants cover a spectrum of outcomes, ranging from full immunity (no infection) to full susceptibility (fast infection) and all the intermediate stages in between. Another lifestyle option, although not yet that well understood, is the asymptomatic colonization of the host (Williamson et al. 2007; Shaw et al. 2016; Veloso and van Kan 2018). The outcome of the interaction with the host depends on the host species and its capacity to prevent fungal growth, the *B. cinerea* strain and its growth characteristics, and the climate conditions which can be more favorable for the host or the pathogen. Notably, *B. cinerea* field populations are characterized by high levels of variability in morphology and preferred mode of reproduction, virulence, spectra of secondary metabolites produced, and fungicide resistance. The genetic variability can be achieved through sexual reproduction, hyphal anastomosis, transposable elements,

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heterokaryosis, heteroploidy, or viruses (Coley-Smith et al. 1980; Elad et al. 2004; Fillinger and Elad 2016). In this regard, it is important to use reference host plants and *B. cinerea* strains in the laboratory for elucidating the mechanisms and regulation of pathogen attack, host immune response, and fungal reproduction. *Phaseolus vulgaris* (French bean) and *Arabidopsis thaliana* are valuable model experimental hosts that exhibit high and low susceptibility to aggressive *B. cinerea* strains such as B05.10 (Fig. 14.1). Thus, the use of the highly sensitive host, *P. vulgaris*, enables the study of the interaction from the fungal perspective by revealing differences between the capacity of *B. cinerea* mutants to enter and colonize plant tissues (Giesbert et al. 2012; de Vallee et al. 2019). In contrast, the role of plant immune responses can be studied in (genetically modified) *A. thaliana*, which represents a "poor host" because of its potent phytoalexin camalexin (Denby et al. 2004; Rowe and Kliebenstein 2008; Mengiste et al. 2009).

The life cycle of *B. cinerea* includes vegetative mycelia for substrate colonization, (macro)-conidia for dispersal, sclerotia for survival and as a prerequisite for sexual development, microconidia as male gametes, and fruiting bodies bearing the sexual spores (Fig. 14.2). The relationship with the host further includes the development of penetration structures and the active secretion of factors for killing host cells and for obtaining nutrition from the dead tissue.

14.1.1 Penetration Structures

B. cinerea enters the host by using two different pre-penetration structures (Backhouse and Willets 1987; Choquer et al. 2007). The conidia that are ubiquitously distributed in the air germinate after landing on the plant surface under favorable conditions and form short germ tubes with apical swellings (appressorium-like structures), which directly penetrate the plant surface. Alternatively, hyphae derived from already established mycelia or older elongated germ tubes form multicellular penetration structures, the so-called infection cushions. The penetration process by germ tubes and infection cushions can be easily monitored on onion epidermis by using lactophenol blue staining for distinguishing structures inside and outside of the onion cells (Fig. 14.1).

Several signaling pathways are implicated in the early stages of plant infection. Deletion of the genes encoding the mitogen-activated protein (MAP) kinases, BMP1 and BcSAK1, results in avirulent mutants (Zheng et al. 2000; Segmüller et al. 2007; Schamber et al. 2010). In contrast, deletion of the genes encoding the MAP kinase BMP1 and components of the cAMP cascade leads to mutants with retarded infection due to reduced penetration efficiency (Doehlemann et al. 2006b; Rui and Hahn 2007; Schumacher et al. 2008b). Deletion of genes encoding members of the NoxB (NADPH oxidase) complex results in another interesting phenotype: conidia germinate, form germ tubes and appressorium-like structures, which initiate penetration but without success. New outgrowths appear that are followed by further attempts to penetrate. Components of the NoxA complex are specifically involved in the formation of infection cushions. However, all NOX mutants eventually colonize the host tissue (Segmüller et al. 2008; Marschall et al. 2016). An intact fungal wall cell is critical for the differentiation of functional infections cushions as highlighted by phenotype analysis of Δ*bcchs3a*, Δ*bccrz1*, and Δ*bcskn7* mutants that are more strongly impaired in mycelia-derived than in conidia-derived infection processes (Schumacher et al. 2008a; Arbelet et al. 2010; Viefhues et al. 2015).

Taken together, conidia and mycelia of the same strain may have different penetration capabilities due to the two different kinds of infection structures whose formation is regulated by independent pathways. A recent study identified genes that are upregulated in mature infection cushions including two that encode secreted fasciclin-like proteins that are required for full virulence on *P. vulgaris* (Choquer et al. 2021).

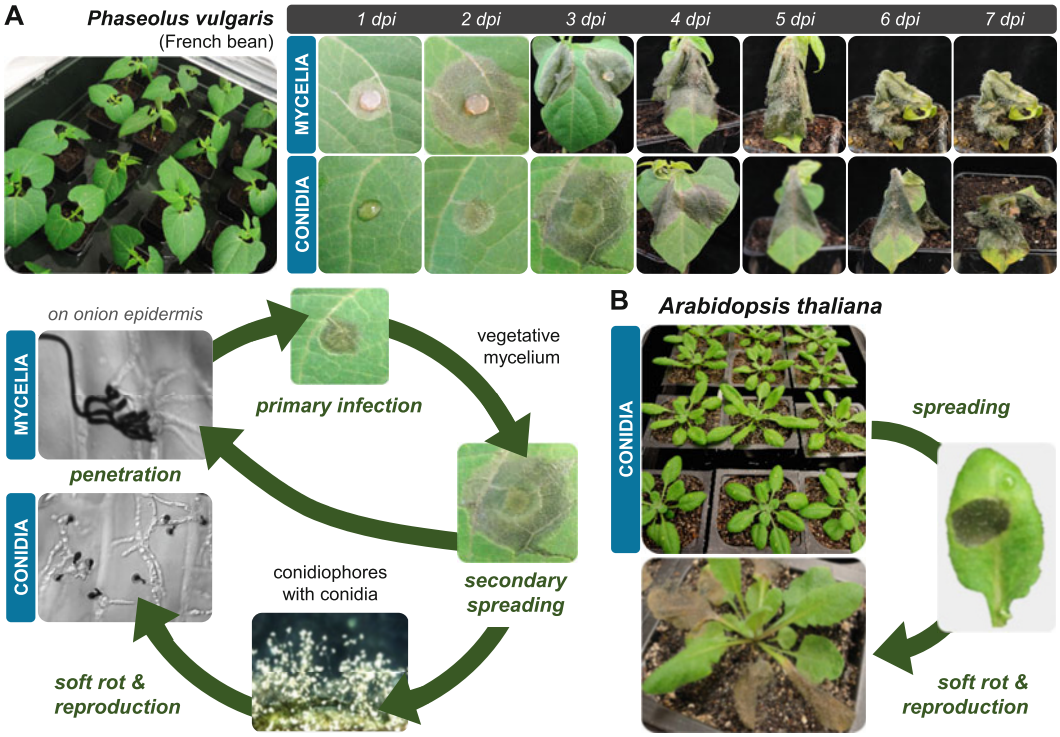

Fig. 14.1 Infection cycle of *B. cinerea* B05.10 under laboratory conditions. (**a**) On *Phaseolus vulgaris* (French bean) as a highly susceptible host. Primary leaves of living plants are inoculated with plugs of vegetative mycelia (two-day-old complete medium (CM) cultures) or conidial suspensions (2×10^5 conidia/ml Gamborg B5 + 2% glucose) and are incubated under humid conditions and natural light conditions at room temperature. The fungus establishes primary (restricted) infections that spread quickly. After one week the whole leaf is infected: the plant tissue collapses and becomes watery (soft rot), which is accompanied by the formation of gray conidiophores and conidia. The penetration process is monitored on isolated onion epidermis. Hyphae deriving from vegetative mycelia produce infection cushions; conidia form short germ tubes that directly penetrate the epidermal cells (hyphae growing inside the onion epidermis are colorless while fungal structures outside the host are stained with lactophenol blue). Dpi—days post-inoculation. (**b**) On the model plant *Arabidopsis thaliana* as a lowly susceptible host. Leaves of living plants can be infected by conidia as described above, resulting in fast spreading and soft rot formation. In comparison to French bean, no defined stages (i.e., primary and secondary infections) can be distinguished. Due to the high level of basal resistance, the outcomes of different assays may vary significantly. *A. thaliana* is likely not a (natural) host for *B. cinerea* outside the laboratory

14.1.2 Virulence Determinants

After penetration, the epidermal and underlying cells die and *B. cinerea* establishes a primary infection that is characterized by collapsed brownish tissue and defined margins. At this stage, hyphae are probably restricted to this region. The subcuticular mycelia may remain inactive in this restricted lesion for an indefinite duration (quiescence) and become active again under specific circumstances, such as during the ripening processes in which fungitoxic compounds decline in concentration, levels of sugars increase and the chemical composition of the cell wall changes (Prusky et al. 2013). Finally, *B. cinerea* overcomes the plant defense barriers and initiates vigorous secondary growth; hyphae grow invasively and decompose the plant tissue very rapidly resulting in soft rot and formation of conidiophores and conidia (Fig. 14.1).

B. cinerea secretes several non-host-specific cell death-inducing factors, which are low

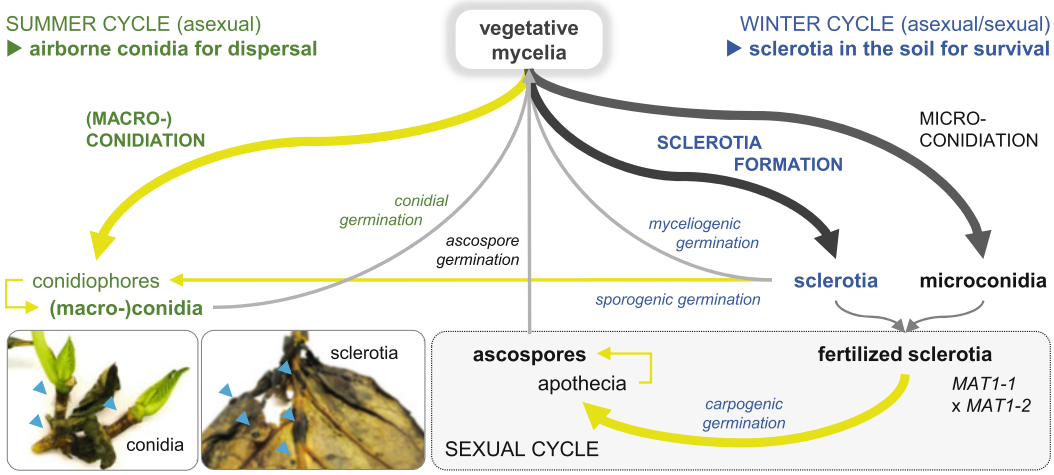

Fig. 14.2 Reproduction cycles of light-responsive *B. cinerea* strains. Young vegetative mycelia may develop either macro-conidia for dispersal (summer cycle, green) or sclerotia for survival (winter cycle, blue). Both conidia and sclerotia germinate asexually under favorable conditions. Fertilization of the sclerotia by microconidia of the opposite mating type is part of the sexual cycle that results in the formation of apothecia as fruiting bodies in spring. The apothecia contain asci with eight ascospores in ordered tetrads. Light promotes conidiation and represses sclerotial development. Sclerotia and microconidia are formed under the same conditions that is the absence of light. Left bottom corner: conidiophores with conidia and sclerotia (blue arrows) formed by *B. cinerea* on *Hydrangea* plants from the greenhouse (left) and a dark cold storage (right)

molecular weight metabolites or proteins (van Kan 2006; Amselem et al. 2011). The expression of these factors may be induced during the fungus-host interaction but synthesis is not restricted to this state. Two families of phytotoxic secondary metabolites, the botryanes (BOT) and the botcinins (BOA) contribute to full virulence (Dalmais et al. 2011; Viaud et al. 2016). Reactive oxygen species (ROS) play a dual role in the relationship as both interacting partners produce ROS. H_2O_2 generation is observed in the penetration structures, in and around the penetrated host cell wall in the early stages and in colonized tissues (Choquer et al. 2007; Heller and Tudzynski 2011). Several secreted necrosis-inducing proteins have been identified of but only a few contribute significantly to virulence. Cell wall-degrading enzymes (CWDEs) may be involved in different stages of plant infection: in penetration by lysing the cuticle or epidermal cell walls, in the manifestation of infection by degrading components of the plant defense, or in nutrition by effective decomposition of dead tissues. Some CWDEs also exhibit necrotizing activity (González et al. 2016). The redundancy of these groups of virulence determinates has been demonstrated by Hahn & co-workers who generated mutants using CRISPR/Cas9 technology where multiple genes encoding cell death-inducing proteins and key enzymes for the synthesis of BOT and BOA were deleted. On all tested tissues, infection efficiency of the mutants decreased with increasing number of deleted genes. However, even mutants with twelve genes deleted were eventually able to colonize the host tissue and to form conidia (Leisen et al. 2021).

A relatively new class of secreted effectors produced by *B. cinerea* are small RNAs (sRNAs) that derive from retrotransposons. They are taken up and recognized by the host resulting in the suppression of defense-related gene expression due to RNA interference (pathogen-induced gene silencing). Deletion of the genes encoding fungal Dicer-like proteins, BcDCL1 and BcDCL2, abolishes the generation

of sRNAs and consequently results in reduced virulence. On the other hand, the mutation of AGO1 in *A. thaliana* that is required for active gene silencing leads to decreased susceptibility, indicating that cellular components of both interaction partners are involved in the process (Weiberg et al. 2013; Wang et al. 2016; Porquier et al. 2021).

Like other necrotrophs, *B. cinerea* induces plant defense responses by pathogen-associated molecular pattern (PAMP) recognition, such as cell wall fortification and production of antifungal compounds that activate JA/ET (jasmonate/ethylene)-dependent signaling pathways. Mutations that abolish JA and ET signaling result in increased susceptibility to *B. cinerea* (Mengiste et al. 2009). In addition, *B. cinerea* triggers SA (salicylic acid) signaling to induce the hypersensitive response (HR), a localized cell death reaction that is effective against biotrophic but supports necrotrophic pathogens (El Oirdi et al. 2011; Rossi et al. 2011). As JA/ET and SA pathways are antagonistic, the induction of SA signaling concurrently results in the suppression of JA/ET-associated defense responses (Robert-Seilaniantz et al. 2007; Mengiste 2012). Furthermore, *B. cinerea* may affect the hormone balance by fungal production of ethylene, auxins, and abscisic acid (ABA) (Sharon et al. 2004). However, the impact of fungal-derived phytohormones in the interaction is still unclear. JA-dependent defense responses against necrotrophs are downregulated in plants in which the shade avoidance response has been initiated. This program is induced by a low red/far-red light ratio which indicates the presence of competitors and the concomitant decrease of photosynthetic yields. The shade avoidance response consequently redirects more carbon resources to promote growth for optimizing photosynthetic yields and allows the plants to finish the life cycle at the expense of defense (Ballaré 2014; Ballaré and Pierik 2017; Liu et al. 2021). Because of its short repetitive infection cycles that yield high numbers of airborne conidia for consecutive infections of "immunocompromised" high-yielding host plant varieties planted in high densities, *B. cinerea* is a highly destructive pathogen in industrial monocultures.

14.2 Adaptations to Light

Sunlight is an important environmental factor in almost all ecosystems as it represents a source of energy, information, and stress. Cyanobacteria, algae and plants all use light for energy generation (photosynthesis). Less apparent is the utilization of light for repair of photodamaged DNA or for establishing proton gradients by microbial opsins. All organisms must protect themselves from the harmful effects of light such as UV radiation, ROS accumulation, heat, and desiccation. Finally, light quality and quantity can be used for signaling (photoinduction, photomorphogenesis), as a guide for directed growth (phototropism) and timing (photoentrainment), processes that include sensing and transduction into intracellular signals.

B. cinerea and other plant pathogens infecting the sun-exposed parts of the plant must cope with the high (UV, blue) light conditions the host plant seeks. Furthermore, the fungus experiences an altered light spectrum ('green gap') when it colonizes shaded parts of the plant. It is depleted for blue and red light that is absorbed by the plant chlorophyll and enriched for green and far-red light that is reflected or transmitted by the plant tissue. As these ambient light conditions trigger the shade avoidance response in the plant, the pathogens may trigger their own "shade response" such as the upregulation of virulence determinants and inoculum production assuming that new host tissues are close and highly susceptible.

14.2.1 Genetic Make-up: Photoperception

Light is perceived by chromophore-containing proteins called photoreceptors (PRs). FAD (flavin adenine dinucleotide), MTHF (5,10-methyltetrahydrofolate), retinal and bilin are

bound by conserved input domains allowing for the distinction of four major PR classes: (i) near-UV/blue-sensing cryptochrome/photolyase-family proteins (CPFs), (ii) blue-sensing light-oxygen-voltage domain-containing proteins (LOVs), green-sensing membrane-associated opsins (OPSs) and red/far-red-sensing phytochromes (PHYs) (Heintzen 2012). Great numbers of PR-encoding genes are found in phototroph-associated microbes—including plant-pathogenic and rock-inhabiting fungi—pointing to the relevance of light in their life cycles (Schumacher and Gorbushina 2020; Losi and Gärtner 2021). *B. cinerea* possesses substantially more PRs than the long-established models in fungal photobiology, including *Aspergillus nidulans* which has three and *Neurospora crassa* which has eight PRs (Dasgupta et al. 2016). Striking is the expansion of PHYs (at least two) in the Leotiomycetes. The presence of two CPFs (BcCRY1, 2), four LOVs (BcWCL1, VVD1, LOV3, 4), two OPSs (BOP1, 2), and three PHYs (BcPHY1, 2, 3) enables *B. cinerea* to sense near-UV, blue, green, red and far-red light. Yet the mechanism and proteins involved for sensing UV (280–315 nm) are unknown and thus the total number of PRs of *B. cinerea* may be even larger. The eleven PRs of *B. cinerea* possess the conserved chromophore binding sites, and all are expressed in response to light. While deletion and overexpression mutants have been generated, characterization of these mutants is just beginning (Schumacher 2017).

14.2.2 Gene Expression: Photoregulation

Changes at the transcriptional level are prerequisites for all photoresponses and indicate that the organisms perceive and respond to light. But not all changes in gene expression will result in obvious phenotypes. Thus, PRs and light-responsive transcription factors (LTFs), which may control the expression of several genes have been studied to elucidate the regulatory networks and genes involved in the photoresponses.

The exposure of vegetative mycelia of *B. cinerea* B05.10 to full-spectrum light significantly alters gene expression (Fig. 14.3). The group of light-induced genes (LIGs) comprises genes involved in photoperception, stress responses, and transcriptional regulation. The group of light-repressed genes (LRGs) is smaller and enriched for genes encoding amino acid transporters (Schumacher et al. 2014). LIG expression occurs with different kinetics: genes are either subjected to photoadaptation or not. LIGs of the first group (early, late) mediate short-term (adaptive) responses. LIGs of the second group exhibit maximal expression after longer exposure to light and may be critical for development (*dev* LIGs). The expression levels of all PR-encoding genes are influenced by light: *bccry1*, *bccry2*, *bcvvd1*, and *bop1* are early LIGs, *bclov3*, *bcphy2* and *bop2* are late LIGs, and *bcphy1*, *bcphy3*, *bclov4*, and *bcwcl1* belong to the group of dev LIGs. Importantly, the blue light-sensing BcWCL1 as part of the White Collar complex (WCC) acts as the primary PR by modulating the expression levels of the remaining PR-encoding genes within a short period of time (Schumacher 2017). To date, eight LTFs out of the identified 30 LTFs (Schumacher et al. 2019) have been functionally characterized including the early LIGs that encode BcLTF3 and BcREG1, the late LIGs that encode BcLTF1, BcATF1 and BcLTF4, 5, 6, and the only studied dev LIG that encodes BcLTF2 (Michielse et al. 2011; Temme et al. 2012; Schumacher et al. 2014; Cohrs et al. 2016; Brandhoff et al. 2017).

14.2.3 Reproduction: Photomorphogenesis and Phototropism

B. cinerea can reproduce asexually via macroconidia and sclerotia, and sexually via ascospores. The reproduction cycles depend on the availability of a host and the environmental conditions –mainly light and its absence, temperature, substrate—and are therefore linked to the seasons. Macroconidia are predominantly formed in the summer as inoculum for new infections

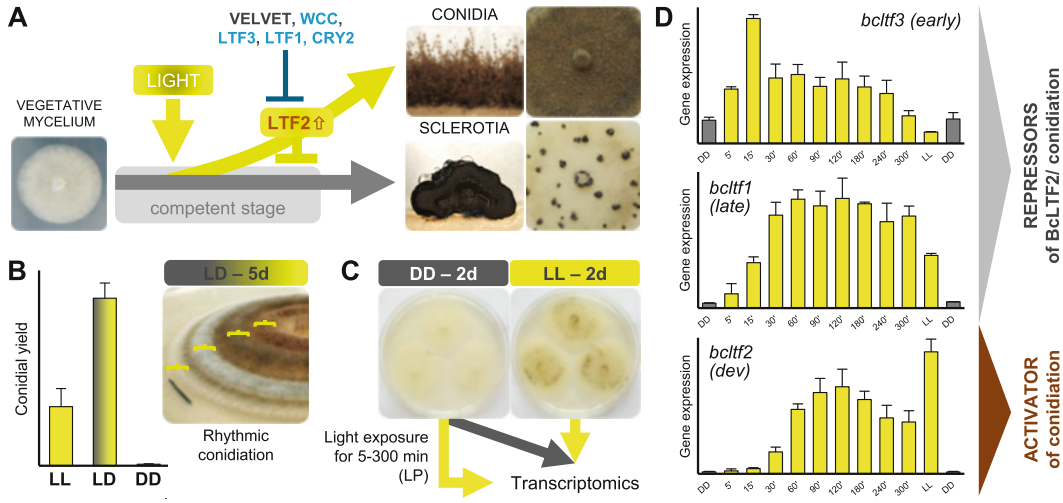

Fig. 14.3 Prominent light responses of *B. cinerea* WT: B05.10—morphogenesis and gene expression. (**a**) Light induces conidiation and represses sclerotium formation via transcriptional activation of BcLTF2. Low light dosages received in the competent stage of vegetative mycelia prevent the formation of sclerotia. Conidia and sclerotia as observed after two weeks of cultivation on solidified CM in 12 h light/12 h dark (LD) or constant darkness (DD) at 20 °C; vegetative mycelium as formed from a mycelial plug after two days of incubation in darkness. (**b**) Light-dark cycles are more effective than constant light (LL) and cause rhythmic conidiation ("rings"). Conidiophores with conidia are formed overnight on aerial mycelia that have been grown during the day (yellow brackets). (**c**) Transcriptional light responses are studied in surface-grown mycelia obtained from cellophane-covered solidified CM. Dark-grown mycelia are exposed to light pulses (LPs) before harvest. (**d**) Gene expression profiles determined by RT-qPCR of three light-responsive transcription factors (LTFs) exhibiting different kinetics. *Bcltf3*, *ltf1*, and *ltf2* represent early, late, and developmental light-induced genes (LIGs) and affect conidiation

(host tissue is available) and sclerotia in autumn for surviving the winter in the absence of a host. Sexual reproduction—fertilization of sclerotia with microconidia and subsequent apothecial development—is promoted by nutrient starvation and lower temperatures and happens in winter, resulting in the appearance of the fruiting bodies (apothecia) the following spring. Non-fertilized sclerotia and ascospores germinate with mycelia and conidia to initiate the summer cycle (Williamson et al. 2007; Amselem et al. 2011) (Fig. 14.2). Field populations of *B. cinerea* are highly diverse regarding the formation of these reproduction structures. Strains may or may not differentiate macroconidia, microconidia, sclerotia, and apothecia with ascospores during their life cycles. In general, *B. cinerea* strains can be classified into light-responsive strains that undergo photomorphogenesis, and blind strains that exhibit the same phenotypes in light and constant darkness (*always mycelia*, *always sclerotia* or *always conidia*). Light responsiveness allows for the formation of all structures—as found in strain B05.10 that is used in most laboratories as the host for genetic modification (Fig. 14.3). Photomorphogenesis is considered a characteristic feature of *B. cinerea* and its absence as the result of mutated regulatory genes.

Vegetative mycelia of *B. cinerea* B05.10 quickly colonize the host tissue (invasive growth) or the agar medium (saprophytic growth). For example, the growth rates on solidified complete medium are 10–15 mm per day so that the area of a standard Petri dish is colonized after three days of incubation under optimal temperature conditions (~20 °C). Little aerial hyphae and biomass accumulates as mycelia consist of thin layers of hyphae. Young vegetative mycelia are fully competent and can develop conidiophores

with macroconidia or sclerotia. Light responsiveness depends on the physiological age and is less effective in aged colonies that have completely colonized the substrate or have already initiated one of the reproduction programs.

The (macro)conidia are short-lived asexual dispersal units. They are multinucleate, contain a two-layered cell wall—the outer wall is melanized—and are formed at denticles that arise from the spherical ampulla located at the tip of a conidiophore (synchronous holoblastic conidiogenesis) (Cole 1981). The development of conidia (conidiation) starting from initial conidiophores through to the presence of mature conidia may be completed within eight hours and requires light. Alternation of black light (near-UV) and white light, or alternation of full-spectrum light and darkness (Fig. 14.3) are most effective in promoting conidiation, while blue light alone inhibits conidiation and causes morphological changes in the various stages of conidiogenesis as mature conidiophores, ampullae at the tips of conidiophores, denticles, and conidial initials de-differentiate into sterile hyphae. Inhibition by blue light can be overcome by subsequent exposure to near-UV or far-red light. Red light does not promote conidiation after blue light inhibition and reduces conidiation in far-red light-treated colonies (Tan 1975; Suzuki et al. 1977).

Conidiophores positively respond to blue light after induction of conidiation by black light (positive phototropism)—most likely mediated through the blue light-sensing WCC—while green, yellow, and red light are ineffective (Jarvis 1972). Three TFs are known to be required for conidiogenesis: *Bcreg1* mutants form conidiophores with ampullae and denticles but fail to form mature conidia, the mutation of *bcltf3* phenocopies the blue light effect as the conidial initials grow out to sterile hyphae, and BcHOX8 is crucial for both proper conidiophore development and conidiogenesis (Michielse et al. 2011; Antal et al. 2012; Brandhoff et al. 2017). Initiation of conidiophore development requires BcLTF2 (sufficient and essential for conidiation), the stress-activated MAPK module, the response regulator BcSKN7, and the cell wall integrity-related MAPK module (Rui and Hahn 2007; Segmüller et al. 2007; Yan et al. 2010; Liu et al. 2011; Yang et al. 2012, 2015; Viefhues et al. 2015; Cohrs et al. 2016; Wang et al. 2018). Conidiation is repressed by the transcription factors BcLTF1, BcWCL1, BcMADS1, BcSTE12, the hybrid-histidine kinases BOS1 and BcSLN1 acting upstream of the BcSAK1 module, and the VELVET complex (Liu et al. 2008; Schamber et al. 2010; Schumacher et al. 2012, 2014, 2015; Canessa et al. 2013; Yang et al. 2013; Zhang et al. 2016; Müller et al. 2018; Ren et al. 2019).

The conidia may stay dormant for long periods, held in check by lack of moisture and nutrients. Germination starts with the swelling of the conidium, followed by the emergence of the germ tube. This is accompanied by nuclear divisions, the induction of genes encoding secreted proteins and lytic enzymes, and the production of an extracellular polysaccharide (EPS) matrix (Gull and Trinci 1971; Doss 1999; Leroch et al. 2013). Differences between isolates were observed regarding the induction of this process; some *B. cinerea* isolates germinate readily in distilled water, while others require sugars and amino acids (Blakeman 1975). Conidial germination on hydrophilic surfaces is stimulated by carbon sources and requires the MAP kinase BMP1, the Gα subunit BCG3 and the adenylate cyclase BAC, but not by the cAMP-dependent protein kinase (PKA), and is accompanied by the rapid degradation of trehalose, an important carbon storage compound and stress protectant (Doehlemann et al. 2006a, 2006b; Schumacher et al. 2008b). Germination on hydrophobic surfaces in the absence of nutrients is controlled by the MAP kinase BMP1 in a cAMP-independent manner (Doehlemann et al. 2006b). Other mutations result in multiple or elongated germ tubes with abnormal shapes, possibly due to defects in surface sensing (Rui and Hahn 2007; Kokkelink et al. 2011; Leroch et al. 2015). Experimental evidence for the secretion of self-inhibitors of germination by *B. cinerea* exist

(Carlile and Sellin 1963; Kritzman et al. 1980); yet the compounds and regulatory networks remain elusive.

A recent study reported on the presence of 1-phenylethanol and 3-phenylpropanol and the absence of farnesol and tyrosol—four commonly involved compounds in quorum sensing in bacteria and fungi—in culture extracts of *B. cinerea* (Rosero-Hernandez and Echeverri 2020). Three forms of conidial fusions can be distinguished: fusions between conidia via a short connective hypha (conidial anastomosis tubes, CATs) (dominant type), fusions between germ tubes and conidia, and fusions between germ tubes directly or indirectly via CATs (Akutsu et al. 1981). Conidial/germling fusions are induced by a lack of nutrients and require the NoxA complex (Roca et al. 2012; Siegmund et al. 2015). Another factor influencing the germination process is light. Conidia germinate less well under green light conditions (Zhu et al. 2013), and germ tubes develop more frequently at the un-illuminated site of the conidium and grow away from the white light source (negative phototropism) (Robinson 1914; Gettkandt 1954; Islam et al. 1998). These observations suggest that light guides the germ tubes to the host surface and prevents conidial germination during the day.

Sclerotia are dark pigmented structures of limited growth, which allow the fungus to survive many years in the absence of suitable hosts or conditions favoring active growth. Sclerotial development consists of the three stages (i) initiation: formation of dichotomous branches, (ii) development: growth to the full size, and (iii) maturation: surface delimitation, internal changes, pigmentation of the peripheral hyphae, and exudation of water. The mature sclerotium has three distinct layers consisting of a melanized outer rind, a cortex of thin-walled pseudoparenchymatous cells, and a large medulla of loosely arranged filamentous hyphae. The formation of sclerotia is influenced by different factors, such as temperature, nutrition, pH, physical damage and growth against mechanical barriers, and is generally promoted by lower temperatures, high humidity, and the absence of light (Coley-Smith et al. 1980). The NoxA complex is essential for sclerotial development, which might be due to the inability of the mutants to form anastomoses (hyphal fusions) as common features of infection cushions and sclerotia (Segmüller et al. 2008; Siegmund et al. 2015). Several other mutants do not produce sclerotia, but this is due to the deregulation of morphogenetic programs rather than due to defects in sclerotial development. For example, inappropriate induction of vegetative growth resulting in *always mycelia* is caused by the overexpression of WCC components or deletion of the Gβγ dimer of heterotrimeric G proteins (Canessa et al. 2013; Tang et al. 2021). Conidiation in the dark by *always conidia* mutants has been described above. Only a few mutations are known to cause the *always sclerotia* phenotype; for example the deletion of *bckdm1* encoding a putative histone 3 lysine 36 (H3K36) demethylase (Schumacher et al. 2019). The sclerotia may remain quiescent for long periods and can germinate in the presence of nutrients and after low temperature activation in three different ways: myceliogenically (by hyphae), sporogenically (by conidiophores), or carpogenically (by apothecia) (Coley-Smith and Cooke 1971) (Fig. 14.2).

Microconidia are the male gametes (spermatia). They are uninucleate, contain a lightly melanized cell wall, and are formed by phialides on conidial germ tubes, more mature hyphae, infection cushions or sclerotia under nutrient limitation conditions. They germinate exclusively in the presence of a female parent of the opposite mating type (Faretra and Antonacci 1987; Fukumori et al. 2004). The process of microconidiation is rather poorly studied. Nevertheless, microconidia and sclerotia are formed simultaneously in the laboratory—that is after two weeks in the absence of light under nutrient-rich conditions—suggesting that both production structures are formed at the same time in the field so that mating can occur to initiate the sexual cycle.

Apothecia (fruiting bodies) contain sexual spores. They arise from sclerotia after the sexual process (spermatization by microconidia) and are 2–20 mm in size. The upper part (disc) consists of a hymenial layer composed of paraphyses and

asci containing the eight uninucleate ascospores in ordered tetrads. *B. cinerea* is self-sterile/heterothallic with the two mating types *MAT1–1* and *MAT1–2*. Strains of the opposite mating types may be reciprocally crossed by using either sclerotia or microconidia. Derivatives of strain B05.10 and SAS56 (*MAT1–1*) can be crossed with the wild strain SAS405 (*MAT1–2*) (Faretra et al. 1988b; Amselem et al. 2011). There is little information about the physiology of the apothecia. Carpogenic germination of sclerotia requires pre-conditioning in cold, moist conditions, moderate temperatures, sufficient air, and light. Thereby, apothecial initials grow toward the light source (positive phototropism). Besides, the origin of the sclerotia plays a role as smaller sclerotia from minimal medium are unable to form apothecia likely due to the shortage of storage substances (Faretra and Antonacci 1987; Faretra et al. 1988a). Mutants may be female-sterile because they are unable to form sclerotia or the sclerotia are non-functional, but this does not imply male sterility (Segmüller et al. 2008; Jonkers et al. 2011; Siegmund et al. 2013). Genes expressed in different stages of apothecial development have been identified. Hydrophobins in maternal tissues and the mating type genes are essential for the regular development of apothecia (Terhem and van Kan 2014; Rodenburg et al. 2018).

The response of vegetative mycelia of *B. cinerea* to light is exceptional, and the switch to conidiation or sclerotial development can be easily monitored to identify the regulatory networks. Sclerotial development is the morphogenetic program that the fungus usually pursues (in the dark). Light prevents further sclerotial development in a competent stage by inducing the formation of aerial hyphae that develop into conidiophores and conidia. As a result, the upregulation of *bcltf2* is sufficient for conidiation even in the dark (*always conidia*). The accumulation of vegetative mycelia (*always mycelia*)—optionally with aerial hyphae—suggests that the conidiation program is turned on and prevents sclerotial development but cannot be completed because of defects in conidiophore differentiation. Importantly, these regulators can be essential for full virulence. Thus, mutations of the BcVEL1-encoding gene were found to cause hyperconidiation (*always conidia*) and reduced virulence in wild strains T4 and 1750 (Schumacher et al. 2012, 2013). Recently, mutations in *Bcin04g03490* encoding a putative transcriptional regulator with Zn_2Cys_6-type DNA binding and acetyltransferase domains were demonstrated to cause a mycelial morphotype and avirulence in *B. cinerea* field isolates (Acosta Morel et al. 2021). In these studies, mutated genes were identified in blind wild isolates. Their deletion in light-responsive B05.10, phenocopied blindness and reduced virulence, while addition of the intact genes from B05.10 rescued the phenotypes in the blind wild strains.

14.2.4 Metabolism: Photoprotection

Pigments are versatile secondary metabolites that protect cells from the detrimental effects of light—especially from UV-induced damage. Often, they have further beneficial functions such as the strengthening of reproduction structures or antimicrobial activities. Common in Ascomycota are the dark 1,8-dihydroxynaphthalene (DHN) melanin and the orange to yellow carotenoids. However, certain species evolved other pigment synthesis pathways leading to their characteristic pigmentations. While the genes for DHN melanogenesis and carotenogenesis are highly conserved in several species, their differential expression profiles reflect the adaptation to environmental light conditions.

Thus, fungi such as *B. cinerea* produce DHN melanin and carotenoids in a spatial and temporal manner while fungi in more extreme sun-exposed habitats usually exhibit constitutive melanogenesis and carotenogenesis leading to the term "black fungi" (Schumacher and Gorbushina 2020). Likewise, the designation "gray mold fungus" for *B. cinerea* is based on the production of DHN melanin during the latter stages of host infection resulting in plant debris covered with a layer of grayish conidiophores and conidia. The sclerotia of *B. cinerea* appear blackish and resemble the

dense microcolonies of black fungi. DHN melanogenesis can be divided into three phases: (i) the *de novo* synthesis of the pentaketide T4HN (1,3,6,8-tetrahydroxynaphthalene), (ii) its modification to DHN, and (iii) the polymerization of DHN resulting in the highly complex DHN melanin (Butler and Day 1998). The genes involved in the first two phases are highly conserved in all fungi and are tightly, partially, or not clustered in the genome. In contrast, different multicopper oxidases (laccases) may carry out the cross-linking of DHN in distantly related Ascomycetes (Jia et al. 2021). Remarkably, *B. cinerea* possesses two different types of melanin polyketide synthases (phase I) mediating DHN melanogenesis together with a shared set of downstream-acting enzymes (phase II), and most likely again with different multicopper oxidases (phase III) in different developmental stages. BcPKS12 directly and BcPKS13 via BcYGH1 provide the precursor T4HN in either sclerotia or conidia and light-stressed mycelia; then T4HN is further converted by the same enzymes in both structures (Schumacher 2016). Melanogenesis is coupled with developmental programs because the genes involved are under the control of the respective master regulators. BcLTF2 as an activator of conidiation positively affects the expression of *bcpks13* (dev LIG) and the core genes (conidial DHN melanogenesis), and *bcltf2* expression is repressed by the WCC, BcLTF1, and the Velvet complex. Expression of *bcpks12* and the core genes, and in turn sclerotial melanogenesis, depends on BcSMR1 (sclerotial melanin regulator) whose expression is predicted to be repressed by a yet unknown WCC-dependent LTF (Cohrs et al. 2016; Zhou et al. 2017). The upregulated expression of *bcltf2*, *bcpks13* and the other melanogenic genes in the multicellular infection cushions suggests that the melanin is of the "conidial/BcPKS13 type" (Choquer et al. 2021). A recent study reported on the compartmentalization of melanogenic enzymes to avoid poisoning by pathway intermediates (Chen et al. 2021). Melanin is dispensable for virulence and the differentiation of the reproduction structures but contributes to their stability and longevity (Schumacher 2016; Zhu et al. 2017; Zhou et al. 2018).

B. cinerea possesses the highly conserved cluster of four genes required for carotenogenesis (*bcphs1*, *bcphd1*), retinal formation (*bccao1*), and green light sensing (*bop2*). The bifunctional BcPHS1 (phytoene synthase and lycopene cyclase) and the phytoene dehydrogenase (BcPHD1) may catalyze consecutive reactions leading to β-carotene and torulene. Retinal—the chromophore for green light absorption by opsins—can be formed by cleavage of β-carotene by the carotenoid oxygenase. The genes are induced by light and in a blue light/WCC- and BcLTF1-dependent manner (Schumacher 2017). In summary, carotenoids may help *B. cinerea* to resist light-induced stresses, for example, by scavenging of singlet molecular oxygen and free radicals and by protecting the integrity of the membranes. Furthermore, they represent the basis for the formation of retinal for the green light-absorbing opsins. In *B. cinerea*, the carotenoids are almost invisible as the faint orange pigmentation is masked by the dark melanin.

A few *B. cinerea* strains exhibit a remarkable red pigmentation due to the synthesis of bikaverin, a polyketide with antimicrobial properties that is typically produced by different *Fusarium* species (Dos Santos and Bicas 2021). The reddish *B. cinerea* strains possess the entire bikaverin gene cluster consisting of six genes, while grayish *B. cinerea* strains such as B05.10 lack the key enzyme-coding gene (Campbell et al. 2012; Schumacher et al. 2013). Bikaverin production by *B. cinerea* is relevant for interactions with bacteria and certain host species rather than for photoprotection (Spraker et al. 2018; Plesken et al. 2021). However, bikaverin synthesis is regulated by the VELVET complex (Schumacher et al. 2013).

