Ralph A. Dean · Ann Lichens-Park
Chittaranjan Kole *Editors*

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ISBN 978-3-662-44052-0 ISBN 978-3-662-44053-7 (eBook)
DOI 10.1007/978-3-662-44053-7
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014946390

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The turn of the century not only ushered in a new millennium, but the age of microbial genomics with the genome sequence of the first plant pathogen *Xylella fastidiosa,* the bacterium that causes citrus variegated chlorosis. At that time, knowledge of genomes of agriculturally significant microbes was extremely limited. In an effort to improve the situation, the United States Department of Agriculture (USDA) offered a competitive grants program in 2000 to support the sequencing of agriculturally relevant microorganisms. In the following year, the United States National Science Foundation (NSF) partnered with USDA to jointly offer a competitive grants program that would support genome sequencing for more diverse microorganisms. The USDA/NSF Microbial Genome Sequencing Program was offered for 10 years. During that time, USDA supported the sequencing of a large number of agriculturally important microbes, including bacteria, viruses, fungi, oomycetes and even a nematode. Some of the most expensive projects were jointly funded by USDA and NSF. Other sequencing projects were completed in collaboration with the United States Department of Energy's (DOE) Joint Genome Institute (JGI). In addition, NSF and DOE supported the genome sequencing of microbes of importance to other areas of science. The last year of the USDA/NSF Microbial Genome Sequencing Program was in 2009, by which time sequencing costs had decreased dramatically and sequencing speed had increased tremendously. USDA's support for microbial genomics had shifted toward functional analysis of the sequenced genomes. This book describes how the availability of the genome sequences of some agriculturally important plant-associated fungi, many of which were first sequenced with support from the USDA, has revolutionized our understanding of these eukaryotic microorganisms. This book also describes how knowledge derived from genomics can be translated into improved ways of managing microbes so as to increase the sustainability of agriculture in the United States and around the world.

Genome sequence information from fungi and oomycetes has laid the foundation for significant increases in knowledge of their lifestyles, their dynamic evolution, and how they interact with plants. New approaches for functional analysis of the genomes are accelerating the progress toward novel understanding and improved management methods.

In this volume on "Genomics of Plant-Associated Fungi: Monocot Pathogens," each chapter describes the genomic analysis of a genus, species

or group of related fungi. Some of these genera contain species that attack dicot hosts, but we include them here because they include important pathogens of cereals and other monocot plants. A companion volume, which we have also edited, is entitled "Genomics of Plant-Associated Fungi and Oomycetes: Dicot Pathogens." A third volume (edited by Dr. Dennis Gross, Dr. Ann Lichens-Park and Dr. Chittaranjan Kole) describes the genomic analysis of plant-associated bacteria. Taken together, these three volumes illustrate some fundamental discoveries about these microbes with regard to the overall structure of their genomes, their lifestyles, and the molecular mechanisms that form the basis of their interactions with plants. Many of the genomes described exhibit considerable variation in DNA content, even among related species, illustrating selective invasion and expansion of repetitive genetic elements. Some genomes, such as those of *Phytophthora* species, show a large degree of conservation in gene content and colinearity (synteny) among related species. On the other hand, species of *Cochliobolus* and *Mycosphaerella* are more diverged but they exhibit mesosynteny, where gene content is conserved within chromosomes but gene order is not. In other genera, gene conservation is minimal. Some fungal genomes, such as *Pyrenophora tritici-repentis* are unstable and dynamic with large differences in genome size and chromosome number within a population. A number of chapters show that fungal genes, DNA segments (*Verticillium*) and even chromosomes (*Fusarium*, *Alternaria* and *Mycosphearella*) can move within species, genera, or even across kingdoms. In many instances, these events affect pathogenicity and host range.

Biotrophic fungi feed on living organisms. Genomics has provided new insights about fungal lifestyles, such as obligate biotrophy. Mildews, rusts, and other fungi that are obligate biotrophs have lost many genes involved in primary metabolism (for example, nitrogen, sulfate and amino acid biosynthesis) and also secondary metabolism. Indeed, *Blumaria* and other powdery mildews have only half of the gene content of related fungi. Necrotrophic fungi live on dead plant material. Genome structural and functional analysis has revealed the necrotrophic lifestyle to be more sophisticated than once thought. Necrotrophs possess effectors, typically gene products that affect the development of diseases on host plants. Moreover, necrotrophs do not contain an excess of genes for degradation of plant material. Regulation of these degradative genes, rather than expansion of them, may be the key to the necrotrophic lifestyle.

Genomics has led to practical advances and the understanding needed to implement fair and effective policies. Beginning in 2013, all fungi must have a single name as determined during the Nomenclature Session at the Botanical Congress in Melbourne. It is no longer acceptable to use the anomorph or teleomorph names. Genome sequences, which are the foundation of modern classification, have clarified the species concept in some instances but in other cases the "One Fungus One Name" concept has prompted much debate and controversy among mycologists. A name has important federal and global implications. For example, clear nomenclature is needed for effective quarantine policies.

Fungal diagnostics has been greatly advanced through genomic technologies. The ability to accurately distinguish between closely related pathotypes is another requirement for effective quarantine policies. This can be very important for tracking devastating plant pathogens, such as the Ug99 pathogen that causes wheat stem rust.

Genomic studies of populations enable accurate reconstruction of previous disease epidemics, notably that of *Phytophthora infestans*, the oomycete pathogen that caused the notorious Irish potato famine. Genomic information enables predictions about the spread and evolution of new races. Knowledge about changes in race structure, including fungicide sensitivity, provides insights that influence fungicide use and contribute to improved plant breeding and cultivar release.

We wish to express our thanks to all of the authors and co-authors who contributed to the chapters in this volume. They have done a tremendous job, clearly describing the novel findings and exciting advances enabled by genomics with regard to the microbes addressed in their chapters. We also wish to specifically thank some current and former employees of USDA, NSF and DOE whose support has been invaluable to the success of the microbial genomics program and to the existence of this volume. These people are Dr. Sonny Ramaswammy, Dr. Colien Hefferan, Ms. Betty Lou Gilliland, Ms. Erin Daly, Mr. Edward Nwaba, Dr. Deborah Sheely, Ms. Cynthia Montgomery, Dr. Michael Fitzner, Dr. Daniel Jones, Ms. Pushpa Kathir, Dr. Anna Palmisano, Dr. Mark Poth, Dr. Maryanna Henkart, Dr. Daniel Drell, and all of the USDA and NSF Program Officers and Staff who worked with Dr. Ann Lichens-Park while the Microbial Genome Sequencing Program was offered. Space limitations prevent us from describing the roles played by each of these individuals but their contributions were significant and we are immensely grateful to all of them.

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Contents

Pyrenophora tritici-repentis: A Plant Pathogenic Fungus with Global Impact

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1.1 Introduction

1.1.1 Morphology and Taxonomic Position

Pyrenophora tritici-repentis (*Ptr*) (Died.) Drechs. (syn. *P. trichostoma* (Fr.) Fuckel), anamorph: *Drechslera tritici-repentis* (Died.) Shoem. (syn. *Helminthosporium tritici-repentis* (Died.)) is a pathogenic fungus and causal agent of the economically important disease, tan spot of wheat. Throughout this chapter, *Pyrenophora tritici-repentis* will be referred to as *Ptr*.

Ptr is a filamentous ascomycete that produces gray/white multinucleate mycelia and propagates both asexually (conidia) and sexually (ascospores). Sequential arrangement and the same transcriptional directionality of the *MAT* genes, *MAT1-1* and *MAT1-2*, in a single locus is consistent with the homothallic nature of sexual reproduction exhibited by *Ptr* (Lepoint et al. 2010). Conidia are borne on straight, cylindrical, olive-black conidiophores, often swollen at the base. These conidiophores are multinucleate and arise as single units or clusters of two or three through stomata, in between epidermal cells, or on wheat straw. Conidia are pale straw-colored (subhyaline), cylindrical and rounded at the apex,

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R. A. Dean et al. (eds.), *Genomics of Plant-Associated Fungi: Monocot Pathogens*,
DOI: 10.1007/978-3-662-44053-7_1,

and characteristically four to seven-septate, and multinucleate (Bockus et al. 2010; Ellis and Waller 1974). These spores have distinct conical basal segments with a characteristic "snakehead-like" appearance that is diagnostic. Black pseudothecia (can be beaked) contain clavate asci, each bearing eight ascospores. The brown, oval to globose ascospores are multinucleate with three transverse septa and slight septal constrictions (the median cell potentially with longitudinal septation) (Bockus et al. 2010; Ellis and Waller 1974).

Ptr belongs to the class Dothideomycetes, which is the most diverse and largest class of ascomycete fungi. It is in the order Pleosporales and family Pleosporaceae and is grouped with other agriculturally relevant species of *Cochliobolus*, *Leptospheria*, and *Stagonospora* (Manning et al. 2013; Schoch et al. 2009) within the Pleosporales. Multi-gene phylogenetic analyses of sequence data of the *MAT1-2* high-mobility group (HMG) box, glyceraldehyde phosphate dehydrogenase (GPD), and internal transcribed spacer (ITS) regions place *Pyrenophora* species in a monophyletic clade (Andrie et al. 2008; Zhang and Berbee 2001) and provide high confidence in *Pyrenophora* species designations (Andrie et al. 2008).

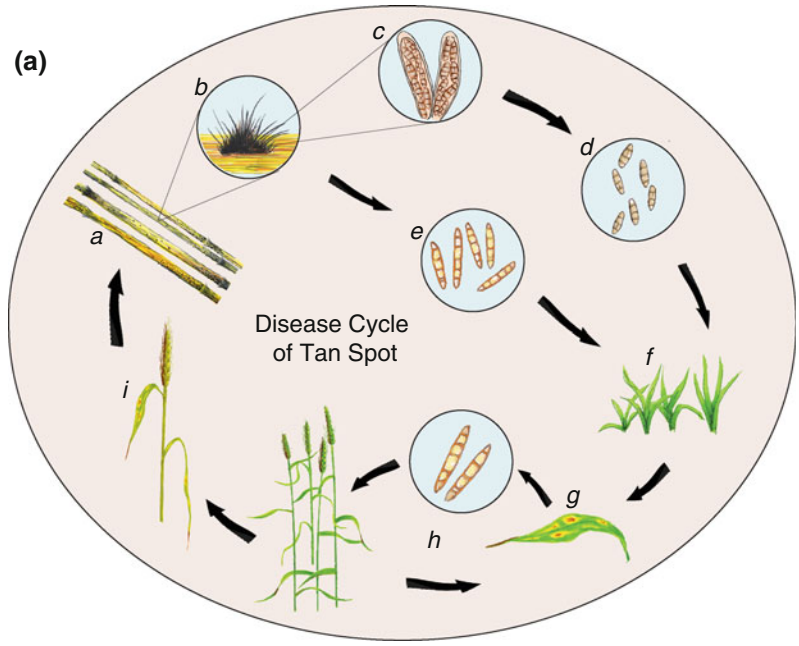
1.1.2 Host Range and Tan Spot of Wheat Disease Cycle

Ptr is mostly associated with tan spot of wheat, but isolated from other cereal and noncereal grasses. All classes of wheat are susceptible including *Triticum aestivum* (bread wheat) and *T. turgidum* (durum wheat). *Ptr* has been isolated from rye (*Secale cereale*), bromegrass (*Bromus* spp.), orchardgrass (*Dactylis glomerata*), and many other graminaceous species including barley (*Hordeum vulgare*) and oat (*Avena sativa*). Native prairie grasses and other grass species are considered to be sources of inoculum and could provide genetic diversity for *Ptr* (Bockus et al. 2010; Ali and Francl 2003; Hosford 1971; Krupinksy 1986, 1992a).

Throughout the disease cycle (Fig. 1.1a), both teleomorph (sexual reproduction) and anamorph (asexual reproduction) stages persist. In the field, following the harvest season, *Ptr* can overwinter and survive saprophytically on wheat stubble where pseudothecia develop and mature. Ascospores (teleomorph state), which are considered the primary inoculum are present throughout the growing season, and infect leaves of young plants in close proximity to the infected plant residue. Conidia (anamorph state), although considered mainly a secondary inoculum with repeated cycles of production during the growing season, can also play a primary role in the initial stages of infection (Krupinsky 1992b). Humidity induces conidial production on leaves within maturing lesions and long-distance dispersal by wind is responsible for spread of the disease (Schilder and Bergstrom 1992). Multiple rounds of inoculum can sustain the severity of tan spot through all stages of plant growth, being especially severe on older leaves. Symptoms induced by *Ptr* are shown in Fig. 1.1b. Infected seed (red smudge) has also been shown to be a source of inoculum where mycelia develop in the pericarp and can remain viable up to 3 years in refrigerated storage (Bergstrom and Schilder 1998; Schilder and Bergstrom 1995).

1.1.3 Relevance to Agriculture

Wheat currently constitutes more than 20 % of the world's caloric intake, and in 2011 approximately 700 million tons were grown over 220 million ha (Fig. 1.2) (CIMMYT 2012; FAOSTAT 2012; USDA_ERS 2012). *Ptr* exhibits the widest geographic range of any known *Pyrenophora* species De Wolf et al. 1998) and though *Ptr* was first identified as an occasional pathogen of wheat (Conners 1937; Dreschler 1923; Mitra 1934), it has spread steadily throughout all major wheat-growing regions of the globe. Moreover, tan spot disease incidence and severity have increased dramatically, with many of the major outbreaks having coincided strongly with implementation of minimal tillage practices (Annone 1998; Oliver et al. 2008; Rees et al. 1988). Accordingly, tan