B. cinerea also produces mycosporine-like amino acids (MAAs) for UV absorption and as compatibles solutes and antioxidants (Volkmann and Gorbushina 2006). Mycosporine-glutamicol—called mycosporine-2—was first isolated as a UV-absorbing compound (P310)

from near-UV-treated cultures that had initiated conidiation. This compound was not detected in dark-grown vegetative cultures leading to the hypothesis that it may have a regulatory effect on conidiation (Tan and Epton 1974; Arpin et al. 1977).

The extracellular polysaccharide (EPS) matrix of *B. cinerea* consists mostly of cinerean [β-(1,3)(1,6)-D-glucan]. The polymer forms a capsule strongly adhering to the germinating conidia and hyphae and contributes to the adhesion to (host) surfaces, protection from light and concomitant heat and desiccation stresses, creation of a microenvironment for extracellular enzymes and as a nutrient reservoir. Thus, *B. cinerea* synthesizes cinerean under carbon surplus conditions and may degrade it by extracellular β-1,3 glucanases under carbon starvation conditions (Dubourdieu et al. 1981; Stahmann et al. 1992; Doss 1999).

14.2.5 Enzyme Systems: Photodamage Repair

The EPS matrix represents the first layer for protecting the cellular contents from environmental stresses, with the two-layered cell walls fortified with DHN melanin, and membranes with incorporated carotenoids providing second and third layers. The cellular lumen contains several primary metabolites functioning as UV screens (e.g., mycosporines), osmoprotectants (e.g., polyols, sugars, amino acids), and/or antioxidants (e.g., tocopherols, ascorbic acid, and carotenoids). Nonetheless, excessive sunlight (UV) may pass these lines of defense and cause mutagenic lesions in the DNA, disadvantageous conformational changes of proteins and the accumulation of harmful ROS. Therefore, enzymatic mechanisms must accomplish the repair of damaged DNA, misfolded proteins, and the detoxification of ROS. In fact, these genes are often upregulated upon different stresses via the stress-activated MAPK module and/or by light in a WCC-dependent fashion.

Photo-damaged DNA is repaired by the CPF protein BcCRY1 using visible light as a source of energy. The second related protein (BcCRY2) exerts a regulatory role in photomorphogenesis and does not exhibit photoreactivation activity (Cohrs and Schumacher 2017). With BcCRY1, a cyclobutane pyrimidine dimer (CPD) photolyase, *B. cinerea* possesses a single photolyase only. Other Leotiomycetes contain an additional (6–4)-pyrimidine-pyrimidone photoproduct (6-4PP) photolyase, and several Chaetothyriales (Eurotiomycetes) and Dothideomycetes even possess a second CPD photolyase (Schumacher and Gorbushina 2020). Expression of *bccry1*, *bccry2*, and *bcuve1* encoding a conserved UV endonuclease for UVDE-dependent excision repair is induced by light in a WCC-dependent manner. Consequently, the Δ*bcwcl1* mutant exhibits a decreased UV tolerance (Cohrs and Schumacher 2017).

Refolding of partially denatured proteins in *B. cinerea* can be mediated by molecular co-chaperones including small heat shock proteins (HSPs). The expression of several genes encoding HSPs, dehydration-induced protein-like (dehydrins), and other stress-related proteins is induced upon exposure to light, as well as to temperature, osmotic and oxidative stress (Heller et al., 2012).

The oxidative stress response (OSR) system of *B. cinerea* comprises detoxifying enzymes, such as superoxide dismutases, catalases, peroxidases, peroxiredoxins, glutaredoxin, and thioredoxins and enzymes reducing glutathione and the thioredoxins (Viefhues et al. 2014). The corresponding genes are upregulated in response to oxidative stress by the transcriptional regulators BAP1 and BcSKN7 (Temme and Tudzynski 2009; Viefhues et al. 2015; Yang et al. 2015). The OSR system has a moderate basal activity that ensures that ROS produced as by-products of various metabolite pathways do not accumulate to toxic amounts. Simultaneous exposure to light and oxidative stress exceeds the buffer capacity, explaining the stronger effect of oxidants on *B. cinerea* in light compared to constant darkness. Both Δ*bcltf1* and Δ*bcwcl1* mutants affected in photoinduction of gene expression are more sensitive to oxidative stress in light (Canessa et al. 2013; Schumacher et al. 2014).

14.2.6 Circadian Clock: Photoentrainment

Several genes involved in coping with light-associated stress in *B. cinerea* are subject to photoregulation by the transcriptional WCC complex and BcLTF1 as a co-activator, as well as by the stress-activated MAP kinase BcSAK1 (Canessa et al. 2013; Schumacher et al. 2014) (unpublished). By this mechanism, the cell activates the needed stress protection measures in a timely fashion and saves resources under non-stressed conditions. Almost all organisms on earth are exposed to a 24 h day/night cycle of changing light and temperature conditions, leading to independent evolution of circadian clocks in the different kingdoms. As molecular machinery entrained by light and temperature cues, they can keep time and enable the organism to anticipate daily changing conditions and to adapt their metabolism in advance. The molecular basis of the fungal circadian clock has been intensively studied in *N. crassa* (Baker et al. 2012), but little is known about the presence and biological functions of the circadian clock in other fungi (Hevia et al. 2016). However, the maintenance of a clock (gain *vs.* loss) may reflect the relevance of light in an ecological niche. A functional circadian clock exists in *B. cinerea* that shares similarity with the *N. crassa* system, comprising the blue light-sensing WCC as the input pathway, and BcFRQ1 (frequency) as the oscillator. *Bcfrq1* transcript and protein levels oscillate in constant darkness, which requires BcWCL1 as part of the WCC. Likewise, the entrainment of the clock by light requires the WCC. The output of the clock is visible on the host *A. thaliana*: larger lesions are formed when leaves are inoculated at dusk than at dawn which is in accordance with increased susceptibility of the host during the night. Δ*bcfrq1* mutants exhibit decreased conidiation accompanied by sclerotial development in the light (*always sclerotia*), suggesting that BcFRQ1 modulates photomorphogenesis in a nutrition-dependent manner (Canessa et al. 2013; Hevia et al. 2015).

14.3 Conclusion: *Botrytis* Exploits the Host for Nutrition and Sun Protection

B. cinerea maintains a highly sophisticated light signaling machinery that senses different light qualities to trigger a variety of responses, that are photoprotection, photomorphogenesis, positive and negative phototropism and photoentrainment. These processes render *B. cinerea* a valuable model to enlighten the role of light in parasitic fungus-plant interactions.

The vegetative mycelium of *B. cinerea* is the core of all infection and developmental programs in that it mediates the rapid colonization of dead plant tissues in terms of nectrotrophic (active killing and colonization) and saprophytic growth (colonization of already dead tissues). The vegetative mycelium is not visibly pigmented and thus considered to be sensitive to different biotic and abiotic stresses. However, the vegetative hyphae have a very limited half-life and are usually restricted to the invasive growth phase in which the hyphae are protected from light by the plant tissue. Fast colonization of fresh tissues and proper nutrient acquisition enables the rapid formation of well-protected and long-lasting reproduction structures (conidiophores with conidia and sclerotia) on the surfaces of rotted plant tissues. Depending on the light and temperature conditions, conidiation or sclerotial development is initiated for spreading of disease (summer cycle) or fungal survival (winter cycle). Taken together, *B. cinerea* uses the light regulatory networks to avoid light whenever possible; for example, by minimizing the half-life of sensitive cells that are hiding in plant tissues and by scheduling critical steps for the night including conidiogenesis, conidial germination, and penetration of plant tissues. Not least, the multinucleate nature of hyphae and conidia helps *B. cinerea* to survive in light-flooded habitats by compensating for detrimental mutations in single nuclei.

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Part V

Genomes and Evolution

Species of *Zymoseptoria* (Dothideomycetes) as a Model System to Study Plant Pathogen Genome Evolution

15

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Abstract

Fungi in the genus *Zymoseptoria* infect a diverse range of grasses, and include the important wheat pathogen *Zymoseptoria tritici*. Natural grasslands in the Middle East are a hotspot of diversity for *Zymoseptoria* species and it is the center of origin for *Z. tritici*. The wheat pathogen emerged by host tracking during the domestication of wheat around 11,000 years ago. Extensive sampling of *Z. tritici* isolates and the sister species infecting wild grasses has allowed detailed analyses of genome evolution associated with speciation and divergent host specialization. The genomes of *Zymoseptoria* species show an overall high extent of synteny, but also comprise highly variable regions and variation in genome content including a large complement of accessory chromosomes. These enigmatic chromosomes represent an ancestral trait in *Zymoseptoria* exhibiting extensive presence-absence variation within species and poorly understood dynamics of chromosome loss and amplification. The study of allopatric populations of *Z. tritici* supports local adaptation processes via positive selection and transposable element expansion. Nonetheless, the shallow population structure of *Z. tritici* suggests continuous gene flow among populations and interspecific hybridization between species leading to introgression and genetic exchange between species. Here, we summarize recent findings from research of *Zymoseptoria* genome datasets, and underline the power of this fungal pathogen to understand the evolution of virulence, host specialization, and the host-shift dynamics of fungal plant pathogens in natural and agricultural ecosystems.

Keywords

Zymoseptoria species complex · Genome content · Selection · Host adaptation · Accessory chromosomes · Transposable elements · Hybridization · Population genetics

15.1 Introduction

The Dothideomycete fungus *Zymoseptoria tritici* causes the disease *Septoria Tritici Blotch* (STB); one of the most devastating wheat diseases that occur in wheat fields across the world (Torriani et al. 2015). *Z. tritici* is a so-called hemibiotrophic pathogen which invades the leaf tissue via open

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stomata to colonize leaf mesophyll tissue. During initial colonization no symptoms are visible, however after approximately one week of "biotrophic" infection, the fungus shifts to a necrotrophic life style leading to the development of necrotic lesions. Host cell death is accompanied by the formation of asexual fruiting bodies (so-called pycnidia) filled with asexual spores that give rise to new secondary infections (Kema 1996; Duncan and Howard 2000; Jing et al. 2008).

Given the economical impact of STB to wheat production, breeders and researchers have set out to identify traits conferring resistance towards the pathogen. *Quantitative trait locus* (QTL) analysis has led to the mapping of 21 Septoria tritici blotch (*Stb*) *resistance genes*, most conferring resistance towards specific pathogen isolates, and some with minor quantitative resistance effects (Arraiano and Brown 2017). The hexaploid nature of the wheat genome has challenged the detailed characterization of resistance genes, however a few genes have been cloned and described in detail, including the *Stb6* gene, which encodes a wall-associated receptor kinase (WAK)-like protein that recognizes a small secreted effector protein (AvrStb6) produced by some isolates of *Z. tritici* (Saintenac et al. 2018).

An important aim of *Z. tritici* research is to discover the underlying molecular determinants of virulence. Only a few effector genes have been identified and functionally characterized, including those that encode LysM effectors, which are produced to bind fungal chitin and thereby avoid recognition by plant immune receptors (Tian et al. 2021). Furthermore, there is evidence that *Z. tritici* actively suppresses plant immune defenses during the extended biotrophic colonization of the wheat tissue before the switch to necrotrophic growth (Seybold et al. 2020). *Z. tritici* produces a multitude of small secreted proteins during early infection (Palma-Guerrero et al. 2017; Haueisen et al. 2019), one of which has been shown to interact with a wheat E3 ubiquitin ligase involved in pathogen recognition and immune signaling (Karki et al. 2021). Metabolome analysis moreover suggests that defense-related hormones and pathways are suppressed by the fungus as it colonizes the mesophyll tissue (Rudd et al. 2015). During the shift from biotrophic to necrotrophic host colonization, the fungus may actively induce plant cell death. Heterologous expression of effector candidates in *Nicotiana benthamiana* has led to the identification of several putative necrotrophic effectors that induce cell death (Kettles et al. 2017). However, this approach has so far not been able to demonstrate the function of necrotrophic effector candidates in wheat.

Zymoseptoria tritici is also an important model experimental species on research of *population genetics* of plant pathogens. In the field, only the asexual fruiting bodies are clearly visible and for many years, it was considered that *Z. tritici* mainly reproduced clonally (McDonald and Martinez 1991). A series of studies using hierarchical sampling of *Z. tritici* at different spatial scales and polymorphic genetic markers, however, provided evidence for extensive recombination among fungal isolates in wheat fields as well as long-distance gene flow mediated by airborne ascospores (Zhan et al. 1998; Linde et al. 2002). More recent population genomic studies have confirmed high levels of genetic variation in populations of *Z. tritici*, frequent sexual recombination and extensive gene flow at regional as well as continental scales (Linde et al. 2002).

As a model species in plant pathogen population genetics, collections of isolates from wheat fields across the world have provided an excellent and unique resource to address the intriguing question; from where did this pathogen emerge and when did it become a pathogen of wheat. Population genetics studies using polymorphism data from *Z. tritici* populations around the world reveal the Middle East as a center of diversity with an enrichment of unique alleles (Banke et al. 2004; Banke and McDonald 2005). Based on the hypothesis that the wheat pathogen emerged in the Fertile Crescent, Banke and colleagues applied demographic modeling and coalescence analyses and found that the most plausible center of origin of *Z. tritici* indeed is the Middle East (Banke et al. 2004). As wheat was domesticated in the Fertile Crescent by Neolithic farmers more than 10,000 years ago (Brown

et al. 2009), *Z. tritici* could have co-evolved as a new pathogen with the new crop host. Plant domestication and an intensification of crop cultivation could more generally have been drivers for the rapid evolution of new plant pathogens.

Intensive sampling of *Zymoseptoria* from cultivated and wild grasses in regions that overlap with the Fertile Crescent has allowed us to test this hypothesis and reconstruct the evolutionary history of *Z. tritici* (Stukenbrock et al. 2007, 2011). These locations have proved to be a hotspot of diversity for *Zymoseptoria* species infecting grasses of the subfamily Pooideae. Several new species have been formally described from these collections including *Z. pseudotritici*, *Z. ardabiliae,* and *Z. brevis,* isolated from grass species belonging to the genera *Elymus*, *Dactylis, Lolium,* and *Phalaris.* Other already described species, *Z. tritici*, *Z. halophila,* and *Z. passerinii*, were collected from wild species of *Aegilops* and *Hordeum* (Table 15.1). In general, the high diversity of these grass-infecting pathogens correlates with a high biodiversity of Pooideae grasses in the Fertile Crescent region, which may be an important driver for the diversification of pathogens (Blumler 1992). Also at other locations, a few species of *Zymoseptoria* have been isolated from non-crop Pooideae species; *Z. crescenta* and *Z. verkleyi*, and to date, the genus *Zymoseptoria* includes eight recognized species with different and overlapping host ranges (Fig. 15.1, Table 15.1) (Quaedvlieg et al. 2011; Crous et al. 2012; GBIF Backbone Taxonomy 2011).

Closely related species of *Zymoseptoria* provide an excellent model system to study the diversification and speciation of pathogens. Different types of "speciation scenarios" have been proposed including host jumps, hybridization and "host tracking" where pathogen emergence is associated with host domestication. Analyses of population histories and speciation using coalescence analysis suggest that the emergence of *Z. tritici* occurred approximately 11,000 years ago and coincided with the domestication of wheat (Stukenbrock et al. 2007). *Z. tritici* was the first example of a plant pathogen that has emerged as a consequence of domestication; several other examples have followed, documenting a general impact of artificial selection on pathogen evolution.

In natural grasslands, the diversity of *Zymoseptoria* species may to a large extent result from recurrent host jumps and hybridization rather than plant–pathogen co-evolution and co-speciation. This is because the phylogenetic relationships of host species do not correlate with the phylogenetic relationship of pathogen species. The origin of the *Pooideae* subfamily, naturally distributed in temperate climates, is dated around 61–77 MY (mean 69 MY) and includes circa 4000 species, among them, commercially important crops like wheat and barley, and forage grasses such as fescues and ryegrass (Soreng et al. 2017; Schubert et al. 2019). The hosts of *Zymoseptoria* species are classified into the tribes *Triticea* (subtribes *Hordeinae* and *Triticinae*) and *Poeae* (subtribes *Aveninae*, *Loliinae*, *Dactylidinae*) (Soreng et al. 2015), and are grouped in at least ten different genera (Table 15.1). The subtribes *Hordeinae* and *Triticinae* contain the most economically relevant host species: *Hordeum vulgare* ssp. *vulgare* and *Triticum aestivum*, domesticated barley and wheat, respectively. *Triticum* species are estimated to have diverged from *H. vulgare* 8 ± 2 MYA (Middleton et al. 2014), much earlier than the divergence estimated between wheat- and barley-infecting species of *Zymoseptoria*, suggesting that speciation of the pathogens occurs at a considerably faster rate than their host species given the high mutation rates and short reproduction cycles (sexual and asexual) of the pathogen (Fig. 15.1) (Thines 2019).

Genome data from multiple species of *Zymoseptoria* allow us to address fundamental questions in the evolution of host-pathogen interactions in wild and agricultural ecosystems. Which evolutionary mechanisms drive adaptive evolution of pathogens? How do new virulence specificities evolve? How do pathogens adapt to their hosts in natural and managed ecosystems? Which traits define reproductive barriers among closely related pathogen species? Comparative genome analyses can be applied to identify commonalities and differences between genomes and to trace signatures of natural selection, which

Table 15.1 Host range and species divergence of *Zymoseptoria* genus and their

Zymoseptoria Species	Host Species Range	Type of host	Reference	Divergence in years ago
Z. tritici (Zt)	*Aegilops cylindrica* *Aegilops tauschii* *Avena sp.* *Calamagrostis sp.* *Triticale sp.* *Triticum durum* *Triticum aestivum* *Elymus repens* *Lolium multiflorum* *Lolium perenne*	*In planta* infection Natural host Natural host Natural host Natural host Natural host Natural host Detached leaf Detached leaf Detached leaf	Not published (Quaedvlieg et al. 2011) (Stukenbrock et al. 2012b) (Stukenbrock et al. 2012b) (Quaedvlieg et al. 2011) (Quaedvlieg et al. 2011) (Kema and van Silfhout 1997) (Stukenbrock et al. 2011) (Stukenbrock et al. 2011) (Stukenbrock et al. 2011)	11,000 (Zt-Zpt) 22,300 ((Zt-Zpt)- Za)
Z. pseudotritici (Zpt)	*Agropyron sp.* *Dactylis glomerata* *Elymus repens* *Dactylis glomerata* *Lolium multiflorum* *Lolium perenne*	Natural host Natural host Natural host Detached leaf Detached leaf Detached leaf	(Stukenbrock et al. 2011) (Stukenbrock et al. 2012a) (Stukenbrock et al. 2012a) (Stukenbrock et al. 2011) (Stukenbrock et al. 2011) (Stukenbrock et al. 2011)	11,000 (Zt-Zpt)
Z. ardabiliae (Za)	*Agropyron sp.* *Agropyron tauri* *Dactylis glomerata* *Lolium sp.* *Elymus repens* *Triticum aestivum*	Natural host Natural host Natural host Natural host Detached leaf Detached leaf	(Stukenbrock et al. 2011) (Stukenbrock and Dutheil 2018) (Stukenbrock et al. 2011) (Stukenbrock et al. 2011) (Stukenbrock et al. 2011)	22,300 ((Zt-Zpt)- Za)
Z. brevis	*Phalaris paradoxa* *Phalaris minor*	Natural host Natural host	(Quaedvlieg et al. 2011) (Quaedvlieg et al. 2011)	
Z. passerinii	*Aegilops tauschii* *Avena* sp. *Hordeum jubatum* *Hordeum vulgare* *Hordeum murinum* *Hordeum bulbosum* *Hordeum distichon* *Elymus repens* *Dactylis glomerata* *Lolium perenne*	Natural host Natural host Natural host Natural host Natural host Natural host Natural host Natural host Detached leaf	(Quaedvlieg et al. 2011) (Quaedvlieg et al. 2011) (Goodwin and Zismann 2001) (Goodwin and Zismann 2001) (Quaedvlieg et al. 2011)	

(continued)

Table 15.1 (continued)

Zymoseptoria Species	Host Species Range	Type of host	Reference	Divergence in years ago
		Detached leaf	Not published (Seifbarghi et al. 2009) (Stukenbrock et al. 2011) (Stukenbrock et al. 2011) (Stukenbrock et al. 2011)	
Z. halophila	*Hordeum glaucum* *Hordeum vulgare* *Hordeum halophilum*	Natural host Natural host Natural host	(Quaedvlieg et al. 2011) (Quaedvlieg et al. 2011) (Quaedvlieg et al. 2011)	
Z. crescenta Abrinbana, Abdollahz. & Crous	*Aegilops triuncialis*	Natural host	(Crous et al. 2018)	
Z. verkleyi Crous, Videira & Quaedvl.	*Poa annua*	Natural host	(Crous et al. 2012)	

may reflect functionally important traits. Several studies have used genome analyses to study genetic variation within and between species of *Zymoseptoria* and to address some of the questions listed above (Stukenbrock et al. 2007, 2011; Hartmann et al. 2018, 2021). In this chapter, we aim to provide an overview of studies of genome evolution in *Zymoseptoria* to a large extent focusing on the important wheat-infecting species *Z. tritici*. We emphasize the distinction of this study system to understand the evolution and population biology of plant pathogens and underline the importance of this type of studies in applied research of crop pathogens.

15.2 Genome Architecture in *Zymoseptoria* Species

15.2.1 "Gold Standard" Reference Genome

The first complete genome assembly of *Z. tritici* was completed by the Joint Genome Institute and published in 2011 (Goodwin et al. 2011). The consortium behind the genome sequencing project supported scaffold assembly of the genome by providing a fine-scale genetic map of *Z. tritici*. The resulting 40 Mb genome assembly was at that time categorized as a "gold standard" reference genome with chromosomes sequenced from telomere to telomere for almost all 21 chromosomes. Even to date, it is one of the best-assembled fungal genomes.

The Dutch isolate IPO323 that was selected for sequencing has become a reference for the community of scientists working on *Z. tritici*. The genome of *IPO323* comprises approximately 18% of repetitive sequence distributed on 13 *core chromosomes* and eight *accessory chromosomes* (Goodwin et al. 2011). These abundant non-essential chromosomes account for almost 12% of the entire genome and occur in different numbers and combinations in different field isolates. The *Z. tritici* genome encodes 11,839 genes distributed across the 21 chromosomes including some regions with higher and some with lower gene density; notably the accessory chromosomes have a lower gene density compared to the core chromosomes and are enriched in genes without a known function and without known protein domains (Grandaubert et al. 2015).

Earlier karyotype studies of *Z. tritici* based on electrophoretic separation of chromosomes had already demonstrated a considerable variation in chromosome content and lengths (McDonald and Martinez 1991). Not only were accessory

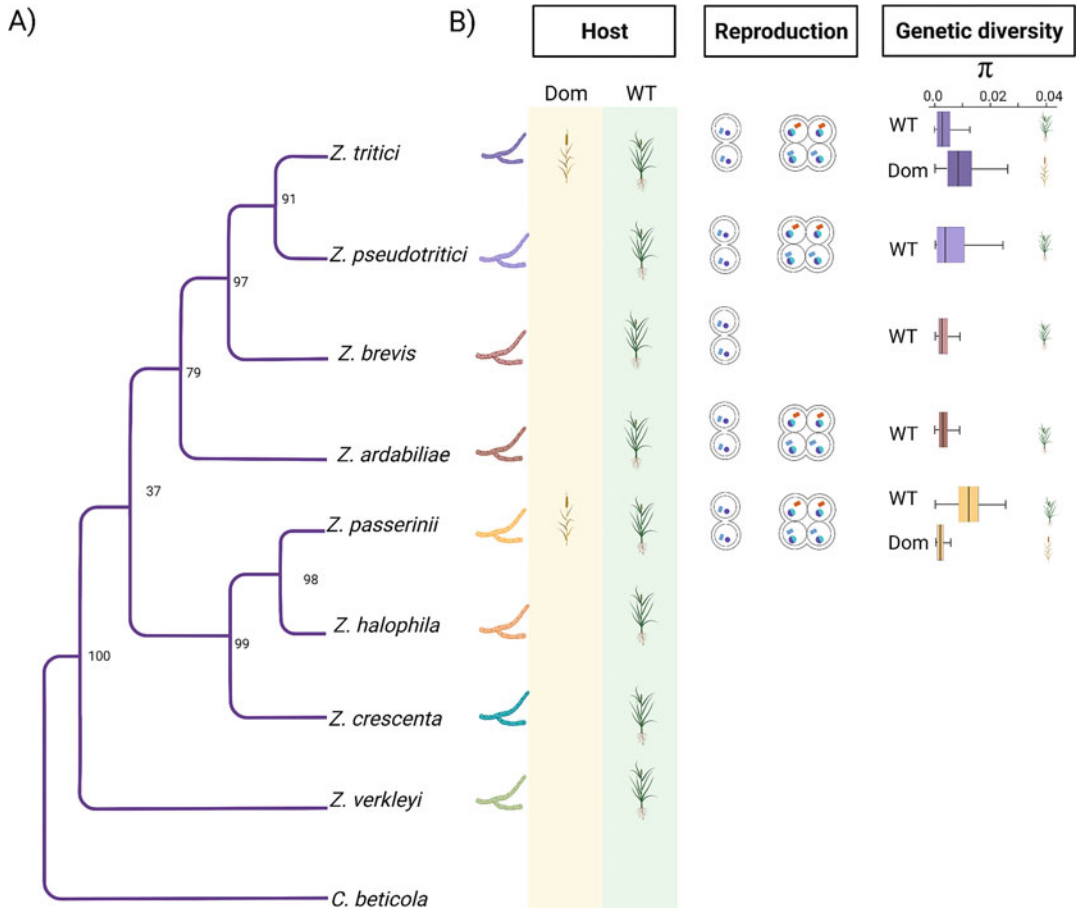

Fig. 15.1 Overview of described species in the *Zymoseptoria* genus. (**a**) Maximum Likelihood Phylogeny rooted with *Cercospora beticola* and built with RAxML (Stamatakis 2014) based on six concatenated nuclear loci: *18S rRNA*, *28S rRNA*, *tub*, *ef-1*, *ITS*, and *rpb2*. Sequences were retrieved from the nucleotide database of NCBI (NCBI Resource Coordinators 2015), and the numbers at each node of the tree show statistical support. (**b**) The first panel indicates the type of host: domesticated (Dom) or wild (WT); the central panel shows the (known) types of reproduction: asexual (two daughter cells) or sexual reproduction (four daughter cells); and the last panel shows genetic diversity (π) for the fungal populations isolated from domesticated (Dom) or/and wild (WT) hosts. The left and right whiskers extend from Q1 and Q3 to the most extreme data points, respectively

chromosomes variable in size, but also the core chromosomes as determined by pulse field gel electrophoresis (PFGE) analyses. More recently, genome sequencing of multiple *Z. tritici* isolates has confirmed the extensive variation in genome architecture and has provided detailed insights into the structural variation. Presence-absence variation is not only conferred to accessory chromosomes, but also includes segments of the core chromosomes (Croll et al. 2013). These accessory segments often encode genes and associate with transposable elements, which likely promote their variability.

By comparing the composition of multiple isolates and recording accessory sequences of every isolate (Tettelin et al. 2005; Hurgobin and Edwards 2017), it has been possible to reconstruct the *Z. tritici* "pangenome" (Plissonneau et al. 2018; Badet et al. 2020). For fungi with varying genome composition, this type of analysis seems particularly relevant to discovering the complete complement of genes. In two different

pangenome studies, the authors used PacBio long read genome assemblies of *Z. tritici* isolates collected from different parts of the world and found an exceptionally large repertoire of "accessory" genes (Plissonneau et al. 2018; Badet et al. 2020). In fact, only 60% of the identified orthogroups (OGs; i.e., groups of genes that share a common ancestor) were shared among all *Z. tritici* isolates implying functional redundancy and a huge repertoire of non-essential genes.

At the interspecific level, a recent study has provided a *comparative genomics* overview of the genome content and structure for five of the eight species recognized within the *Zymoseptoria* genus: *Z. tritici*, *Z. ardabiliae*, *Z. brevis*, *Z. passerinii*, and *Z. pseudotritici* (Feurtey et al. 2020). In this study, Feurtey and colleagues used high-quality genome assemblies from representatives of the five species to compare the content of orthologous genes, transcriptionally active regions, transposable element content and genome architecture. Interestingly, in spite of the extensive structural rearrangements involving accessory segments and chromosomes, the synteny between *Zymoseptoria* species is largely conserved (Feurtey et al. 2020).

The comparative genomics approach also allowed detailed comparisons of the composition and abundance of genes known to be involved in plant–pathogen interactions, such as those encoding putative effector proteins, carbohydrate-active enzymes (CAZymes) and genes involved in secondary metabolite biosynthesis (Feurtey et al. 2020). Molecules in these categories are known to be involved in the interaction with plant immune pathways, as well as defining pathogen lifestyles and interactions with other plant-associated microorganisms (Zhao et al. 2013; Lo Presti et al. 2015; Snelders et al. 2018; Shi-Kunne et al. 2019). Biosynthetic gene clusters (BGCs) and CAZymes are overall conserved among *Zymoseptoria* species and are mainly encoded by genes on core chromosomes, implying the importance of these genes in the biology of the fungus. Interestingly, and in contrast to findings from other pathogen species with accessory chromosomes (reviewed in Tsuge et al. 2013; Yang et al. 2020), predicted effector genes localize to the core chromosome and not the accessory chromosomes. Effector candidate genes in *Z. tritici* however often show presence-absence variation within as well as between species, which may reflect differences in gene duplications and/or transposable element activity (based on the same effector annotation approach, there are 274 predicted effectors in *Z. brevis* and 637 in *Z. ardabiliae*). So far, no study has conducted a systematic analysis of effectorome evolution among *Zymoseptoria* species, which in part is due to the challenge of recognizing sequence-based homology for this group of rapidly evolving genes.

An important finding from the comparative genome study of the five *Zymoseptoria* species was that many genes showing presence-absence variation among isolates of *Z. tritici*, also vary in frequency between species. This suggests that presence-absence variation is a type of ancestral polymorphism that may play a role in species diversification and divergent host specialization.

15.2.2 Transposable Element Content

Transposable elements (*TEs*) are a major component of *Zymoseptoria* genomes and shape genomic architecture and content. TEs are repetitive DNA elements that can change their location within eukaryotic genomes through transposition and can contribute to increasing genetic diversity by producing new haplotypes through minor or major rearrangements (Bourque et al. 2018). In fungal plant pathogens, TEs have been shown to be important drivers of accelerated evolution through their mutational effects and the interplay of TE activity and genome defense mechanisms such as repeat-induced point mutation (RIP) and epigenetic features such as DNA methylation (Raffaele and Kamoun 2012; Seidl and Thomma 2014; Galazka and Freitag 2014; Möller and Stukenbrock 2017; Möller et al. 2021).

The content of TEs is in large part determined by the efficacy of genome defenses, the extent of clonal versus sexual reproduction, and the effective population size of the organism. Some fungal genomes are very TE-rich, e.g., the genome of the mildew fungus *Blumeria graminis,* which is comprised of 74% TEs (Frantzeskakis et al.

2018). In the reference *Z. tritici* IPO323 genome, there are more than 2500 low-copy repeat families, including 70% Class I TEs (LTRs and LINEs) (Dhillon et al. 2014). With the availability of more high-quality *Zymoseptoria* genome assemblies, it has been possible to analyze and compare in detail the content of repetitive elements. The TE content and composition vary extensively between *Z. tritici* isolates implying that active TEs contribute to genome evolution. Comparing the overall TE content of isolates from different regions show that this ranges from 5.50 Mb in an Iranian isolate (14.0% of the genome) to 8.80 Mb in an Australian isolate (21.5% of the genome) (Lorrain et al. 2021). The fact that the TE proportion varies across different *Z. tritici* isolates suggests independent and recent expansions and losses among genomes (Lorrain et al. 2021). Much of the structural variation in the genome of *Z. tritici* relates to TE activity as rearrangements often are associated with TEs (Möller et al. 2019; Badet et al. 2020). The TE content in some of the sister species of *Z. tritici* is even higher, suggesting divergence in the efficacy of controlling TEs or in population genetic structure, including population size and reproduction mode. For example, the genome of the *Z. passerinii* isolate Zpa63 comprises 31% TEs and the *Z. ardabiliae* isolate Za17 only 18% (Lorrain et al. 2021).

TE activation and transposition can be triggered in response to environmental stress, as experimentally demonstrated with *Z. tritici* during axenic growth. By growing the fungus in a nutrient-poor and nutrient-rich medium, Fouche and co-workers demonstrated that nutrient status of the fungus can induce transposition events of some TE families in the genome (Fouché et al. 2020). Interestingly, the overall expression of TEs is upregulated at the early stage of plant infection, which coincides with the expression of co-localized effector genes (Soyer et al. 2019; Meile et al. 2020). The overlapping expression patterns of TEs and effectors very likely relate to the relevance of chromatin-based gene regulation whereby transcriptional silencing in facultative heterochromatin under some conditions is relieved. So far, the external plant signaling promoting chromatin-based gene regulation is unknown.

Several effector genes co-localize with TEs, and mutational effects mediated by TE activity have been shown to impact fitness of fungal isolates, either with respect to virulence or fungicide resistance (Omrane et al. 2017; Stewart et al. 2018; Fouché et al. 2020). In some cases, the association of TEs with particular genes has been directly favored by natural selection. This appears to be the case for a TE insertion on chromosome 12 at a locus that also encodes a gene involved in fungicide resistance (Oggenfuss et al. 2021). The particular TE on chromosome 12 has increased in frequency in North American *Z. tritici* populations, which experienced the first systematic application of fungicides in the 70s followed by the evolution and spread of fungicide resistance in the subsequent decades. While most studies have focused on the advantageous effects of TE insertions near functionally relevant genes, we also expect that TE insertions can have negative fitness effects. Often such events are not detected because the individuals carrying these mutations are sorted out by natural selection. However in a few cases, such events have been identified. For example, the gene *REP9–1* co-localizes within a transposon class II element. When the gene is expressed, the fungus is less virulent and produces less pycnidia (Wang et al. 2021). Under field conditions, these mutations would likely disappear and be outcompeted by more fit individuals without the TE.

In fungi, one of the primary host genome defense mechanisms against repetitive sequences is Repeat-Induced Point (RIP) mutations, first described in *Neurospora crassa* (Selker 2002). RIP mutations can be recognized as transition mutations that increase AT content. While RIP has so far not been experimentally tested in *Z. tritici*, some repetitive sequences contain extensive RIP signatures. Interestingly, the efficacy of RIP (recognized as transition mutations from C:G to T:A nucleotide base pairs in repeated sequences and neighboring regions) varies considerably between species (Lorrain et al. 2021). This may be due to the fact that different evolutionary mechanisms are in operation in the

different species (clonal versus sexual reproduction), but may also reflect that genome defense mechanisms themselves are evolving. An intriguing example of this is DNA methylation, which has been lost recently in *Z. tritici* while still functioning in the other *Zymoseptoria* species and in field isolates occurring at the center of origin in the Middle East (Möller et al. 2021). Cytosine methylation in these fungi occurs in TEs and is associated with extensive deamination and C to T mutations conferring efficient inactivation and silencing of TEs.

15.2.3 Accessory Chromosomes

Accessory chromosomes, also known as supernumerary, conditionally dispensable chromosomes or "B" chromosomes are present in many eukaryotes and have distinct features from those of core chromosomes that encode essential products and are indispensable (Habig and Stukenbrock 2020). Accessory chromosomes show non-Mendelian segregation and are present in some but not all individuals of a given species, leading to presence-absence variation among individuals of a population. Overall, fungal accessory chromosomes are generally enriched with repetitive DNA sequences, either satellite repeats or active and mutated transposons, and have a lower gene density when compared to the core genome (Galazka and Freitag 2014; Möller and Stukenbrock 2017; Bertazzoni et al. 2018).

In plant pathology and fungal genomics, accessory chromosomes have received particular attention due to specific features encoded in these genome compartments in some pathogen species. In some pathogen species, accessory chromosomes have been shown to play an important role in virulence. The first, and one of the best-characterized virulence traits encoded on an accessory chromosome, was described in the fungal pathogen *Nectria haematococca* (anamorph *Fusarium solani*) (Miao et al. 1991; Hirschi and VanEtten 1996; Han et al. 2001; Temporini and VanEtten 2002; Coleman et al. 2009). Chromosome 14 of *N. haematococca* is accessory and encodes a cluster of pea pathogenicity (*PEP*) genes that are required to detoxify phytoalexins produced by pea plants, conferring virulence on pea by isolates carrying the chromosome (Miao et al. 1991; Hirschi and VanEtten 1996; Han et al. 2001; Temporini and VanEtten 2002; Coleman et al. 2009). Likewise, in the tomato fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici*, virulence determinants that define host range are also encoded on accessory chromosomes (Ma et al. 2010; van der Does et al. 2016).

To date, based on information of genome sequencing and gel electrophoresis analyses, *Z. tritici* is the fungus with the largest complement of accessory chromosomes. The haploid 40 Mb genome of the reference isolate IPO323 comprises eight accessory chromosomes, however other isolates can have less (Goodwin et al. 2011; Plissonneau et al. 2016). Interestingly, an isolate without accessory chromosomes has never been encountered. Accessory chromosomes in *Z. tritici* show extensive *presence-absence variation* among isolates (Fig. 15.2) and fully assembled chromosomes range in size from 400 kb to 1 Mb in length, and exhibit specific hallmarks that distinguish them from core chromosomes (Habig and Stukenbrock 2020). First of all, the proportion of repetitive sequences (e.g., TEs) is significantly higher for the accessory genome of *Z. tritici* than the core genome; 21% of the total proportion of repeats in IPO323 locate on the accessory chromosomes, although these only comprise 12% of the total genome size (Dhillon et al. 2014; Lorrain et al. 2021). Second, accessory chromosomes have a lower gene density compared to core chromosomes and are enriched in genes without predicted function or known protein domains (79.3% of the genes on these chromosomes) (Goodwin et al. 2011; Grandaubert et al. 2015; Plissonneau et al. 2016). Third, accessory chromosomes show much lower transcriptional activity compared to core chromosomes both in vitro and in planta, in which, on average, the genes encoded on these chromosomes are expressed at 13-fold lower levels than the genes present on core chromosomes (Kellner et al. 2014). Most pronounced, accessory chromosomes show an enrichment of the histone methylation mark

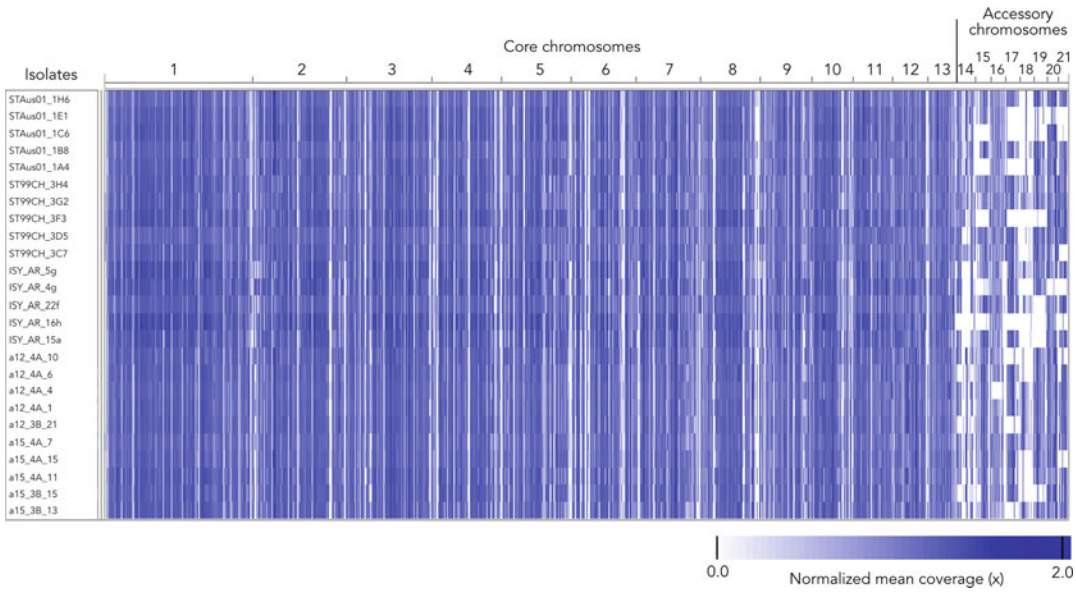

Fig. 15.2 *Zymoseptoria tritici* isolates show large Presence-Absence Variation (PAV) of accessory chromosomes within and between populations. Chromosome presence-absence variation was analyzed by read mapping to the *Z. tritici* IPO323 reference genome (Goodwin et al. 2011). Heatmap represents normalized mean coverage of reads mapped to each position of the reference genome. Isolates represent a subset of worldwide *Z. tritici* populations published previously (Hartmann et al. 2017). Darker blue color indicates sequences of higher coverage than single copy regions, such as repetitive sequences. "STAus01" = Australia population; "ST99CH" = Switzerland population; "ISY_Ar" = Israel population; "a12" = Oregon, US population 1 ("Madsen" wheat cultivar); "a15" = Oregon, US population 2 ("Stephens" wheat cultivar)

H3K27me3 (tri-methylation of the lysine 27 residue of the H3 protein) that is associated with facultative heterochromatin (Schotanus et al. 2015). Schotanus and colleagues used chromatin immunoprecipitation and sequencing (ChIP-seq) with antibodies against three histone modification marks: dimethylated H3K4 (H3K4me2), trimethylated H3K9 (H3K9me3), and trimethylated H3K27 (H3K27me3) to determine the distribution of euchromatin and heterochromatin across the *Z. tritici* genome (Schotanus et al. 2015). In fungi, the chromatin mark H3K27me3 is known to associate with facultative heterochromatin, which is a more dynamic type of heterochromatin (Connolly et al. 2013). While the accessory chromosomes are enriched with H3K27me3 and H3K9me2, the core chromosomes are associated, to a large extent, with the euchromatin mark H3K4me2, reflecting the gene-rich landscape and transcriptional activity (Galazka and Freitag 2014; Schotanus et al. 2015; Studt et al. 2016).