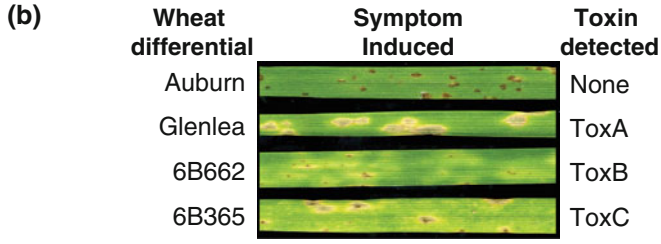


Fig. 1.1 Disease cycle of tan spot caused by *P. tritici-repentis*. **a** The schematic depicts the major steps of wheat infection by *P. tritici-repentis*. *P. tritici-repentis* overwinters saprophytically on wheat straw (*a*) where pseudothecia (*b*) develop and ascospore-containing asci (*c*) mature. In spring, mature ascospores (*d*) are released and serve as a primary inoculum to infect young plants (*f*). Asexual conidia (*e*) may also be present in the early stages of disease induction. Mature lesions (*g*) are the source of newly produced conidia that serve as secondary inoculum throughout the growing season. Conidia, dispersed by wind and rain, promote infection of new plants and re-infection of the source plants (*h*). Depending on environmental and climatic conditions, infection of foliage, stems, and/or spikes can occur (*i*). **b** The standard set of differential wheat lines and a resistant cultivar were inoculated with a race 8 isolate of *Ptr* that produces ToxA, ToxB and ToxC. No symptoms are produced on resistant cultivar Auburn; Glenlea is sensitive to ToxA and has a susceptible reaction to the isolate with characteristic necrotic lesions. 6B662 and 6B365 are sensitive to ToxB and ToxC, respectively, and have susceptible reactions with characteristic chlorosis

spot is now considered an economically important disease of wheat worldwide (Fig. 1.2) (Ciuffetti and Tuori 1999; De Wolf et al. 1998; Strelkov and Lamari 2003).

In 2003, tan spot was identified as the most prevalent leaf spot disease of wheat in Canada, and in the US tan spot ranks consistently amongst the top three most prevalent fungal pathogens found to reduce wheat yields (Fernandez et al. 2012; Bockus et al. 2001; Chu et al. 2008a; Tekauz et al. 2004). Yield losses in the US have been estimated at 2–15 % chronically, with

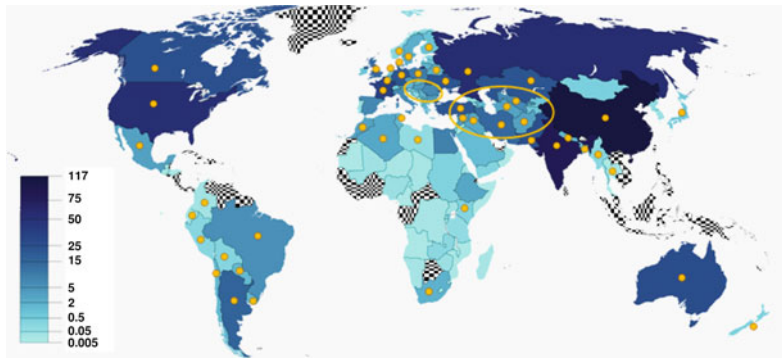


Fig. 1.2 Maps overlaying global wheat production and incidence of *P. tritici-repentis*. Global wheat yields of individual countries for 2011 (wheat-producing countries are *shaded*; *darker shading* represents greater annual wheat production; *checkered areas* represent countries with negligible or no wheat production), with nations (*dots*) and regions (*encircled areas*) of the world that have reported tan spot disease of wheat. Global wheat production data sourced from http://www.usda.gov and http://faostat.fao.org

maximum recorded losses of approximately 50 % (Evans et al. 1999; Hosford et al. 1982; Wegulo et al. 2009). Australia is currently heavily impacted by the disease, where major industry losses due to tan spot are estimated to exceed $200 million annually (Murray and Brennan 2009). South American and African nations have reported some of the heaviest crop damage due to this pathogen (Gamba et al. 2012; Gilchrist et al. 1984; Mamluk 1993; Moreno and Perello 2010). In the Indo-Gangetic Plains region, current yield losses due to Helminthosporium leaf blight, the combined effects of tan spot and spot blotch (*C. sativus*), average 15 % annually and can reach more than 30 % (Duveiller 2004; Sharma et al. 2004; Sharma et al. 2003).

Identification of tan spot within the last decade in regions previously thought to be free of the disease, including regions of Europe and Asia (Ali et al. 2001; Postnikova and Khasanov 1998; Singh 2007; Todorova 2006), along with increasing disease severity in certain wheat-growing nations, suggests that the impact of *Ptr* on the global wheat supply will continue to grow in the near future. The global effects of climate change on *Ptr*, wheat crops, and tan spot disease severity are expected to vary depending upon latitude, elevation, organism-specific life cycles, and other factors. However, current climate change models favor a global increase in distribution area, infection rates, and severity of fungal plant pathogens such as *Ptr* (Duveiller et al. 2007; Luck et al. 2011; Rosenzweig et al. 2000, 2001). Therefore, monitoring of tan spot symptoms, and early detection of *Ptr* will be imperative for tracking and potentially containing the spread of this economically important wheat disease.

Currently, frequent crop rotations and fungicide applications, along with planting of resistant wheat cultivars are common strategies employed to reduce tan spot spread and severity in the field (Jorgensen and Olsen 2007; Lamey and McMullen 2011; Singh et al. 2012). Still, high levels of crop loss due to infection by *Ptr* show that tan spot mitigation presents a continuing challenge to wheat growers worldwide. Utilizing *Ptr* host-selective toxins (HSTs) rather than *Ptr* conidial inoculations to screen seedlings early in wheat breeding programs could provide a more direct and optimized approach to identify resistant (insensitive) cultivars for advancement. Identification of new, yet-to-be-characterized HSTs secreted by pathogenic *Ptr* races will also provide breeders with additional tools to more effectively select for resistant wheat cultivars, reducing the likelihood of crop damage and pathogen spread, and the need for fungicides. Decreased use of expensive and hazardous agricultural chemicals reduces grower costs, and leads to healthier environments and economies. Characterization of the genomes of

Ptr isolates obtained from all geographic regions where tan spot has been identified will be instrumental in defining the range of pathogenicity factors present in the species, which will aide in future attempts to mitigate the disease.

1.1.4 Pathogen Population Diversity

1.1.4.1 Physiologic Specialization in *P. tritici-repentis*

The occurrence of physiologic specialization in *Ptr* has long been established. Hosford (1971) demonstrated that wheat genotypes vary in their resistance to tan spot, while isolates of the fungus vary in their pathogenicity. However, while recognized in some early studies, physiologic specialization was described only in quantitative terms, through the measurement of parameters such as lesion size (Cox and Hosford 1987; Misra and Singh 1972), lesion number, percent infection (da Luz and Hosford 1980), and percent leaf necrosis (Nagle et al. 1982; Schilder and Bergstrom 1990). These approaches did not distinguish between qualitatively different types of foliar lesions. It was not until Lamari and Bernier (1989a, 1991) showed that the necrosis and chlorosis symptoms associated with tan spot are distinct, and result from highly specific interactions between *Ptr* and its wheat host, that physiologic specialization in the tan spot fungus began to be well understood.

The recognition that isolates of *Ptr* can cause necrosis and/or chlorosis in a highly specific manner allowed for their classification into pathotypes based on a differential ability to induce these symptoms on the leaves of selected wheat hosts (Lamari and Bernier 1989a). Thus, isolates of the fungus were initially classified as pathotype 1 (necrosis$^+$ chlorosis$^+$), pathotype 2 (nec$^+$ chl$^-$), pathotype 3 (nec$^-$ chl$^+$), or pathotype 4 (nec$^-$ chl$^-$), the latter of which was considered to be nonpathogenic. However, while this symptom-based classification system enabled the grouping of isolates according to qualitatively different lesion types, it could not accommodate situations in which isolates of *Ptr* could induce the same symptom, but on different host genotypes. This limitation became evident when pathotype 3 isolates were identified from North Africa that possessed a virulence pattern distinct from previously described North American isolates of pathotype 3; while both groups caused only chlorosis, they caused this symptom on different wheat hosts (Lamari et al. 1995). In order to accommodate isolates of *Ptr* with distinct virulence patterns, a race-based classification system was proposed, in which isolates of the fungus were classified according to their virulence pattern on a wheat differential set (Lamari et al. 1995). This system has been widely adopted and is used to characterize pathogenic diversity on *Ptr* (Lamari and Strelkov 2010). The differential set consists of six wheat lines and cultivars, including the hexaploids 'Glenlea', line 6B662, line 6B365 and 'Salamouni', and the tetraploids 'Coulter' and line 4B1149. Of these hosts, however, only 'Glenlea' and lines 6B662 and 6B365 have been effective for distinguishing the currently defined races of *Ptr*.

1.1.4.2 Race Structure and Geographic Distribution

Thus far, eight races of *Ptr* have been characterized, based on the reaction of the three effective differential hosts, 'Glenlea', 6B365 and 6B662, to spore inoculation and the HSTs produced (Table 1.1, Fig. 1.1B) (Lamari and Strelkov 2010; Strelkov and Lamari 2003). To date, three *Ptr*-produced HSTs have been characterized, and they are designated as Ptr ToxA (syn. Ptr necrosis toxin, Ptr toxin, and ToxA) (Ciuffetti et al. 1998; Ballance et al. 1989; Tomas et al. 1990; Tuori et al. 1995; Zhang et al. 1997), Ptr ToxB (syn. Ptr chlorosis toxin) (Ciuffetti et al. 1998; Orolaza et al. 1995; Strelkov et al. 1999), and Ptr ToxC (syn. Ptr chlorosis toxin) (Ciuffetti et al. 1998; Effertz et al. 2002), according to nomenclature agreed upon by the community of researchers working on the wheat-*Ptr* pathosystem (Ciuffetti et al. 1998). HSTs are described in greater detail in Sect. 1.1.6. Throughout this chapter Ptr ToxA, Ptr ToxB, and Ptr ToxC will be referred to as ToxA, ToxB, and ToxC, respectively.

Table 1.1 Toxin production and reaction of three wheat differential hosts to the eight currently defined races of *Pyrenophora tritici-repentis*

Toxin produced[a]				Differential host[b]		
Race	ToxA	ToxB	ToxC	'Glenlea'	6B662	6B365
1	■		■	S(N) [c]	R	S(C)[c]
2	■			S(N)	R	R
3			■	R	R	S(C)
4				R	R	R
5		■		R	S(C)	R
6		■	■	R	S(C)	S(C)
7	■	■		S(N)	S(C)	R
8	■	■	■	S(N)	S(C)	S(C)

[a] Toxins produced by each race are marked with black boxes
[b] The three hexaploid wheat genotypes listed are effective for distinguishing the eight current races of *Ptr*
[c] S(N) = susceptible (necrotic reaction); S(C) = susceptible (chlorotic reaction)
R = resistant Adapted from Lamari and Strelkov (2010)

Races 1 (produces ToxA and ToxC) and 2 (produces ToxA), which correspond to the original pathotypes 1 and 2, respectively, appear to be the predominant races in the Great Plains of North America (Lamari et al. 1995, 1998; Ali and Francl 2003; Aboukhaddour et al. 2013; Singh et al. 2007) and the Southern Cone Region of South America (Gamba et al. 2012). These races have also been identified in the wheat center of diversity (Lamari et al. 2005), with race 1 also recently reported from North Africa (Benslimane et al. 2011). Race 3 (produces ToxC), corresponding to the original pathotype 3, has been identified (usually at fairly low frequencies) from North America (Lamari et al. 1998; Ali and Francl 2003; Singh et al. 2007; Aboukhaddour et al. 2013) and the Caucasus region (Lamari et al. 2005). Race 4, which consists of nonpathogenic or avirulent isolates of *Ptr* and produces no known HST, corresponds to the original pathotype 4 and has been reported from North America (Lamari et al. 1998; Ali and Francl 2003; Singh et al. 2007; Aboukhaddour et al. 2013) and North Africa (Benslimane et al. 2011). Like race 3, race 4 generally is found at low frequencies on wheat, although it was the most common race identified from noncereal grasses in the Great Plains (Ali and Francl 2003). Race 5 (produces ToxB) was first found in Algerian collections of *Ptr* (Lamari et al. 1995), and subsequently reported from the United States (Ali et al. 1999), Canada (Strelkov et al. 2002), Azerbaijan and Syria (Lamari et al. 2005). Nonetheless, despite this wide distribution, extensive surveying suggests that race 5 is rare in North America. Race 6 (produces ToxB and ToxC), has only been identified from pathogen populations in Algeria (Strelkov et al. 2002), while races 7 (produces ToxA and ToxB) and 8 (produces ToxA, ToxB, and ToxC) were originally reported from the Middle East and the Caucasus (Lamari et al. 2003, 2005), and have since been reported from Algeria as well (Benslimane et al. 2011). In *Ptr* populations from Australia, where tan spot is one of the most damaging diseases of wheat, the *ToxA* gene (encoding the necrosis-inducing HST ToxA) was found to be ubiquitous, while *ToxB* (encoding the chlorosis-inducing HST ToxB) was absent, consistent with a predominance of races 1 and/or 2 on that continent (Antoni et al. 2010). As would be expected, the greatest diversity in the race composition of *Ptr* appears to occur in regions corresponding to the wheat center of diversity (Lamari et al. 2005; Lamari and Strelkov 2010).

In addition to the eight well-characterized races of *Ptr*, there have been a number of reports suggesting the existence of other races or novel

virulence patterns. Andrie et al. (2007) described two isolates of the fungus that, while producing infection phenotypes consistent with classification as races 2 and 8, were genotypically distinct from those races. Specifically, the isolates did not possess all of the toxin-encoding genes expected for races 2 and 8. Thus, to validate the presence/absence of the *ToxA* and *ToxB* genes it was proposed that, in addition to phenotypic analyses, genotypic analyses (PCR or Southern analysis) are conducted to ensure that a particular isolate does or does not conform to the current classification system (Andrie et al. 2007). Unfortunately, no molecular test is currently available for the identification of ToxC; therefore, classification must rely on the reaction on the differential line 6B365 alone. Necrosis-inducing isolates of *Ptr*, which apparently lack the *ToxA* gene coding for the necrosis-inducing toxin ToxA, were also reported from Arkansas (Ali et al. 2010). In another study characterizing *Ptr* populations from North Africa, five isolates were identified that caused disease on tetraploid but not hexaploid wheat, suggesting that these isolates could represent a new race of the fungus (Benslimane et al. 2011). There have been other suggestions of the existence of new races of *Ptr,* but these have yet to be published as full reports in the refereed literature. The continued identification of novel races of the tan spot fungus is expected. Currently, the ability to detect races is limited by the size and effectiveness of the differential set. As such, the discovery of new races will likely be facilitated in the future by careful expansion of the host differential set (Lamari and Strelkov 2010), and/or by the identification of additional HSTs produced by *Ptr*.