Earlier studies were directed at understanding why the accessory chromosomes of *Z. tritici* are unstable and show presence-absence variation. Accessory chromosomes can be lost during meiosis (Wittenberg et al. 2009; Croll et al. 2013; Fouché et al. 2018) as well as during mitotic cell divisions in vitro and *in planta* (Möller et al. 2018). This phenomenon is not only constrained to *Z. tritici* as small chromosomes in *Z. ardabiliae* show a similar loss rate during mitotic growth (Möller et al. 2018). Chromosome instability could arise from dis-functional centromeres or telomeres. However, the characterization of centromeres based on ChIP-seq targeting the centromere-specific histone protein CenH3 showed that centromeres of accessory chromosomes share the same characteristics as centromeres of core chromosomes (Schotanus et al. 2015). Moreover, sequenced-based analyses

of repeats in subtelomeric regions did not show any evidence of distinct telomere repeat composition on accessory chromosomes (Schotanus et al. 2015). These results suggest that accessory chromosomes have all the required features expected to be essential for proper chromosome segregation.

Given the wide distribution of H3K27me3 on the accessory chromosomes, Möller and colleagues addressed the role of this histone modification in chromosome loss in *Z. tritici* (Möller et al. 2019). The authors generated deletion mutants of the histone methyltransferases Kmt6 and Kmt1 responsible for the H3K27me3 and H3K9me3 modifications, respectively. Using experimental evolution, they showed that accessory chromosomes in the *kmt6* deletion mutant, depleted in H3K27me3, had increased stability of accessory chromosomes. Overall the chromosome loss rate in the mutants during in vitro mitotic propagation was only 1.7% compared to a loss rate of 6.6% in the wild-type strain (Möller et al. 2019). In contrast, the loss of H3K9me3 introduced large-scale genomic rearrangements accompanied by TE activation and redistribution of H3K27me3, leading to additional chromosome instability (Möller et al. 2019). This indicates that facultative heterochromatin plays a role in the instability of the accessory chromosomes, possibly even a mechanism that facilitates their loss.

Accessory chromosomes, although highly unstable, have been maintained over long evolutionary periods in *Z. tritici*. Given the high rate of chromosome losses, a paradox was how these chromosomes can be maintained given the high rate of chromosome loss during mitotic cell division. This question was addressed using experimental mating of *Z. tritici* isolates with known chromosome complements (Habig et al. 2018). *Z. tritici* is a haploid heterothallic fungus and requires two individuals of different mating types (mat1–1 and mat1–2) to form a diploid zygote and complete sexual mating (Kema et al. 1996, 2018). By dissecting the chromosome content in the meiotic products obtained from the mating of *Z. tritici* isolates, using tetrad analysis, it is possible to determine the fate of individual chromosomes during meiosis. If one of the parental strains lacks an accessory chromosome, the resulting diploid zygote produced by mating will consequently contain an unpaired chromosome. If the transmission of accessory chromosomes follows a Mendelian segregation pattern, any unpaired accessory chromosome is expected to be present in 50% of the resulting ascospores. However, from extensive tetrad analysis of meiotic products from independent mating experiments, it was found that unpaired accessory chromosomes exhibited a surprisingly higher-than-expected ratio of transmission. On the other hand, the paired accessory chromosomes showed Mendelian segregation with some loss events (Habig et al. 2018). Intriguingly, for the unpaired accessory chromosomes, the unexpected transmission advantage occurred only when the unpaired chromosome was derived from the female parent (the parental strain donating the mitochondria). This unexpected pattern provided evidence for a female-specific meiotic chromosome drive mechanism that can increase the frequency of accessory chromosomes during sexual mating. The chromosome drive suggests a hitherto unknown mechanism whereby unpaired accessory chromosomes are amplified during meiosis to ensure the maintenance of accessory chromosomes in *Z. tritici* (Habig et al. 2018).

Gene annotation and transcriptome studies do not indicate functional relevance of accessory chromosomes in *Z. tritici*, however given the high number of these small chromosomes, it has been tempting to speculate that they have a role in the biology of the pathogen. This question was addressed by Habig and collaborators who used carbendazim treatment to eliminate accessory chromosomes in *Z. tritici* (Habig et al. 2017). They generated a chromosome-deletion strain for each accessory chromosome and tested the impact of the individual chromosomes on virulence in three different wheat cultivars. In contrast to the findings from other plant–pathogen systems, the chromosome-deletion strains in some wheat cultivars showed an increased number of pycnidia suggesting a fitness cost of the accessory chromosomes in a wheat cultivar-specific manner (Habig et al. 2017). Hence, although virulence factors have not yet been

identified in accessory chromosomes, these accessory regions contribute to the overall fitness of the fungus *in planta*, possibly by encoding specific avirulence factors that increase host resistance.

15.3 Adaptive Evolution Within and Between *Zymoseptoria* Species

Genome data provides insights into the evolutionary history of populations and species. In research on fungal pathogens, population genomic analyses have proven of fundamental importance to dissect the origin and population history of pathogens (e.g., Islam et al. 2016; Menardo et al. 2016). Hereby, genome studies can support the assessment of future disease emergence and spread as well as the development of sustainable control strategies (Stukenbrock and McDonald 2008; de Vries et al. 2020). Focusing on the impact of natural selection in shaping genetic and functional traits in pathogen genomes, genome analyses can use information from population data and from between species comparisons to identify signatures of recent and past adaptive evolution.

15.3.1 Signatures of Selection in *Z. tritici* Genomes

For *Z. tritici*, evolutionary analyses have been used to elucidate the origins of this important fungal pathogen and to pinpoint important traits for its rapid adaptation and dissemination. With the growing availability of large-scale genome data, particular interest has been given to identifying signatures of *positive selection*, which in turn may reveal functional traits that have been important for adaptive evolution (Nielsen 2005).

Regions in the genome that are shaped by either positive or negative (purifying) selection can be recognized by the distribution of genetic variants among individuals. Evolutionary analyses to detect selection build on a null model of neutral evolution where deviations from this "*neutral model*" may indicate selection (Kimura 1968). Deviation from neutrality can be detected by analysis of the frequency of alleles across a particular region in comparison to the overall distribution of alleles frequencies. To this end, population genomic data can be used to compute the site frequency spectrum (SFS) of genetic variants and thereby compare the observed SFS with the expected SFS under neutral evolution (Braverman et al. 1995; Ronald and Akey 2005; Smith and Haigh 1974). While selection shapes the SFS, the frequency of rare and common alleles in a population is in large part also determined by the history and demography of the organism. The SFS alone can therefore not be used to detect signatures of selection along a genome sequence, but can be combined with other statistics such as linkage disequilibrium (LD) and the nucleotide diversity to reveal signatures of positive selection. Positive selection of a particular allele leaves a "selective sweep" footprint along a genome sequence. Such selective sweeps can be recognized by a depletion of genetic variation, high LD, and an excess of low-frequency alleles (Kaplan et al. 1989; Braverman et al. 1995; Smith and Haigh 1974).

A study using *population genomics* data of a global collection of *Z. tritici* isolates was performed using different genomic scans to detect genome-wide signatures of selective sweeps in four populations of the pathogen collected in wheat fields in different geographical regions (Hartmann et al. 2018). The study used different types of analyses to identify and compare selective sweeps including extended haplotype homozygosity (EHH) tests and Composite Likelihood Ration (CLR) tests, which detect different "strengths" of selective sweeps. The EHH test can be used to reveal incomplete selective sweeps, whereby the favorable allele is not entirely fixed while the CLR test allows detection of hard selective sweeps where the favorable allele is fixed by selection (Vitti et al. 2013; Pavlidis and Alachiotis 2017).

In the global collection of *Z. tritici* isolates, different geographical populations show common but also specific signatures of selection

(Hartmann et al. 2018). Approximately 5% of the *Z. tritici* core genome shows signs of positive selection, including selective sweeps widely distributed along the 13 core chromosomes. Combining the genome scans with annotation data allowed the authors to make inferences about functional traits involved in adaptive evolution. On average, more than 300 genes have experienced recent selection, and these genes are enriched in *Gene Ontology* (*GO*) terms associated with protein transport and localization. The selective sweep regions also comprise a large number of genes predicted to be involved in plant–pathogen interactions such as genes encoding plant cell wall-degrading enzymes (CWDEs), peptidases, and other small secreted proteins (SSPs) lacking conserved domains as well as genes located in secondary metabolite gene clusters (De Jonge et al. 2012; Zhong et al. 2017; Hartmann et al. 2017). The population specific selective sweeps likely reflect local adaptation to different selection pressures imposed in agricultural ecosystems over time, such as fungicides, local climate conditions, and host genotypes (Zhan et al. 2005, 2006; Zhan and McDonald 2011). The broad range of gene functions detected in the selective sweep regions indicates a broad suite of traits important for adaptation to local environments beyond host defense avoidance (Hartmann et al. 2018).

Adaptive evolution can be studied for individual genes, but can also be quantified more broadly across the genome and between populations of species (Nordborg et al. 1996; Eyre-Walker and Keightley 2007, 2009; Galtier 2016). This type of analysis can be applied to address if, e.g., adaptation occurs more rapidly in agricultural environments, or which genome features correlate with adaptive evolution and which do not; questions that are highly relevant to understand evolutionary dynamics of plant pathogens.

Population genomic data of *Z. tritici* has been applied to compute rates of adaptation and to determine which genome features specifically correlate with natural selection (Grandaubert et al. 2019). The authors used information from protein-coding genes to first determine the proportion of non-synonymous and synonymous variants, the *Pn/Ps* ratio. In a population, *purifying selection* is expected to eliminate non-synonymous variants, and thus the *Pn/Ps* ratio can be considered as a proxy for purifying selection. In the genome of *Z. tritici*, the *Pn/Ps* ratio correlates negatively with the recombination rate whereby genes located in regions of the genome with high recombination have a low *Pn/Ps* ratio. This correlation is expected when recombination acts in concert with selection to eliminate any non-advantageous mutation at non-synonymous sites in coding sequences (Marais and Charlesworth 2003). Interestingly, genes predicted to encode effector proteins overall have a higher *Pn/Ps* ratio compared to all other genes, which may reflect a lower efficacy of purifying selection in these genes and possibly on sites linked to positively selected mutations in this gene category (Grandaubert et al. 2019).

To quantify adaptive evolution, the authors computed two statistics; α (the proportion of non-synonymous divergence attributable to adaptive evolution) and ω_a (the rate of adaptive evolution). Interestingly, measures of both α and ω_a are comparable to values obtained from other organisms such as different mammal species suggesting that a plant pathogen such as *Z. tritici* does not exhibit exceptional rates of adaptive evolution (Galtier 2016). By correlating these measures of adaptive evolution, it was possible to show that adaptive evolution likewise correlates with recombination rate. *Recombination* allows the efficient and rapid fixation of beneficial alleles as predicted by evolutionary theory (Marais and Charlesworth 2003; Galtier 2016). On the other hand, there is no correlation of TE content and adaptive evolution, suggesting that TEs may accelerate the formation of new alleles, but they are not drivers of adaptation in the genome of *Z. tritici* (Grandaubert et al. 2019). The correlation of selection with recombination suggests a pervasive effect of Hill-Robertson interference in *Z. tritici*. *Hill–Robertson interference* (also known as selective interference) implies that the linkage between selected mutations tends to "interfere" with the effectiveness of selection at any one of the linked loci, reducing their probability of fixation in a natural

population dependent on the rate of recombination (Hill and Robertson 1966; Felsenstein 1974; Comeron et al. 2008). In other words, the efficacy of selection at linked sites is considerably reduced in regions with low recombination (Hill and Robertson 1966; Felsenstein 1974; McVean and Charlesworth 2000) such as the accessory chromosomes in *Z. tritici* (Stukenbrock and Dutheil 2018). Altogether, this study provides evidence that *background selection* and selective interference are widespread in the *Z. tritici* genome and shape the adaptive evolution in this rapidly evolving fungal pathogen.

15.3.2 Signatures of Selection Between Species of *Zymoseptoria*

Divergence between species can reflect divergent adaptation to specific niches, among plant pathogens, for example, adaptation to distinct host species. Inference of loci that have experienced divergent adaptive selection may elucidate traits that have been important for ecological speciation (Giraud et al. 2010). This type of analysis has been used to develop hypotheses about divergent specialization to different hosts and environments.

Different methods have been developed to detect selection at the interspecific level, including codon-based methods that compare the proportion of synonymous and non-synonymous divergence in protein-coding sequences. An excess of non-synonymous substitutions can indicate that positive selection has acted on a gene. Extended methods compute the *dN* (non-synonymous substitution rate) and *dS* (non-synonymous substitution rate) based on models of codon evolution to determine the most likely scenario explaining substitution patterns in genes, hereunder branch-specific models that assess selection in particular branches of a tree or site models that determine selection on particular sites in a protein-coding sequence (Miyata et al. 1979; Li et al. 1985; Nei and Gojobori 1986; Suzuki and Gojobori 1999; Yang and Nielsen 2002; Eyre-Walker 2006). The McDonald-Kreitman (MK) test and methods based on the MK test use information from within and between species, polymorphisms, and substitutions, respectively to detect an excess of non-synonymous substitutions that may reflect divergent adaptation (McDonald and Kreitman 1991; Alachiotis and Pavlidis 2018; Moutinho et al. 2020).

Different studies have identified footprints of divergent selection among species of *Zymoseptoria* based on comparative analyses of genome-wide gene sequences. One of the first genome-based studies of *Z. tritici* (Stukenbrock et al. 2010) used data from the sister species *Z. pseudotritici* (as used in the original paper yet not described as a distinct species and is therefore referred to as S1). An advantage of the study system is the relatively close relatedness; at the nucleotide level, the overall divergence between *Z. tritici* and *Z. pseudotritici* is only 7% allowing the alignment of whole genome sequences. Patterns of evolution in genes were estimated by the rates of synonymous (*Ks*) and non-synonymous (*Ka*) substitutions for 9521 aligned protein-coding genes (Nei and Gojobori 1986), revealing considerably higher rates of evolution of genes on accessory chromosomes compared to core chromosomes. The study allowed a first crude search of genes with signatures of divergent positive selection between the two *Zymoseptoria* species. Indeed 43 genes, all with unknown protein function, had a significantly higher rate of non-synonymous changes compared to synonymous changes ($Ka > Ks$), a first dataset of candidate genes potentially involved in divergent host specialization.

Sequencing of additional genomes of *Z. tritici*, *Z. pseudotritici*, *Z. ardabiliae,* and *Z. passerinii* allowed new analyses and comparisons to be conducted (Stukenbrock et al. 2011). The expanded dataset allowed for more detailed analyses of gene evolution using phylogenetics and branch-specific estimates of *dN/dS*. Interestingly, these first comparative analyses of gene evolution among four *Zymoseptoria* species revealed an overall higher branch-specific *dN/dS* ratio on the terminal branch of *Z. tritici* (*dN/*

$dS = 0.045$) compared to the most closely related species *Z. pseudotritici* ($dN/dS = 0.041$).

In many crop plants, it has been shown that one consequence of domestication is an excess of non-adaptive, non-synonymous mutations. This is because strong selection at a few loci in the genome not only reduces overall variation in the genome of domesticated species, but also increases linkage disequilibrium around selected traits (Kantar et al. 2017). Would the same signature of "co-domestication" be visible in the genome of the wheat pathogen *Z. tritici*? Based on a set of genomes of each of the three species *Z. tritici*, *Z. pseudotritici,* and *Z. ardabiliae*, it was possible to compare the proportion of non-synonymous and synonymous polymorphisms among the three species. The *Pn/Ps* ratio reflects, as specified above, the efficacy of purifying selection. In contrast to the general pattern observed for crops, including wheat, the speciation of *Z. tritici* has not entailed such a "domestication cost." On the contrary, the comparison of *Pn/Ps* ratios among the three species indicated an overall stronger efficacy of purifying selection in the domestication-associated pathogen *Z. tritici* compared to the two "wild" pathogen species. Moreover, these results suggest that the higher *dN/dS* values observed for the *Z. tritici* branch are due to higher overall fixation of adaptive substitutions rather than mere accumulation of deleterious mutations (Stukenbrock et al. 2011). Genes identified to be under positive selection in the three species *Z. tritici*, *Z. pseudotritici,* and *Z. ardabiliae* were enriched in genes encoding secreted proteins and putative effectors. A subsequent study used gene deletion and infection assays to investigate the functional relevance of a subset of these genes and indeed found that three of the genes had an impact on pathogenicity in wheat (Poppe et al. 2015). This study demonstrates the value of evolutionary predictions in identifying functionally relevant traits in genome data.

15.4 Genetic Variation Between *Zymoseptoria* Species

15.4.1 Recurrent Hybridization Shapes Genetic Variation in *Zymoseptoria* Genomes

Population genetic studies of *Z. tritici* have documented extensive *gene flow* involving recombination between distinct genotypes (Zhan et al. 2003). There is ample evidence that gene flow and recombination also occur between species of *Zymoseptoria*. Genome analyses show that interspecific hybridization occurs at the center of origin of these pathogen species. The sympatry between *Zymoseptoria* sister species and the infection of co-existing grasses may facilitate genetic exchange between species, and perhaps even be advantageous events that promote host jumps. The outcome of *hybridization* can be the establishment of a new hybrid lineage with properties, e.g., a host niche, that is distinct from that of the parents. It is also possible that hybridization is accompanied by backcrossing to the parental species, whereby only segments from the hybridization event remain as introgressed regions (Stukenbrock 2016).

Interspecific hybridization between species of *Zymoseptoria* was first identified in the species *Z. pseudotritici* (Stukenbrock et al. 2012a; b). The genome of *Z. pseudotritici* is a mosaic pattern of highly variable regions interspersed between regions with no variation at all. In fact, in a genome alignment of five individuals, several segments of up to 100 kb show no single polymorphism. The highly variable regions, on the other hand, comprise only two haplotypes with no variation between individuals sharing the same haplotype. This genome pattern is consistent with a scenario where two individuals of distinct, but closely related species, gave rise to a new hybrid by sexual mating. The species *Z. pseudotritici* has thereby emerged from this single hybridization event. By correlating the recombination rate with the length of variable segments, it was possible to date the timing of the hybridization event

to approximately 400–500 generations ago (Stukenbrock et al. 2012a, b).

In a population genomic study of *Z. tritici*, Feurtey and co-workers detected a similar mosaic pattern of highly variable regions interspersed between regions showing much less variation (Feurtey et al. 2020). The authors took a phylogenetic approach to test the hypothesis that the highly variable regions in *Z. tritici* result from introgression from other species of *Zymoseptoria*. By comparing the topology of phylogenetic trees in windows along a genome alignment of *Z. tritici*, *Z. pseudotritici*, *Z. ardabiliae,* and *Z. passerinii,* the authors could show that the highly variable regions systematically coincided with non-monophyletic tree topologies. It was not possible to identify a unique source for the introgressed regions, suggesting that hybridization occurs readily between different species of *Zymoseptoria.* Introgression can be adaptive and involve the transfer of specific alleles from one species to another, however, in *Z. tritici* the highly variable introgressed regions only involve a few genes and often associate with TEs. The functional relevance of the few genes that have been affected by introgression is so far not known. But the study also points to a mechanism whereby TEs can move between species leading to the high diversity of element families observed among genomes of *Zymoseptoria* (Lorrain et al. 2021). Evidence for the spread of DNA elements via hybridization has also been shown in a study of spliceosomal introns that identified patterns consistent with introgression between *Zymoseptoria* species (Wu et al. 2017). Consistent with the finding of Feurtey and colleagues, these specific intron elements are in many cases flanked by sequences showing a higher extent of variability compared to regions not affected by introgression.

Lastly, in a study of DNA methylation in *Z. tritici*, it was found that isolates in the Middle East differ from isolates originating from other places of the world. DNA methylation is in general a lost trait in *Z. tritici* as the DNA methyltransferase gene *dim2* is non-functional (Dhillon et al. 2014). The non-functionalization occurred as a consequence of gene duplication followed by extensive RIP mutations. Sister species of *Z. tritici* still maintain a functional copy of *dim2* and have DNA methylation, and so do *Z. tritici* isolates collected in the Middle East (Möller et al. 2021). For these Middle East *Z. tritici* isolates, there is ample evidence that the functional copy has been acquired de novo by introgression from sister species of *Zymoseptoria* occurring on wild grasses. This study exemplifies the functional relevance of hybridization between the grass pathogens, a phenomenon that also may confer the exchange of pathogenicity-related traits.

Overall, genome studies in *Zymoseptoria* demonstrate an important role of interspecific mating and hybridization in the formation of new genetic diversity. The relevance of hybridization in host jumps has so far not been addressed, but could be investigated with experimental mating and plant infection assays. One challenge of studying hybridization with experimental approaches is the difficulty of crossing *Zymoseptoria* under controlled conditions.

15.5 Concluding Remarks

Zymoseptoria tritici is one of the most devastating fungal wheat pathogens. Research of this pathogen is of utmost importance in order to understand the evolution and spread of new adaptive traits, as well as the underlying molecular biology of plant–pathogen interactions. *Zymoseptoria* isolates are laboratory-amenable and can be genetically modified by *Agrobacterium tumefaciens*-mediated transformation (Bowler et al. 2010; Fagundes et al. 2020), whereby the functional relevance of genes of interest can be assessed. For some species, *Z. tritici* and *Z. passerinii*, infection assays are also established with domesticated and wild cereal species to investigate *in planta* functions. We still do not have robust protocols to generate sexual crosses and rely on experiments under specific conditions to induce sexual mating. Protocols for in vitro mating would allow detailed studies of meiotic events under controlled conditions.

Z. tritici has proven an excellent model organism to study the genome evolution of eukaryotic microbial pathogens. The small haploid genome size makes whole genome sequencing less costly, and hundreds of genome sequences are already available in publicly accessible databases (*Zymoseptoria*records—NCBI). The genome of *Z. tritici* provides fascinating features including a balance of TE activity with efficient silencing and mutagenesis of some elements, while other elements under some conditions can be active and generate new variation. Known mechanisms of genome defenses include RIP, DNA methylation (for some isolates), and histone methylation. This fungus provides an intriguing model to understand how these mechanisms are regulated and how they evolve over short evolutionary time scales Zymoseptoria-SRA-NCBI (2022).

The large complement of accessory chromosomes in *Z. tritici* is not observed in other fungal genomes—although accessory chromosomes per se are found in many species. It appears that these chromosomes may be selfish genetic elements and that the varying complement that we observe among individuals reflects a dynamic of chromosome loss (during mitosis) and amplification (during meiosis). Which mechanisms are at stake to drive this dynamic are poorly understood, but calls for detailed research of cell and chromosome biology in this fungus.

Lastly, *Z. tritici* and its close relatives provide a unique model system to study the evolutionary biology of pathogens. A set of closely related species originating from wild grasses at the center of origin of these species are now described (Quaedvlieg et al. 2011; Stukenbrock et al. 2012b; Feurtey et al. 2020). The genomes are so closely related that they can be aligned and thereby provide an excellent resource to study genome evolution associated to speciation, host jumps and host tracking. Importantly, it allows for the detailed comparison of evolutionary processes in natural and managed ecosystems and thereby to test the impact of agriculture on pathogen evolution.

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Accessory Chromosomes of the *Fusarium oxysporum* Species Complex and Their Contribution to Host Niche Adaptation

16

Houlin Yu, Dilay Hazal Ayhan, Domingo Martínez-Soto, Shira Milo Cochavi, and Li-Jun Ma

Abstract

The genome of *Fusarium oxysporum,* an ascomycete fungus, can be divided into two compartments: core chromosomes (CCs) and accessory chromosomes (ACs). CCs are conserved, vertically transmitted from parent to offspring, and involved in essential housekeeping functions, whereas lineage- or strain-specific ACs are horizontally transmitted and associated with specialized functions. These two genomic compartments differ in terms of gene density, transposable element distribution, and epigenetic markers. Although commonly observed in eukaryotes, the functional importance of ACs stands out among phytopathogenic fungi, especially in relation to their pathogenicity and adaptability to hosts and other environmental conditions. Recent studies confirmed that these structural and functional variations observed at the genomic level contribute to the colonization of both plant and human hosts by different *F. oxysporum* strains, most likely through coordination and crosstalk between these two compartments. In this review, we focus on the cross-kingdom fungal pathogenicity of *F. oxysporum*, providing a summary of the genome dynamics of *F. oxysporum* and describing how these dynamics shape the host niche through molecular dialogues.

Keywords

Accessory chromosome · Cross-kingdom fungal pathogenicity · Fungal genome compartmentalization · *Fusarium oxysporum* · Genome crosstalk · Host niche adaptation

16.1 *Fusarium oxysporum*: A Species Complex Occupying Diverse Ecological Niches

The genus *Fusarium* contains over 300 phylogenetically closely related species that inhabit different ecological niches (Aoki et al. 2014), including plants (Dean et al. 2012; Fones et al. 2020) and humans (O'Donnell et al. 2007). As a cross-kingdom pathogen that causes devastating vascular wilts in many crops and severe infectious diseases in humans, *Fusarium oxysporum* is one of the most important pathogenic *Fusaria* (Ma et al. 2013; Michielse and Rep 2009; Ploetz 2015; Edel-Hermann and Lecomte 2019; Pegg et al. 2019; Zhang et al. 2020). While a sexual stage (teleomorph) has been described for some *Fusarium* species, it has not been observed for *F. oxysporum*. In the absence of sexual reproduction, these organisms cannot strictly be defined as a biological species. The term *F. oxysporum*

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species complex (FOSC) is used to describe all *F. oxysporum* strains based on morphology and phylogeny using molecular markers (Ma et al. 2013). Members within the FOSC are classified as different *forma speciales* based on their specific adaptation to particular plant hosts (Armstrong and Armstrong 1981). For instance, strains within *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *cubense* cause wilt disease on tomato (*Solanum lycopersicum*) and banana (*Musa* spp.) plants, respectively. Over 120 *forma speciales* have been described in the FOSC.

As *Fusarium* wilts cause substantial economic losses in many economically important crops, including potato (*Solanum tuberosum*), sugarcane (*Saccharum* spp.), bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), banana, and date palms (*Phoenix dactylifera*), *F. oxysporum* is considered to be among the top five most economically important plant pathogens (Dean et al. 2012). The fungus impairs the vascular transport of nutrients and water in the infected plant, causing wilting and, eventually, death (Gordon 2017; Husaini et al. 2018).

A common reason for the devastation of *Fusarium* wilts is the lack of effective means to remove the pathogen from infected fields. The fungus produces three types of asexual spores: microconidia (Fig. 16.1), macroconidia, and chlamydospores. All of these spore types contribute to the persistence of the disease (Gordon 2017; Husaini et al. 2018). Microconidia or propagules are abundantly produced and can be found in the soil. Macroconidia are usually formed on plant stems or leaves. Thick-walled resistant chlamydospores are produced upon the exhaustion of nutrients and can survive in the soil for many years (Nelson 1981).

One of the most severe epidemics of agricultural crops to date involved *Fusarium* wilt of banana (Viljoen et al. 2020), historically referred to as Panama disease. In the 1950s, millions of "Gros Michel" banana plants were destroyed by race 1 of *F. oxysporum* f. sp. *cubense* (*Foc*) in Central America (Stover 1962). Almost a century later, a new *Foc* race, tropical race 4 (TR4), was detected as the causal agent of *Fusarium* wilt in "Cavendish" bananas, the *Foc* race 1-resistant cultivar that revived the Central American banana export industry and became the most economically important banana cultivar in global markets (Ploetz 2015). TR4 was first described in Southeast Asia (Boehm et al. 1994) and has spread rapidly in Australia (Shivas et al. 1995), Africa (Visser et al. 2010), the Middle East (Maymon et al. 2018), Europe (Özarslandan and Akgül 2020), and Latin America (García-Bastidas et al. 2020). The history and ongoing problems of this disease illustrate the ability of *F. oxysporum* to overcome host resistance and adapt quickly to changing environments (Martin et al. 2006; Seidl and Thomma 2014).

Even though there are more reports on plant-pathogenic FOSC strains, nonpathogenic *F. oxysporum* strains, including some biocontrol agents, are also prevalent in nature. For instance, a comprehensive study of *Fusarium* wilt suppressive soils from the Châteaurenard region in France established a novel approach to control *Fusarium* wilt (Alabouvette 1999). Similarly, the biocontrol *F. oxysporum* strain CS-20 was reported to induce host defense and reduce wilt symptoms in tomato plants (Shcherbakova et al. 2016).

In addition to being plant pathogens, some *F. oxysporum* strains are also human pathogens and can cause severe skin, nail, or disseminated infections, known as fusariosis (O'Donnell et al. 2007; Zhang et al. 2020). Much more invasive than other infectious fungal pathogens, the infective agent can be detected in blood samples among approximately 50% of fusariosis patients (Nucci and Anaissie 2007). Because *Fusarium* spp. are broadly resistant to most of the clinically available antifungals, fusariosis has a high mortality rate (Scheel et al. 2013; Nucci et al. 2014; Prajna et al. 2016). Indeed, a 100% mortality rate was reported among persistently neutropenic fusariosis patients (Boutati and Anaissie 1997; Nucci and Anaissie 2002). *Fusarium* species were also reported as the most common causative agents of fungal keratitis in India (Lalitha et al. 2015; Hassan et al. 2016), China (Wang et al. 2009; He et al. 2011), South Africa (O'Sullivan et al. 1997), and Brazil (Ibrahim et al. 2009).

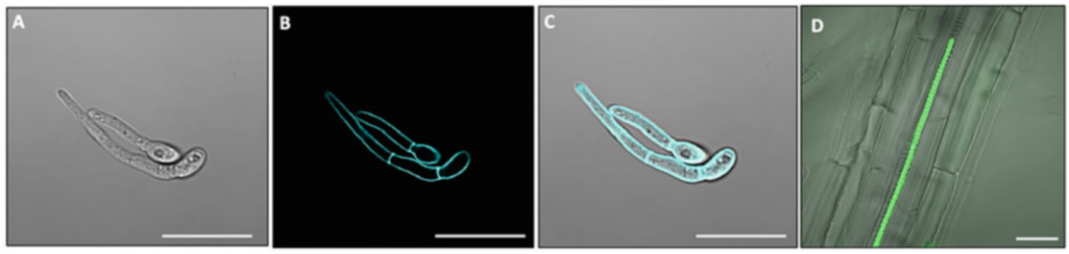

Fig. 16.1 Morphology and plant colonization of *Fusarium oxysporum* strain Fo5176, phytopathogen of *Brassicaceae*. (**a**–**c**), Germination of microconidia. (**a**), Bright field; (**b**), Fluorescence of fungal cell wall stained with Calcofluor white (excitation at 380 nm and emission at 475 nm); and (**c**), Merge. (**d**), Fo5176 colonization of a lateral root xylem of *Arabidopsis thaliana* at 5 days post-infection. Fungal structures (shown in green) were stained with WGA-Alexa Fluor 488 and detected with excitation at 488 nm and emission at 500 to 540 nm. Scale bar, 20 μm

Collectively, fusariosis has been listed as an emerging fungal disease and the second most common opportunistic mold infection after aspergillosis (Guarro 2013; Nucci et al. 2014).

16.2 Accessory Chromosomes: Contributors of Host-Specific Pathogenicity Among the FOSC

The broad host range and host-specific pathogenicity of the FOSC have been attributed to the existence of diverse and horizontally transmitted accessory chromosomes (ACs) that result in highly dynamic and compartmentalized genomes (Ma et al. 2010, 2013; Rep and Kistler 2010; Kistler et al. 2013; van der Does and Rep 2012) (Fig. 16.2). In contrast to core chromosomes (CCs), which are conserved among all *F. oxysporum* genomes, ACs are highly variable (Ma et al. 2010, 2013). First reported in the early 1990s, fungal ACs are also referred to as supernumerary chromosomes, conditionally dispensable chromosomes, and lineage-specific chromosomes (Croll and McDonald 2012; Croll et al. 2013; Ma 2014; Bertazzoni et al. 2018; Yang et al. 2020) and are thought to be horizontally transferred (Masel et al. 1996; Akagi et al. 2009).

The presence of ACs in *F. oxysporum* was solidified in a 2010 study examining the genome of the tomato pathogen *F. oxysporum* f. sp. *lycopersici* strain 4287 (Fol4287) (Ma et al. 2010). Four ACs lacking homologous counterparts in two closely related species, *F. graminearum* and *F. verticillioides*, were reported in Fol4287. This study also demonstrated the discordance in phylogenetic relationships of genes between CCs and ACs and further tested the hypothesis that ACs are transferred horizontally. The accessory sequences of *F. oxysporum* can exist as separate chromosomes (i.e., ACs) or can be attached to CCs. The Fol4287 genome contains four separate ACs and additional accessory sequences located at the ends of CCs, including chromosomes 1 and 2. In the genome of *F. oxysporum* f. sp. *cubense* TR4, all accessory sequences are attached to the ends of CCs (Zhang 2019).

The existence of ACs and their contribution to fusariosis were reported in the genomes of two human-pathogenic *F. oxysporum* isolates, NRRL 32931 and NRRL 47514, a clinical strain isolated from a leukemia patient, and a strain associated with the USA 2005/06 *Fusarium* keratitis outbreak, respectively (Zhang et al. 2020), 10 years after the initial report of ACs in a *F. oxysporum* plant-pathogenic strain (Ma et al. 2010).

ACs were reported to determine host-specific pathogenicity among plant-infecting *F. oxysporum* isolates, including other *F. oxysporum* f. sp. *lycopersici* strains (Vlaardingerbroek et al. 2016), *F. oxysporum* f. sp. *melonis* (Schmidt et al. 2016), *F. oxysporum* f. sp. *cepae* (Armitage et al. 2018), *F. oxysporum* f. sp. *radicis-cucumerinum*

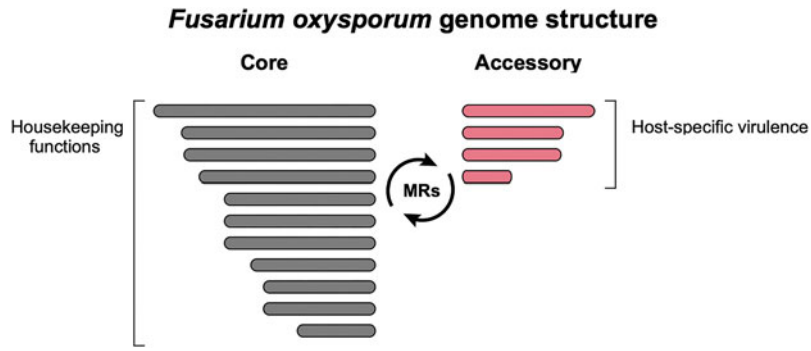

Fig. 16.2 *Fusarium oxysporum* genome structure. This diagram demonstrates the genome compartmentation of *F. oxysporum*. Core chromosomes contain genes that are highly conserved and fulfill all essential housekeeping functions. Accessory chromosomes contain genes that are lineage- or strain-specific and often perform functions related to adaptation to a unique host (e.g. host specific virulence). Active crosstalk goes on between core and accessory chromosomes through master regulators (MRs)

(Van Dam et al. 2017), legume-infecting strains (Williams et al. 2016), and an Arabidopsis-infecting strain (Thatcher et al. 2012) (Fig. 16.1). Experimentally, horizontal transfer of whole or partial ACs was confirmed in Fol007 and the *F. oxysporum* f. sp. *radicis-cucumerinum* isolate Forc016 (Ma et al. 2010; Van Dam et al. 2017; Li et al. 2020). A direct link between ACs and pathogenicity was documented in the laboratory by introducing pathogenicity into the nonpathogenic strain Fo47 after the migration of one of the ACs, Chromosome 14, from Fol007 (Ma et al. 2010; van der Does and Rep 2012). A later study demonstrated that only part of the small arm of Chromosome 14 was sufficient to induce virulence symptoms (Li et al. 2020). The observation that these ACs can be horizontally transferred explains the polyphyletic nature of *forma speciales* groups (O'Donnell et al. 1998; Katan 1999; Alves-Santos et al. 1999; Abo et al. 2005).

ACs are characterized by a low gene density and high repeat content, and are enriched in diverse transposable elements (TEs). In the Fol4287 genome, 74% of all transposons occur in ACs, and Fol4287 ACs contain 95% of the DNA transposons (Ma et al. 2010). High repeat content may contribute to the plasticity of ACs and explain the high frequency of large chromosomal duplications and partial or complete chromosomal losses among ACs (Ma et al. 2010; Vlaardingerbroek et al. 2016; Li et al. 2020). For example, the genome of Fol4287 has a large segmental duplication between Chromosomes 3 and 6 (Ma et al. 2010).

In addition, CCs and ACs show distinct epigenetic patterns (Fokkens et al. 2018). In the Fol4287 genome, CCs are enriched in H3 lysine 4 dimethylation (H3K4me2), a histone marker for euchromatin, in non-centromeric and non-subtelomeric sequences. By contrast, ACs are enriched in the heterochromatic signal H3 lysine 27 trimethylation (K3K27me3). Furthermore, the differences in histone modifications correspond to differences in transcriptional regulation. H3K27me3-associated regions show lower transcript levels, whereas H3K4me2-enriched regions are characterized by high gene density and exhibit higher transcription levels than the rest of the genome (Fokkens et al. 2018), as described in other filamentous fungi (Connolly et al. 2013; Soyer et al. 2014; Galazka and Freitag 2014).