1.1.4.3 Genetic Analyses of Diversity in *P. tritici-repentis*

While there has been a significant effort to characterize pathogenic diversity in *Ptr*, other studies have focused on understanding genetic diversity in populations of the fungus. In one of the earliest such studies, random amplified polymorphic DNA (RAPD) markers were used to examine the relationship between isolates from North America and North Africa that represented races 1–6 of *Ptr* (Aung 2001). Significant differences were found between necrosis-inducing isolates of races 1 and 2, which produce ToxA, and necrosis noninducing isolates of races 3, 5, and 6, which do not produce ToxA. These findings were consistent with the results of a more recent study, in which a global collection of 80 isolates was examined with 31 simple sequence repeat (SSR) markers designed from the *Ptr* genome (Aboukhaddour et al. 2011). In this latter analysis, which included isolates representing the eight races of the pathogen, a significant genetic differentiation was detected among populations, and isolates could be grouped into four distinct populations based on their region of origin. Moreover, ToxA nonproducing isolates clustered together, but were distantly related to ToxA-producing isolates, indicating that the host-specificity imposed by the *Ptr* toxins may lead to differentiation among isolates of *Ptr* (Aboukhaddour et al. 2011).

In contrast to the above findings, an amplified fragment length polymorphism (AFLP)-based analysis of a collection of 97 isolates of *Ptr*, which included races 1–4 of the pathogen, suggested no relationship between isolate grouping and race or geographic origin (Friesen et al. 2005). This led the authors of that study to conclude that the *Ptr* population is preferentially outcrossing and that spread of the pathogen is recent or constant, and worldwide (Friesen et al. 2005). The apparent discrepancy between the results obtained by Aung (2001) and Aboukhaddour et al. (2011) on one hand, and Friesen et al. (2005) on the other, may reflect, at least in part, the differential origin of the isolates studied. The isolates analyzed by Friesen et al. (2005) originated in North America, South America, and Europe, while those analyzed by Aung (2001) came from North America (specifically Canada) and Algeria, and those analyzed by Aboukhaddour et al. (2011) included isolates from Syria and Azerbaijan as well as Canada and Algeria. Moreover, beyond the origin of the isolates analyzed, the type of markers used in the studies may have also impacted the results. The SSR markers used by Aboukhaddour et al. (2011) were designed to represent

each chromosome of *Ptr*, with some of the SSR loci located more than 2 Mb apart when on the same chromosome. It is likely, therefore, that the results of the SSR analysis provided a more representative picture, relative to earlier studies, of the diversity of the entire *Ptr* genome (Aboukhaddour et al. 2011). This suggestion is supported by a recent analysis of more than 400 isolates of *Ptr* with 12 SSR markers, in which moderate to high population differentiation was found between continents (Gurung et al. 2013).

In addition to the generally high degree of genetic variability that has been observed with respect to populations of *Ptr*, many reports have suggested that greater differences exist between pathogenic and nonpathogenic isolates of the fungus. Aung (2001) found that pathogenic isolates shared only 26 % similarity with nonpathogenic isolates, while Abboukhaddour et al. (2011) reported only 25 % similarity between pathogenic isolates and one nonpathogenic isolate. Similarly, an analysis of the diversity of the mating type locus (*MAT*) in a collection of 88 isolates, representing races 1–5 of *Ptr*, revealed two distinct phylogenetic groups (Lepoint et al. 2010). One of the groups was more homogenous than the other and was associated with isolates causing "typical" tan spot lesions. The second group was more heterogeneous and included race 4 isolates as well as isolates that caused mostly small lesions on wheat and other hosts (Lepoint et al. 2010). Likewise, a comparison of the genomes of isolates representing races 1, 4, and 5 of *Ptr* revealed greater genetic divergence between the pathogenic and nonpathogenic strains, suggesting their genetic segregation (Manning et al. 2013). Chromosome-based characterization of *Ptr* isolates has also shown a high level of genome plasticity in the fungus (Lichter et al. 2002; Aboukhaddour et al. 2009), with one study reporting greater karyotype polymorphisms between pathogenic and nonpathogenic strains (Lichter et al. 2002). Collectively, these studies seem to provide fairly strong evidence for the existence of significant differences between pathogenic and nonpathogenic isolates of *Ptr*. Differences between pathogenic and nonpathogenic isolates will be presented in greater detail in Sect. 1.3.1.

1.1.5 Genetics of Susceptibility and Resistance

1.1.5.1 Tan Spot Susceptibility Conforms to an Inverse Gene-for-Gene Relationship

Sensitivity to HSTs produced by some necrotrophic pathogens such as *Ptr* is conditioned by dominant host sensitivity (susceptibility) genes that result in a compatible interaction and disease. This is in contrast to host interactions involving pathogens with biotrophic lifestyles that follow classical gene-for-gene relationships where pathogen-produced effectors (avirulence gene products) are recognized by dominant host resistance (R) genes resulting in incompatible (resistant) interactions (Flor 1956). Therefore, some plant–necrotroph interactions, such as the wheat–*Ptr* interaction, are the inverse of the classical gene-for-gene system observed in host-biotroph systems (Friesen et al. 2008; Lamari and Strelkov 2010; Strelkov and Lamari 2003; Wolpert et al. 2002).

Consistent with the inverted gene-for-gene system and in contrast to the classic characterization of disease resistance loci, genetic loci in wheat that condition sensitivity to HSTs are generally considered as susceptibility loci because compatible host–HST interactions are highly correlated with disease. The genes *Tsn1*, *Tsc1*, and *Tsc2*, were designated as such because *Tsn1* (*Tan spot necrosis*) confers sensitivity to a necrosis-inducing HST (ToxA) and *Tsc1* and *Tsc2* (*Tan spot chlorosis*) confer sensitivity to chlorosis-inducing HSTs (ToxC and ToxB, respectively). Additional qualitative tan spot resistance (susceptibility) genes have been observed through conidial inoculations that are not (yet) associated with reaction to specific HSTs (Singh et al. 2008; Singh and Hughes 2006; Tadesse et al. 2006a, b). For these genes, the tan spot research community in 2007 adopted the designation '*Tsr*' (*Tan spot resistance*) (McIntosh et al. 2008). Genes associated with a specific reaction to a HST would continue to be given the *Tsn* or *Tsc* designations depending on the symptom elicited by the HST. For a more

comprehensive description of tan spot resistance and sensitivity, see (Faris et al. 2013).