The presence of ACs and CCs compartmentalizes the *F. oxysporum* genome. While CCs share homologous chromosomes in sister species, ACs may have size and sequence polymorphisms and disrupt the chromosomal collinearity even among isolates of the same *forma speciales* (Bertazzoni et al. 2018; Yang et al.

2020), introducing different evolutionary speeds within the same species genome. Fokkens et al. (2021) revealed an even more complex "multi-speed" genome structure by identifying AC patterns on the three smallest CCs, termed "fast-core chromosomes". These chromosomes have similar gene density and repeat content as the other CCs but are on a faster evolutionary track (Fokkens et al. 2018). These findings give rise to many intriguing questions about the evolvability and function of the epigenetic machinery in *F. oxysporum*.

16.3 Determinants of Host-Specific Pathogenicity

To explore how members of the FOSC interact with a broad range of hosts, researchers have examined the virulence-related genes that contribute to pathogenicity and host specificity, the interplay between CCs and ACs, and the regulation across these two compartments within the same genome (Ma et al. 2013; Yang et al. 2020; Zuriegat et al. 2021). Below, we discuss the genomic and genetic characteristics of *F. oxysporum*, focusing on the kinases that mediate signal transduction involved in host recognition, transcription factors (TFs) that are activated by the signal transduction pathways and then regulate other genes, and effectors produced in response to host signals (Fig. 16.3).

16.3.1 An Expanded Kinase Family Enhances Environmental Sensing

Kinases are essential components of host–fungal interactions, playing key roles in host sensing and signal transduction and mediating an effective response to the environmental stimuli. A comprehensive survey of the *F. oxysporum* kinome (i.e., a complete set of kinases encoded in the genome) revealed an expansion of kinase proteins among members of the FOSC, facilitated in part by the presence of ACs (DeIulio et al. 2018). This study also highlighted 99 "core" kinase families highly conserved among ascomycetes fungi.

The "core" kinase families include the mitogen-activated protein kinase (MAPK) signaling pathway, one of the most evolutionarily conserved eukaryotic cellular mechanisms of extracellular information perception and transduction. In fungi, the MAPK pathway is involved in mating, sporulation, cell cycle, morphogenesis, cell wall integrity, autophagy, cell–cell interactions, fungus–host interactions (neutral or beneficial), the response to stress stimuli (such as the damage-associated molecular pattern (DAMP) response), the DNA damage response (Milo-Cochavi et al. 2019), and pathogenesis (reviewed by Martínez-Soto and Ruiz-Herrera 2017). Three *F. oxysporum* MAPKs, Mpk1, Hog1, and Fmk1, have been functionally characterized. Mpk1 and Hog1 are involved in the response to stress, colonization, and virulence, while Fmk1 is required for host adhesion and penetration (Segorbe et al. 2017). Mpk1 is the key element in the chemotropic response pathway of *F. oxysporum* (Turrà et al. 2015), as it senses nutrients of root exudates and responds rapidly by promoting germination and guiding the germ tubes and hyphae toward the stimulus (Turrà and Di Pietro 2015; Turrà et al. 2015).

The expanded *F. oxysporum* kinome includes atypical kinases and histidine kinases. One expanded kinase family is the target of rapamycin (TOR) kinase belonging to the atypical kinase family and is a key regulator involved in nutrient sensing, the cell cycle, survival, growth, and fungal development. The expansion of the histidine kinase family is equally interesting, as they function in signaling pathways that sense the condition of the external environment (Loewith and Hall 2011; DeIulio et al. 2018). The observed kinome expansions among members of the FOSC appear to have equipped the pathogens to survive in diverse hosts. The comparative kinome analysis among FOSC suggests a convergent evolution that shapes individual *F. oxysporum* isolates with an enhanced and unique capacity to perceive the environment and activate the associated downstream responses (DeIulio et al. 2018).

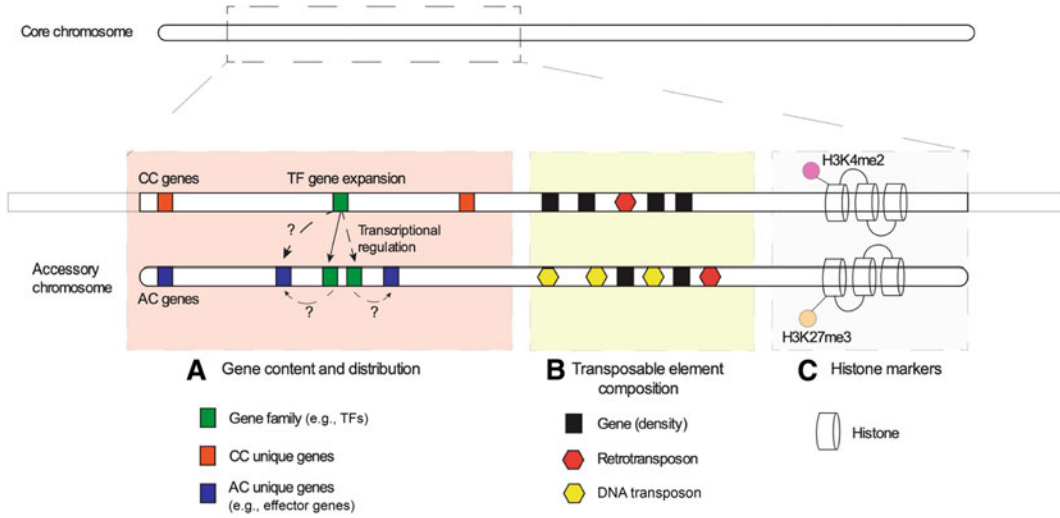

Fig. 16.3 Characteristics that differentiate accessory chromosomes from core chromosomes in *Fusarium oxysporum* (reproduced from Yang et al. 2020). This diagram compares a small fraction of a single core chromosome with a complete accessory chromosome, demonstrating that core chromosomes are more gene enriched than accessory chromosomes. (**a**) Functional division of coding sequences. While core genes, which are highly conserved and fulfill all essential housekeeping functions, are vertically transmitted in diverse taxa, accessory genes are lineage- or strain-specific and often perform functions related to adaptation to a unique host. Gene family expansions were observed when similar functions were predicted for some accessory and core genes. For gene families (green boxes) that are represented in both core and accessory chromosomes, it is postulated that the core genes tend to be the predominant functional genes in terms of regulating downstream targets such as effector genes, but this is not always the case. (**b**) Disproportionate distribution of transposable elements (TEs). Accessory chromosomes are enriched in TEs. In the *F. oxysporum* genome, core chromosomes have fewer repetitive sequences relative to genome size, and they are often ancient, mostly decayed class I RNA TEs, whereas ACs are densely populated with active class II DNA TEs. Effector genes are usually associated with TEs such as miniature inverted-repeat transposable elements. (**c**) Structural divergence of epigenetic signals. Core and accessory chromosomes have different histone marks. Fungal genes associated with the same type of histone marks may be coordinately expressed and could be under similar evolutionary pressure

16.3.2 Crosstalk Coordinates the Functions of Core and Accessory Chromosomes

Signaling pathways are part of a complex and holistic network, involving the interconnected activation or deactivation of master regulators (*e.g.*, TFs) and the subsequent induction or repression of genes (*e.g.*, effectors) in response to the environment, such as plant stimuli (Husaini et al. 2018). Even in a compartmentalized genome, *F. oxysporum* ACs and CCs do not function as two autonomous entities. Rather, crosstalk between these two compartments is likely to be coordinated by mediators such as TFs (Michielse et al. 2009; Yang et al. 2020), with a key set of TFs functioning during host colonization in *F. oxysporum* (Guo et al. 2014, 2021).

One example documenting the importance of host-specific pathogenicity accomplished through such crosstalk is the expression of *SIX* (*Secreted In Xylem*) effector genes located in the ACs of *F. oxysporum* f. sp. *lycopersici.* The expression of *SIX* effector genes is directly regulated by the TF Sge1 (SIX gene expression 1), which is located on a CC (Michielse et al. 2009). The expression of *Sge1* is, in turn, regulated by the AC-encoding TF Ftf1 (Fusarium TF1) (van der Does et al. 2016; Niño-Sánchez et al. 2016).

Another expanded TF family is Ebr (Enhanced Branching), which regulates general metabolism

and virulence (Jonkers et al. 2014; Zhang et al. 2020). Three paralogous copies of this gene, *Ebr2, Ebr3*, and *Ebr4*, are encoded in ACs and regulated by *Ebr1*, which is encoded in the core genome. Knocking out the core *Ebr1* gene reduced pathogenicity and resulted in growth defects (Jonkers et al. 2014), suggesting the importance of this ortholog. However, the AC encoding *Ebr2* rescues the *Ebr1* knockout mutation when controlled by the *Ebr1* promoter, indicating some functional redundancy among members of this family.

In addition to Ftf (Niño-Sánchez et al. 2016) and Ebr groups (Jonkers et al. 2014; Zhang et al. 2020), an expansion of the alkaline pH-responsive TF PacC/Rim1p was observed in the human-pathogenic strains NRRL 32931 (isolated from a leukemia patient) and NRRL 47514 (a strain associated with the *Fusarium* keratitis outbreak) (Zhang et al. 2020). These expansions are expected to play a role in the fungal response to high pH and other extreme environmental conditions of the human host (Zhang et al. 2020). Similar to the kinases, an expansion of TFs facilitated in part by the acquisition of ACs was also observed, which may suggest that regulatory networks involved in environmental adaptation are fine-tuned to support complex cross-kingdom interactions (Ma lab, unpublished).

Although roughly 5% of the genes in the *F. oxysporum* genome encode TFs (Ma et al. 2010), only 26 TFs have been functionally characterized in *F. oxysporum* (Zuriegat et al. 2021). Further research is much needed.

16.3.3 Effectors Disarm Host Defense

Plants have sophisticated defense mechanisms against pathogen invasion (Glazebrook 2005; Ponce de León and Montesano 2013; Nishad et al. 2020). For successful colonization, a phytopathogen must break down the plant cell wall and suppress plant immunity. Secreted fungal effectors are major players at the plant–microbe interface (De Wit et al. 2009; Lo Presti et al. 2015), contributing to processes ranging from host recognition to intracellular invasion of the plant tissue (Stergiopoulos and de Wit 2009; Giraldo and Valent 2013).

Members of the FOSC employ effectors throughout the course of infection, including during penetration of the plant root epidermis, propagation through the cortical cell layers, and eventually during colonization of the root vasculature. Many effectors are encoded by ACs. For instance, the SIX proteins, the first set of effectors described in *F. oxysporum* (Rep et al. 2004), are encoded by genes localized on Chromosome 14, an AC (Ma et al. 2010; Schmidt et al. 2013). Among them, SIX1, SIX3, SIX5, and SIX6 confer full virulence to Fol4287 (Rep et al. 2004; Ma et al. 2013; Gawehns et al. 2014), and SIX1 suppresses plant immunity (Tintor et al. 2020). Another important group of effectors in the FOSC are enzymes involved in the degradation of plant compounds (such as carbohydrate-active enzymes, *i.e.*, CAZymes) (Ma lab, unpublished). Widely dispersed in the fungal world, particularly in phytopathogens (Zhao et al. 2013), CAZymes play crucial roles in degrading plant compounds during appressorial penetration, nutrient uptake, and plant tissue colonization (Stergiopoulos and de Wit 2009; Dodds and Rathjen 2010; Giraldo and Valent 2013).

A pan-genomic analysis of all predicted FOSC effector genes revealed tremendous effector diversity (Ma Lab, unpublished; van Dam et al. 2016; Constantin et al. 2021) and suggest that the effector gene repertoire in each *formae speciales* is directly related to the fungal lifestyle. For example, genomes of *F. oxysporum* plant pathogens such as Fol4287 and Fo5176 maintain more genes encoding SIX effectors and CAZymes than endophytic and human-pathogenic strains (Ma lab, unpublished).

Candidate effectors can be predicted based on their small size (typically <300 amino acids in length), the presence of a secretion signal peptide, and enrichment for cysteine residues. Based on this simple definition, *F. oxysporum* effector genes can be found in both CCs and ACs. Effectors encoded by CCs are conserved in both pathogenic and endophytic *Fusarium* strains. A recent study demonstrated that CC-encoded

effectors determine endophytic growth and multi-host plant compatibility for both endophytic and pathogenic *F. oxysporum* (Redkar et al. 2022), further reinforcing the notion that AC effectors specifically determine host-specific pathogenicity. Additional experimental work is needed to decipher the functions of different effectors.

16.3.4 Convergent Points Highlight Adaptation to Both Abiotic and Biotic Stresses

Studies of *F. oxysporum*, a cross-kingdom pathogen, are usually focused on fungal pathogenesis. However, as a cosmopolitan fungus, members of the FOSC have been isolated from diverse ecological niches, including soil, air, plants, animals, and even the International Space Station (Urbaniak et al. 2019; Schuerger et al. 2021). Signaling pathways involved in the fungus's response to biotic and abiotic stresses are distinct, with adaptation to abiotic stresses influencing how microbes interact with their hosts. Possessing a remarkable capacity to adapt to changing environmental stresses, members of the FOSC could be a model for exploring both biotic and abiotic stress signaling pathways and the intersection between them. Here, we summarize some research on temperature and pH adaptation, both of which are tightly controlled physiological parameters that can be considered to be abiotic stresses.

Significant shifts in phenotypic traits and expression profiles were observed when a plant-pathogenic strain of *F. oxysporum* was subjected to high temperature (37 °C) (Segorbe et al. 2017). By contrast, this temperature did not affect the growth rate of the human pathogen *F. oxysporum* MRL8996 (NRRL 47514) (Zhang et al. 2020; Ayhan 2021). The expression profile shift of this human pathogen in response to high temperature is different from that of the plant-pathogenic strain (Ma lab, unpublished).

The genome of the human-pathogenic *F. oxysporum* strain NRRL 32931 contains four ACs with 812 genes that are significantly enriched in genes encoding metal ion and cation transporters (Zhang et al. 2020). These genes could be important for overcoming human nutritional immunity by sequestering trace minerals to limit microbial infection. The genome of this fungus contains additional copies of the TF encoding *PacC/Rim1*, which is involved in the response to pH changes and other stresses (Peñalva and Arst, Jr. 2004; Zhang et al. 2020). Both phenotypic and genotypic observations support the importance of genetic weapons gained by the FOSC, perhaps via ACs, in the fungus's response to different environmental stresses. We anticipate that this topic will be investigated further using diverse approaches. One such approach is experimental evolution, which enables adaptation trajectories to be observed in a controlled environment (Garland and Rose 2009). This approach has been successfully applied to study microbial adaptation under in vitro (Barrick and Lenski 2013) and in vivo (Lescat et al. 2017) conditions, and provides an opportunity to study the crosstalk between microbial responses to biotic and abiotic stresses. For instance, this approach could be used to analyze the adaptation of an isolate to elevated temperature and pH, which is thought to facilitate opportunistic infection through the bloodstream of humans.

16.4 Mechanisms of Niche Adaptation: Genome Evolution and Maintenance

Natural selection of genetic variations is the basis of evolution. By monitoring biological processes such as mutagenesis, selection, adaptation, and speciation, biologists predict patterns that give rise to diverse organisms and track changes in these patterns over time. Genome plasticity is a driving force in the "arms race" between a pathogen and its host. The capacity to colonize more than 100 different hosts highlights the genomic plasticity of FOSC members.

Meiotic recombination is a well-known process underlying genomic variation. With the increasing availability of sequenced genomes, it has become clear that asexual species, such as *F. oxysporum*, also show a high degree of genetic

variability (Takken and Rep 2010; Karasov et al. 2014; Perez-Nadales et al. 2014; Gladieux et al. 2018). In addition to the horizontally transmitted ACs, there is tremendous interest in studying the evolvability of members within the FOSC that operate under the model of predominant clonal evolution. Such studies mostly involve inspecting patterns of mutation contributed by single nucleotide variations (SNVs), insertions and deletions (INDELs), transposon insertion and excision variations, large segmental deletions, duplications, and translocations in the genome (Ayhan 2021).

Exposure to DNA-damaging agents triggers a range of stress-related responses across the tree of life (Fry et al. 2005). SNVs occur when damaged DNA is not completely repaired either before or after DNA replication. Several conserved DNA repair mechanisms are responsible for repairing the damage and maintaining genome stability. DNA damage repair in fungi has mostly been studied in *Saccharomyces cerevisiae* (Workman et al. 2006; Smolka et al. 2007; Shalem et al. 2008; Fu et al. 2008; Bandyopadhyay et al. 2010; Guénolé et al. 2013). Even though many pathways are shared among yeast and filamentous fungi, there are important differences. Several DNA-damaging agents, such as methyl methanesulfonate (MMS), UV radiation, and hydroxyurea, were used to study the processes involved in DNA damage tolerance and repair capacity in the FOSC (Milo-Cochavi et al. 2019).

MMS exposure induces some strong and shared responses in *S. cerevisiae* and *F. oxysporum*, including the activation of the Chk1-Chk2 signal transduction pathway, proteasome components, and the Xbp1-Yap1 networks (Milo-Cochavi et al. 2019). However, some clear differences were reported in a recent study (Milo-Cochavi et al. 2019), including unique upregulation of genes encoding the splicing module, the basic transcription machinery, and several RNA pol II-associated proteins in *F. oxysporum* but not in *S. cerevisiae*. This study suggested that *F. oxysporum* responded to the chronic DNA damage by recycling the transcription machinery in response to stalled RNA polymerases and consequently reactivated the splicing machinery (Milo-Cochavi et al. 2019). The other observed difference was the regulation of ribonucleotide reductase (RNR), a key enzyme that mediates the synthesis of deoxyribonucleotides (dNTPs) in response to DNA damage. In contrast to *S. cerevisiae* and other organisms, *F. oxysporum* did not upregulate the expression of RNR. Accordingly, dNTP pools in *F. oxysporum,* but not in yeast, were decreased in response to the DNA damage agent hydroxyurea (Cohen et al. 2019).

UV radiation is probably the most common toxic environmental mutagen. Genes involved in repairing UV-induced DNA damage are important for reducing premutagenic lesions in DNA and transcription errors. A recent study (Milo-Cochavi et al. 2019) reported that *F. oxysporum* responded to UV radiation through a developmentally regulated oscillation in the expression of the UV repair genes photolyase (*Phr1*) and UV endonuclease (*Uvde*). Both gene products can specifically bind to damaged DNA caused by UV radiation. At the early stages of germination, *Phr1* expression was induced, while *Uvde* expression was reduced. The trend was reversed at 14 hours post-inoculation when spores were fully germinated and filaments were established (Milo-Cochavi et al. 2019). This observation led to the hypothesis that *F. oxysporum* operates a photolyase-based, transient, and precise UV repair machinery, and that the fungus only actives this machinery upon UV exposure to minimize the cost of transcription when it is not necessary (Milo-Cochavi et al. 2019). We should note that most of the current knowledge about DNA damage repair in *Fusarium* is based on patterns of gene expression. While induction of a certain gene by DNA damage may indicate a functional role in damage tolerance and survival, other processes, mostly post-translational modifications such as phosphorylation, neddylation, ubiquitination, SUMOylation, and PARylation, may also play important roles and should be comprehensively studied.

Chromatin state also has a crucial role in DNA damage and repair (Dabin et al. 2016; Stadler and Richly 2017; Allshire and Madhani 2018). The heterochromatic as well as transposon- and

repeat-rich environment of *F. oxysporum* ACs may affect their mutation rate and repair efficiency and have either beneficial or adverse effects on the genomic plasticity required for genetic changes associated with plant–fungal interactions.

In addition to SNVs, large- and small-scale genome rearrangements and TEs can also promote rapid adaptation to new environments. Transposons enriched among ACs contribute significantly to fungal pathogenicity. For instance, the insertion of a Hornet-like DNA transposon into the *SIX3* effector gene enables the pathogenic *F. oxysporum* f. sp. *lycopersici* to evade plant immunity and evolve into a new disease-causing race (Inami et al. 2012). Members of another DNA transposon family, the miniature Impala elements or MIMPs, are associated with promoter regions. Miniature Fot5 associates with the downstream *SIX* genes in an AC, Chromosome 14 of Fol4287 (Schmidt et al. 2013). Similarly, Helitrons are found upstream of *SIX9* homologs in the Arabidopsis-pathogenic strain Fo5176 (Chellapan et al. 2016).

How these mechanisms impact on mutational patterns in an organism, particularly asexual fungi, needs to be investigated further. These data, which have been mostly obtained from observations of only a few *F. oxysporum* strains, should be expanded to other fungal relatives to reveal what aspects are genus-, species-, or even strain-specific.

16.5 Conclusion and Perspective

The presence of fungal ACs and their contribution to fungal phytopathogenicity are widely accepted (Ma et al. 2010; Croll and McDonald 2012; Bertazzoni et al. 2018). A recent study provided clear evidence of the link between fungal ACs and adaptation to human host conditions (Zhang et al. 2020), extending the contribution of fungal ACs to adaptation beyond plant hosts. There is still much to learn about the genome dynamics of ACs. Here, we discuss a few unresolved questions.

16.5.1 Improving the Quality of AC Assemblies

Due to the high level of repetitive sequences, ACs are rarely assembled to the chromosomal level when sequenced. A strategy incorporating long-read sequencing, such as PacBio and Nanopore reads, with high-throughput chromosome conformation capture (Hi-C), has been successful in producing chromosomal scale assemblies of *F. oxysporum* strains Fo47 and Fo5176 (Wang et al. 2020; Fokkens et al. 2021). An alternative strategy is to sequence one chromosome at a time (Peng et al. 2019). Single ACs can be separated using contour-clamped homogeneous electric field electrophoresis and then extracted from the gel (O'Brien et al. 2006) or flow-sorted from the genomic DNA pool (Jain et al. 2016).

16.5.2 Exploring the Origin of ACs

The evolutionary origin of ACs is an intriguing puzzle for the fungal community. Horizontal transfer is one widely accepted hypothesis for the acquisition of fungal ACs, and conidial anastomosis during germination is postulated to mediate conidial fusion and facilitate intra- and interspecies genetic exchange (Gabriela Roca et al. 2005; Mehrabi et al. 2011). Mobile genetic elements, such as plasmids, can be horizontally transferred. The mobility of ACs suggests that they have a plasmid-like nature. The known sizes of fungal ACs are mostly below 2 Mb, and plasmids with insert sizes larger than 1 Mb have also been reported (Finan et al. 1986; Harrison et al. 2010), which suggests that fungal ACs may be novel mobile genetic elements. However, ACs have a linear structure, with a centromere and telomeric sequences (Fokkens et al. 2021), rather than a circular structure, as is typical of plasmids. To test this hypothesis, a detailed survey might be necessary.

ACs can also be generated from CC genome duplication and partial loss (possibly different processes are involved back and forth). Active links are observed between ACs and the

telomeres of CCs, as reflected by their shared enrichment of effector genes and other genes involved in host invasion (Ma and Xu 2019; Peng et al. 2019), supporting the alternative hypothesis that ACs can originate from the non-disjunction of duplicated chromosomes (*e.g.*, CCs) followed by mutations and/or degradation (Croll et al. 2013). Of course, we cannot exclude the possibility that different mechanisms may contribute to different ACs.

Many questions remain to be explored, such as: Why is the FOSC so versatile in acquiring extra genetic material? Are there critical factors that determine the fate of an AC once it emerges in a new genomic landscape? Do evolutionary events (*e.g.*, changes in the ability to maintain chromosome stability) underly the origin/acquisition of ACs? The distinct genomic nature, as well as differences in the rate of evolution between CCs and ACs, suggest that different mechanisms maintain these types of chromosome. Does the heterochromatic structure of ACs affect the recruitment and accessibility of repair factors to the damage site? Do the same repair factors function across the genome, or do they differ by type (*e.g.*, CCs vs. ACs)? Do CCs and ACs show different DNA repair efficiency, and if so, how does this affect the mutation rate and evolution of the ACs?

Mutagenesis experiments coupled with experimental evolution, genetics, and genomic studies of DNA repair of different damage types will shed light on this intriguing aspect. In addition, constructing a *F. oxysporum* pan-genome that incorporates a substantial number of high-quality genome assemblies, or a comprehensive comparative analysis of ACs belonging to the same *formae speciales*, will be necessary to track the evolutionary origin and trajectory of ACs.

16.5.3 Effective Regulation of ACs

There seems to be a proximal association between the expansion of TFs and their target genes. For instance, *Ftf1* genes are close to clusters of *SIX* genes on the Fol4287 pathogenicity-conferring chromosome (Schmidt et al. 2013). de Vega-Bartol et al. (2011) postulated that the different number of TFs and effector genes may be related to the fine-tuning of the host-specific infection process, and this may be achieved through copy number and sequence variations of Ftf1 binding sites on *SIX* gene promoters (Zhao et al. 2020). This possibility remains to be tested, preferably by chromatin immunoprecipitation sequencing (Park 2009) or DNA affinity purification sequencing (Baumgart et al. 2021), which would not only reveal the actual binding sites but also identify the genome-wide targets.

Crosstalk between core and accessory components of the genome is critical for the coordinated response to environmental changes and the switch between vegetative growth and pathogenicity. What is the relationship between the regulatory roles of CC and AC TFs? Do AC TFs regulate core genes, and do they compete for the same binding sites? What determines the expansion and contraction of gene regulators during evolution? Aside from TFs, protein kinases are also largely expanded in ACs of *F. oxysporum*. How do CC kinases regulate the biological activity of AC-encoding proteins, or *vice versa*? Undoubtedly, the relationship and crosstalk between TFs and protein kinases in ACs and CCs will impact on the global regulatory networks of the fungus.

16.5.4 Novel Therapeutic and Management Strategies

The severity of the problem caused by this cross-kingdom pathogen underscores the urgency in developing novel therapies and disease management strategies. It is of great interest to explore novel targets, as most marketed antifungal agents have the same mode of action, perturbing cell membrane integrity.

Loss of epigenetic marks can potentially change the transcriptional program and consequently affect many aspects of the life and disease cycle of pathogenic fungi, including resistance to stresses and pathogenicity. A link between histone acetylation, antifungal resistance, pathogenicity, and stress tolerance was reported in several

important fungal pathogens, such as *Candida albicans* and *Cryptococcus neoformans* (Robbins et al. 2012; Lamoth et al. 2015; Li et al. 2015; Garnaud et al. 2016; Brandão et al. 2018). Several histone deacetylase (HDAC) inhibitors also showed antifungal activity, particularly when they were combined with existing fungicides (Mai et al. 2007; Pfaller et al. 2009; Lamoth et al. 2015; Brandão et al. 2018). A recent study showed a potential connection between epigenetics and metabolism in *F. oxysporum*, focusing on Sirtuin, a type of HDAC and one of the primary consumers of cellular NAD^+. Disrupting the NAD^+ biosynthesis salvage pathway inhibited growth and reduced the biomass of *F. oxysporum*, revealing a potential approach to develop novel antifungals (Anand et al. 2019).

The absence of a specific DNA repair enzyme from a specific phylogenetic group of fungi or individual species may indicate that members of this group are more vulnerable to DNA damage agents. A comparative genomic approach, such as the one performed by Milo et al. (2019), can be a starting point for designing functional experiments to identify synthetic lethal interactions that can be further exploited for developing sophisticated new fungicides. The unorthodox behavior of RNR upon DNA damage in one strain of *F. oxysporum* may suggest that this fungus has increased sensitivity to RNR inhibition; therefore, RNR could be a promising candidate for the development of species-specific pesticides (Cohen et al. 2019).

Comparative systems, *e.g.*, of endophytic and pathogenic *Fusarium*, have illustrated the power of distinguishing the key events associated with fungal pathogenicity and plant host defense (Guo et al. 2021). Additional comprehensive omics approaches, such as proteomics and metabolomics, will provide valuable insight into host–pathogen interactions. Different post-translational modifications, such as phosphorylation, glycosylation, and ubiquitination, that are related to alterations in both the pathogen and host proteome can also be elucidated. In plants, pathogenic *F. oxysporum* travels through many cell layers, starting at the epidermis and moving through the cortex, endodermis, and root vasculature to establish the infection. Investigating gene transcription regulation at single-cell resolution in response to the fungal pathogen is of high research interest and is now feasible through single-cell RNA sequencing (Plant Cell Atlas Consortium et al. 2021; Cole et al. 2021). Collectively, a better understanding of fungal pathogenicity and host defense mechanisms will be the key to controlling these infections. Technological advancements mentioned above will make this endeavor feasible.

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Part VI

Global Pandemics and Food Security

Global Landscape of Rust Epidemics by *Puccinia* Species: Current and Future Perspectives

17

Melania Figueroa, Peter N. Dodds, Eva C. Henningsen, and Jana Sperschneider

Abstract

Rust fungi include many significant crop pathogens that belong to the *Puccinia* genus (order Pucciniales, phylum Basidiomycota). Historically, *Puccinia* species have posed a threat to members of the Poaceae plant family; however, recent outbreaks have raised awareness of their destructive nature. This chapter focuses on *Puccinia* species that have caused epidemics affecting crops that account for half of the global food production. Advances in high-throughput sequencing technology and computational approaches have increased our understanding of virulence evolution in rust fungi and the molecular factors behind disease epidemics. Pioneering research has revealed the first few avirulence (*Avr*) genes from *Puccinia* species, and novel mechanisms behind the diversification of pathogen populations, which enable the coevolutionary arms race between *Puccinia* members and their hosts. The majority of *Avr* genes in *Puccinia* species remain to be identified and additional research is required to develop a broad picture of the mechanisms these pathogens use for host manipulation. These topics are likely to remain a strong focus for future research; however, the development of high-quality genomic resources is imperative.

Keywords

Puccinia · Epidemics · Virulence · Effectors · Genome · Chromosomes · Avirulence

17.1 An Introduction to Rust Fungi and the *Puccinia* Species

Rust fungi are a diverse group of fungi that parasitise plant hosts and are classified in the order Pucciniales (formerly Uredinales) within the Basidiomycota phylum (Kolmer et al. 2018; Aime et al. 2017). There are Pucciniales species that infect virtually every family of plants, and many are important pathogens of major agricultural and horticultural crops. Many of the most significant pathogens fall within the *Puccinia* genus, which includes over 4000 species that infect diverse hosts, including deciduous trees, grasses, and herbaceous plants (Ainsworth 2008). The high degree of host specificity often observed among rust fungi led to the distinction of formae speciales (abbreviated f. sp.—plural ff. spp.), which is a subdivision of the species nomenclature defining groups of isolates that have different host ranges (Eriksson 1894). For instance, *Puccinia striiformis* f. sp. *tritici* and

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Table 17.1 Plant pathogens discussed in this chapter, with distribution and host information

Crop/host	*Puccinia* species (disease name)	Pathogen distribution
Wheat (*Triticum aestivum*)	*P. graminis* f. sp. *tritici* (wheat stem rust)	North and South America, Australia, Asia, Africa, Europe
	P. striiformis f. sp. *tritici* (wheat stripe rust)	North and South America, Australia, Asia, Africa, Europe
	P. triticina (wheat leaf rust)	North and South America, Australia, Asia, Africa, Europe
Barley (*Hordeum vulgare*)	*P. graminis* f. sp. *tritici* (wheat stem rust)	North and South America, Australia, Asia, Africa, Europe
	P. striiformis f. sp. *hordei* (barley stripe rust)	North and South America, Africa, Asia, Europe
	P. hordei (barley leaf rust)	North and South America, Australia, Asia, Africa, Europe
Oat (*Avena sativa*)	*P. coronata* f. sp. *avenae* (oat crown rust)	North and South America, Australia, Asia, Africa, Europe
	P. graminis f. sp. *avenae* (oat stem rust)	North and South America, Australia, Asia, Africa, Europe
Maize (*Zea mays*)	*P. sorghi* (common corn rust)	North and South America, Australia, Asia, Africa, Europe
	P. polysora (Southern corn rust)	North and South America, Australia, Asia, Africa
Sugarcane (*Saccharum officinarum*)	*P. kuehnii* (orange rust)	North and South America, Australia, Asia, Africa
	P. melanocephela (common rust)	North and South America, Australia, Asia, Africa
Sorghum (*Sorghum bicolor*)	*P. purpurea* (sorghum rust)	North and South America, Australia, Asia, Africa, Europe

P. striiformis f. sp. *hordei* define separate groups of this species that preferentially infect wheat or barley, respectively. This taxonomical system is commonly used across *Puccinia* species and is coupled with race designations based on virulence profiles that were first established in the early 1900s and have been updated over time (Hoerner 1919; Stakman and Levine 1922; Stakman et al. 1929). Of all the *Puccinia* species and formae speciales, a handful are of special importance for their potential to cause significant yield losses in agronomically important crops such as wheat, oat, barley, corn, sorghum, sugarcane, etc. Some are even considered ecological threats.

This chapter focuses on some key *Puccinia* species that have caused epidemics at regional, national, or even global scales and are recognised as threats to agriculture. To bring focus to this chapter, we discuss the *Puccinia* species (Table 17.1) that affect primary crops that have recently accounted for half of global food production (FAO Statistical Yearbook 2021). These include sugarcane (18% of the total, with 1.9 billion tonnes), maize (13%, 1.4 billion tonnes), wheat (8%, 0.9 billion tonnes), barley (1.4%, 0.2 billion tonnes), oat (0.2%, 0.03 billion tonnes), and sorghum (0.6%, 0.06 billion tonnes). We aim to highlight the advantages that new high-throughput sequencing technology and the genomics era have brought to the scientific community and the opportunities for research on rust fungi. These advances can help address long-standing questions such as the mechanisms of virulence evolution, which is the main hindrance to the effective management of these devastating diseases.

17.2 Important Crops and Ecosystems Affected by *Puccinia* Species

Although there are thousands of *Puccinia* species, some are the focus of much more intensive research than others due to their high potential for economic and ecological destruction. Below we will discuss a few important hosts for *Puccinia* species including wheat, barley, oat, corn, sorghum, and sugarcane (Fig. 17.1).

17.2.1 Wheat

Wheat (*Triticum aestivum*) is one of the most important staple grains around the world; hence, wheat production is under increasing pressure to meet the demand of the planet's population growth rate (Figueroa et al. 2018). The *Puccinia* species that infect wheat have attracted a lot of attention since the Roman empire. Pests and diseases of wheat, including rust fungi, result in yield losses of approximately 10–16% globally (Oerke 2006; Strange and Scott 2005; Dean et al. 2012); thus, the destructive potential of pathogens to the world's economy cannot be ignored. There are three main wheat rust diseases: stem, stripe and leaf rust, all caused by members of the genus *Puccinia*, named *P. graminis* f. sp. *tritici* (*Pgt*), *P. striiformis* f. sp. *tritici* (*Pst*), and *P. triticina* (*Pt*) (McIntosh et al. 1995). Global annual losses to wheat rust fungi can be profound with calculations of their value reaching between US $4.3 and 5.0 billion (Figueroa et al. 2018). The effect of *Puccinia* species on wheat production has been recorded for thousands of years, since the domestication of wheat (Roelfs et al. 1992). For instance, spores with morphological features of *Pgt* have been identified in archaeological sites located in Israel, providing evidence of the presence of this pathogen in 1300 BC (Kislev 1982). Ancient Greek and Roman literature alludes to the devastating consequences of rust diseases in agriculture (Chester 1946; Zadoks 1985). Fearing the grain yield losses inflicted by rust diseases, Romans held the Robigalia festival annually to appeal to Robigus, the god of rust, that he might spare their crops. Epidemics by these wheat rust fungi are described in detail in a subsequent section.

17.2.2 Barley

Barley (*Hordeum vulgare*) is one of the oldest crops, domesticated about 10,000 years ago (Badr et al. 2000). Barley has been adapted for

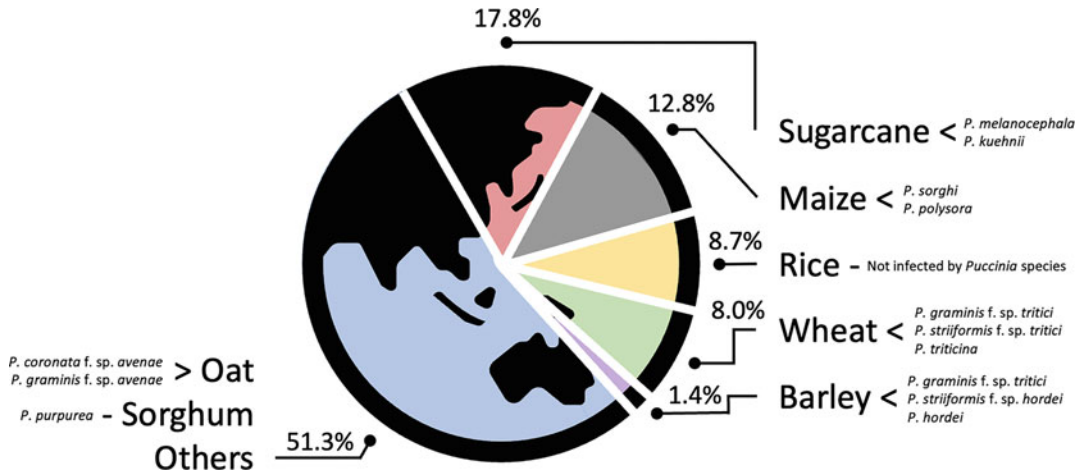

Fig. 17.1 Major crops by tonnes produced globally, as estimated from FAO 2020 data (https://www.fao.org/faostat/en/#data/QCL accessed on 03/11/2022) and *Puccinia* species that effect these crops as discussed in this chapter

almost every climate zone and elevation, so it is often planted in regions where corn and rice cannot be cultivated (Baik and Ullrich 2008; Hadado et al. 2010; Pasam et al. 2014). Currently, barley ranks fifth (tonnes harvested) of cereal crops in the world (FAO accessed on 03/11/2022). Most of the barley production is used for stock feed or malting in the brewing industry, and only a small portion is used for human consumption (Briggs 1978). Barley production is affected by several rust diseases, including *Puccinia striiformis* f. sp. *hordei* (*Psh*), *P. hordei*, and *Pgt*, which can infect barley in addition to its primary host, wheat. Barley stripe rust (*Psh*) historically only affected crops in Asia and Europe, until its introduction to other barley-producing regions such as South America (Colombia, 1975) and North America (Texas, 1991) (Stubbs 1985; Dubin and Stubbs 1986; Roelfs et al. 1992). Barley leaf rust (caused by *P. hordei*) was considered relatively unimportant until around the 1970s, when cropping practices shifted to the advantage of the pathogen (Clifford 1985). Currently, it is most damaging in temperate regions, like parts of North America, Europe, Australia, and New Zealand. Stem rust of barley (caused by *Pgt*) is the least damaging of the three *Puccinia* species; reactions in barley tend to be less severe than on wheat (Steffenson et al. 1985). In addition, barley is often planted earlier and matures earlier than the wheat crop, and thus escapes severe infection.

17.2.3 Oat

Another important cereal is oat (*Avena sativa*), which is a valuable crop both for human consumption and animal feed (Murphy and Hoffman 1992). Oat is produced globally, with the top producers for 2020 being Europe (Russia, Poland, Spain) and the Americas (Canada, US, Brazil) (FAO Crops and Livestock Products, 2022). Oat production is hindered by crown rust and stem rust diseases, caused by *P. coronata* f. sp. *avenae* (*Pca*) and *P. graminis* f. sp. *avenae* (*Pga*), respectively. Both diseases can lead to significant yield losses and reduced grain quality. Oat crown rust causes yield losses in oat from 5 to 40% (Nazareno et al. 2018), although entire oat fields can be lost if infection occurs early in the season (Simons 1985). *Pca* is present in nearly all oat-growing areas. Oat stem rust is likewise a destructive disease of oat, with yield losses from 5 to 100% in the right conditions (Martens 1978). Stem rust mainly affects oat production in Australia, Northern Europe, South Africa, and North America (Harder 1994; Adhikari et al. 2000; Berlin et al. 2012; Boshoff et al. 2019).

17.2.4 Sugarcane

Sugarcane rust is an important new disease that affects *Saccharum officinarum* and *S. officinarum* hybrids. Sugarcane is grown almost exclusively for sugar and biofuel production (Thirugnanasambandam et al. 2018). The top ten sugarcane producers for 2020 in descending order are Brazil, India, China, Pakistan, Thailand, Mexico, the USA, Australia, Indonesia, and Guatemala (FAO stats accessed on 03/17/2022). Several viruses, fungi, and bacteria species hinder sugarcane production (Ricaud et al. 2012) including two *Puccinia* species, *P. kuehnii* and *P. melanocephala* (Purdy 1985). These have had variable importance over time, with both species initially being restricted in their geographic distribution and subsequent expansion. Currently, both rust fungi are now distributed in most areas where sugarcane is produced and epidemics involving both species will be discussed below.

17.2.5 Maize

Another cereal crop of the grass family Poaceae that is affected by rust is maize/corn (*Zea mays*). Given its multiple uses in industry as raw material, livestock feed, human food, and biofuel, this crop plays an important economic role in many countries, with global production reaching 1.2 billion tonnes in 2020 (FAO accessed on 03/21/2022). According to the current records, the top five corn-producing countries in the world are the US, China, Brazil, Argentina, and Ukraine.

Puccinia sorghi and *Puccinia polysora* cause the two main rust diseases in maize (Hooker 1985). *P. sorghi* causes common corn rust and it is typically found in regions of high altitude, but it can also sometimes occur in tropical zones. The development of common corn rust disease is favoured by extended periods of cool temperatures (15–25 °C) (Hooker 1985). As a result of *P. sorghi* infection, corn ears are often damaged, and the plant weight and height are reduced, as well as the oil and protein content (Hooker 1985). In temperate geographic areas such as Argentina and the US Corn Belt, yield losses up to 25% have been attributed to *P. sorghi* infections (Hooker 1985). If rust infection occurs at early plant growth stages such as in Hawaiian corn fields, grain yield losses can average 35% (Kim and Brewbaker 1976). According to various studies, a 10% increase in common rust severity results in a 2–7% yield penalty in sweet corn varieties (Pataky 1987; Shah and Dillard 2006).

Southern corn rust caused by *P. polysora* is more common in areas with higher temperatures than those characteristics of *P. sorghi*. The distribution of *P. polysora* is tropical to subtropical; however, under favourable conditions, it can produce infection in corn fields of temperate regions (Ramirez-Cabral et al. 2017). *P. polysora* also infects *Erianthus alopecuroides*, teosinte, and wild species related to corn such as *Tripsacum dactyloides*, *T. lanceolatum*, *T. laxum,* and *T. pilosum* (Hooker 1985). If infection by *P. polysora* occurs in late-season planting, the flowering time can be affected and an entire corn field can be lost (Futrell 1975; Rodriguez-Ardon et al. 1980). *P. polysora* was not identified before 1941 in the Western Hemisphere, but herbarium samples from 1879 suggest the pathogen was present before then (Cummins 1941).

17.2.6 Sorghum

Sorghum (*Sorghum bicolor*) is another member of the Poaceae family and represents the sixth most important cereal crop after sugarcane, maize, rice, wheat, and barley (Saballos 2008; FAO Stat accessed on 03/17/2022). Sorghum is a widely adapted crop grown in arid and semi-arid tropical areas and is commonly used as a food and feed crop. Presently, sorghum is ranked as the second source of grain-based ethanol in the US market (after maize); thus, the potential applications in biofuel production of sorghum suggest growing needs for this crop. Under warm and humid conditions, sorghum can be infected by *Puccinia purpurea* (Smith and Frederiksen 2000; Miller and Cruzado 1969; Tarr 1962; House 1985).

17.3 The Life Cycle of *Puccinia* Species and Its Role in Disease Epidemics

The rust fungal life cycle is an important factor in the ability of these fungi to cause epidemics, spread, and adapt to the current crop environment. *Rust fungi are obligately biotrophic pathogens*, and as such require living plant tissue to acquire nutrients and are unable to complete their life cycle outside of their hosts (Cummins and Hiratsuka 2004). The establishment of the biotrophic interaction between rust fungi and plant hosts involves the development of specialised structures, completion of complex infection cycles and the ability to suppress host defense responses (Lorrain et al. 2018). *Puccinia* species have a high degree of variation in their life cycles, which are classified as macrocyclic, demicyclic, or microcyclic according to the stages represented (Fig. 17.2) (Petersen 1974; Lorrain et al. 2019). The full life cycle, with both asexual and sexual reproduction is present in macrocyclic rusts, which possess all five recognised spore stages (Petersen 1974; Lorrain et al. 2019). These rust fungi can be either autoecious, having a single host for both sexual and asexual life stages, or heteroecious, having two different and unrelated hosts for the sexual and asexual phases (Lorrain et al. 2019). Microcyclic rusts are by necessity autoecious, as they can only complete the telial cycle (teliospores and basidiospores) and cannot produce urediniospores or aeciospores. Similarly, demicyclic rusts lack the urediniospores. The difference in the life cycles of

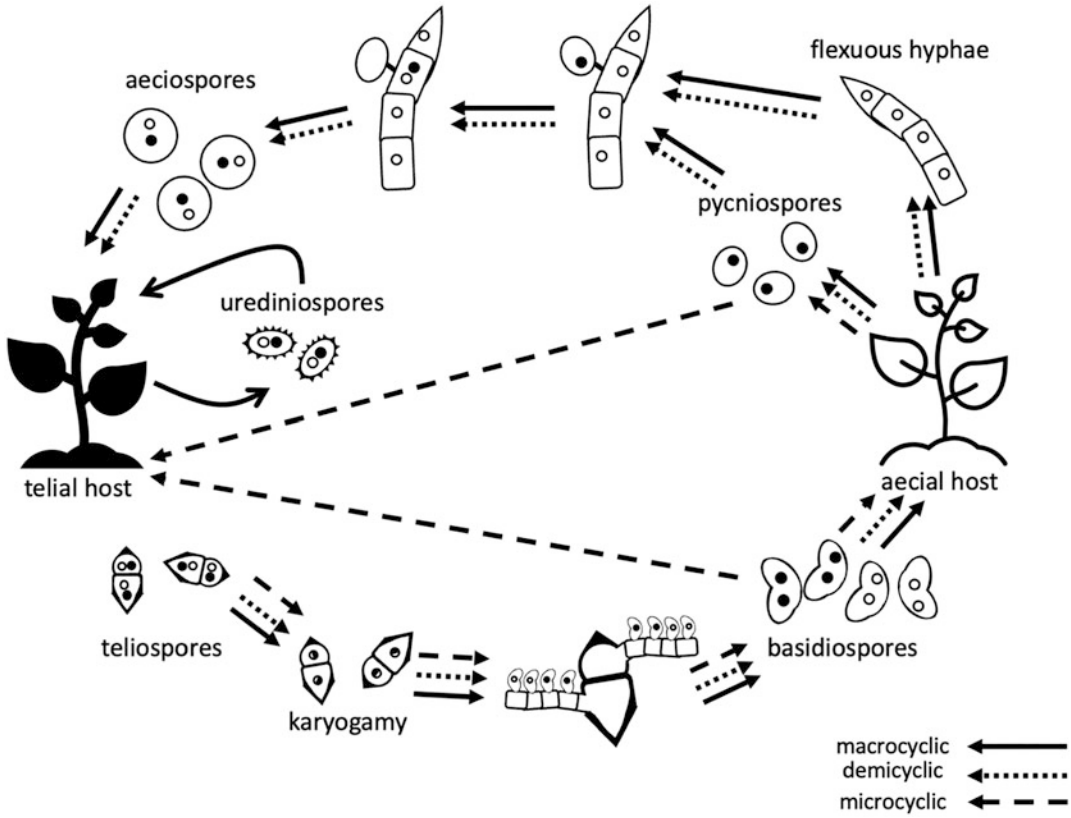

Fig. 17.2 Life cycle of rust fungi can be autoecious (a single host for both sexual and asexual life stages) or heteroecious (two distinct for the sexual and asexual phases). The life cycle of *Puccinia* species can also be classified as macrocyclic, demicyclic, or microcyclic. Macrocyclic rusts display asexual and sexual reproduction and five recognised spore stages. Microcylic rusts only have the telial stage and can produce only two spore types: basidiospores and teliospores, or pycniospores and teliospores. Demicyclic rusts lack the uredinial stage and can be autoecious or heteroecious. Most rust fungi display asexual and sexual reproduction cycles and therefore produce five different spores as illustrated in the diagram: urediniospores ($n + n$), teliospores ($2n$), basidiospores (n), pycniospores (n), aeciospores ($n + n$). Briefly, asexual urediniospores are produced continuously from uredia during the growing season of the telial host. Towards the end of the growing season, the uredium transitions to a telium to produce teliospores. These overwinter/oversummer, undergo karyogamy and then meiosis to produce basidiospores. These infect the aecial host, where sexual reproduction occurs. The infections result in pycnia, where flexuous hyphae or pycniospores are produced. When a pycniospore fuses with a flexuous hypha of the correct mating type, an aecium is produced and from this aeciospores bud and infect the telial host, completing the cycle

rust fungi affects how genetic diversity is generated within each species and which control methods may be effective to reduce the damage caused by the disease (Figueroa et al. 2020). Alternative methods of generating genetic diversity in some microcyclic rusts have been observed (Ono 2002).

The host on which rust fungi complete the asexual cycle is known as the telial host (Newcombe 2004). Urediniospores are produced on and can re-infect telial host plants when weather conditions are favourable. During this uredinial stage, *the fungus is dikaryotic*, meaning that each cell contains two distinct haploid nuclei (karyons) that were derived from either sexual parent. The sexual cycle of rust fungi is completed when monokaryotic (haploid) basidiospores, which germinate from teliospores,

infect the aecial host, produce pycniospores, fertilise, and produce aeciospores. Finally, the aeciospores re-infect the telial host and the cycle is completed (Fig. 17.2). Many of the rust fungi important to agriculture are heteroecious, with the cereal crop being the telial host (Petersen 1974; Lorrain et al. 2019). The alternate (aecial) host is critical for undergoing sexual reproduction, which plays a pivotal role in generating genetic diversity. However, *in the absence of sexual reproduction these pathogens* can still reproduce indefinitely via the asexual cycle as clonal lineages, and also *diversify through somatic processes* such as mutation and other more cryptic mechanisms (Figueroa et al. 2020). Given that sexual reproduction can generate new combinations of beneficial alleles and purge deleterious alleles and asexual reproduction can propagate genotypes with high fitness, rust fungi with mixed reproduction modes display high evolutionary plasticity and rapid host adaptation, which can result in sudden disease outbreaks.

Another mechanism that can contribute to new diversity in clonal populations is *somatic hybridisation*. This phenomenon was first described in laboratory studies in the mid-1900s, where simultaneous infection of a host plant with urediniospores of two rust isolates of the same species sometimes gave rise to spores with different virulence profiles to the original inoculant (Flor 1964; Bartos et al. 1969). In some cases, only two non-parental classes were observed suggesting that a nuclear exchange occurred between the dikaryons, while others suggested the possibility of further recombination between the genotypes (Watson 1957; Ellingboe 1961). However, whether this process occurs in the field and contributes to rust diversity was difficult to assess until the development of advanced genomic approaches. In fact, Li et al. (2019) found that one of the most epidemiologically significant races of wheat stem rust, Ug99 (see below), arose through just such a process, with the exchange of nuclei between parental isolates. Phylogenetic analysis of additional *Pgt* isolates suggested that several additional clonal lineages were also derived by such nuclear exchange events.

Rust fungi propagate asexually (clonally) through multiple infection cycles mediated by urediniospores. This infection cycle begins when urediniospores land on a leaf of a susceptible plant, imbibe water, and germinate (Staples and Macko 1984) (Fig. 17.2). Soon after germination, the germ tube grows perpendicularly to the venation of the leaf and locates a stomate on the host leaf via thigmotropism prior to differentiating an appressorium (Hoch and Staples 1987). The appressorium is a morphological differentiation of the hyphal apex. The appressorium produces a penetration peg, which allows the fungus to grow within the host leaf. Haustorial mother cells differentiate from infection hyphae adjacent to mesophyll plant cells, and haustorial formation is initiated. *The haustorium is a critical structure* for the pathogen, as it is the closest interface between the fungus and the host where nutrients are derived (Szabo and Bushnell 2001; Garnica et al. 2014). Once fungal colonies mature, they form erumpent pustules in the leaf surface which release urediniospores that can spread and restart the infection cycle.

17.4 Important Epidemics Caused by *Puccinia* Species

Multiple disease management strategies including the use of genetic resistance and fungicides have been instrumental to control rust diseases (Ellis et al. 2014; Oliver 2014; Chen 2014). However, the menace of rust fungi to crop production is still present as the pathogen's population evolves and for some species the evolutionary capacity seems extremely high. In species and regions where virulence evolution occurs quickly, the "*boom and bust*" model explains why management strategies like resistance gene breeding can break down quickly and sudden outbreaks of rust diseases occur (Fig. 17.3). A common situation that enables epidemics is the wide deployment of a new resistant crop cultivar. This scenario creates an extremely high selective pressure on the pathogen, allowing rare virulent individuals to succeed on the new cultivar and propagate. The large number of susceptible plants

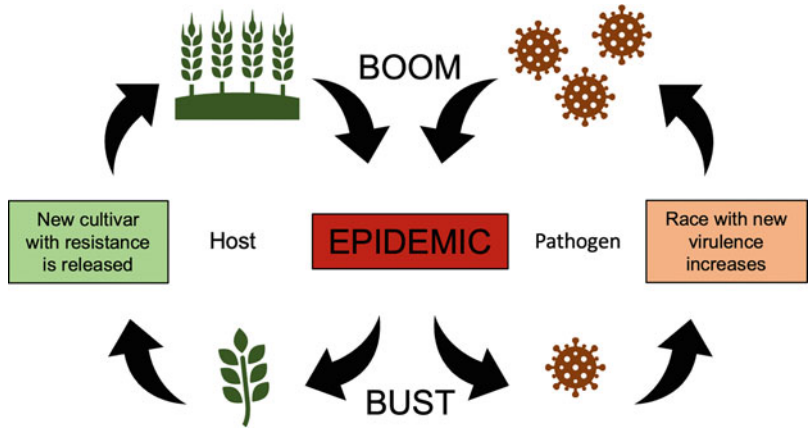

Fig. 17.3 Diagram of the boom-and-bust cycle frequently observed in crop plants affected by *Puccinia* species. As a new crop cultivar with single-gene resistance is released and widely planted, only individuals of the corresponding *Puccinia* species with virulence to the new resistance gene (termed a race) survive and reproduce due to high selection pressure. Once the abundance of the virulent race increases to high enough levels, an epidemic occurs and the coverage of the once-effective resistant crop cultivar diminishes. The cycle repeats when a new single-gene crop cultivar is introduced

and large amount of virulent inoculum (the boom) results in an epidemic, leading farmers to reduce acreage of the new cultivar, which also reduces the frequency of the pathogen (the bust). This model explains the epidemiology of many, but not all, of the rusts of crop plants. Below we discuss in detail epidemics and losses known for the *Puccinia* species that affect important cereal crops as mentioned earlier.

17.4.1 Wheat Rusts

17.4.1.1 Stem Rust

The causal agent of black or stem rust, *Puccinia graminis* f. sp. *tritici* (*Pgt*), has a wide geographical distribution around the globe (Leonard and Szabo 2005). Although *Pgt* is not as prevalent as the other two wheat rust species *P. striiformis* f. sp. *tritici* (*Pst*), and *P. triticina* (*Pt*), *Pgt* remains as a prominent pathogen, which under appropriate weather conditions such as warmth and high moisture may cause substantial grain losses (Kolmer et al. 2018; Leonard and Szabo 2005). A stem rust epidemic can colonise an entire susceptible wheat field in 21 days with yield losses of up to 50–60%, and in extreme epidemics near total losses (Peturson 1958; Roelfs 1978; Shank 1994). *Pgt* is a heteroecious species, relying on wheat as the telial host and barberry (*Berberis* spp.) as the aecial host (Roelfs 1985). *The presence of barberry generally increases disease severity by providing an early and local source of spring inoculum and enabling sexual recombination* (Roelfs 1985; Olivera et al. 2019). However, the absence or low prevalence of barberry in many parts of the world means that most *Pgt* populations are largely asexual.

Significant losses due to stem rust have affected regions in North America, South America, Europe, India, China, Australia, and South and East Africa at different times (Leonard and Szabo 2005). The devastating effects of *Pgt* in North America are demonstrated by an epidemic in 1916, which destroyed approximately 300 million bushels of wheat across the United States and Canada, and in 1935 which destroyed more than 135 million bushels of wheat in South and North Dakota and Minnesota (Leonard 2001). In 1953 and 1954, another stem rust epidemic in the US led to USD $365 million losses (USDA-ARS 2021 Mar 19). The need to control and minimise stem rust disease in North America is such that it set in motion US legislative orders such as a

multi-decade program to eradicate the sexual host of *Pgt* (barberry) and global advances like the introduction of semi-dwarf rust-resistant wheat, which is one of the cornerstones to the Green Revolution in the 1950s and 1960s.

The barberry eradication program began in the USA after the severe stem rust epidemic in 1916, with federal and state governments hiring labour to locate and remove barberry plants from 1918 to 1980 (Peterson 2001; Figueroa et al. 2016). The eradication program not only removed the early spring inoculum source of *Pgt,* but also eliminated the potential for sexual recombination to occur between individuals. Common barberry (*Berberis vulgaris*) was introduced to North America by Europeans in the seventeenth century and was used for medicine, fruit production, and as wind breaks adjacent to wheat fields (Peterson 2003). The close proximity of the telial and aecial hosts created a perfect environment for large, diverse populations of the stem rust pathogen, which resulted in frequent epidemics (Roelfs 1985). The success of the barberry eradication program is demonstrated by the dramatic drop in the number of stem rust races reported in the USA each year during the annual survey (Liberatore 2017, 2018; Fajolu 2021).

In Australia, stem rust epidemics have been recorded intermittently (Shipton 1966; Park 2008) and *Pgt* remains as a potentially damaging foliar pathogen of wheat (Murray and Brown 1987), third after wheat stripe rust and leaf rust (Murray and Brennan 2009). The 1973 epidemic is considered as one of the most detrimental events to the Australian grain industry in history (Watson and Butler 1984). In response to the 1973 stem rust epidemic, Australia launched the National Rust Control Program, which is currently known as the Australian Cereal Rust Control Program (ACRCP) (Park 2008) and remains active involving partners at the University of Adelaide, the University of Sydney and the Commonwealth Scientific and Industrial Research Organisation (CSIRO). In China, severe epidemics were reported in 1948, 1951, 1952, and 1956 (Roelfs 1977). Furthermore, severe epidemics in Europe were documented in 1932 and 1951 (Zadoks 1963).

In recent years, stem rust disease has once again emerged as a global threat. The detection of race Ug99 (TTKSK) in Uganda in 1998 and its geographical spread in Africa and the Middle East exemplify the evolution of new virulence traits in *Pgt* populations (Pretorius et al. 2000; Singh et al. 2008; Singh et al. 2011). Given that approximately 90% of wheat varieties in the world were ranked as susceptible to Ug99 (Singh et al. 2011) the high risk of *Pgt* to destabilise the world's economy became extremely concerning. These concerns triggered a coordinated international response to identify effective sources of stem rust resistance and develop cultivars suitable for deployment and risk mitigation. In 2005, the Borlaug Global Rust Initiative (BGRI) was established by Nobel Peace Prize Laureate Dr. Norman E. Borlaug including multiple institutions such as the Indian Council of Agricultural Research (ICAR), the International Center for Agricultural Research in the Dry Areas (ICARDA), the International Maize and Wheat Improvement Center (CIMMYT), the United Nations Food and Agricultural Organization (UN-FAO), and Cornell University to support wheat research and direct efforts to battle rust (McIntosh and Pretorius 2011). The enormous efforts of the scientific community led to the delivery of new resistant wheat germplasm (Pretorius et al. 2015). As noted above, the origin of the Ug99 lineage was related to a somatic hybridisation event, highlighting the potential of this process to contribute to the emergence of new races with highly significant impacts.

Since the discovery of Ug99, this lineage has diversified through mutation to *at least 13 clonally derived races* with novel virulence profiles (Singh et al. 2011; Olivera et al. 2012, 2015; https://bgri.cornell.edu/). In addition to Ug99, other unrelated *Pgt* races have emerged in different parts of the world. Sporadic disease reports in European countries including Denmark and the UK have occurred after the 2013 stem rust epidemic in Germany (Hovmøller 2017). A devastating epidemic in Ethiopia in 2014 was caused by the so-called "Digalu" race (TKTTF), so named due to it overcoming the *SrTmp* resistance gene in this widely used cultivar (Olivera et al. 2015), which

shows similarities to isolates detected in Germany (Olivera Firpo et al. 2017). Between 2015 and 2016, Western Siberia experienced devastating epidemics of stem rust that caused up to 40% yield losses (Hovmøller 2018a; b; Shamanin et al. 2016). More recently, an important *Pgt* outbreak took place in Sicily in 2016 by a broadly virulent race (known as TTTTF) (www.rusttracker.cimmyt.org; Bhattacharya 2017). In 2017, after decades without a stem rust outbreak, Sweden suffered from a stem rust epidemic, which appeared to involve a sexual population of the pathogen propagating on local barberry (Berlin 2017).

17.4.1.2 Stripe Rust

Wheat stripe (yellow) rust disease is caused by *P. striiformis* f. sp. *tritici* (*Pst*), a pathogen which has become more problematic in the past two decades. Infection by *Pst* generally requires cool and humid conditions and the disease traditionally impacted wheat production mostly in the middle east and North Africa (Yahyaoui et al. 2000). However, the emergence of *Pst* isolates with tolerance to warmer temperatures has supported a broad global geographic expansion more recently, which has raised serious concerns (Chen 2005; Beddow et al. 2015; Hubbard et al. 2015; Milus et al. 2009). Wheat stripe rust epidemics have resulted in global losses estimated to reach at least 5.5 million tons per year (Beddow et al. 2015). In general wheat yield losses caused by stripe rust range from 10% to 70% depending on susceptibility of the cultivar, time of infection, rate of disease development, and duration of disease (Chen 2005). However, 100% yield losses can occur, especially when infection occurs early, and the disease is established during the wheat growing season.

In North America, stripe rust was first detected in 1915 and later studies using herbarium samples determined the pathogen had been present in the region for over two decades (Line 2002). Based on outbreak reports from epidemics in the US, it seems that the effects of stripe rust on wheat production decreased until the 1950s (Chen 2005). Then, the pathogen was detected again and yield losses were recorded. The first record of damage by stripe rust in the USA was made in 1958 when 2.9 million bushels was lost in the state of Washington due to the disease (Chen 2005). Subsequent stripe rust epidemics in Washington caused losses that reached 7.5 million bushels in 1960 and 15 million bushels in 1961. Other states in the USA such as Oregon, Idaho, Montana, and California have also been intermittently affected by *Pst*. In Europe, *Pst* caused severe outbreaks in 1977 across Italy, Tunisia, and eastern Algeria, and in 1978 in Spain (Zadoks 1963).

Molecular and phenotypic characterisation of *Pst* isolates from Europe, Australia as well as North and South America indicated that, prior to the year 2000, *Pst* populations were mostly clonal and the underlying mechanism of evolution was through single stepwise mutations (Hovmøller et al. 2002, 2016; Enjalbert et al. 2005; Chen 2005; Steele et al. 2001; Chen et al. 2010; Ali et al. 2014; Hubbard et al. 2015). For example, *Pst* was first reported in Australia in 1979 and it is postulated that the pathogen arrived to the country from Europe. Subsequently, stepwise changes in virulence generated more than 20 distinct races in this clonal lineage (Wellings and McIntosh 1990; Steele et al. 2001). The only region that showed genetic variation in *Pst* populations was the Himalayan (Nepal and Pakistan) and near-Himalayan (China) regions, where genetic recombination through sexual reproduction of the pathogens was known to occur on *Berberis* species (Duan et al. 2010; Mboup et al. 2009; Ali et al. 2014). It is worthwhile to note that *Berberis* species also serve as alternate hosts for *Pgt* so the impact of these species in wheat rust epidemics is remarkable (Leonard and Szabo 2005). After the year 2000, researchers investigating stripe rust detected significant changes across USA (Chen et al. 2002; Markell and Milus 2008), Europe (Hovmøller and Justesen 2007), and Australia (Wellings 2007). New races of *Pst* identified in Europe (Hovmøller et al. 2016) caused severe epidemics in fields of durum and bread wheat in regions such as Italy (Sicily, Sardinia, Puglia, Lazio, and Emilia Romagna) where the occurrence of the disease was atypical.

The first post-2000 *Pst* strains that were detected include PstS1 and PstS2 (Chen et al. 2002; Markell and Milus 2008; Hovmøller and Justesen 2007; Wellings 2007). These strains showed increased aggressiveness and high-temperature adaptation, which likely contributed to their rapid spread (Milus et al. 2009). In 2011, additional new *Pst* races reached Europe (www.wheatrust.org), causing serious concerns to the grain industry. Two of these races, "Warrior" and "Kranich," named after the wheat cultivars whose resistance the new races overcame, derived from the vicinity of the Himalayan region. A third race, which is virulent on Triticale, shares commonalities with races from the Middle East and Central Asia (Lucas 2017) and is also commonly found across Europe (Ali et al. 2017; Hovmøller et al. 2016; Hubbard et al. 2015).

17.4.1.3 Leaf Rust

P. triticina (*Pt*) is the causal agent of wheat leaf rust (Anikster et al. 1997; Bolton et al. 2008), the most common and widely distributed of the three *Puccinia* species affecting wheat, although it generally results in lower yield losses than stem or stripe rusts (Huerta-Espino et al. 2011; Bolton et al. 2008). The fungus is heteroecious, and the aecial host is *Thalictrum speciosissimum* or *Isopyrum fumaroides*. These alternate host species show limited global distribution meaning that the pathogen is largely restricted to asexual reproduction in most wheat-growing regions (Bolton et al. 2008).

Monitoring of the population of *Pt* has been extensive in some countries such as the USA, Canada, and Australia, where national rust surveys started in 1926, 1931, and 1920, respectively (Johnston et al. 1968; Johnson 1956; Waterhouse 1952). More than 70 races of *Pt* are described each year in North America, which highlights the high genetic diversity of the pathogen (Kolmer et al. 2007). High levels of diversity are also reported for Europe. For instance, in France, between 30 and 50 races are identified each year (Goyeau et al. 2006). It is thought that the high diversity of races and the recurrence of common races in each year reported in countries like Egypt (Saari and Wilcoxson 1974) and in the Southern and Central Plains of the USA is consistent with oversummer survival of the pathogen.

Leaf rust outbreaks cause serious production losses in almost all wheat production areas of the USA on a yearly basis. In the USA, leaf rust epidemics are common, especially in regions where soft winter red wheat is used as a rotation crop (Anzalone 1985). From 2000 to 2004, losses in the USA due to *Pt* were more than three million tonnes, which was equivalent to approximately US$350 million (Huerta-Espino et al. 2011). In the state of Kansas, losses of winter wheat reached 13.9% in 2007, 4.7% in 2008, and 1.4% in 2009. In the eastern prairies of Canada, yield reductions range from 5 to 15% when wheat-grown cultivars that are susceptible are widely planted (Samborski 1985). However, as mentioned for other wheat rusts, early timing of infection can mean higher losses. Between 2000 and 2009, Canada lost approximately ten million hectares of wheat grown annually. Wheat leaf rust epidemics in Mexico have also been significant. Northwest Mexico suffered up to 40% yield reductions during 1976–1977 due to a *Pt* outbreak (Dubin and Torres 1981).

Wheat production in the Southern Cone (Argentina, Brazil, Chile, Paraguay, and Uruguay) is often affected by *Pt* (Germán et al. 2007). It is calculated that yield losses reached $172 million in the region during 1996–2003 (Germán et al. 2004). In Eastern Europe, a yield reduction caused by *Pt* infection ranges between 3 and 5%; whereas in Western Europe the pathogen has little impact on wheat production (Samborski 1985). In Russia, *Pt* is responsible for yield losses in the Volga Basin, as well as the North-Caucasian and Central Chernozem regions (Huerta-Espino et al. 2011). The disease is also important in South Africa (Terefe et al. 2009; Visser et al. 2011; Terefe et al. 2014) where the population seems to only diversify through clonal means (Kolmer et al. 2019; Selinga 2015).

The significance of leaf rust disease is also documented for Ethiopia and India, where average losses are approximately 3% (Dmitriev and Gorshkov 1980; Saari and Wilcoxson 1974; Samborski 1985). In 1973, Pakistan wheat-producing areas experienced high *Pt* infection

ranging from 40 to 50% and reaching 100% in some cases (Hassan et al. 1973). A severe epidemic led to the loss of 830,000 tonnes of Pakistani wheat in 1978 (Hussain et al. 1980).

Leaf rust epidemics have caused significant problems in China, especially in the Northern Plains, the middle to lower Yangtze River Valley, and southwest and northwest regions where the pathogen is commonly found (Liu and Chen 2012). Among the severe *Pt* epidemics in Northern China, it is worth citing those which occurred in 1969, 1973, 1975, 1979, 2012, 2013, 2015, and most recently 2020 (Hu and Roelfs 1985; Zhang et al. 2015; Zhou et al. 2013; Wu et al. 2021a; b; Zeng et al. 2022). In Australia, the effects of leaf rust have been recorded since early days in all wheat-growing regions, particularly in Northern New South Wales and Queensland territories (Watson and Luig 1962). Leaf rust epidemics occurred in Western Australia between 1990 and 2000 intermittently, and in South Australia and Victoria in 1999 (Huerta-Espino et al. 2011). Australian losses to the diseases are estimated to reach AU$12 million and have the potential to escalate to AU$197 million (Waterhouse 1952; Murray and Brennan 2009).

17.4.2 Barley Rusts

17.4.2.1 Stem Rust

P. graminis f. sp. *tritici* is a pathogen barley shares with wheat, and similarly stem rust is an important disease in most places where barley is grown. In general, the epidemics of stem rust on barley tend to mirror those of wheat. However, in the USA the barley and wheat crops had different trajectories after the 1940s, due to the discovery of a *durable stem rust resistance gene in barley, called Rpg1* (Shands 1939; Steffenson 1992). While wheat breeders had to continuously introduce new resistance genes until the last major wheat stem rust epidemic in 1955, barley cultivars remained completely resistant to stem rust until 1989, when a race virulent on *Rpg1* barley (race QCC) emerged (Martens et al. 1989). Despite the arrival of QCC, losses in barley were minimal and *Rpg1* maintains a record of practical efficacy of about 80 years (Steffenson 1992). Even in other barley-producing areas, such as Australia, Africa, Europe, and Central Asia, wheat stem rust affecting barley was not prioritised in breeding due to the relatively low occurrence of epidemics (Luig 1985; Steffenson et al. 2017).

Stem rust continues to impact barley production throughout the rest of the world. The stem rust epidemics caused by Ug99 and derivative isolates illustrate the threat; these were found to be virulent on more than 95% of tested barley varieties (Singh et al. 2011; Steffenson et al. 2017). Renewed efforts to characterise barley stem rust resistance genes since 1999 has resulted in the discovery of two new genes (*Rpg6* and *Rpg7*) for a total of nine known resistance gene in barley, which still pales in comparison to the 60+ genes identified in wheat (McIntosh et al. 1995; Fetch et al. 2009; Sharma 2012; Komugi Wheat Genetic Resources Database 2013; Henningsen et al. 2021). Thanks to the lower susceptibility of barley, however, major stem rust epidemics so far seemed to have failed to materialise in recent years.

17.4.2.2 Stripe Rust

Another agronomically important rust is *P. striiformis* f. sp. *hordei* (barley stripe rust) (Figueroa et al. 2018). The barley stripe rust pathogen is present on all continents except Australia and Antarctica. In Europe, stripe rust is present on barley but epidemics are rare due to agricultural practices; the only major epidemics of note occurred in 1961 in Central Europe and from 1960 to 1965 in England (Hassebrauk 1962; Macer and Driessche 1966; Stubbs 1985). Barley stripe rust epidemics are likewise uncommon in Asia, except for severe epidemics in Japan from 1950 to 1956 (Kajiwara et al. 1964; Stubbs 1985). An epidemic also occurred in Myanmar (then Burma) in 1938 (Seth 1939).

Stripe rust was not present in the Americas until the 1970s, so upon arrival in South America, stripe rust caused epidemics on barley as it spread: first in Colombia in 1975, then to Ecuador in 1976, Peru in 1977, Bolivia in 1978, Chile in

1980, and finally Argentina in 1982 (Dubin and Stubbs 1986). Stripe rust also spread northward to Mexico, causing an epidemic on barley in 1987 (Calhoun et al. 1988), and then began causing epidemics in the USA (Line 2002). Texas was the first state, in 1991, and then the pathogen spread to Oklahoma, New Mexico, Colorado, Arizona, Montana, Idaho, California, Utah, Oregon, and Washington between 1991 and 1995 (Brown et al. 1993; Chen et al. 1994; Marshall and Sutton 1995; Line and Chen 1996). Stripe rust returned to California in 1996 to cause another major epidemic (Line 2002). Stripe rust was still an important disease of barley in the USA into the 2000s, but there have not been any epidemics reported more recently (Line 2002; Chen 2008). Stripe rust likewise occurs on barley in Canada, but it has not reached epidemic levels (Xi et al. 2015). Although stripe rust of barley is not present in Australia, an unusual form of *P. striiformis* that affects mainly wild *Hordeum* species was identified in 1998 (Wellings et al. 2000).

17.4.2.3 Leaf Rust

Barley leaf rust (caused by *P. hordei*) is present worldwide in barley-producing regions (Clifford 1985). Despite being widespread, losses are restricted to specific regions; elsewhere epidemics are uncommon. In New Zealand, economically important losses occurred in 1974 (Arnst et al. 1979). Australia had several epidemics in the 1920s, with none occurring again until 1978, 1983, 1984, 1988, and 1990 (Waterhouse 1927; Cotterill et al. 1992). Barley leaf rust epidemics occurred in the southeastern USA from the 1940s to the 1960s, (Griffey et al. 1994). In recent years, small-scale epidemics have occurred in the states of Virginia, Texas, and Washington, but there have been no nationwide epidemics (Hughes 2016; Liberatore 2017).

In Europe, major epidemics happened in England in 1970–1971 and minor outbreaks happened in the Czech Republic in the 1980s (Melville et al. 1976; Dreiseitl 1987). Leaf rust epidemics occurred on barley in 1992, 1997, 1998, and 1999 in South Africa (van Niekerk et al. 2001).

17.4.3 Oat Rusts

Oat crown rust infections occur under high humidity conditions and moderate temperatures (21–25 °C) (Carson 2011). The negative effects of crown rust infection extend from foliar damage to stunted roots and poor drought tolerance in plants (Gnanesh et al. 2014). Oat crown rust (caused by *P. coronata* f. sp. *avenae,* hereafter *Pca*) has caused epidemics resulting in 50% yield loss, and in some circumstances near-total losses have been reported (d'Oliviera 1942; USDA-ARS CDL 2014). Like with *Pgt*, disease severity is generally increased in areas where the alternate host is present. The high prevalence of common buckthorn (*Rhamnus cathartica*) in North America is an important epidemiological factor for oat crown rust as spring inoculum enables *sexual recombination* on buckthorn and therefore the propagation of recombinant genotypes (Simons 1985; Miller et al. 2020). Buckthorn was introduced from Europe to North America sometime during the nineteenth century for its desirable medicinal and later ornamental properties; however, buckthorn is extremely invasive and has colonised all suitable environments in the USA and into Canada (Kurylo and Endress 2012; Knight et al. 2007).

The diverse *Pca* genotypes resulting from sexual recombination are subject to strong selection when single-resistance gene oat varieties are released. Those that are virulent on new single-resistance gene cultivars amplify to epidemic levels, causing a boom as illustrated in Fig. 17.3. The fallout results in the new cultivar being abandoned, and the pathogen numbers decrease again in a bust (Simons 1985; Mundt 2014). Despite the negative ecosystem impacts of buckthorn and the connection between buckthorn and *Pca* diversity and virulence, large-scale programs to eradicate the invasive plant have not occurred and current efforts are small-scale and voluntary.

Due to the high diversity of oat crown rust, epidemics occur regularly around the world (McCartney et al. 2011; Park 2013; Nazareno et al. 2018; Leonard and Martinelli 2005). The

high levels of polymorphisms in oat crown rust are not exclusive to sexual populations as asexual populations from geographic regions where the alternate host is absent, such as Australia and Brazil, also display highly polymorphic virulence traits. This is most likely due to the widespread prevalence of wild oats which can maintain high *Pca* populations outside of commercial crops.

Oat crown rust epidemics in the USA have occurred intermittently. An important race of *Pca* is the race 45 group (race 45A now known as race 203), which caused severe epidemics in the early 1950s, resulting in the deployment of the resistant oat cultivar Victoria (Welsh et al. 1954). The Victoria cultivar is now known for being extremely susceptible to Victoria blight (caused by *Cochliobolus victoriae*), which had never been observed in oat before (Welsh et al. 1954). Currently, it is believed that the gene that confers resistance to oat crown rust is also the receptor for the victorin toxin of *C. victoriae*, but further work is needed to confirm this (Lorang et al. 2007). From 1949–1953, *Pca* caused between 8 and 30% yield losses in Iowa, and in 1979 losses reduced the crop by 2% in Minnesota, and by 3% in both North and South Dakota (Sherf 1954; Simons 1985). A recent epidemic in 2014 caused losses as high as 50% in Minnesota (USDA-ARS CDL 2014). Forage oats have also been affected by crown rust epidemics as it has been recorded for New Zealand (Eagles and Taylor 1976).

Oat stem rust is caused by *Puccinia graminis* f. sp. *avenae*, which is also a highly variable pathogen (Martens 1985). In North America, there were severe epidemics of oat stem rust in 1904, 1916, 1923, 1927, 1935, 1938, 1943, 1949, 1953, 1955, and 1977 (Craigie 1957; Roelfs and Long 1980). Of these, the greatest losses occurred in 1953 when Minnesota, Iowa, Wisconsin, North Dakota, and South Dakota reported losses of 25, 10, 7, 5, and 4%, respectively, reaching a total of 947,450 tonnes. Oat stem rust epidemics were also reported for Canada in 1944, 1945, 1947, and 1950 (Green et al. 1961), 1970, 1977, 1981. The Canadian epidemic in 1977 caused a loss of 385,000 tonnes of grain (Martens 1978). In Australia, the oat stem rust epidemic in 1973 caused between 10 to 35% total yield losses in some areas whereas others lost up to 80% of the crop in some regions (Park et al. 2000). In 2001, another severe stem rust epidemic also caused severe yield losses.