1.1.5.2 Toxin Sensitivity Loci

Tomas and Bockus (Tomas and Bockus 1987) were the first to show that a necrosis-inducing toxic component of *Ptr* culture filtrate, later designated as *Ptr* ToxA (Ciuffetti et al. 1998), was cultivar specific, which suggested that genetic variation for sensitivity to the HST existed among wheat lines. Sensitivity was strongly correlated with susceptibility to tan spot (Tomas and Bockus 1987) and was shown to be controlled by a single dominant gene, *Tsn1*, located on the long arm of chromosome 5B (Faris et al. 1996; Lamari and Bernier 1989b). Anderson et al. (1999) demonstrated that the functional *Tsn1* allele was indeed a susceptibility gene. Positional cloning and characterization of *Tsn1* by Faris et al. (2010) revealed a gene that encodes a protein with conserved nucleotide binding (NB) and leucine-rich repeat (LRR) domains, as well as a serine/threonine protein kinase (S/TPK) domain, all of which are commonly found in classical R genes (Eitas and Dangl 2010). However, *Tsn1* was unique in that all three of the major domains occurred in the same protein (Faris et al. 2010).

While sensitivity to ToxA is sufficient on its own to confer disease (Ciuffetti et al. 1997), other determinants of disease can exist, including the potential of other HST interactions; therefore, sensitivity to ToxA does not always completely define susceptibility (Singh et al. 2008; Cheong et al. 2004; Chu et al. 2010, 2008b; Faris et al. 2012; Faris and Friesen 2005; Friesen et al. 2003). Under these circumstances, it influences disease severity to varying degrees depending on the genetic background of the host and the toxin compliment of the pathogen.

A single dominant gene was shown to confer susceptibility to a race 5 isolate of *Ptr* that produces ToxB and sensitivity to the partially purified toxin (Orolaza et al. 1995). Friesen and Faris (2004) showed that the ToxB sensitivity gene, designated *Tsc2*, was on the short arm of wheat chromosome 2B and accounts for 69 % of the disease variation caused by a race 5 isolate, demonstrating that, like the *Tsn1*–ToxA interaction, the *Tsc2*–ToxB interaction plays an important role in causing tan spot disease.

The *Tsc1*–ToxC interaction appears to be more complex than the *Tsn1*–ToxA and *Tsc2*–ToxB interactions. A quantitative trait locus (QTL) on the short arm of wheat chromosome 1A (*QTsc.ndsu-1A*) explained as much as 35 % of the variation associated with resistance to chlorosis induced by a race 1 isolate (Faris et al. 1997, 1999) and was also associated with resistance to chlorosis induced by race 3 (Effertz et al. 2001). Partially-purified ToxC from a race 1 isolate showed that insensitivity to the HST was governed by a single gene (*Tsc1*) on chromosome arm 1AS that coincided with the position of *QTsc.ndsu-1A* (Effertz et al. 2002). To date, studies have not conclusively established the mode of inheritance of sensitivity/insensitivity to ToxC.

1.1.5.3 Additional Tan Spot Resistance Loci

Although race 3 and 5 isolates are known to cause chlorosis in hexaploid wheat lines due to the *Tsc1*–ToxC and *Tsc2*–ToxB interactions, respectively, Gamba and Lamari (1998) showed that some isolates of races 3 and 5 caused necrosis in some tetraploid wheat lines. It was shown that single recessive genes, *Tsr2* and *Tsr5*, that were mapped 8.3 cM apart on durum chromosome 3BL, confer resistance to a race 3 and 5 isolate, respectively (Singh et al. 2008).

Evaluation of a set of synthetic hexaploid wheat lines for reaction to tan spot caused by a race 1 isolate identified three resistance genes now designated *Tsr3a*, *Tsr3b*, and *Tsr3c*, all located on the short arm of chromosome 3D (Tadesse et al. 2007). Additionally, tan spot resistance in the hexaploid wheat landrace Salamouni and the winter wheat cultivar Red Chief to a *Ptr* race 1 isolate was due to a single recessive gene on chromosome 3A designated *Tsr4* (Tadesse et al. 2006b, 2010). The fact that recessive alleles of the genes *Tsr2, Tsr3, Tsr4*, and *Tsr5* confer resistance would suggest that

they likely confer insensitivity to yet unidentified HSTs; at least two different groups have identified novel HSTs produced in *Ptr* cultures (Meinhardt et al. 2003; Tuori et al. 1995).

1.1.5.4 Quantitative Resistance

Tan spot reactions can be largely affected by genetic backgrounds, environmental conditions, and other factors, which is why the quantitative tan spot reaction rating scale developed by Lamari and Bernier (1989a) is widely used. Prior to the discovery by Lamari and Bernier (1991) that chlorosis and necrosis were controlled by independent factors, studies on the heritability of tan spot resistance showed that resistance was highly heritable, but quantitatively controlled (Elias et al. 1989; Nagle et al. 1982). QTLs have been identified that corresponded to locations of known HST sensitivity genes including *Tsn1* (Cheong et al. 2004; Chu et al. 2008b; Faris et al. 2012; Singh et al. 2008), *Tsc1* (Effertz et al. 2001; Sun et al. 2010), and *Tsc2* (Li et al. 2011), but also revealed numerous additional QTLs with significant effects for resistance/susceptibility to tan spot. Additionally, race-nonspecific resistance/susceptibility QTLs have been identified on wheat chromosome arms 1BS and 3BL (Faris and Friesen 2005), and 2AS, 5AL, and 5BL (Chu et al. 2008b). Taken collectively, the identification of at least two novel HSTs, the finding of race-nonspecific resistance/susceptibility QTLs, numerous QTLs with minor resistance effects, and additional monogenic recessive resistance genes indicate the strong potential for the existence of additional HST/host gene interactions and perhaps other genetic mechanisms associated with resistance and/or susceptibility.

1.1.6 Host–Pathogen Interaction

1.1.6.1 Infection by *P. tritici-repentis*

The interaction of a plant and microbe involves a complex array of signals (Harrison and Baldwin 2004; Glazebrook and Ton 2007) that result in a diversity of responses with corresponding changes of gene expression in both partners. As mentioned earlier, *Ptr* exists as both saprophyte and parasite. In the latter regard, it is generally considered to be a necrotrophic pathogen because it facilitates pathogenesis through the expression of HSTs that kill host cells in advance of its growth. Cytological studies of infection by *Ptr* (Dushnicky et al. Dushnicky et al. 1996, 1998a, b; Larez et al. 1986; Lamari and Bernier 1989b) have demonstrated that the infection process is more complex than might be expected for a necrotroph, and that infection by *Ptr* is not exclusively necrotrophic and includes a reduced biotrophic phase. The infection process starts with conidial germination and penetration of the epidermal layer, usually completed within 24 h. Following penetration, hyphal growth is restricted to the epidermal cells and vesicle-like structures are formed that give rise to numerous secondary hyphae formed within epidermal cells. This intracellular infection of the epidermis occurs in both compatible/susceptible and incompatible/resistant genotypes with epidermal cells capable of occasionally producing cell wall depositions suggestive of a biotrophic interaction. Following this "reduced biotrophic" phase, secondary hyphae emerge from the epidermis and begin to grow intercellularly among the mesophyll cells. At this stage, the distinction between a susceptible and resistant interaction can be observed. In the resistant interaction, infection is restricted to a limited number of mesophyll cells and growth of the fungus ceases. Symptoms are visible as small black spots with no chlorosis or necrosis. In the susceptible interaction, mesophyll cells appear to die and hyphae continue extensive intercellular growth, which leads to subsequent macroscopic symptom development and ultimately sporulation of the pathogen.