17.4.4 Corn Rusts

Corn rusts caused by *P. sorghi* (common corn rust) and *P. polysora* (southern corn rust) have resulted in losses of up to 35 and 80%, respectively (Reyes 1953; Kim and Brewbaker 1976). In recent times, there have been increased reports of southern corn rust in the USA, which may be related to an increase in the planting of susceptible varieties (Crouch and Szabo 2011). Epidemics of southern corn rust have occurred in the USA, West Africa, and Asia (Futrell 1975; Rhind et al. 1952; Chen et al. 2004; Reyes 1953). Prior to 1972, *P. polysora* was prevalent only in the lower Mississippi River Valley in the USA; however, multiple epidemics across various USA states between 1972 and 1979 demonstrated the geographic expansion of the pathogen in North America (Raid et al. 1988). For instance, southern corn rust was recently reported in South Dakota (Byamukama 2020). Data regarding economic losses due to the corn rust diseases is not well recorded; however, the potential economic damage of *P. polysora* is between 45–75% yield losses. In the state of Minnesota, a minimum economic loss of three million US dollars was estimated due to common rust in an epidemic that attacked sweet corn in 1977, and in the US state of Georgia, southern rust was responsible for the loss of more than 18 million US dollars in 2014 (Groth et al. 1983; Little 2014). It is worth highlighting the emergence of a new race of southern corn rust that is virulent to Rpp9, which was used to provide effective resistance to southern rust in the Southern USA (Brewbaker et al. 2011).

Epidemics of *P. polysora* in West Africa in 1950 affected Liberia, Ivory Coast, Gold Coast, Dahomey, and Nigeria. It is estimated that during this epidemic up to 50% of the crop was lost in some regions (Rhind et al. 1952). Around 1953,

outbreaks of southern corn Rust were also reported in the Philippines where yield losses reached 80–84% (Reyes 1953). Southern corn rust epidemics also occurred in Northern China in 1998, which resulted in yield losses of 42–53% (Chen et al. 2004).

17.4.5 Sorghum Rust

P. purpurea, which causes the sorghum rust disease, is widely distributed around the globe and is present in almost all sorghum-growing areas, particularly East Africa, India, as well as South and Central America. The host range of *P. purpurea* also includes *S. sudanense* (sudangrass and other grass sorghums), *S. halpense* (johnsongrass), *S. almum* (columbusgrass), *S. virgatum* (tunisgrass), *S. nitidum*, *S. verticillifloru*, *S. nitens*, and *S. arundinaceum* (Johnston and Mains 1931; Tarr 1962). Sorghum rust is more severe in the tropics than in drier temperate areas. A significant problem is that infection by *P. purpurea* predisposes sorghum to other secondary diseases such as Fusarium stalk rots, charcoal rot, and grain moulding (Frederiksen 1986). In the USA, sorghum rust is more important in Hawaii and the gulf coast region than in the major crop-producing areas which include Colorado, South Dakota, Oklahoma, and Texas (Hooker et al. 1985). In Australia, sorghum rust is not considered an important disease since most epidemics take place late in the season, thus causing minor yield losses of grain (White et al. 2012).

17.4.6 Sugarcane Rusts

Sugarcane production is affected by *Puccinia melanocephela,* which causes common rust disease, and *P. kuehnii,* which causes orange rust disease (Cummins and Hiratsuka 2004; Boddy 2015). *P. melanocephala* was first reported on sugarcane in 1949 in India (Patel et al. 1950) and has spread since to other important sugarcane-producing areas. Sugarcane rust epidemics were not particularly concerning before 1978 when the Dominican Republic experienced a drastic epidemic by *P. melanocephela* (Presley et al. 1978). One year later, *P. melanocephela* was present in the Caribbean and the Americas, including important sugarcane-producing areas like Mexico, and US states such as Florida, Louisiana, Mississippi, and Texas (Purdy 1985). In Mexico, a total of 1,344,168 tonnes of sugarcane were lost due to the *P. melanocephela* outbreak in 1979–1980 (Osada and Reyes 1980). In the USA, the sugarcane yield loss caused by a common rust epidemic in 1987 was 20% (Raid and Comstock 2000). Cuba lost 40% of its sugarcane production area in 1979, and 28% in 1980 also due to common rust epidemics (Purdy 1985).

Orange rust was traditionally known as a minor problem of sugarcane in Asia and Australia. However, in the year 2000, Australian sugarcane fields suffered a significant epidemic of orange rust that caused yield losses of up to 40–45%, with a total cost exceeding AU$ 200 million (Apan et al. 2003; Braithwaite et al. 2004; Magarey et al. 2008). Seven years later, *P. kuehnii* was found in sugarcane fields of Florida, Costa Rica, Guatemala, Nicaragua, and in South America (Chavarría et al. 2009; Glynn et al. 2010; Cadavid et al. 2012; Chaves et al. 2013), potentially as airborne introductions from Africa (Rott et al. 2016). In 2007, orange rust caused an estimated 10% loss on susceptible sugarcane in Florida. Total sugar losses reaching up to 50% or more have been reported in sugarcane-producing countries around the globe (Comstock et al. 2010; Raid et al. 2011; Rott et al. 2016).

17.4.7 Myrtle Rust

Puccinia psidii, recently reclassified as *Austropuccinia psidii* (Beenken 2017), is another rust fungus that has attracted attention due to its economic impact in the forest industry. This rust species also *known as* Eucalyptus or Myrtle rust *provides an example of ecological and industrial concerns that can be inflicted due to the geographic expansion of this pathogen.* Myrtle rust appeared in Australia in 2010 on the central coast

of New South Wales and is a threat to the *Myrtaceae* family, which encompasses ~10% of Australia's native plants. It had spread across the entire east coast of Australia within five years, impacting the growing lemon myrtle industry with up to 70% yield losses (Lancaster et al. 2016). Myrtle rust is also a direct threat to the profitable Australian tourism industry, which depends on the conservation of Australia's iconic landscapes and wildlife. Myrtle rust spread further to New Zealand in 2017, possibly by wind from Australia. In New Zealand, the incursion of myrtle rust is a threat to many significant native plants such as mānuka, famous for mānuka honey, and rātā, a long-lived tree with cultural importance to Māori people.

To date, *A. psidii* is also one of the major threats to Eucalyptus plantations, which cover more than 20 million hectares around the world, primarily in South America and southern Asia (IUFRO et al. 2018). The disease was first noted in guava trees (*Psidium guajava*) from Brazil in 1884 and it was first reported in Eucalyptus plantations around the 1950s (Grgurinovic et al. 2006; Alvares et al. 2017), where it causes an economic impact associated through reduction of wood productivity (Coutinho et al. 1998). *Presently, A. psidii* is present in Central America and the Caribbean, Mexico, and the USA including the Hawaiian Islands, South America, parts of Asia, South Africa, and Oceania (Grgurinovic et al. 2006). While the entire host range of *A. psidii* has not been determined; scientists regard all genera of the Myrtaceae family as potential hosts (Carnegie and Lidbetter 2012; Morin et al. 2012; Pegg et al. 2014).

17.5 Molecular Basis of Rust Virulence

A major strategy for the control of rust diseases in cereals is the development of disease-resistant cultivars through breeding (Ellis et al. 2014) Such genetic resistance to rust infection in host plants is usually mediated by an innate immunity system that activates defenses to confer disease resistance upon pathogen recognition. *Dominant resistance (R) genes* encoding immunoreceptors in the host plant confer recognition of proteins encoded by specific *Avirulence (Avr) genes* from the pathogen, as described in the gene-for-gene model (Flor 1971). *R* genes often encode intracellular receptor proteins belonging to the *nucleotide-binding leucine-rich-repeat receptor (NLR)* class (Dodds and Rathjen 2010). These receptors recognise pathogen "effector" proteins that are delivered into host cells during infection to suppress host basal defenses and facilitate infection (Periyannan et al. 2017). This type of resistance is known as race-specific resistance, as it is effective to combinations of Avr factors (effectors) that define a specific race of the pathogen. Effective *R* genes in a crop provide strong selection for the pathogen to evolve and escape recognition, therefore variation in effector gene content and sequence can be responsible for the emergence of new virulence traits and breakdown of disease resistance in the field (Figueroa et al. 2020). This gene-for-gene interaction has been a fundamental concept for breeding disease-resistant crop varieties and represents the underlying model of host-pathogen co-evolution (Ellis et al. 2014).

The *gene-for-gene model* was first developed in the flax rust disease system (Flor 1971). Flax rust disease is caused by *Melampsora lini*, which while not a *Puccinia* species, does belong to the same order Pucciniales and has served as *a model to understand the evolution of virulence* in rust fungi and other biotrophic and hemibiotrophic pathogens. The *R* genes in flax were among the first to be identified in plants (Lawrence et al. 1995) and the corresponding *Avr* genes from *M. lini* were the first rust Avr effectors that were identified (Dodds et al. 2004). These advances paved the way for more recent *R* and *Avr* gene discoveries in cereals and their pathogens (Fig. 17.4). Four polymorphic loci (*L*, *M*, *N,* and *P*) in flax encode R proteins of the NLR immune receptor type, with nineteen variants of different recognition specificity discovered in the late 1990s and early 2000s (Lawrence et al. 1995, 2010; Anderson et al. 1997; Dodds et al. 2001a, b). Later, the first *Avr* effector genes from flax rust were cloned, with a total of six *Avr* loci now identified in *M. lini* (Dodds et al.

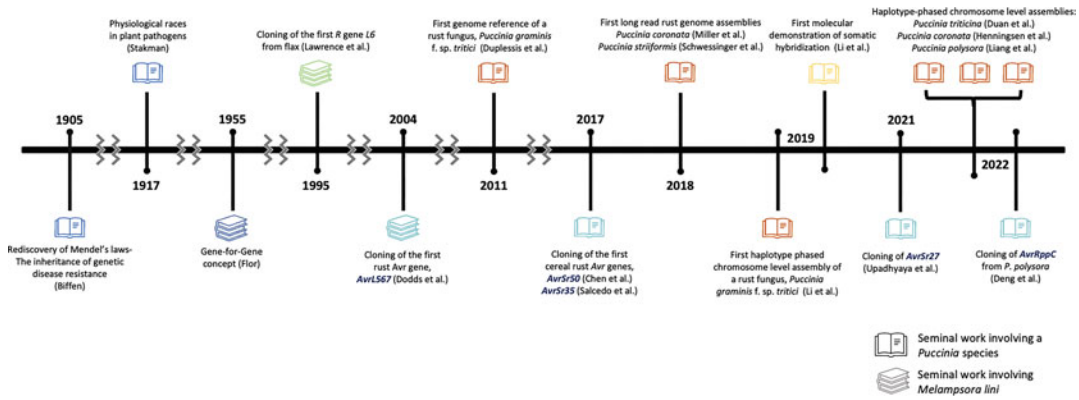

Fig. 17.4 Timeline describing research milestones that have transformed our understanding of the genetics and molecular components of plant pathology, and the involvement of rust fungi and *Puccinia* species. From R.H. Biffen, who demonstrated the application of Mendelian laws in the inheritance of disease resistance while studying cereal rust disease, including *P. striiformis* f. sp. *tritici*, to the most recent advances in avirulence (*Avr*) effector gene discovery powered by the arrival of the genomics era. The definition of physiological races by E.C. Stakman, again using cereal rusts, and the postulation of the gene-for-gene concept by H. H. Flor working with the flax rust pathosystem set the stage for modern plant pathology and the discovery of resistance (*R*) genes and their corresponding *Avr* effector genes as demonstrated by G. Lawrence and P.N. Dodds, respectively, in the flax rust system

2004; Catanzariti et al. 2006; Barrett et al. 2009; Anderson et al. 2016). These all encode *small, secreted proteins that are expressed in haustoria* and recognised by the flax receptors after delivery into host cells during infection. Thus, these proteins appear to represent a subset of "*effector*" proteins that are delivered to host cells during infection to manipulate host physiology (Figueroa et al. 2021), which have become targets for host recognition. This paradigm has underpinned efforts to define candidate effector and *Avr* genes in other rust fungi including *Puccinia* species.

Three *Avr* loci in flax (*AvrP4*, *AvrM14*, *AvrP123*) contain a single gene with multiple allelic variants, while three other loci (*AvrL567*, *AvrM,* and *AvrL2*) contain duplicated gene copies with copy number variation between haplotypes. In three cases, direct interaction between the Avr protein and the corresponding host NLR immune receptor has been shown (Dodds et al. 2006; Catanzariti et al. 2010; PN Dodds unpublished data). In general, the *Avr* gene variants that lead to escape of recognition encode proteins with variations in amino acids that are exposed on the surface of the protein structures (Wang et al. 2007; Zhang et al. 2018; Ve et al. 2013), which prevent interaction with the corresponding NLR R proteins. This is consistent with direct R-Avr recognition causing the selection of *Avr* gene variants that retain their pathogenicity function but lose recognition by the immune receptors. Indeed, there is evidence for diversifying selection acting on these loci to maintain high genetic diversity (Dodds et al. 2006; Barrett et al. 2009).

A critical factor in the success of *Avr* gene identification in *M. lini* was the existence of a segregating family that allowed genetic mapping of the *Avr* loci. Such resources are difficult to develop and maintain, so progress in other rust fungi has taken longer, but advances in genomic analysis (Figueroa et al. 2020 and see below) have allowed the utilisation of natural and induced mutants which have now resulted in the isolation of four *Avr* effector genes from cereal rust fungi (Chen et al. 2017; Salcedo et al. 2017; Upadhyaya et al. 2021; Deng et al. 2022). As in *M. lini*, these all encode secreted proteins expressed during infection and are recognised by intracellular host immune receptors reinforcing this paradigm as the basis of rust virulence/avirulence.

The first of these were *AvrSr35* and *AvrSr50* identified from the wheat stem rust fungus *Pgt* (Chen et al. 2017; Salcedo et al. 2017). In both cases, genomic comparison of mutant virulent isolates to their wildtype (avirulent) parental isolates allowed identification of the *Avr* gene, with function confirmed by co-expression with the corresponding *R* gene (*Sr35* and *Sr50* respectively). As observed in the flax rust system, recognition of these effectors by the NLR immune receptor is mediated by direct protein interactions. Ortiz et al. (2022) examined diversity in AvrSr50 alleles across a range of *Pgt* isolates and identified 14 different allelic variants characterised by amino acid sequence variation. Most of these were still recognised by Sr50, but a single amino acid change in one variant was sufficient to block recognition. Structure analysis indicated that this was exposed on the surface of the protein and suggests that it has an important role in defining the physical interaction with the immune receptor. Additional virulence alleles of *AvrSr50* involve gene knockout events, including a common allele containing a 26 kbp insertion within the gene. Another allele contains a C-terminal protein extension due to a mutation in the stop codon, which leads to loss of recognition in transient assays. Salcedo et al. (2017) also found amino acid sequence variation in AvrSr35, but this was not correlated with virulence phenotypes, with the most common virulence allele in *Pgt* containing a small MITE (miniature inverted-repeat transposable element) insertion. Another virulence allele present in Ug99 contains a larger insertion of about 57 kbp in the *AvrSr35* coding sequence.

The *AvrSr27* locus in *Pgt* was recently identified by a similar mutational approach (Upadhyaya et al. 2021) and consists of two adjacent related genes (paralogs) encoding secreted effector proteins. In this case, the virulence allele contains a single copy of this gene. All three AvrSr27 protein variants can be recognised by Sr27 in transient expression assays; however, the virulence allele gene copy shows much lower expression which may explain its lack of recognition during natural infection. Other virulent races have arisen in the field as a result of multiple independent DNA deletion events in which both genes at the avirulence locus have been lost (Upadhyaya et al. 2021). Thus, apparent knock-out mutations (deletions, insertions, and expression level polymorphisms) underly most field-derived virulence in *Pgt*, rather than sequence variation as observed in *M. lini*. This may be a consequence of *Pgt* populations being mainly clonal due to the lack of an alternate sexual host in most wheat-growing areas. This means that most virulent isolates result from spontaneous mutations, rather than reassortment of allelic variation through sexual recombination (Figueroa et al. 2020). *M. lini* on the other hand is an autoecious rust and completes a sexual cycle on the same host (flax) every season.

Most recently, the *AvrRppC* gene was identified from southern corn rust (*P. polysora*) (Deng et al. 2022). In this case, an effector screening approach was used, with about 120 predicted secreted effectors of *P. polysora* tested by co-expression with the maize *RppC R* gene. Again, recognition of AvrRppC by RppC was shown to be based on physical interaction, and sequence variation was observed between allelic variants of AvrRppC. The six most common variants included three that are recognised by RppC and three that were not recognised and define virulence alleles. It is not yet clear to what extent sexual recombination contributes to *P. polysora* population diversity.

Another strategy to identify *Avr* loci in *Puccinia* species is the use of genome-wide association studies (GWAS) on sexually derived populations. For instance, Miller et al. (2020) showed that the North American population of the oat crown rust fungus *Pca* shows high levels of sexual recombination, due to the widespread presence of the alternate host in this region. A GWAS analysis of a population of 60 *Pca* isolates identified genomic regions with significant association with fifteen different crown rust *R* (*Pc*) genes in oats. Importantly, according to the GWAS analysis, these fifteen *Pc* genes detected only seven regions in the *Pca* genome associated with changes in virulence, with several groups of *Pc* genes detecting the same loci. These findings may have multiple explanations: 1) the same gene

or alleles of the same gene have been deployed multiple times, 2) effectors recognised by distinct immunoreceptors are genetically linked and occur in a cluster, or 3) one effector is recognise by multiple immunoreceptors. Examination of the gene annotation in these regions of the *Pca* genome assemblies (Miller et al. 2018; Henningsen et al. 2022) has identified a small number of candidate effector genes that are likely to confer these *Avr* phenotypes.

Despite these advances, the vast majority of *Avr* genes in *Puccinia* species remain to be identified and further investigation is needed to develop a broad picture of the mechanisms these pathogens use for host manipulation. These are likely to remain a strong focus for future research efforts but will rely on improvements in genomic resources for these fungi as well as advances in effector identification strategies.

17.6 Genomic Resources to Study Virulence Evolution

Rusts typically have larger genome sizes than other fungi (haploid genome sizes range from ~80 Mb to ~2 Gb; Table 17.2), have high repeat content, and are recalcitrant to culturing. These features have caused them to lag behind other plant-pathogenic fungi in the development of genomic resources (Figueroa et al. 2020; Tavares et al. 2014; Aime et al. 2017; Williams 1984). In addition, *the dikaryotic nature of these fungi* during the uredinial phase, which is the main propagating phase of *Puccinia* species that infect cereals, has also *posed challenges for accurate assembly of genomes*. This is because the two haplotype sequences are prone to become collapsed in regions where they are highly related while they may be assembled separately in regions of high sequence divergence. Until recently, this has generally resulted in most rust genome assemblies representing imperfect representations that neither capture the full diversity of the two haplotypes nor provide a unique haploid genome representation. However, *long-read sequencing* and *Hi-C chromatin contact* approaches have now made fully nuclear-phased chromosome level assemblies achievable for rust fungi. Unlike many other fungi, long-read sequence data alone is still not sufficient for chromosome-scale assemblies in rust fungi, and Hi-C data is required for scaffolding. In addition, the location of the two haplotypes in two separate nuclei results in a strong Hi-C phasing signal due to the physical separation of the homologous chromosomes, allowing accurate assignment of genomic sequences to each nucleus (Duan et al. 2022).

17.6.1 Evolution of Rust Genome Assembly Approaches

Early attempts at rust genome assembly relied on short reads, sometimes supplemented with Sanger sequencing of cloned fragments (e.g. fosmid clones). These assemblies were limited by being highly fragmented and by containing an undefined mix of collapsed haploid and duplicated diploid content. For example, two short-read assemblies of *Pt* races 77 and 106 have more than 44,000 contigs (Kiran et al. 2016), while the *Pt* isolates 1–1 BBBD Race 1 and Pst78 were assembled into 24,838 contigs and 17,295 contigs, respectively (Cuomo et al. 2017), all with unresolved haplotypes. The advent of *long-read sequencing technologies presented an opportunity to accurately assemble and phase dikaryotic rust genomes*. In Table 17.2, we list the long-read-based genome assemblies that have been published to date, along with a rating (Platinum, Gold, Silver, Bronze) according to their completeness, phasing, and contiguity standards. The first attempts at haplotype phasing were carried out for oat crown rust (*Pca*) and wheat stripe rust (*Pst*) using PacBio FALCON-unzip assemblies (Miller et al. 2018; Schwessinger et al. 2018). In these assemblies, the primary contigs represent an unphased haplotype, while the haplotigs represent an incomplete, partially-collapsed haplotype. Wu et al. (2020) used a similar approach to generate a partially phased assembly for *Pt*, in which the primary contigs contained a high percentage of duplicated BUSCO genes (~12%), suggesting the haploid

Table 17.2 Summary of long-read-based genome assemblies for *Puccinia* species and assigned standard based on completeness, quality, and haplotype phasing

Species	Isolate name	Standard	Genome size	Assembly level	Haplotype assignment	Sequence technology	Assembly	Reference
P. triticina	Pt76	Platinum	$2n = 260$ Mbp	chromosome ($n = 18$)	nuclear phased	PacBio-HiFi	Canu, NuclearPhaser	Duan et al. (2022)
P. graminis f. sp. *tritici*	Pgt21–0	Gold	$2n = 176$ Mbp	chromosome ($n = 18$)	nuclear phased	PacBio	Canu, parental/HiC	Li et al. (2019)
P. coronata f. sp. *avenae*	Pca203	Gold	$2n = 210$ Mbp	chromosome ($n = 18$)	nuclear phased	PacBio	Canu, HiC	Henningsen et al. (2022)
P. graminis f. sp. *tritici*	Ug99	Silver	$2n = 176$ Mbp	contigs $n = 514$, N50 = 970kbp	nuclear phased	PacBio	Canu, parental	Li et al. (2019)
P. polysora	GD1913	Silver	$2n = 1.65$ Gbp	chromosome ($n = 18$)	haplo-separated	PacBio-HiFi	Canu	J. Liang (Pers Comm)
P. triticina	Pt64	Silver	$2n = 295$ Mbp[a]	chromosome ($n = 18$)	haplo-separated	PacBio	Falcon-unzip/ Falcon-phase, HiC	Wu et al. (2021a, b)
P. striiformis	Pst134 E16 A+ 17+ 33+	Silver	$2n = 167$ Mbp	chromosome ($n = 18$)	haplo-separated	Nanopore	Canu, HiC	Schwessinger et al. (2022)
P. coronata f. sp. *avenae*	12SD80	Bronze	$p = 99$ Mbp $h = 51$ Mbp	contigs $p = 603$, N50 = 270kbp	partially haplo-separated	PacBio	Falcon-unzip	Miller et al. (2018)
P. coronata f. sp. *avenae*	12NC29	Bronze	$p = 105$ Mbp $h = 61$ Mbp	contigs $p = 777$, N50 = 220kbp	partially haplo-separated	PacBio	Falcon-unzip	Miller et al. (2018)
P. striiformis f. sp. *tritici*	Pst104E	Bronze	$p = 83$ Mbp $h = 73$ Mbp	contigs $p = 156$, N50 = 1.5Mbp	partially haplo-separated	PacBio	Falcon-unzip	Schwessinger et al. (2018)
P. striiformis f. sp. *tritici*	PST130	Bronze	$p = 85$ Mbp $h = 66$Mbp	contigs $p = 151$, N50 = 1.4Mbp	partially haplo-separated	PacBio	Falcon-unzip	Vasquez-Gross et al. (2020)
P. striiformis f. sp. *tritici*	DK0911	Bronze	$p = 74$ Mbp $h = 52$ Mbp	contigs $p = 94$, N50 = 1.5 Mbp	partially haplo-separated	Pacbio	Falcon-unzip	Schwessinger et al. (2020)
P. triticina	Pt104	Bronze	$p = 140$ Mbp[a] $h = 128$ Mbp	contigs $p = 162$, N50 = 2.0 Mbp	partially haplo-separated	Pacbio	Falcon-unzip	Wu et al. (2020)
P.hordei	Ph560	–	~$1.3n = 207$ Mbp	contigs $n = 858$, N50 = 405 kbp	not separated, partially collapsed	Pacbio	SMARTdenovo	Chen et al. (2019)
P. striiformis f. sp. *tritici*	93–210	–	85 Mbp	contigs $n = 493$, N50 = 295 kbp	collapsed haploid	Pacbio	Metassembler (Canu, Falcon, Sparse)	Xia et al. (2018)
P. striiformis f. sp. *hordei*	93TX-2	–	77 Mbp	contigs $n = 562$, N50 = 218 kbp	collapsed haploid	Pacbio	Metassembler (Canu, Falcon, Sparse)	Xia et al. (2018)

[a]overestimate—contains duplicated content

genome size was overestimated. In all these assemblies, FALCON-unzip returned only partially phased assemblies where the primary and haplotig assemblies do not represent the two nuclear genomes but are a random mosaic of the two haplotypes.

The integration of Hi-C read sequencing into the assembly process has now resulted in *fully-phased* chromosome level assemblies for three rust fungi: the wheat stem rust fungus (*Pgt*) (Li et al. 2019), the wheat leaf rust fungus (*Pt*) (Duan et al. 2022) and the oat crown rust fungus (*Pca*) (Henningsen et al. 2022). The first of these was *Pgt*, where PacBio RSII long-read sequence data was assembled using Canu (Koren et al. 2017; Li et al. 2019), producing an almost fully diploid raw assembly. Gene synteny was used to identify paired sequences representing alternative haplotypes and parental information from a natural hybridisation event involving a single nucleus exchange between isolates allowed for the assignment of each sequence to a nuclear haplotype. A relatively small number of assembled contigs contained phase switches between the two haplotypes, but these could be corrected on the basis of the parental data. Hi-C chromatin contact information was used for scaffolding of contigs to chromosome level and revealed a unique and highly useful feature: there was a much higher number of read pairs indicating contact of DNA sequences within nuclei than between nuclei, producing *a strong signal for nuclear assignment*. This characteristic was used as a basis to develop an optimised pipeline (*NuclearPhaser*) for chromosome assembly and nuclear phasing based on long-read assembly and detection of phase information and phase switches using Hi-C chromatin contact data (Duan et al. 2022). In this case, both Nanopore MinION and PacBio HiFi sequence data were compared for assembly and phasing. Although the long Nanopore reads allowed assembly of large contigs and scaffolding to the chromosome scale, phase switches between the two nuclear haplotypes were highly prevalent throughout the assembly and were too numerous to allow accurate detection of boundaries and efficient phase switch correction. Thus, the currently high error rate of Nanopore sequence calling algorithms precludes nuclear phasing by this approach. However, the highly accurate PacBio HiFi reads were also sufficient for assembly and scaffolding to chromosome scale, despite the overall shorter read lengths compared to Nanopore data and contained only a very small number of phase switches within contigs. These were predominantly located at haplotig boundaries and could be readily corrected using the Hi-C contact information. The HiFi sequence assembly also accurately resolved genomic regions with low heterozygosity that were collapsed in the Nanopore assembly. Subsequently, we applied the same assembly pipeline to generate fully phased chromosome level assembly for an isolate of the oat crown rust pathogen, Pca203 (Henningsen et al. 2022). In this case, first-generation PacBio reads rather than HiFi reads were used, which resulted in more phase switches in the raw assembly compared to the leaf rust assembly, but a still manageable number for manual phase switch correction. The three fully assembled, phased genomes of *Pgt*, *Pca,* and *Pt* all contain 18 chromosomes per haploid genome but differ in haploid genome size (~86 vs ~104 vs ~134 Mbp, respectively), primarily due to differences in repeat element content. Nevertheless, they all show a *high level of gene synteny* despite low overall sequence identity (Henningsen et al. 2022). Likewise, a recent *Pst* assembly based on nanopore data also contains 18 chromosomes, although this was not nuclear phased due to the high error/phase switch rate in nanopore data (Schwessinger et al. 2022). Similarly, A Pacbio-HiFi/Hi-C based assembly of the maize southern corn rust *P. polysora* also contained 18 chromosomes that showed synteny with *Pgt*, *Pca* and *Pt*, despite being up to ten times larger in total genome size (Liang et al. 2022). Given the conserved chromosome number and high synteny between homologous chromosomes, we have proposed that a *uniform chromosome numbering* convention for *Puccinia* species should be established in which the chromosome number is assigned based on synteny to the equivalent chromosomes in *Pgt*, which was

the first chromosome level assembly generated (Li et al. 2019).

17.7 Conclusions and Perspectives

The *Puccinia* genus includes a large group of pathogens that infect diverse plant species and, as highlighted in this chapter, important cereals and grasses of the Poaceae family. From a food security and industrial perspective, the *Puccinia* species represent a challenge for disease management. All have histories of causing recurring epidemics that can be very severe in their agronomic impact, often due to the emergence of new strains with altered virulence or survival traits. Human activities associated with agriculture such as the plantation of monocultures, changes to native ecosystems and variations in climate patterns are among the factors contributing to the emergence of new rust strains or their proliferation in recent decades.

Progress in understanding the biology, particularly mechanisms of virulence and evolution of *Puccinia* species has been slow partly due to the lack or limited access to genetic and genomic resources. However, the underlying mechanisms of evolution have begun to be elucidated at the molecular level. Recent advances in DNA long read sequencing and acquisition of genome-wide three-dimensional data (Hi-C) coupled with sophisticated computational pipelines have provided a gateway to study the evolution of virulence in the *Puccinia* species. Advances such as haplotype-phased genome assemblies have enabled the characterisation of genetic variation in a few *Puccinia* species (*Pgt*, *Pca*, *Pt*, and *P. polysora*). Important technical and biological lessons have come from this research. It is now evident that substantial genetic variation can be present within the haplotypes of *Puccinia* species, and that somatic hybridisation can contribute to the emergence of new variants, including the devastating stem rust strain Ug99. This was only revealed by the very recent development of haplotype level genome assemblies, so we now question whether additional hybridisation events have also been involved in the emergence of other rust races and behind other epidemics. Future comparisons of haplotypes across *Puccinia* species and beyond will answer this question. However, we must bear in mind that high-accuracy sequencing platforms are required to prevent phase switches in genome assemblies that would be misleading during comparative analyses.

Sustainable agriculture demands crop protection strategies that effectively reduce the use of fungicides and protect our environment. Accurate and reliable in field pathogen surveillance, pathogen diagnosis, and virulence prediction are essential components for biosecurity programs and tailored responses to prevent rust disease and outbreaks. Monitoring disease agents such as *Puccinia* species, which are transmitted by airborne routes can be challenging. However, recent breakthroughs in sensor-based methods through sequencing and phenotyping technologies combined with advances in Modelling and Artificial Intelligence offer opportunities to develop robust and pathogen detection techniques as well as disease forecasting methods. Field-based sequencing platforms will benefit from the high-quality genomics resources that are now being generated as well as the creation of an inventory of *Avr* allelic variants and associated phenotypes for *Puccinia* species. The identification of Avr effectors and their allelic variants bring the opportunity to revolutionise biosecurity programs. In comparison to other fungal pathogens, the discovery of Avr effectors in rust fungi has also been slow. As more chromosome-level assemblies become publicly available, the community would have the capacity to expand the *Avr* gene catalogue of *Puccinia* species and develop the tools for detailed molecular pathotyping.

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Magnaporthe oryzae and Its Pathotypes: A Potential Plant Pandemic Threat to Global Food Security

18

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Abstract

Food security for the growing world population can be affected by many different socio-economic and food production variables including pest outbreaks. Plant disease epidemics historically played a significant role in the starvation and displacement of the world population. Despite huge progress made by researchers in managing diseases of staple commodities, the threat level remains very high, as disease-causing organisms adapt to new hosts, become more virulent by changing their genetic makeup, and show increased resistance against fungicide products. The history of blast disease, which affects one of the world's staple foods, rice, goes back centuries and has been a continual problem for rice production worldwide with the recent inclusion of wheat blast. Extensive studies on the biology, pathogenicity, and population genetics of *Maganaporthe oryzae* (Synonym: *Pyricularia oryzae*), the causal agent of blast disease, have enriched our understanding of the potential threat that this pathogen poses to rice and wheat production, and therefore world food security. Based on host specificity, mating ability, and genetic relatedness, *M. oryzae* is divided into several subgroups or pathotypes (different crop-adapted lineages). The genome structure of *M. oryzae*, characterized by instability, parasexual recombination, and the presence of transposon elements, enabling this pathogen to evolve rapidly and jump from one host to another, has raised real concerns for scientists, growers, and food policy makers. All available options such as forecasting and mapping of disease and pathogen race distribution, early and reliable quick diagnostics, biological and chemical control measures, inclusion of cultivars with resistance genes, and development of blast-resistant variety using CRISPR-Cas genome editing should be considered and deployed as a package for successful control of *M. oryzae*.

Keywords

Rice blast · Wheat blast epidemic · *Magnaporthe oryzae* · Climate change · Host-jump · CRISPR-Cas technology · Disease resistance

18.1 Introduction

Food security for the increasing world population is firm on the agenda of the World Food Program of the United Nations. This agenda item is facing

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considerable scrutiny, as one-in-four people globally are moderately or severely affected by food security. Food security is of fundamental importance for human existence. For a healthy and active life, all people should have economic and physical access to sufficient safe and nutritious food at all times according to their food preferences to meet daily dietary needs (FAO 2003). The world population has been increasing steadily till now and is estimated to reach over 9 billion by 2050 (FAO 2009; Godfray et al. 2010). Consequently, the demand for food is expected to increase up to 50% by 2050 (van Dijk et al. 2021), and agricultural production needs to improve by 100% and 70% in the developing countries and globally, respectively, by 2050 on currently cultivated land compared to that in 2009 (FAO 2011). Meanwhile, frequency and intensity of extreme weather events and the outbreaks of different biotic stresses have been increasing due to global climate change, which affects the yield and quality of crops including staple grains. This opposing trend between crop production and demographic demand may further worsen global food security (Deng et al. 2017). The effect of global warming on crop disease intensity on a global scale has been projected by Chaloner and colleagues (Chaloner et al. 2021). Approximately 8000 species of fungi and oomycetes have been shown to cause plant disease to date, and some of them cause significant yield loss of grains (Fisher et al. 2020; Saunders 2021). It has been documented that over the last decades, food security has further been jeopardized worldwide by new biotic stresses, especially the outbreaks of fungal diseases (Fisher et al. 2012, 2018, 2020; Islam et al. 2016). Among them, blast is the most devastating and widespread disease caused by the haploid, ascomycete fungus *Magnaporthe oryzae* B. Couch (Synonym *Pyricularia oryzae*). It infects more than 50 species from the Poaceae family, which includes rice, wheat, maize, finger millet, and pearl millet, and destroys enough food supply that could sustain millions of people (Ou 1985; Sesma and Osbourn 2004; Wilson and Talbot 2009; Pennisi 2010; Liu et al. 2014; Islam et al. 2016, 2020; Fishers et al. 2012, 2020; Pordel et al. 2021). Rice blast destroys each year enough rice to feed 60 million people (Skamnioti and Gurr 2009). However, strategies for economically feasible and sustainable management of blast diseases remain elusive.

Although rice (*Oryza sativa* L.) has been the main host of *M. oryzae* (Saleh et al. 2014; Zhang et al. 2016), one of its pathotypes can cause disease in wheat (*Triticum aestivum* L.) based on reports from South America, Africa and South Asia (Igarashi et al. 1986; Islam et al. 2016, 2019, 2020; Tembo et al. 2020). Although the blast-causing fungal pathogen is distributed all over the world, it is mostly host-specific at the pathotype level (Ou 1985; Gladieux et al. 2018). Globalization of food production systems has led to high volumes of international trade of plants and plant products, through which pathogens can readily disperse to new areas (Fisher et al. 2012). One recent example is the introduction of the deadly wheat blast disease into Bangladesh, likely through contaminated wheat grain import from Brazil (Ceresini et al. 2018), which has caused yield losses of up to 100% (Islam et al. 2016).

The distinct pathotypes of the hemibiotrophic fungal pathogen, *M. oryzae*, are regarded as severe threats to millions of hectares of rice, wheat, maize, pearl millet, and finger millet growing areas all over the world due to its potential of severely affecting productivity and quality of these cereal crops (Goulart and de Paiva 1992; Goulart et al. 2007; Kohli et al. 2011; Maciel 2011; Islam et al. 2016; Chakraborty et al. 2020a, b; Islam et al. 2020; Chakraborty et al. 2021; Jacob 2021; Nayaka et al. 2021; Pordel et al. 2021). More importantly, it is now well proven that the blast pathogen has the potential to cause an epidemic on plant species by jumping to hosts outside its primary host range (Tosa et al. 2004; Inoue et al. 2017; Maekawa and Schulze-Lefert 2017). Nevertheless, disease intensity and extent of damage may vary from place to place and year to year, mostly influenced by the changing weather conditions, time, and crop stage of initial infection (Ceresini et al. 2018; Islam et al. 2019, 2020; Hosahatti et al. 2021). Some good reviews specifically on rice and wheat blast

diseases have recently been published (Wilson and Talbot 2009; Cruz and Valent 2017; Ceresini et al. 2018, 2019; Islam et al. 2019, 2020; Valent 2021; Valent et al. 2021). This chapter provides a comprehensive review of our understanding of the biology and genomic events taking place during the development of a new pathotype of the blast pathogen, and ways it can be sustainably managed to ensure food and nutritional security for an ever-increasing world population.

18.2 History of Blast Disease Outbreaks

The blast pathogen was initially designated as *P. oryzae* by Cavara in Italy (Cavara 1892) and later on by Shirai (1896) in Japan. However, the name *P. oryzae* refers to the asexual stage of the blast fungus, *M. oryzae*. In 1673, a blast-like disease was first reported on rice by Soong Ying-Shin in China (Ou 1985; Manibhushanrao 1994). Later, it was reported for the first time in Japan by Tsuchiya in 1704 (Goto 1955). The presence of rice blast in Brazil has been reported since 1912 (Averna-Sacca 1912). The disease was reported several centuries ago in Asia and spread all over the rice-growing areas of the continents (Tharreau et al. 2009). Phylogenetic analysis indicates that the rice and foxtail millet population of blast originated due to a host shift from a *Setaria* population about ~7000 years ago (during early domestication) in the middle of the Yangtze valley in China (Couch et al. 2005). Typical symptoms of rice blast disease in the field conditions are shown in Fig. 18.1.

In contrast, the occurrence of wheat blasts is relatively recent and for the first time was detected in the Brazilian state of Paraná in the mid-1980s (Igarashi et al. 1986) and from there spread fast to the warmer and humid zones of central and southern Brazil. In 2002, wheat blast was found in Paraguay (Viedma 2005), and in 2007 in the province of Formosa in north-eastern Argentina (Cabrera and Gutiérrez 2007; Perelló et al. 2015). The pathogen spread to eastern Bolivia in 1996 (Barea and Toledo 1996). A large-scale epidemic (100% head infection of wheat) was reported for parts of the northern Paraná State in 2009. This epidemic affected approximately one-third of the total wheat-growing acreage in Brazil and attracted global attention on the potential danger to global wheat production (Duveiller et al. 2016). Important wheat-producing areas of Argentina were threatened by blast disease in 2012. Later, it was confirmed in Buenos Aires Province (Perelló et al. 2015). Wheat blasts appeared for the first time in Asia in 2016 with reports of severe outbreaks in Bangladesh (Islam et al. 2016; Islam 2018). It affected more than 15,000 ha of wheat located in eight districts of south-western and southern parts of the country with up to 100% yield reduction (Callaway 2016; Islam et al. 2016; Malaker et al. 2016). Within two years of the outbreak in Bangladesh, wheat blast was reported from Zambia in 2018, where growers experienced severe disease in the rainfed production system (Tembo et al. 2020).