1.1.6.2 Host-Selective Toxins and Their Role in Pathogenesis

To gain a comprehensive understanding of plant host–pathogen interactions, it is essential to identify and characterize the factors that play a primary role in the ability of microorganisms to cause plant disease (pathogenicity or virulence

factors). *Ptr* produces multiple HSTs that play a key role in disease development on susceptible (sensitive) wheat and HSTs are known to be involved in pathogenesis because they are toxic only to hosts susceptible to the fungus but not to resistant plants or nonhosts (Walton 1996; Wolpert et al. 2002; Yoder et al. 1997; Ciuffetti et al. 2010; Lamari and Strelkov 2010; Friesen et al. 2008; Moreno et al. 2012). Additionally, HSTs have been demonstrated to play a causal role in pathogenesis through conventional genetic analyses of toxin production and pathogenicity; as well as, through the identification and cloning of the genes responsible for their production. Of the three currently characterized *Ptr* HSTs, ToxA, and ToxB are proteins. Characterization of ToxC is not complete and its isolation and characterization has proven to be difficult; however, ToxC appears to be a nonproteinaceous, polar, nonionic, low-molecular-mass molecule (Effertz et al. 2002).

1.1.6.3 Host-Selective Toxin, *Ptr* ToxA

ToxA, the most studied of the *Ptr* HSTs, was the first proteinaceous HST to be identified, purified, and characterized (Tomas and Bockus 1987; Ballance et al. 1989; Tomas et al. 1990; Tuori et al. 1995; Zhang et al. 1997). ToxA is the product of a single gene (*ToxA*) and encodes a pre-pro-protein. The pro-domain (4.3-kDa) is required for proper folding and maximal activity and the mature 13.2-kDa ToxA protein is secreted, following cleavage of both the pre- (23-amino acid signal peptide) and pro-domains (Tuori et al. 2000; Ballance et al. 1996; Ciuffetti et al. 1997). Analysis of the primary amino acid sequence of the mature ToxA protein revealed the presence of a conserved RGD (Arg-Gly-Asp) motif, known to be important for protein–protein interactions (Ciuffetti et al. 1997; Ballance et al. 1996; Zhang et al. 1997). Genetic transformation of nonpathogenic ToxA-minus isolates has demonstrated that the expression of *ToxA* is 'both necessary and sufficient' for pathogenesis (Ciuffetti et al. 1997).

Sarma et al. (2005) determined the high-resolution crystal structure of ToxA. Analysis of the three-dimensional structure supports a single domain protein with a β-sandwich fold of novel topology. In crystal form, ToxA exists as a strong interacting trimer, described as a three-bladed pinwheel with the RGD loop at the tips of the blades. In the absence of trimer formation, the monomeric structure of the RGD-containing, solvent-exposed loop is predicted to be larger and more mobile. In solution, ToxA exists predominantly as a monomer and the significance of a possible trimer in the interaction of ToxA with the host site-of-action is currently unknown. Structural studies revealed a surprising similarity with the classic mammalian RGD-containing motif, the fibronectin type III (FnIII) domain. Similar topologies and the positional conservation of the RGD-containing loop allows for the possibility that ToxA may be distantly related to mammalian FnIII proteins and may gain entry into the cell through interaction with a receptor via the RGD motif of ToxA (Sarma et al. 2005).

Additional lines of evidence provide support for the hypothesis that ToxA binds extracellularly and gains entrance into the cell via the RGD motif. Mutational analyses of the RGD motif and amino acids surrounding this motif, and within the solvent-exposed loop, were shown to be essential for ToxA activity (Sarma et al. 2005; Meinhardt et al. 2002; Manning et al. 2004). Mapping of the mutantional data onto the ToxA stucture further strengthened the importance of the RGD and surrounding sequence to activity (Sarma et al. 2005). Additionally, physiological studies provided direct support that the first contact of ToxA with its host occurs through an abundant, saturable, high-affinity binding site (putative receptor) that recognizes the solvent-exposed, RGD-containing loop on ToxA. Also, the RGD motif was shown to be required for ToxA internalization into sensitive (susceptible) mesophyll cells (Manning et al. 2008). The amount of ToxA internalization in susceptible wheat directly correlated with the extent of symptom development (Manning et al. 2008; Manning and Ciuffetti 2005). One current hypothesis is that genotype-specific uptake of ToxA occurs via receptor-mediated endocytosis (RME).

ToxA has been shown to be capable of crossing the plasma membrane and being internalized by toxin-sensitive wheat genotypes. Cytological analyses that incorporated immunocytochemical localization techniques coupled with direct observation of the sub-cellular distribution of green fluorescent protein (GFP)-labeled ToxA have demonstrated that once ToxA enters sensitive mesophyll cells it localizes to the chloroplast (Manning and Ciuffetti 2005). While toxin could not be detected in insensitive cells, internalization could occur, but toxin may not accumulate at levels sufficient for detection. Internalization and chloroplast localization of ToxA into sensitive genotypes is consistent with the cytoplasmic localization of the R gene-like ToxA-sensitivity gene product, Tsn1 (Faris et al. 2010), and the interaction of ToxA with two chloroplast localized proteins (Manning et al. 2007, 2010; Tai et al. 2007).

Intracellular expression of ToxA leads to cell death in both sensitive and insensitive wheat lines, barley, and tobacco (Manning and Ciuffetti 2005; Manning et al. 2010; Tai and Bragg 2007). The lack of *Tsn1* in all but sensitive wheat lines suggests that Tsn1 could be involved (indirectly) in toxin uptake, cytoplasmic accumulation, or chloroplast localization. Alternatively, the cell death induced in non-*Tsn1*-containing plant leaves may be due to additional ToxA activities that are not host-specific. Regardless, multiple lines of evidence support the role of chloroplasts as central players in ToxA-induced necrosis in sensitive wheat (Manning et al. 2007; Manning and Ciuffetti 2005; Pandelova et al. 2009, 2012; Tai et al. 2007; Manning et al. 2009, 2010). These studies suggest that there are multiple components that contribute to ToxA-induced necrosis in sensitive wheat that are complex and likely interconnected.

1.1.6.4 Host-Selective Toxin, *Ptr* ToxB

ToxB was the second proteinaceous HST from *Ptr* to be identified, purified, and characterized and is produced by select races (races 5, 6, 7, and 8) of *Ptr* (Orolaza et al. 1995; Strelkov et al. 1999, 2002; Martinez et al. 2001; Lamari et al. 2003). *ToxB* encodes an 87 amino acid pre-protein (Lamari et al. 2003; Martinez et al. 2001). Following cleavage of the pre-domain (23 amino acid, signal peptide), the mature 6.5-kDa ToxB protein is secreted (Strelkov et al. 1999; Figueroa Betts et al. 2011). In contrast to the single copy nature of *ToxA*, *ToxB* was shown to be present in multiple copies that range from 2 to 10, dependent on the race and/or isolate (Lamari et al. 2003; Martinez et al. 2004). Furthermore, it was shown that *ToxB* expression levels are proportional to copy number (Amaike et al. 2008). Genetic transformation of a non-pathogenic *ToxB*-minus isolate demonstrated that expression of *ToxB* rendered this isolate pathogenic and as with natural isolates that express *ToxB*, virulence of transformants was proportional to *ToxB* copy number (Strelkov et al. 2002; Ciuffetti et al. 2010). There are no known conserved functional protein domains encoded by the gene, but there are four cysteine residues, one in close proximity to the N- and another at the C-terminus (Martinez et al. 2001; Strelkov and Lamari 2003).