Blast was reported on finger millet [*Eleusine coracana* (L.) Gaertn. subsp. *Coracana*] in Somalia (Mohamed 1980), Malaya (Burnett 1949), Tanzania (Kuwite and Shao 1992), Zambia (Muyanga and Danial 1995), Sri Lanka (Park 1932), Nepal (Thompson 1941), Ethiopia, Kenya, and Uganda (Dunbar 1969; Adipala 1992). It is a severe disease of pearl millet in the Southern Coasts of the USA (Wilson and Gates 1993). Blast disease on the first domesticated grain crop, foxtail (Italian) millet (*Setaria italica*) (Sharma et al. 2013), as well as perennial ryegrass (*Lolium perenne*) (Tosa et al. 2004) and Italian ryegrass (*L. multiflorum*) (Xue et al. 2017) has also been reported.

18.3 Pathotype Delineation Among *M. oryzae* Isolates

Despite morphological identity among *M. oryzae* pathogens infecting different graminaceous hosts, there is genetic variation among them that enables infection of specific host groups. Extensive phylogenetic analyses of different fungal pathogen groups have been performed that revealed larger phylogenetic divergence among pathogens of

Fig. 18.1 Blast disease symptoms in rice. (**a**) Break down of infected panicles and entire inflorescences due to neck blast (arrows). (**b**) Severe leaf blast symptoms (**c**) Typical eye-shaped lesion (arrows) on rice leaf. (**d**) Completely bleached panicles from the neck region (arrow)

grasses compared to other diverse hosts (Gladieux et al. 2018). As such, based on host specificity, mating ability, and genetic relatedness, *M. oryzae* was divided into several subgroups or pathotypes (Urashima et al. 1993; Kato et al. 2000; Gladieux et al. 2018). For example, the *Oryza* pathotype is pathogenic on rice (*O. sativa*), the *Setaria* pathotype infects foxtail millet (*S. italica*), the *Panicum* pathotype infects common millet (*P. miliaceum*), the *Eleusine* pathotype infects finger millet (*E. coracana*), and the *Digitaria* pathotype infects crabgrass (*D. sanguinalis*) (Kato et al. 2000). The *Triticum* pathotype is pathogenic on wheat (*T. aestivum*), triticale (× *Triticosecale*), barley (*Hordeum sativum*) (Urashima et al. 2004), rye (*Secale cereale*), durum wheat, signalgrass (*Urochloa brizantha*) and more than 10 other grass species (Urashima et al. 1993; Castroagudín et al. 2016). While the *Avena* pathotype is pathogenic on oats, and the *Lolium* pathotype is pathogenic on perennial ryegrass (Oh et al. 2002). *Avena* and *Lolium* pathotypes can infect wheat as well (Oh et al. 2002; Tosa et al. 2004; Farman et al. 2017). Diversity of pathotypes in *M. oryzae* as deduced by Gladieux et al. (2018) is shown in Fig. 18.2.

A number of phylogenetic analyses since 2010 have reexamined the association between *Pyricularia*-like species and *Pyricularia*, resulting in significant alterations to the grouping of fungi within the order Magnaporthales (Hirata et al. 2007; Choi et al. 2013; Luo and Zhang 2013; Klaubauf et al. 2014; Murata et al. 2014). Interestingly, taxonomists have proposed that the name *Magnaporthe* spp. be replaced by *Pyricularia* spp. thereby eliminating the genus *Magnaporthe* totally (Luo and Zhang 2013; Klaubauf et al. 2014; Murata et al. 2014). Moreover, previous multi-locus phylogenetic analysis of *Pyricularia* species by Couch and Kohn (2002) using portions of actin, beta-tubulin, and calmodulin genes classified *P. grisea* (Hebert) Barr. species into two different clades. One clade is *P. oryzae*, associated with cereals (cultivated grasses) and another clade is *M. grisea*, related with the grass genus *Digitaria* (Couch and Kohn 2002). A recent multi-gene-based phylogenetic study of 128 *Pyricularia* spp. collected from sympatric populations of wheat, rice, and grasses growing in or near wheat fields identified the existence of *P. graminis-tritici* (Pygt), a new blast species associated with wheat and several

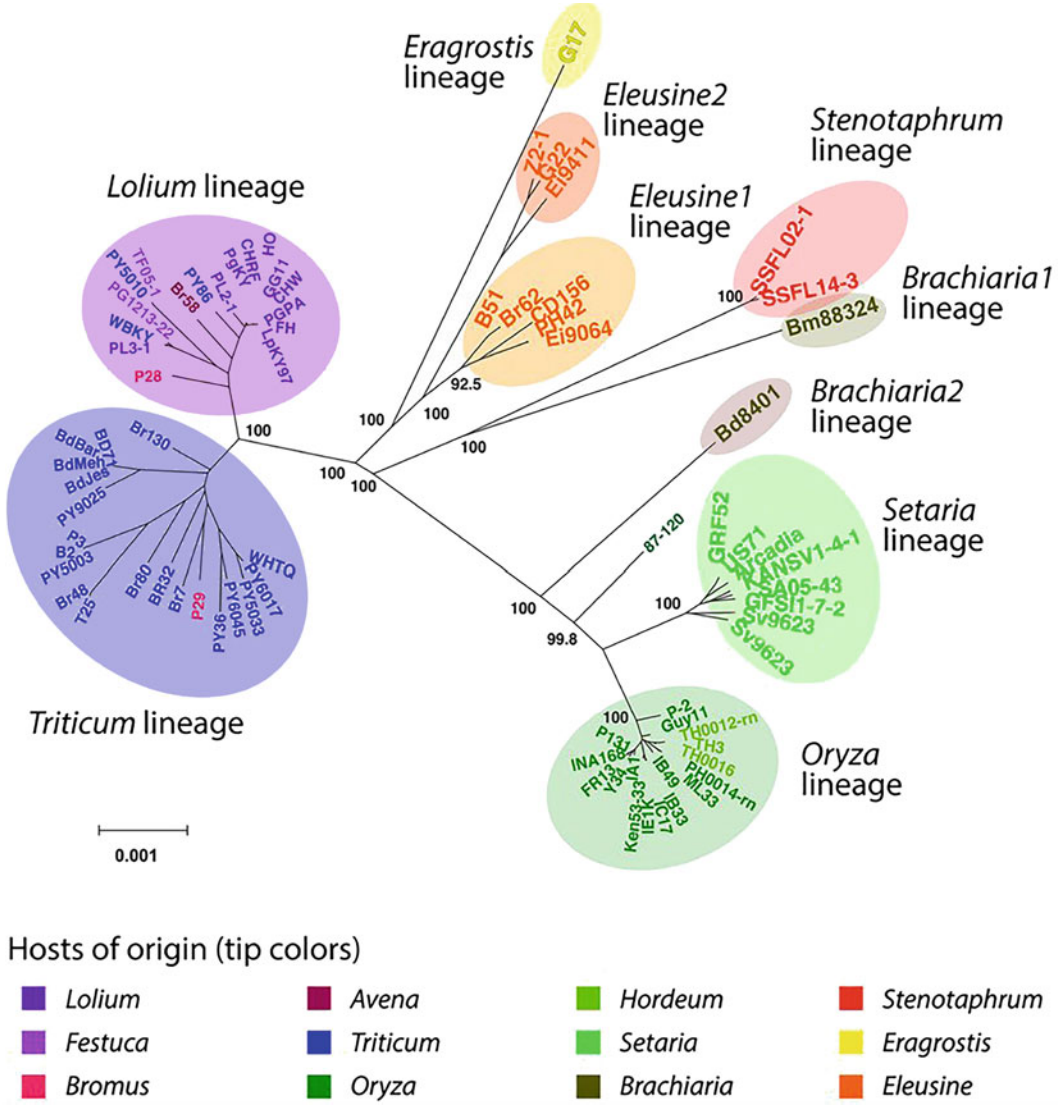

Fig. 18.2 Various lineages or pathotypes in blast fungus *Magnaporthe oryzae* (reproduced from from Gladieux et al. 2018)

other hosts (Castroagudín et al. 2016). However, Valent et al. (2019) argued that Pygt is not the correct species name for the wheat blast fungus. A report from Gladieux et al. (2018) revealed that blast pathogen genotypes associated with rice and wheat aligned in separate clades with significant variation.

18.4 Biology of the Pathogen

18.4.1 Sexual Reproduction

The sexual (teleomorphic) stage of the blast pathogen is elusive and has not been observed in nature. However, some members are able to reproduce sexually *in vitro* (Yaegashi and

Udagawa 1978). Hebert (1971) was the first to report the perfect or sexual stage of the blast pathogen by crossing two isolates originating from crabgrass. *M. oryzae* is recognized as a heterothallic Ascomycete where mating is controlled by two different alleles of a single locus, *MAT1*. Consistent with sexual reproduction in other Ascomycetes, the blast fungus produces ascospores in asci that are found within a specialized structure called a perithecium with long cylindrical necks (Yaegashi and Udagawa 1978). Perithecia and asci within can be produced *in vitro* by incubating opposite mating types on oatmeal agar under light at about 20 °C for 2–3 weeks. Perithecia are dark brown to black in color, are produced singly or in groups, and have a diameter of 60–300 μm. Unitunicate asci inside perithecia contain hyaline, crescent-shaped ascospores measuring 16–25 × 4–8 μm, each with three septa, four cells, and a haploid nucleus (Valent et al. 1986). It has been shown that sexual spores of blast pathogens were capable of infecting rice, wheat, turf grass, and finger millet (Tredway et al. 2003; Consolo et al. 2005; Le et al. 2010; Takan et al. 2012; Saleh et al. 2012; Maciel et al. 2014). Therefore, sexual recombination appears to be an underlying factor for high genetic variability in the blast pathogen, and the emergence of new pathotypes involved in causing diseases on new hosts through host shifts. The underlying molecular mechanism of sexual reproduction in blast fungus will be an interesting topic for ongoing research.

18.4.2 Asexual Reproduction

The asexual or imperfect stage of *M. oryzae* is known as *P. oryzae*. Generally, the mycelium of *M. oryzae* is branched, and septate with mostly uninucleate hyphae. Conidiophores are mostly unbranched, produced as single or as multiple fascicles that are grayish in color. The slender, septate, denticulate conidiophores bear conidia at their tips. The conidia are produced in succession, one at a time (Agrios 2005; David et al. 2012). Although it produces both macro- and microconidia asexually, the production of macroconidia is most common. The cells of conidia are uninucleate, containing two large and two small chromosomes in each nucleus. Conidiophores produce conidia under high humidity, which are then dispersed by wind on the same or to neighboring plants. Microconidia of *M. oryzae* were first detected by Kato et al. (1994), and are produced from dark pigmented phialides. Microconidia are uninucleate, non-septate, hyaline, and lunate with a thin cell wall. A mass of microconidia can accumulate at the apex of the phialide followed by continued growth and splits. Mature microconidia are crescent-shaped (Kato et al. 1994).

18.4.3 Disease Cycles

M. oryzae can attack plants regardless of the developmental stage, and can infect all parts including panicles, nodes, stems, and leaves. The three-celled conidium of *M. oryzae* attaches to the leaf cuticle by means of an adhesive that initiates the infection process (Subramanian 1968; Talbot 2003; Chakraborty et al. 2021). This adhesive is usually released during hydration from an apical compartment at the conidial tip (Hamer et al. 1988). Upon germination, a narrow germ tube is produced followed by flattening to a hook at its tip and subsequently differentiating into an appressorium that is usually dome-shaped (Talbot 2003). As the single-celled appressorium matures, the conidium starts to collapse and dies through autophagic cell death. The necessity for autophagic cell death in the rice blast fungus is well-established (Veneault-Fourrey et al. 2006). The appressorium at this stage creates enormous turgor pressure on the plant surface through melanization. Formation of a narrow penetration peg at the base of the appressorium coupled with physical force helps to puncture the cuticle and allow entry into the epidermis. Bulbous, invasive hyphae subsequently invade the plant tissue by a process of invagination at the cell membrane of epidermal cells (Kankanala et al. 2007). The details of the cell and developmental biology of plant infection by the rice blast fungus *M. oryzae* have recently been illustrated by Eseola et al.

(2021) and Cruz-Mireles et al. (2021a, b) (Fig. 18.3). The penetrating hyphae of blast fungus releases effectors into the plant cells from their cap-like biotrophic interfacial complex (BIC) structure (Kankanala et al. 2007; Mosquera et al. 2009; Giraldo et al. 2013). Effectors are virulence determinant secretory proteins of pathogenic fungi. Some previously identified effector proteins secreted by *M. oryzae* to suppress host resistance are *Avr-Pita*, *Avr-Pii*, *Avr-Piz-t*, *Pwl1*, *ACE1*, *Bas1–4*, *Slp1*, and *Mc69* which target multiple components of host immunity (Mentlak et al. 2012; Liu et al. 2013; Chen et al. 2014). Furthermore, concurrent phytotoxin secretion by *M. oryzae* helps successful biotrophic fungal growth within the host cells (Iwasaki et al. 1969; Yun et al. 2015; Jacob et al. 2017). A large accumulation of reactive oxygen species (ROS) is induced by fungal infection, which triggers host cell death and formation of necrotic lesions thereby facilitating further fungal growth in those cells (Gupta et al. 2021). After the death of the host, *M. oryzae* usually overwinters on alternative hosts such as weed grasses followed by dissemination to new host plants by dewdrop splash, wind, infected seeds, and/or crop residues (Ou 1985; Urashima et al. 1999; Talbot 2003; Wilson and Talbot 2009; Valent et al. 2021).

18.5 Blast of Major Cereals: Wheat and Rice

Although blast used to be a major disease of rice, wheat blast caused by a new pathotype, *M. oryzae Triticum* (MoT), has emerged as a new serious threat to wheat production worldwide (Islam et al. 2016, 2020; Islam 2018; Islam and Kamoun 2018; Tembo et al. 2020). It can reduce the yield of wheat by up to 100% (Islam et al. 2016). An appreciable amount of research has been directed to understand the development and spatiotemporal spread of wheat blast, which is posing a significant risk to food security as wheat is the third largest cereal grown in the world (FAO, http://faostat.fao.org). It is now considered a long-term threat to wheat production in South Asian countries, especially in Bangladesh with the potential to spread to other major wheat-growing countries in the region (Islam et al. 2016, 2019, 2020). The recent occurrence of this disease in an African country Zambia is also a big concern for the food security of that continent (Tembo et al. 2020).

The wheat blast-causing pathotype of *M. oryzae* is predominantly a spike disease, however, it can attack all aerial plant parts of wheat (Igarashi et al. 1986, Islam et al. 2020), although leaf infection has been reported only from highly susceptible wheat varieties (Maciel 2011; Duveiller et al. 2016). Infected seeds followed by airborne spores are thought to be the primary sources of infection and the fungus can survive in infected crop residues (Urashima et al. 1999). Spike infection is the most conspicuous symptom of this disease in a wheat field although it can sometimes be confused with Fusarium head blight (FHB) infection. Careful observation is required to differentiate the symptoms of these two destructive diseases in the field (Duveiller et al. 2016). The blast pathogen infects the base or upper part of the rachis of the wheat plant rather than individual spikelets, which is common in FHB infection. Typical wheat blast symptoms on spikes are premature bleaching of spikelets above the infection site and blackish bright spots (arrow in Fig. 18.4d) on the rachis (Igarashi 1990; Urashima 2010; Islam et al. 2016, 2019, 2020). Head infections during the grain filling stage may produce small, deformed, shriveled, lower-weight, and discolored seeds (Islam et al. 2016, 2020; Surovy et al. 2020). Necrotic lesions containing gray centers, of typical eye shape, are observed on the leaves of a highly susceptible variety in severely infected wheat fields (Igarashi 1990; Cruz et al. 2015; Islam et al. 2016; Valent et al. 2021). Characteristic symptoms of wheat blast disease are shown in Fig. 18.4.

Rice blast causes 66 billion US$ global loss annually, which is estimated as loss of enough rice to feed 60 million people (Pennisi 2010). It is the second largest cereal crop produced in the world after corn (FAO, http://faostat.fao.org). Rice blast is concerning not only to people who depend on rice as the staple food, but it also creates a threat to export markets since it is a

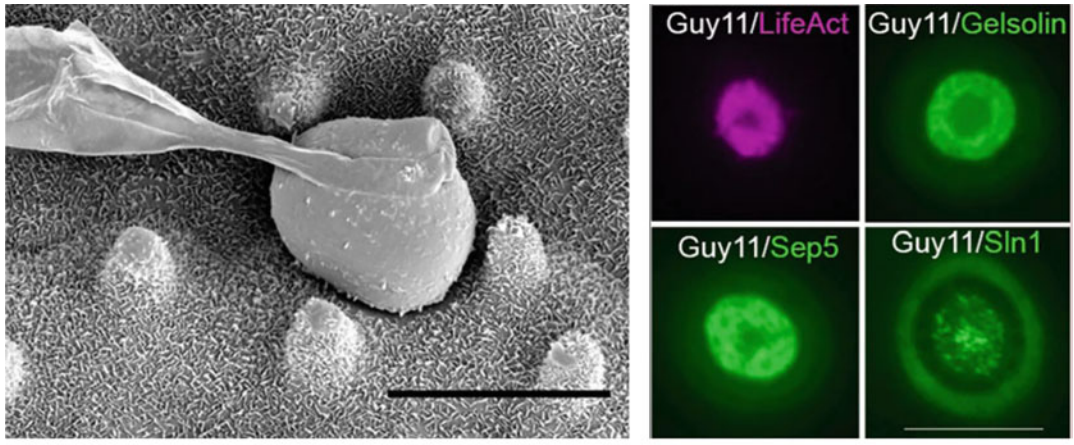

Fig. 18.3 Scanning electron micrograph of a dome-shaped appressorium in the rice blast fungus. (**a**) Scanning electron micrograph showing an appressorium on the rice leaf surface. The conidium has already collapsed due to autophagic cell death. Bar = 10 μm. Micrograph by Kim Findlay, John Innes Centre Bio-Imaging. (**b**) Reorganization of the fungal cytoskeleton at the appressorium pore. An F-actin toroidal network can be visualized by expression of LifeAct-mCherry and Gelsolin-GFP. This is scaffolded by a septin ring, shown here by expression of Sep5-GFP. The turgor sensor kinase Sln1-GFP localizes to the appressorium pore. Bar = 10 μm (Adapted from Cruz-Mireles et al. 2021b)

seed-borne disease (Nalley et al. 2016; Asibi et al. 2019; Hosahatti et al. 2021; Yin et al. 2021). *M. oryzae* pathotype *Oryzae* (MoO) infects all aboveground parts of rice plants at any growth stage. Due to some typical characteristics, MoO is recognized as a model experimental fungus for research on host-pathogen interactions. The cell biology of pre- and post-infection development of this fungus has been well documented (Hamer and Talbot 1998; Saunders et al. 2010; Yan and Talbot 2016; Eseola et al. 2021; Cruz-Mireles et al. 2021a, b). The morphogenetic basis of the host cell invasion (from appressorium to transpressorium) by the blast fungus has been documented through live cell imaging in rice (Cruz-Mireles et al. 2021b). Appressorium development at the tip of the germ tube is dependent on three important prerequisites. First, the *M. oryzae* Pmk1 mitogen-activated protein kinase (MAPK) signaling pathway is activated in response to pathogen interaction with the hard hydrophobic surface of the plant (Dean 1997). A well-coordinated phosphorylation cascade is then triggered by sensory proteins that involve three levels of MAPKs including Mst11 (mitogen-activated protein kinase kinase kinase, MAPKKK), Mst7 (mitogen-activated protein kinase kinase, MAPKK), and Pmk1 (MAPK). The three-celled conidium finally undergoes autophagy and an iron-dependent programmed cell death process called ferroptosis, followed by trafficking of its contents to the appressorium (Veneault-Fourrey et al. 2006; Shen et al. 2020). Leaf blast becomes visible as very small brown-colored lesions on the leaf surface followed by elongation over time and then changes into a spindle-shaped structure measuring several centimeters long and about 0.5–1.0 cm wide. A susceptible cultivar under favorable conditions, starts showing more lesions from the initial infection or by secondary spread. Rapidly enlarging lesions can merge together leading to complete drying of the leaf and even the whole plant (Ribot et al. 2008; Zewdu 2021). Node blast usually starts from infection of the lower nodes of plants. Brown to black patches may appear on diseased rachis and glumes, especially where the pathogen remains on branches. In addition, panicle blast initiates at the rice booting stage. Infection of the neck region is considered the most damaging as infected panicles and entire

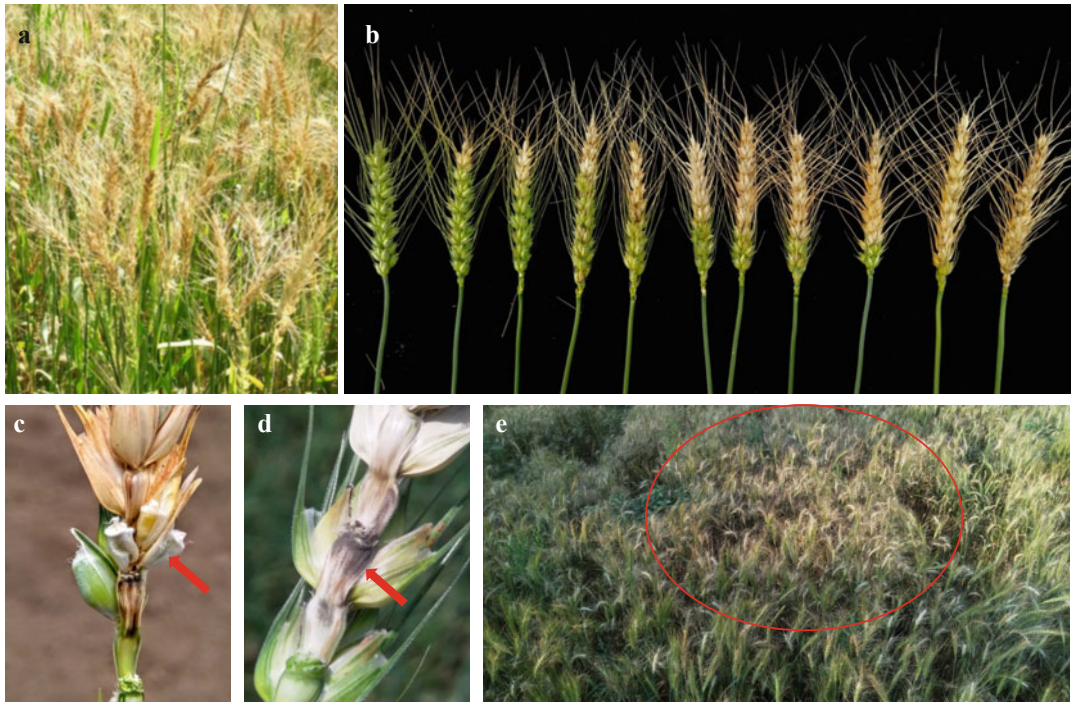

Fig. 18.4 Blast disease symptoms in different parts of wheat plant. (**a**) Blast-affected field showing silvery bleached spikes with green canopy. (**b**) Complete to partial infections (right to left) in wheat spikes collected from blast-affected field. (**c**) Two shriveled grains (whitish color, arrow) and a normal (green)-colored unaffected grain just below the infection point in a spike. (**d**) Dark-gray sporulation (arrow) of the fungus MoT on the rachis. (**e**) A characteristic patch (see inside the circle) of blast infection indicating the early stage of field infection

inflorescences frequently break down causing a total loss. As a result, grain development is hampered above the infected region and looks whitish from a distance (Valent 2021). Under favorable conditions, spore germination occurs rapidly with a chance of secondary infection and spread. This makes blast disease polycyclic where the infection cycle is repeated many times throughout the growing season depending on the crop stage of initial infection (Fig. 18.5) (Park et al. 2009; Yang et al. 2011).

18.6 Molecular Cross-talks between Host and *Magnaporthe oryzae*

The molecular cross-talks between the wheat plant and MoT that determines the outcome of infection and disease development are poorly understood. However, the rice-MoO pathosystem has been studied extensively as described in the earlier section. Upon entry into the plant tissue, *M. oryzae* produces the mycotoxin tenuazonic acid, an antibiotic that suppresses host immunity and also a wide range of phytotoxic secondary metabolites for successful biotrophic growth (Nukina 1999; Kankanala et al. 2007; Patkar et al. 2015; Yan and Talbot 2016). At the beginning of fungal attachment with the host, the cell wall acts as a barrier. During germination, cell division and cell stress responses are governed by the mitotic exit network (MEN) and the cell wall integrity (CWI) pathways, respectively. The cell wall integrity pathway component, MoMkk1 (a component of MAPK), is phosphorylated initially by the cell cycle-related kinase MoSep1, which links MEN with CWI signaling. The MEN and CWI cross-talk is mediated by

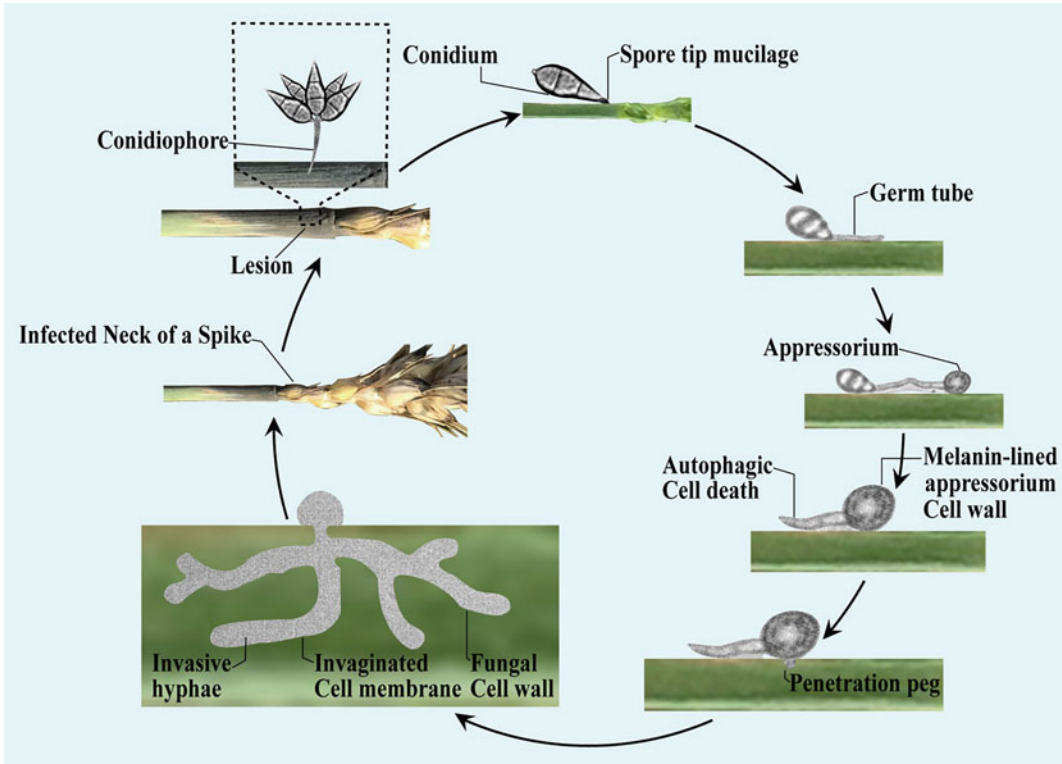

Fig. 18.5 Putative wheat to wheat infection cycle of *Magnaporthe oryzae Triticum* (MoT) pathotype (Overwinters in alternate host plants like weed grasses, spreads through wind, dewdrop splashes, infected seeds and/or infected crops residues to new host plants). Scale bar not used (Adapted from Chakraborty et al. 2021)

MoSep1-MoMkk1 to determine the virulence of *M. oryzae*. This finding indicated that the initial growth and pathogenicity of blast fungus is mediated by CWI in response to cell cycle-related stress (Feng et al. 2021). The trehalose-6-phosphate synthase 1 (Tps1)-mediated genetic switch in *M. oryzae* controls the primary metabolic change during appressorium development in response to glucose-6-phosphate levels and the NADPH/NADP balance of the cells (Badaruddin et al. 2013). It has been observed that two novel transcription factors Som1 and Cdtf1 are required for appressorium formation and sporulation (Liu et al. 2011). Appressorium development is interrupted in the *Mst1* mutant due to a delay in the deposition of lipid bodies and transportation of glycogens to the appressorium, which is regulated by cyclic adenosine monophosphate (cAMP) signaling (Soanes et al. 2012). It appears that cross-talk between the blast pathogen and the plant cell occurs by way of the cAMP pathway through the G-subunit protein MagB. The G proteins MagA and MagB appear to modulate the cAMP response pathway by interacting with the Pth11 G protein-coupled receptor. A putative chitin-binding protein with a signal peptide encoded by the *Cbp1* gene plays a critical role in host surface recognition and development of a normal functional appressorium on the leaf surface and an abnormal appressorium on artificial surfaces (Kamakura et al. 2002; Liu et al. 2011).

The second phase of cross-talk occurs immediately after the pathogen penetrates the cell. In general, plants possess an efficient resistance response system to counteract pathogen infection, which can be influenced by a variety of factors. Plants elicit a pathogen-associated molecular pattern (PAMP)-triggered immune response

immediately after detecting the conserved PAMP of the infecting pathogen (Zipfel and Felix 2005). There is also effector-triggered immunity (ETI) that is activated by cognate intracellular immune receptors (Jones and Dangl 2006; Kamoun 2009). Both types of immunity (PAMP-triggered immunity [PTI] and ETI) play a vital role in plant pre-invasive and post-invasive resistance, respectively to prevent the colonization of cells by invading pathogens (Lee et al. 2009; Chen and Ronald 2011; Liu et al. 2012; Li et al. 2014).

Among the many plant-produced biochemicals, jasmonic acid (JA) is an important compound that incites plant defense responses against pathogens. The underlying mechanism of JA production involves conversion of α-linoleic acid to hydroxyoctadecadienoic acid, which is influenced by *M. oryzae* infection and colonization of the host plant (Zhang et al. 2018). Likewise, some specific microRNA (miRNA) can play a significant role in providing immunity against blast pathogens, since biotic stress can activate miRNA in plants (Zhai et al. 2011a; Khraiwesh et al. 2012; Li et al. 2017). Li et al. (2014) reported that overexpression of miR160a and miR398b enhanced the resistance of rice plants against *M. oryzae*. A wide array of genes and gene products are involved in the cross-talk between host and *M. oryzae* from initial contact to expression of disease symptoms (Valent 2021). However, further study is necessary to gain a better understanding of the molecular mechanisms that determine the outcomes of different pathotypes of *M. oryzae* and host interactions.

18.7 Host Species Specificity

Multiple pathotypes with limited host-specificity in the blast fungus are well recognized. The evolutionary biology related to the development of the wheat blast fungus pathotype MoT through a host-jump has been revealed (Inoue et al. 2017). Several examples of single gene-regulated host specificity in *M. oryzae* have been described since the initial report by Yaegashi in 1978 (Yaegashi 1978). For example, the *PWL1* and *PWL2* genes in finger millet and rice blast pathogens, respectively (Kang et al. 1995; Sweigard et al. 1995) prevent these strains from infecting weeping lovegrass (*Eragrostis curvula*). Tosa and co-workers identified five avirulence (*AVR*) effector genes (*PWT1–5*) in the population of rice, oat, and Setaria pathogens that independently block infection of wheat (Murakami et al. 2000; Tosa et al. 2006; Takabayashi et al. 2002). These effector genes seem to play a very important role in host specificity as Inoue et al. (2017) reported that loss of the *PWT3*/*Rwt3* gene interaction may have contributed to the first emergence of wheat blast in Brazil. Additional circumstantial evidence is provided by the fact that wheat varieties planted before the 1980s contained the *R* (resistance) gene *Rwt3*, which may have blocked *Lolium* and oat isolates with the corresponding *PWT3* gene from infecting wheat. But in the 1980s, new *rwt3* wheat varieties lacking this *R* gene were planted in a new region of Brazil where weather was conducive to blast (Igarashi 1990; Inoue et al. 2017). Subsequent mutation in *M. oryzae* resulted in loss of *PWT3* function allowing the emergence of a *Triticum* population capable of causing disease on all wheat varieties. However, all differences in host species specificity are not likely due to *AVR*-like genes. Further studies are needed to precisely elucidate the underlying molecular mechanisms of host-specificity and host-range of *M. oryzae* pathotypes, and factors involved with the host-jumps.

18.7.1 Effectoromics of *M. oryzae*

In addition to PTI, host plants have evolved a second line of defense called ETI, which includes the recognition of specific *AVR* effectors by plants. The presence of matching Avr-R protein pairs can lead to programmed cell death with the subsequent development of a distinct hypersensitive response (HR) in the host (Dai et al. 2021). The blast fungus produces a wide array of effector proteins to overcome host resistance and successfully infect and colonize hosts (Table 18.1) (Yan and Talbot 2016). Due to the arms race between

Table 18.1 Major features of rice blast fungus effectors

M. oryzae effector	Molecular mechanism for gain of virulence	Function	Reference
Avirulence effectors			
Avr-Pib	·	Recognized by *R* protein *Pib*	Wang et al. (1999); Zhang et al. (2015)
Avr-Pita1/ AVR-Pita2	*AVR-Pita1*: (i) Point mutations in coding sequence; (ii) several deletions from 100 bp to >10 kb; (iii) 1.8 kb insertion of Pot3 transposon in promoter. *AVR-Pita* family members flanked by transposable elements	Recognized by *R* protein *Pi-ta*	Bryan et al. (2000); Orbach et al. (2000)
Avr-Pi9	·	Recognized by *R* protein *Pi9*	Qu et al. (2006); Wu et al. (2015)
Avr-Piz-t	Suppresses Bax-programmed cell death	Recognized by *R* protein *Piz-t*	Li et al. (2009)
ACE1	Insertion of 2 kb Line1-like retrotransposon in last exon	Recognized by *R* protein *Pi33*	Böhnert et al. (2004); Collemare et al. (2008)
Avr-Pii	·	Recognized by *R* protein *Pii*	Yoshida et al. (2009)
Avr-Pik/ km/kp	·	Recognized by *R* protein *Pik/km/kp*	Yoshida et al. (2009)
Avr-Pia	·	Recognized by *R* protein *Pia*	Yoshida et al. (2009)
Avr1-CO39	Deletion of 19 kb surrounding gene, transposable elements define borders of deletion	Recognized by *R* protein *Pi-CO39*	Farman et al. 2002; Ribot et al. (2012)
Avr-Pi54		Recognized by *R* protein *Pi54*	Ray et al. (2016)
PWL1 and PWL2	*PWL2*: single bp substitution or large deletions in spontaneous mutants. *PWL1–4* loci: associated with transposable elements	Prevents infection of weeping love grass	Kang et al. (1995); Sweigard et al. (1995)
Biotrophy-associated secreted proteins			
BAS1	·	Accumulates in BIC and translocates into infected rice cells, even moving to neighbor un-invaded cells	Mosquera et al. (2009)
BAS2	·		
BAS3	·	Localizes near cell wall crossing points	
BAS4	·	Resides on chromosome VI and surrounds growing invasive hypha	
BAS107	·	Localizes in cytoplasm	Giraldo et al. (2013)
Secreted proteins			
Slp1	·	Suppresses rice pattern-triggered immunity by interfering with rice chitin elicitor receptor protein CEBiP-mediated signaling	Mentlak et al. (2012)
MC69	·	Required for *M. oryzae* infection; mutation of MC69 prevents development of invasive hyphae after appressorium formation in rice leaf sheath	Saitoh et al. (2012)

host and pathogen, these effectors display a very high level of genetic diversity and can be classified according to the site of their activity. These secreted effectors suppress host immunity by manipulating the physiology and immune responses of the host (Kamoun 2009; Khan et al. 2018; Kim et al. 2020; Qian et al. 2021). Their major targets are host receptors or host defense components to suppress the initiation of resistance against the pathogen (Kamoun 2009; Thomma et al. 2011; Pradhan et al. 2021).

Cytoplasmic effectors (pathogen secreted and translocated into the plant cytoplasm) and apoplastic effectors (secreted to the outside of plant cells) are two distinct types of effectors based on their site of activity in host plants (Kamoun 2009). A few cytoplasmic effectors, specifically Avr-Pita, Avr-Piz-t, Pwl1, and Pwl2 preferentially aggregate in the BIC, which is a plant-derived structure and usually develops at the tip of early invading hyphae (IH). Bas83 is a unique cytoplasmic effector associated with rice membranes and is secreted by *M. oryzae* through a Golgi-independent secretion pathway (Giraldo et al. 2013). Although most effectors help the pathogen to evade host resistance, Pwl2 was reported to prevent *M. oryzae* infecting a second host grass, weeping lovegrass (Kang et al. 1995). Some apoplastic effectors such as BAS4, Avr1-CO39, and Slp1 may reach plant extra-invasive hyphae membrane (EIHM) compartments through bulbous intracellular IH. Using the BAS4-GFP fusion protein, an outline of the IH can be discerned (Mosquera et al. 2009). Recent studies identified factors involved with molecular cross-talk required for the secretion of effector proteins. For example, Qian et al. (2021) reported that MoErv29, a coat protein complex II (COPll) receptor is required for the delivery of effector proteins through recognition and binding of amino-terminal tripeptide motifs. It was also shown that MoErv29 is a pre-requisite for the secretion of apoplastic effectors. Although no nuclear effectors from fungal pathogens have been well characterized, a recent study by Kim et al. (2020) identified and characterized two nuclear effectors, MoHTR1 and MoHTR2 of *M. oryzae* capable of modulating host susceptibility through transcriptional alteration of genes associated with immunity in rice.

Mini chromosomes (small dispensable supernumerary chromosomes) were identified in field isolates of *Triticum* pathotype (such as B71) by Peng et al. (2019) through sequence assemblies. Interestingly, these mini chromosomes contain a very high percentage of repetitive sequences (52.8% in B71 isolate of MoT) compared to core chromosomes (9.7%). Three mini-chromosomes from two different *Triticum* isolates were found to contain shared and different putative effector genes, *AVR* effector homologs, and other sequences found on core chromosome ends in other strains, suggesting cross-talk between these two chromosomal compartments (Raffaele and Kamoun 2012; Peng et al. 2019). Further studies are needed to elucidate the roles of mini chromosomes in MoT. A list of effectors from rice pathogenic *M. oryzae* with major features is presented in Table 18.1.

18.8 Management Strategies

18.8.1 Restricting Movement of Seeds from Diseased Areas to Disease-free Areas

Since *M. oryzae* is a seed-borne pathogen that can spread over a long distance from a diseased area to a non-diseased area, the primary prevention measure is to ensure the health of seeds destined for new production areas (Goulart and de Paiva 1990; Urashima et al. 1999; Silva et al. 2009; Maciel et al. 2014; Cruz and Valent 2017; Islam et al. 2020). Strict enforcement of quarantine and biosafety regulations can make a difference in this regard (Valent et al. 2021). As wheat blast is still limited to relatively small geographic areas of South American countries *viz*. Brazil, Argentina, Bolivia, Paraguay, Bangladesh in South Asia and Zambia in Africa, special care should be taken during the international trade of wheat seeds and grains for restricting the further spread of the wheat blast fungus to new wheat-growing areas of the world.

18.8.2 Early Detection and Disease Forecasting

Early and accurate detection methods for diagnosis of the blast fungus in seeds, asymptomatic plants and alternate hosts is necessary to prevent the spread of inoculum to new areas and to manage blast disease more efficiently (Islam et al. 2020). Reliable and efficient morphological and molecular characterization of the blast pathogen may help in surveillance and disease control in the field. For example, disease symptoms and other characteristics to differentiate wheat blast and Fusarium head blight (FHB) are essential at the field level. Recent advances in detection technology of *M. oryzae* using a commercially available kit have made it possible to confirm the presence of the pathogen in less than 8 h (Harmon et al. 2003). Other techniques such as single spore isolation for obtaining a pure culture of *M. oryzae* from rice and other grass species is now available (Jia 2009). Recently, Gupta et al. (2020) developed a suitable, user-friendly, and cost-effective method for isolation, culture, and storage of MoT from infected wheat samples by modifying a monoconidial isolation technique. These methods could be used for any pathotype of *M. oryzae*. However, a microscopic detection method is unable to identify the specific pathotype of the blast fungus. Therefore, nucleic acid-based methods are recommended.

Whole genome analysis was used to identify DNA markers that differentiate MoT isolates from other host-specific pathotypes (Pieck et al. 2017). A PCR-based diagnostic assay using the marker of MoT3 shows specificity and sensitivity in laboratory studies and is now being developed as a tool for detecting the wheat blast pathogen in the field and in wheat seed or grain lots from affected areas (Pieck et al. 2017). However, Gupta et al. (2019) assessed the MoT3 diagnostic assay and found that it did not distinguish between wheat and rice blast isolates from Bangladesh. They cautioned against the indiscriminate use of this assay to identify wheat blasts and encouraged further development of the assay to ensure its value in diagnosis. Recently, a rapid, specific, and convenient diagnostic method was developed for wheat blast pathogen, MoT pathotype using genome-specific primers (MoT-6098 and MoT-6099) and Cas12a-mediated technology (Kang et al. 2021; Sánchez et al. 2022). To make the tool more useful in field conditions, they developed a loop-mediated isothermal amplification (LAMP) method to detect MoT without the need for using a thermal cycler machine. In another development, they used guide RNAs (gRNAs) and Cas12a protein to target the MoT-6098 and MoT-6099 sequences. Upon activation, Cas12a showed single-stranded deoxyribonuclease (ssDNase) activity without any ability to discriminate pathotypes. At the final stage, target-dependent Cas12a-activated ssDNase was combined with nucleic acid lateral flow immunoassay (NALFIA) and recombinase polymerase amplification (RPA) to develop the Cas12a-mediated diagnostic technology. However, further improvements of this rapid test should make it more cost-effective and convenient for its worldwide application in plant quarantine, early detection in seed lots and in the field. The Cas12a-mediated technology is gaining popularity for point-of-care rapid and specific diagnosis of not only MoT but also various diseases caused by phytopathogens (Lu et al. 2020; Wheatley and Yang 2021; Sánchez et al. 2022; Yao et al. 2022). Taking a different approach, Islam and co-workers successfully applied field pathogenomics, open data sharing, and open science approaches that were successful in rapidly and precisely determining the origin and genetic identity of the pathogen that first caused the wheat blast epidemic in Bangladesh (Islam et al. 2016; Islam and Kamoun 2018; Kamoun et al. 2019). The field pathogenomics approach provides a quick turn around as it does not involve traditional isolation and culture-based time-consuming methods. A high-resolution pathogen population dynamic can also be obtained by this approach directly from the field. This in turn provides new insights into population structure, pathogen biology, and pathogenesis. The open data sharing and open science coupled with field pathogenomics approach could be used in

tackling any plant health or health of any organism including humans in a rapid way (Kamoun et al. 2019).

Conducive weather plays a very crucial role in the development of blast disease. Therefore, systematic monitoring of weather parameters should help in predicting the occurrence and severity of blast disease (Cardoso et al. 2008). Brazilian scientists developed a predictive model, known as Sisalert (Plant disease Epidemic Risk Prediction System) for calculating the risk of a wheat blast outbreak based on weather variables (available at: https://dev.sisalert.com.br/monitoramento/?page_id = 14; Fernandes et al. 2017). Later, the model was adapted for the USA, which indicated that favorable weather conditions do exist for a wheat blast outbreak in 25% of the winter wheat growing regions, including Louisiana, Mississippi, and Florida (Cruz et al. 2016a) in 70% of the growing seasons. This automated weather-based model is very suitable for providing a warning of an imminent blast infection risk (West and Kimber 2015; West et al. 2017), facilitating a real-time decision support system for fungicide applications. As wheat blast is predominantly a head disease and remains asymptomatic at the vegetative stages, disease forecasting should be expanded to all blast-affected countries for reducing crop failure risk through the adoption of well-timed mitigation measures.

18.8.3 Cultural and Sanitary Practices

Alternative hosts such as weedy grasses and crop residues can be a source of secondary inoculum bridging two cropping seasons (Urashima and Kato 1998; Castroagudín et al. 2017). Deep ploughing is beneficial to reduce the initial inoculum from crop residues (Igarashi 1990; International Maize and Wheat Improvement Center-CIMMYT Wheat Program 2016). Crop rotation with non-grass crops and altering the sowing date to avoid the blast host and favorable weather patterns for pathogen growth, respectively may help in managing blast disease (Mehta et al. 1992; Santos et al. 2000; Mehta 2014; Coelho et al. 2016).

18.8.4 Chemical Control

The application of commercial fungicide is the most common traditional method for controlling fungal plant pathogens. There are many commercially available fungicides that provide good control from well-timed applications. Application timing can be determined from disease prediction models. Among the commercially available fungicides, some systemic fungicides are known to be effective against blast pathogens, which target the specific growth stages of *M. oryzae*. These include melanin biosynthetic inhibitors (MBIs), anti-mitotic compounds, choline biosynthesis inhibitors (CBIs), plant defense activators, Quinone outside inhibitors (QoI), and ergosterol biosynthesis inhibitors (EBI) (Pooja and Katoch 2014). Usman et al. (2009) found rabicide (tetrachlorophthalide), Nativo (tebuconazole + trifloxystobin), and Score (difenoconazole) were the most effective fungicides in the field to control rice blast.

18.8.5 Development of Fungicide Resistance in *M. oryzae*

As rice blast management is heavily reliant on chemicals, the rise of fungicide resistance is a real concern. Mutation is the most common natural phenomenon in microbial pathogens to overcome the fungicidal effect. Several studies reported resistance development in *M. oryzae* against fungicides such as triazole (tebuconazole and epoxiconazole) and strobilurin (QoI, azoxystrobin, and pyraclostrobin) (Kim et al. 2003; Castroagudín et al. 2015; Oliveira et al. 2015; Ceresini et al. 2018). Castroagudín et al. (2015) reported a widespread distribution of QoI resistance in *M. oryzae* populations collected from wheat fields and graminaceous hosts across central and southern Brazil, where mutation of the cytochrome b (*cyt b*) gene was common. In Brazil, 72 fungicides are currently registered for

controlling rice blasts (AGROFIT 2019) that include DMIs, QoIs, and MBIs. Although MBI active ingredients are of low risk of developing resistance, MBI-D-resistant *M. oryzae* strains have already been reported, which is associated with a single point mutation of the *SDH* (succinate dehydrogenase) gene (Yamaguchi et al. 2002; Sawada et al. 2004; Dorigan et al. 2019). Like other site-specific fungicides, reports related to resistance development in the rice blast pathogen, as well as the closely related *M. oryzae* subgroup pathogenic to other Poaceae against QoI are available (Vincelli and Dixon 2002; Avila-Adame and Koller 2003; Castroagúdin et al. 2015). These QoI-resistant isolates were found to tolerate a high concentration of active ingredients due to nucleotide substitutions in the *cyt b* gene (Kim et al. 2003). Thus, resistance management in fungicides such as following label directions and rotating chemistry may play an important role in the sustainable management of blast disease.

18.8.6 Breeding for Blast-resistant Varieties

Breeding for blast-resistant varieties is an effective and environment-friendly method of managing *M. oryzae* since chemical control is expensive, harmful to the environment and other non-target organisms (Miah et al. 2013). Conventional methods such as pedigree, backcrossing, recurrent selection, and mutation breeding have been used frequently for developing blast-resistant varieties. Recurrent selection has been used to generate blast-resistant upland rice cultivars like CG-91 (Guimaraes and Correa-Victoria 1997). Many major resistance genes have been found by researchers during many years of research. Some of those genes such as *Pib*, *Pita*, *Pia*, *Pi1*, *Pikh*, *Pi2(t)*, *Pigm,* and *Pi4(t)* have already been successfully introgressed for the development of blast-resistant rice varieties using traditional breeding methods (Korinsak et al. 2011; Deng et al. 2017).

The ETI is an emerging mechanism that can be utilized in blast-resistant genotypes. Consistent with the gene-for-gene model (Flor 1942), ETI is activated in the plant upon recognition of the corresponding *M. oryzae* effector protein by a host *R* protein. The *AVR* in *M. oryzae* frequently encodes effector proteins (Flor 1956). The *R* genes serve as the basis for resistance breeding. A total of 145 *R* genes or loci have been found in rice so far, with approximately 36 *R* genes successfully identified and cloned (Table 18.2) (Sharma et al. 2012; Ashkani et al. 2016; Li et al. 2019a, b, 2020). Many of the *R* genes such as *Pb1*, *Pi25*, and *Pi64*, confer resistance to panicle blast, whereas the majority of the cloned *R* genes offer resistance to leaf blast at the seedling stage (Hayashi et al. 2010; Chen et al. 2011; Ma et al. 2015; Cao et al. 2019; Mao et al. 2021). However, genes providing resistance to the rice blast fungus were linked to reduced rice yield. Deng et al. (2017) analyzed the molecular basis of a blast-resistant rice variant with high yield that may help in overcoming the problem related to yield and resistance. They found that the rice *Pigm* locus contains a cluster of genes that encode nucleotide-binding leucine-rich repeat (NLR) receptors to confer durable resistance to *M. oryzae* without any yield penalty. From a series of experiments, Deng et al. (2017) also demonstrated that epigenetic regulation of antagonistic receptors confers rice blast resistance while balancing yield.

Although wheat blast is a relatively new disease, considerable research has been carried out to identify novel resistance sources in wheat germplasms since the initial appearance of wheat blast in 1985. A total of 10 genes including *RmgTd(t)*, *Rmg1(Rwt4)*, *Rmg2*, *Rmg3*, *Rmg4*, *Rmg5*, *Rmg6*(*Rwt3*), *Rmg7*, *Rmg8*, *RmgGR119,* and a chromosomal fragment from *Aegilops ventricosa*, the 2*NS* translocation, have been identified as sources of resistance to wheat blast to date (Table 18.3) (Islam et al. 2020). A genotype, GR119, carrying the *RmgGR119* gene was found to be almost immune to MoT isolates (Islam et al. unpublished personal communication). Some of these genes have already been incorporated into the elite wheat varieties by Japanese scientists (Wang et al. 2018). Introgression of *Rmg8* and *RmgGR119* genes into the elite

Table 18.2 Detailed list of 36 cloned blast resistance (*R*) genes in rice

R gene name	Chromosome	Source cultivar (country)	Cloning method	Encoding protein	References
Pi37	1	St. No. 1 (China)	MB	NBS–LRR	Lin et al. (2007)
Pit	1	K59 (Japan)	MB	CC–NBS–LRR	Hayashi and Yoshida (2009)
Pish	1	Akihikari and Shin-2 (Japan)	Mutant screening	CC–NBS–LRR	Takahashi et al. (2010)
*Pi35**	1	Hokkai 188 (Japan)	MB	NBS–LRR	Fukuoka et al. (2014)
Pi64	1	Yangmaogu (Japan)	MB	CC–NBS–LRR	Ma et al. (2015)
Pi-b	2	Tohoku IL9 (Japan)	MB	NBS–LRR	Wang et al. (1999)
*Pi21**	4	Owarihatamochi (Japan)	MB	Proline-rich metal-binding protein	Fukuoka et al. (2009)
*Pi63/ Pikahei-1 (t)**	4	Kahei	MB	CC–NBS–LRR	Xu et al. (2014)
Pi-9(t)	6	IR31917 (Philippines)	MB	NBS–LRR	Qu et al. (2006)
Pi2	6	C101A51	MB	NBS–LRR	Zhou et al. (2006)
Piz-t	6	Toride 1 (Japan)	MB	NBS–LRR	Zhou et al. (2006)
Pi-d2	6	Digu (China)	MB	B-lectin receptor kinase	Chen et al. (2006)
Pi-d3	6	Digu (China)	*In silico* analysis	NBS–LRR	Shang et al. (2009)
Pi25	6	Gumei 2 (China)	MB	CC–NBS–LRR	Chen et al. (2006)
Pid3-A4	6	A4 (*Oryza rufipogon*)	MB	NBS–LRR	Lü et al. (2013)
Pi50	6	Er-Ba-zhan (EBZ)	MB	NBS–LRR	Zhu et al. (2012), Su et al. (2015)
Pigm	6	Gumei 4 (China)	MB	NBS–LRR	Deng et al. (2017)
Pi36	8	Q61 (China)	MB	CC–NBS–LRR	Liu et al. (2007)
Pi5	9	RIL260 (Philippines)	MB	CC–NBS–LRR	Lee et al. (2009)
Pii	9	Hitomebore (Japan)	Mutant screening	NBS–LRR	Takagi et al. (2013)
Pi56	9	Sanhuangzhan No. 2	MB	NBS–LRR	Liu et al. (2013)
Pi54	11	Tetep (India)	MB	NBS–LRR	Sharma et al. (2005)
Pikm	11	Tsuyuake (China)	MB	NBS–LRR	Ashikawa et al. (2008)
*Pb1**	11	Modan (Japan)	MB	CC–NBS–LRR	Hayashi et al. (2010)
Pik	11	Kusabue (China)	MB	CC–NBS–LRR	Zhai et al. (2011b)
Pik-p	11	K60 (China)	MB	CC–NBS–LRR	Yuan et al. (2011)
Pia	11	Aichi Asahi (Japan)	MB and mutant screening	CC–NBS–LRR	Okuyama et al. (2011)
Pi1	11	C101LAC (USA)	MB	CC–NBS–LRR	Hua et al. (2012)
Pi54rh	11	*Oryza rhizomatis* (nrcpb 002)	MB	NBS–LRR	Das et al. (2012)
Pi-CO39	11	CO39 (USA)		NBS–LRR	Cesari et al. (2013)

(continued)

Table 18.2 (continued)

R gene name	Chromosome	Source cultivar (country)	Cloning method	Encoding protein	References
Pi54of	11	*Oryza officinalis* (nrcpb004) (India)	MB	NBS–LRR	Devanna et al. (2014)
PiK-h	11	K3 (India)		NBS–LRR	Zhai et al. (2014)
Pik-e	11	Xiangzao143 (China)	MB	CC–NBS–LRR	Chen et al. (2015)
Pik-s	11	Shin 2 (Japan)		NBS–LRR	GenBank: AET36547.1, AET36548.1
Pi-ta	12	Yashiromochi (USA)	MB	NBS–LRR	Bryan et al. (2000)
Pitr	12	Katy	MB	Atypical protein with an armadillo repeat	Zhao et al. (2018)

MB = Map-based; NBS-LRR = Nucleotide-binding site leucine-rich repeat; CC-NBS-LRR = Coiled-coil-nucleotide-binding site leucine-rich repeat
* Indicates *R* genes showing partial resistance

wheat varieties (including a variety having the *2NS* translocation) of Bangladesh are in progress by the researchers of the Institute of Biotechnology and Genetic Engineering (IBGE) of Bangabandhu Sheikh Mujibur Rahman Agricultural University (Islam et al. unpublished personal communication).

New biotechnological tools including sequence-specific nucleases (SSNs) may play an important role in crop improvement by significantly enhancing the speed and success. Clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) is the most effective SSNs toolkit that can be used to develop durable blast-resistant rice/wheat varieties (Shan et al. 2015; Wang et al. 2016; Haque et al. 2018; Islam 2019). The CRISPR-Cas genome modification and transcription activator-like effector nuclease-based approaches should be more effective in the development of transgene-free disease resistant crop varieties for the management of plant diseases including blast of major cereals (Malzahn et al. 2017; Zhou et al. 2021).

18.8.6.1 Mutation Breeding

Mutation breeding is another practical strategy for developing novel alleles that do not exist in germplasm pools. It is now possible to tag mutant genes, pyramid them into a single novel breeding line, and then utilize them in breeding programs (Shu 2009). For example, the radiation-induced glutinous mutant RD6 was created from the well-known non-glutinous cultivar Khao Dawk Mali 105 (KDML105). The chemical mutagen, ethylmethane sulfonate (EMS), was used at the rate of 0.1 and 0.2% to mutate blast *R* genes in the high-yielding variety Ratna (IR8/TKm 6) (Kaur et al. 1975). A highly blast-resistant mutant rice variety, Zhefu 802, was developed from the variety Simei 2 by applying gamma radiation in China (Ahloowalia et al. 2004). However, the application of mutation breeding for developing blast-resistant varieties of wheat has not been accomplished yet. Guo et al. (2021) found rice blast loss-of-function mutant alleles in the wheat genome that can be used as a new strategy for durable wheat blast resistance breeding.

18.8.6.2 Genome Editing Using CRISPR-Cas Technology for Developing Blast Resistance

The use of CRISPR-Cas-mediated genome editing has increased in recent years in a wide spectrum of biological research including the improvement of crops for disease resistance (Mei et al. 2016; Islam 2019; Zhou et al. 2021). The CRISPR-Cas genome editing toolkits can be used as a new potential technology for developing durable blast-resistant rice (Wang et al. 2016;

Table 18.3 List of cloned blast resistance (*R*) genes in wheat

R genes	Source cultivar	Blast fungal isolates	Location on chromosome	Effectiveness	Corresponding avirulence gene in *M. oryzae*, where known	References
RmgTd(t)	*Triticum dicoccum* KU109 (Tat 4)	Not a field isolate	–	–	–	Takabayashi et al. (2002)
Rmg1(Rwt4)	*T. aestivum* cv. Norin 4 (hexaploid)	*Avena* isolate Br58	1D	Not effective against wheat blast pathogen	–	Takabayashi et al. (2002)
Rmg2	*T. aestivum* cv. Thatcher	*Triticum* isolate Br48	7A	Effective at seedling stage and istemperature-sensitive	–	Zhan et al. (2008)
Rmg3	*T. aestivum cv.* Thatcher	*Triticum* isolate Br48	6B	Effective at seedling stage and is temperature-sensitive	–	Zhan et al. (2008)
Rmg4	*T. aestivum* cv. Norin 4	*Digitaria* isolate	4A	Not effective against wheat blast pathogen	–	Nga et al. (2009)
Rmg5	*T. aestivum* cv. Red Egyptian	*Digitaria* isolate	6D	Not effective against wheat blast pathogen	–	Nga et al. (2009)
Rmg6 (*Rwt3*)	*T. aestivum* cv. Norin 4	*Lolium* isolate TP2	1D	Confers resistance at both seedling and heading stage, but is ineffective above 25 °C	–	Vy et al. (2014)
Rmg7	*T. dicoccum* (tetraploid wheat), KU112 (St17), 120 (St24), KU122 (St25)	*Triticum* isolate Br48	2A	Confers resistance at both seedling and heading stage	*Avr-Rmg8*	Tagle et al. (2015); Anh et al. (2018)
Rmg8	*T. asetivum* cv. S-615	*Triticum* isolate Br48	2B	Confers resistance at both seedling and heading stage	*Avr-Rmg8*	Anh et al. (2015, 2018)
RmgGR119	Albanian wheat accession GR119	*Triticum* isolate Br48	–	Confers resistance at heading stage	–	Wang et al. (2018)
2NS/2AS translocation	Chromosomal segment of wheat wild relative *Aegilops ventricosa*	*Triticum* isolate Br48 but not BR71	–	Displays up to 72% reduction of disease symptoms	–	Cruz et al. (2016b)

Zhou et al. 2021) and wheat varieties (Islam 2019; Islam et al. 2020). It could be done by either deletion or disruption of *S* (susceptibility) genes or transcription factors, or by insertion of *R* genes in the genome of popular commercial cultivars. Accomplishment of this goal will need perfectly designed base editing, and gene disruption through simple donor vector constructs (Arazoe et al. 2015; Wang et al. 2016; Nekrasov et al. 2017; Peng et al. 2017; Kim et al. 2019; Yamato et al. 2019).

Through years of research, it has become clear that some genes are linked to increased rice blast disease susceptibility. These represent a wide range of gene functionality that seem to regulate the plant immune response negatively or that encode host proteins used by pathogens for their entry and spread within the plant tissue. The barley *Mlo* locus, a widely used recessive disease resistance gene is an example of a susceptibility factor used by the pathogen for its advantage (Büschges et al. 1997). Alteration or mutation of these genes may lead to enhanced resistance to the disease. An appreciable number of rice *S* genes such as *OsRAC4/5/B*, *OsWAK112d*, *OsMAPK5*, *OsWRKY28/76*, *OsERF922*, *OsGF14b*, *SPL11*, *OB-fold* gene, *OsPLDbeta1*, and *OsSSI2* have so far been characterized (Zeng et al. 2004; Jung et al. 2006; Vega-Sánchez et al. 2008; Yamaguchi et al. 2009; Chen et al. 2010; Delteil et al. 2016; Grand et al. 2012; Liu et al. 2016; Chujo et al. 2013; Yokotani et al. 2013; Xie et al. 2014; Wang et al. 2016). Successful examples of editing the wheat genome by CRISPR-Cas9 include the development of powdery mildew-resistant wheat by disruption of the *TaMLO-A1*, *TaMLO-B1*, and *TaMLO-D1* genes (Wang et al. 2014).

Development of blast-resistant mutagenized rice lines via CRISPR-Cas9 technology has been reported (Wang et al. 2016). Researchers targeted the rice gene *OsERF922* which regulates rice blast resistance negatively (Liu et al. 2012). Likewise, CRISPR-Cas technology was used to knock out the *OsSEC3A* rice gene, and as a result, researchers observed an improved blast defense response and resistance in the rice genotype (Ma et al. 2017). These results indicate that non-transgenic mutated rice/wheat with acceptable blast resistance can be obtained just by "knocking out" the *S* genes or transcription factor-encoding genes. This will help in releasing edited lines quickly for practical application without following strict biosafety guidelines (Wang et al. 2016; Langner et al. 2018; Islam et al. 2020). Recently, Bhowmik et al. (2018) established CRISPR-Cas9-based targeted mutagenesis in wheat microspores that may ease the complication in transformation of wheat although further efforts will be needed to optimize crop-specific CRISPR-Cas9 protocols. Rapid advances in CRISPR-Cas methods should facilitate the use of this revolutionary technology for blast-resistant cereal crop variety development including highly complex hexaploid wheat (Islam et al. 2020; Islam and Molla 2021).

18.8.7 Biological Control

Biological control is the most environment-friendly and sustainable strategy to control the blast fungus. Several genera of fungi and bacteria have shown strong antagonistic activities against the blast fungus *M. oryzae*. The occurrence and severity of blast in rice was reduced significantly by various isolates of *Bacillus*, *Pseudomonas*, *Acidovorax*, *Cryseobacterium*, and *Sphingomonas* species (Shimoi et al. 2010; Chakraborty et al. 2021). Rice seed treated with *B. amyloliquefaciens* UASBR9 resulted in lower disease severity compared to untreated control plants (Amruta et al. 2018). Moreover, *Bacillus* spp. KFP-17, KFP-7, and KFP-5 significantly decreased blast infection in two aromatic rice varieties, super basmati and basmati-385 (Rais et al. 2018). Recently, Li et al. (2021) reported that *B. amyloliquefaciens* HR-2 showed an 82.3% inhibition effect against *M. oryzae*.

The *AVR P. oryzae* and *Bipolaris sorokiniana* (incompatible with rice) reduced blast disease by triggering induced resistance in rice plants (Manandhar et al. 1998). Furthermore, two yeast isolates, CMY018 and CMY045, were strong suppressors of *M. oryzae* in rice (Kunyosying et al. 2018). *Trichoderma* spp. was also reported to control blast disease (Kalpana et al. 2016; Konda et al. 2016). Biological control of cereal blast by various antagonistic bacteria has been comprehensively reviewed by Chakraborty et al. (2021).

The popularity of bioactive natural products is increasing over time in plant protection (Paul et al. 2022). Botanicals are widely used bioactive compounds due to their availability, biodegradability, and low toxicity (Vu et al. 2015). Triterpenoid saponins, such as maejaposide A,

maejaposides A-1, maejaposides C-1, C-2, and C-3 originating from *Maesa japonica*, significantly reduced the development of rice blast disease by 85–98% with minimum inhibitory concentration (MIC) values ranging from 4 to 32 μg/mL compared to the untreated control (Ngo et al. 2019). Previously, four triterpenoid saponins (TPG1, TPG2, TPG3, and TPG5) isolated from the methanol extract of *Trevesia palmata* with MIC values between 4 and 16 mg/mL completely inhibited the mycelial growth of rice blast fungus (Kim et al. 2018). Interestingly, the growth and development of *M. oryzae* is governed by protein kinase C (PKC), linked with the downstream target and cell integrity pathways. Therefore, targeting PKC can be an effective measure to control blast pathogens. Chelerythrine chloride originating from a herbaceous perennial plant *Chelidonium majus* acts as a selective inhibitor of PKC that inhibits germ tube formation and appressorial development of *M. oryzae* in rice (Penn et al. 2015; Chakraborty et al. 2022).

Chakraborty et al. (2020a; 2022) reported that oligomycin B and F, and kinase inhibitor, staurosporine isolated from *Streptomyces* spp. were able to inhibit mycelial growth of the wheat blast pathogen. Moreover, five non-cytotoxic linear lipopeptides such as gageotetrin B, gageopeptide A, B, C, and D, extracted from a marine strain *B. subtilis* 109GGC020, showed an inhibitory effect on the growth of wheat blast pathogen either by hindering conidiogenesis and germination of conidia, or by interfering with the development of germ tubes and appressoria (Chakraborty et al. 2020b). A list of antagonistic microorganisms, secondary metabolites, and plant products that can suppress blast pathogens is presented in Table 18.4.

18.8.8 Integrated Management

All available options such as forecasting and mapping of disease and pathogen race distribution, early and reliable quick diagnostics, biological and chemical control measures, as well as the inclusion of cultivars with resistance genes based on pathogen genetic diversity and virulence, should be considered and deployed as a package for successful control of *M. oryzae* (Zhang et al. 2022). Moreover, along with these strategies, agronomic methods such as crop rotation with non-grass species, removal of crop residues from near and around the field, avoiding broadcast planting and double cropping, water management, and the elimination of yield-limiting variables should be combined for successful control of the blast pathogen for achieving global food security.

18.9 Conclusions and Future Prospects

18.9.1 Population Structure and Evolution of *M. oryzae* Pathotypes

Blast diseases of cereals caused by *M. oryzae* and its pathotypes (different crop-adapted lineages) are of major concern to the policy makers working with food security for the increasing world population. *M. oryzae* genome structure, instability, parasexual recombination, transposable elements in the genome, and evolution that enable them to jump from one host to another have been the subject of numerous studies to date. Host specialization within *M. oryzae* may have occurred likely due to gene gains or losses from repetitive DNA elements (Yoshida et al. 2016; Zhong et al. 2016). In addition, genes related to effector protein secretion can also be gained or lost through chromosomal rearrangements, which may determine the success or failure of the fungus causing disease on a new host or a certain genotype of the same host. The evolution of new pathotypes is influenced by the presence of multiple divergent lineages within *M. oryzae*, which is preferentially associated with a single host genus. These facts indicate an incipient speciation from host range expansion or host shift, and the makeup of multiple lineages within *M. oryzae* may have occurred due to genetic exchanges. A wide variation of effector candidate (EFC) and

Table 18.4 List of antagonistic microorganisms, and inhibitory secondary metabolites and plant products against various pathotypes of blast fungus

Antagonist	Activity	Source of isolation	Blast inhibition	References
Bacillus velezensis Zw-10	Inhibits mycelial growth of MoO	Sichuan basin neutral purplish soil	82.9% *in vitro*	Chen et al. (2020)
B. subtilis 5	Reduces disease severity caused by MoO	Soil from rice field	83.9% in planta	Chen et al. (2019)
B. velezensis P42	Inhibits mycelial growth of MoS	Bacteriology lab, UAS, GKVK, Bengaluru	100% *in vitro*	Mallikarjuna et al. (2020)
B. amyloliquefaciens UASBR9	Reduces disease severity caused by MoO	Rhizosphere soil and roots	96% in planta	Amruta et al. (2018)
B. licheniformis strain BC98	Inhibits mycelial growth of MoO	Rhizosphere soil	97% *in vitro*	Tendulkar et al. (2007)
B. subtilis SPS2	Inhibits mycelial growth of MoO	Rhizoplane or from roots of healthy rice plants	100% *in vitro*	Naureen et al. (2009)
B. subtilis SYX04	Inhibits spore germination, appressorium formation and alters the structures of hyphae and conidia of MoO	Rice leaves	93% *in vitro*	Sha et al. (2016)
B. subtilis UASP17	Inhibits mycelial growth of MoO	Rhizosphere soil	91% *in vitro*	Prasanna Kumar et al. (2017)
Enterobacter sp. B41 (SPR7)	Inhibits mycelial growth of MoO	Rhizoplane or from roots of healthy rice plants	90% *in vitro*	Naureen et al. (2009)
Gordonia terrae JSN1.9	Inhibits mycelial growth of MoO	Rhizosphere soil	88% *in vitro*	Harsonowati et al. (2017)
Pseudomonas fluorescens pf7–14	Inhibits mycelial growth of MoO	Soil from rice field	100% *in vitro*	Gnanamanickam and Mew (1992)
P. fluorescens Pf 2	Reduces disease severity caused by MoE	Rhizosphere soil	96% *in planta*	Kumar and Kumar (2011)
P. fluorescens 551 (Pf 1)	Reduces disease severity caused by MoE	Rhizosphere soil	92% *in planta*	Kumar (2011)
P. fluorescens AUPF25	Inhibits mycelial growth, and promotes fungal lysis of MoO	Rice rhizosphere	100% *in vitro*	Shyamala and Sivakumaar (2012)
Rhizobacterial isolates, Rizo-55	Inhibits mycelial growth of MoO	Rhizosphere soil	95% *in vitro*	Filippi et al. (2011)
Serratia marcescens SPR4	Inhibits mycelial growth of MoO	Rhizoplane or roots of healthy rice plants	95% *in vitro*	Naureen et al. (2009)
Streptomyces albolongus SKB2.3	Inhibits mycelial growth of MoO	Rhizosphere soil	88% *in vitro*	Chaiharn et al. (2020)
S. palmae PC 12	Inhibits mycelial growth of MoO	Rice rhizosphere soil	87.3% *in vitro*	Chaiharn et al. (2020)
S. erythrochromogenes 3–45	Abnormal appressorium formation and inhibits melanization of appressoria of MoO	Field soil	95% *in vitro*	Tamura et al. (2019)
S. globisporus JK-1				Li et al. (2011)

(continued)

Table 18.4 (continued)

Antagonist	Activity	Source of isolation	Blast inhibition	References
	Inhibits conidial germination and reduces appressorium formation of MoO	Contaminated fungal culture plates	88% *in vitro*	
S. philanthi RM-1-138	Inhibits mycelial growth of MoO	Rhizosphere soil	89% *in vitro*	Boukaew and Prasertsan (2014)
Streptomyces sp. isolate UPMRS4	Inhibits mycelial growth of MoO	Soil from rice field	98% *in vitro*	Awla et al. (2016)
Absidia sp.	Inhibits mycelial growth of MoO	Rice seed	100% *in vitro*	Atugala and Deshappriya (2015)
Epicoccum sp.	Inhibits mycelial growth of MoO	Organic field soil	98% *in vitro*	Sena et al. (2013)
Phaeosphaeria oryzae strain MKP5111B	Inhibits conidial germination and mycelial growth of MoO	Rice leaves	100% *in vitro*	Kawamata et al. (2004)
Trichoderma asperullum	Inhibits mycelial growth of MoS	Rhizosphere soil	100% *in vitro*	Konda et al. (2016)
Trichoderma sp. T2 + *B. subtilis* UKM1	Inhibits mycelial growth of MoO	Field soil	95% *in vitro*	Ali and Nadarajah (2014)
Surfactin	Inhibits mycelial growth of MoO	*B. licheniformis* BC98	97% *in vitro*	Tendulkar et al. (2007)
Blasticidin S	Inhibited the protein synthesis of MoO	*S. griseo-chromogene*	95% *in vitro*	Yamaguchi (1982)
Kasugamycin	Inhibited the protein synthesis of MoO	*S. kasugaensis*	95% *in vitro*	Yamaguchi (1982)
Oligomycin A	Inhibits conidial germination and appressorium formation of MoO	*Streptomyces* sp. AMA49	100% *in vitro*	Buatong et al. (2019)
2-Methylpyrazine	Inhibits mycelial growth, sporulation, and conidial germination of MoO	*P. putida* BP25	100% *in vitro*	Patel et al. (2021)
Antifungalmycin 702	Inhibits mycelial growth, suppresses conidial germination and appressorium formation of MoO	*S. padanus* JAU4234	90% *in vitro*	Xiong et al. (2013)
Pyrrolo [1,2-a] pyrazine-1,4-dione	Inhibits mycelial growth of MoO	*Streptomyces* sp. isolate UPMRS4	98% *in vitro*	Awla et al. (2016)
Ascherxanthone B	Inhibits mycelial growth of MoO	*Aschersonia luteola* BCC 8774	86% *in vitro*	Chutrakul et al. (2009)
Alternariol monomethyl ether	Inhibits appressorium formation of MoO	*Alternaria tenuissima*	80–100% *in vitro*	Jeon et al. (2010)
HDFO	Inhibits conidial germination and blast lesion formation of MoO	*Biscogniauxia* sp. O821	99% *in vitro*	Nguyen et al. (2018)
HDFO	Inhibits conidial germination and appressorium formation of MoO	*Biscogniauxia* sp. strain O-811	93.5% *in vitro*	Moriguchi et al. (2019)
Griseofulvin	Inhibits mycelial growth of MoO	*Xylaria* sp. Strain F0010	95% *in vitro*	Park et al. (2005)
GKK1032A2	Inhibits conidial germination of MoO	*Penicillium* sp. IBWF-029-96	100% *in vitro*	Becker et al. (2012)
Tanzawaic Acid K	Inhibits conidial germination of MoO	*Penicillium* sp. IBWF104–06	90% *in vitro*	Sandjo et al. (2014)
ß-1,3-glucanase 1				

(continued)

Table 18.4 (continued)

Antagonist	Activity	Source of isolation	Blast inhibition	References
	Degrades the fungal cell walls of MoO	*B. subtilis* NSRS 89–24	84–100% *in vitro*	Leelasuphakul et al. (2006)
Triterpene glycosides TPG1	Inhibits mycelial growth of MoO	*Trevesia palmata*	100% *in vitro*	Kim et al. (2018)
Maejaposide A	Suppresses disease development caused by MoO	*Maesa japonica*	85–98% *in planta*	Ngo et al. (2019)
Camptothecin	Inhibits mycelial growth and conidial germination of MoO	*Camptotheca acuminata*	100% *in vitro*	Xu et al. (2019)
Bayogenin 3-*O*-cellobioside	Inhibits conidial germination and appressorium formation of MoO	*Oryza sativa*	100% *in vitro*	Norvienyeku et al. (2019)
Pannellin	Inhibits germ tube formation of MoO	*Aglaia edulis*	95% *in vitro*	Engelmeier et al. (2000)
Tricyclazole	Inhibits melanin biosynthesis of MoO	Eli Lilly Company, Greenfield, Indiana	100% *in planta*	Woloshuk et al. (1983)
Tricyclazole	Inhibits mycelial growth of MoO	*Neostapfia colusana*	96% *in vitro*	Froyd et al. (1976)
Allicin 84	Inhibits conidial germination of MoO	*Allium sativum*	90% *in vitro*	Curtis et al. (2004)
Allicin	Inhibits conidial germination of MoO	*A. sativum*	99% *in vitro*	Fry et al. (2005)
Isobavachalcone (IBC)	Inhibits mycelial growth by cell wall degradation of MoO	*Psoralea corylifolia* L	92% *in vitro*	Liu et al. (2018)
Trans-2-hexenal (leaf aldehyde)	Reduces disease severity caused by MoO	Chisso Petrochemical Co., Chiba, Japan	100% *in planta*	Tajul et al. (2012)
Amalab-E	Inhibits mycelial growth of MoO	*Aegle marmelos*	98% *in vitro*	Rout and Tewari (2012)
Plant Tonic9 (EOX-SOV)	Inhibits mycelial growth and conidial germination of MoO	*Elaeis guineensis*	95% *in vitro*	Abed-Ashtiani et al. (2018)
Oligomycins	Inhibits mycelial growth, conidiogenesis, conidial germination, and supresses MoT	*Streptomyces* sp.	98% *in vivo*	Chakraborty et al. (2020a)
Linear lipopeptides	Inhibits mycelial growth, conidiogenesis, conidial germination, and supresses MoT	*Bacillus subtilis*	98% *in vivo*	Chakraborty et al. (2020b)

MoT: *Magnaporthe oryzae* Triticum; MoO: *M. oryzae* Oryzae; MoE: *M. oryzae* Eleusine; MoS: *M. oryzae* Setaria

AVR repertoires among *M. oryzae* pathotypes is likely caused by adaptation to individual hosts that exerted a wide range of selection pressures. Contributing factors in the evolution of effector genes in the *M. oryzae* population need to be explored more precisely. To find vulnerabilities in the fungus that stops its explosive plant damaging nature, some major scientific questions need to be solved. These research questions are (i) how the blast fungus precisely controls the gene expression of biotrophic invasion-specific effector genes; (ii) how the effectors function to overcome host resistance and increase disease severity; (iii) how the lesion develops from dying tissues followed by fungal sporulation; and (iv) how dramatic hyphal morphogenic transitions, environmental sensing, signaling, and highly coordinated gene expression take place that characterize all phases of the disease cycle.

18.9.2 Management of Blast Disease

M. oryzae is an economically important plant pathogen with the potential of causing an epidemic on major cereals like rice and wheat, inflicting food and nutritional insecurity for millions in the world. This fungal pathogen causes devastating yield reduction of rice on a regular basis, and lately on wheat, although the disease severity largely depends on the prevailing field conditions such as temperature, relative humidity, level of disease resistance of the cultivar and crop stage during infection. Most studies related to the potential of the blast pathogen to cause disease epidemics were focused mainly on rice with only recent attention on wheat blast, which is now also considered as an emerging threat for global food security but disease dynamics is poorly understood. The significant gaps in our understanding of epidemiology and host-pathogen interactions will need to be addressed in order to develop long-term management strategies. Further studies are needed to identify the major sources of primary and secondary inoculum, predisposing factors, and relative contributions of inoculum types to the outbreak of an epidemic for developing more effective management recommendations. Deployment of recently developed rapid diagnostic tools using genome-specific markers and Cas12a-mediated technologies for assessing seed health and quarantine would restrict further spread of wheat blast disease. Development of cultivars with durable resistance is of utmost necessity by utilizing the information on *M. oryzae* population structure and genetic information. Marker-assisted selection and CRISPR-Cas technology should speed up breeding efforts and provide an opportunity for the incorporation of cloned and characterized *R* genes into susceptible cultivars, respectively. In addition, agro-eco system-specific integrated disease management options encompassing resistant varieties, nutrient management like application of recommended nitrogen and silicon fertilizers, biological and chemical control should be developed and deployed. Open science and open data sharing approaches should bring the scientific community together in real time to combat the threat of this devastating disease and thereby should help in attaining global food and nutritional security under the threat of global climate change.

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