Additional *ToxB*-like genes are present within the *Ptr* species, the *Pyrenophora* genus, and other genera in the Pleosporaceae, and a distant homolog has been identified in *Magnaporthe oryzae* (Strelkov and Lamari 2003; Andrie et al. 2007; Martinez et al. 2004; Strelkov et al. 2006). At least some race 4 *Ptr* isolates contain a gene, *toxb,* which is present in single copy, is 86 % similar to *ToxB*, and encodes a protein with one additional amino acid compared to ToxB (Martinez et al. 2004; Strelkov and Lamari 2003). Though *toxb* is transcriptionally active, *toxb*-containing isolates do not cause symptoms on ToxB-sensitive hosts (Amaike et al. 2008). Heterologous expression of *toxb* and infiltration of ToxB-sensitive cultivars indicate that *toxb* is inactive (does not induce chlorosis) (Andrie and Ciuffetti 2011; Figueroa Betts et al. 2011; Kim and Strelkov 2007). A race 3 isolate of *Ptr* was shown to contain a *ToxB*-like gene that is similar to *ToxB* from race 5 isolates; however, the signal peptide is modified, which may lead to improper processing or protein folding (Strelkov et al. 2006). Transcripts of the race 3 *ToxB*-like gene in

Ptr have thus far only been detected in conidia (Strelkov et al. 2006). Isolates of the sister-species to *Ptr*, *P. bromi*, express *ToxB*-like genes (*Pb ToxB*) whose coding regions are ~89 % similar to *ToxB* and are also often present in multi-copy; however, unlike *ToxB*, these sequences are variable, both within and between isolates (Andrie and Ciuffetti 2011; Andrie et al. 2008). Interestingly, heterologous expression of several *Pb ToxB* loci revealed that Pb ToxB proteins do not induce symptoms on their host grass species, *B. inermis*, but some will cause chlorosis on ToxB-sensitive wheat cultivars (Andrie and Ciuffetti 2011). The detection of *ToxB*-like genes in other fungal species, including those in the orders Dothideomycetes and Sordariomycetes, suggests that this gene has a long evolutionary history, and unlike many HSTs that are thought to be transferred horizontally, was vertically transferred from a common ancestor (Andrie et al. 2008).

Structure-function studies of ToxB are hindered because there are no conserved protein motifs as there are in ToxA. However, one can take advantage of the activity and amino acid differences, between ToxB and toxb (active vs. inactive, 81 % identical) to derive those differences that impact function and thus provide clues to the structural requirements for activity (Andrie and Ciuffetti 2011; Figueroa Betts et al. 2011; Martinez et al. 2004; Andrie et al. 2008; Kim and Strelkov 2007). To this end, site-directed mutants based on amino acid differences, and chimeric genes formed between regions of ToxB and toxb were heterologously expressed and analyzed for activity. Results indicated that variation in any part of the protein affects full ToxB activity; however, sequence surrounding the first cysteine residue is required for proper folding of the protein and the N-terminal region of the protein is of particular importance for activity (Figueroa Betts et al. 2011). A comparison based on three dimensional structures derived from a Nuclear Magnetic Resonance (NMR)-based approach confirms the importance of the context of the disulfide bonds in ToxB and elucidates possible negative impacts of an additional amino acid in toxb on its function (Barbar and Ciuffetti unpublished data).

Structural (Figueroa Betts et al. 2011; Ciuffetti and Barbar, unpublished data) and biochemical analyses (Strelkov et al. 1999; Ciuffetti and Figueroa, unpublished data) suggest that ToxB shares characteristics with apoplastic effectors produced by other plant pathogenic fungi (Rep 2005; Stergiopoulos and de Wit 2009). The protein is small, with a relatively high percentage of cysteine residues that contribute to its compact and stable form (Martinez et al. 2001; Strelkov and Lamari 2003). Additional protein characteristics such as resistance to heat, organics and some proteolytic enzymes would provide an advantage in the inhospitable environment of the plant apoplast (Orolaza et al. 1995). Interestingly, there is evidence that ToxB may play a role outside of the apoplast in appressoria development (Amaike et al. 2008; Aboukhaddour et al. 2012). Thus, in addition to the role of ToxB as a HST, ToxB might fulfill additional functions related to basic pathogenic fitness, which may explain its presence and maintenance in other fungi.

1.1.6.5 Impact on Host Metabolism

The apparent purpose of necrotrophic pathogenicity factors, as with the HSTs of *Ptr*, is to induce cell death for the benefit of the pathogen that produces them. It has been proposed that susceptibility to some HST-producing necrotrophs requires host responses similar to those that result in resistance induced by biotrophic effectors (Wolpert et al. 2002; Lorang et al. 2012). Typically, host resistance responses to biotrophic effectors (avirulence determinants) involve R-gene mediated regulation of defense-related genes followed by cell death. A complete understanding of how HSTs condition plant disease susceptibility requires not only characterization of the HSTs but also determination of the nature of the host responses triggered. The strict requirement for HSTs for pathogenesis and disease susceptibility makes them an excellent tool to clarify the nature of the host response in susceptibility.

Symptoms produced by ToxA and ToxB on sensitive wheat leaves are distinct (necrosis and chlorosis, respectively) and develop at different rates and intensities, ultimately leading to cell

death (Pandelova et al. 2012). ToxA is rapidly internalized and cell death can be detected as early as 9 h after toxin treatment (Pandelova et al. 2012; Manning and Ciuffetti 2005). Early studies suggested that plant metabolism is actively involved in ToxA-induced effects and that signaling events initiated by toxin perception induce changes in gene expression that lead to cell death (Kwon et al. 1998; Rasmussen et al. 2004). Subsequent studies confirmed that major transcriptional reprogramming in response to ToxA occurs prior to symptom development and continues over time to the point of tissue collapse (Pandelova et al. 2009; Adhikari et al. 2009).

Early transcriptional changes due to ToxA treatment include induction of defense-related genes and transcription factors known to be important for their regulation. Furthermore, products of defense-related genes and pathways could be correlated with their transcript up-regulation (Pandelova et al. 2009; Adhikari et al. 2009; Vincent et al. 2012). Induction of defense responses by ToxA is likely due to activation of the product of the ToxA-sensitivity gene, *Tsn1*, which has structural characteristics similar to resistance genes, reinforcing the idea that susceptibility and resistance have overlapping pathways (Faris et al. 2010; Wolpert et al. 2002). That the ethylene biosynthesis pathway was also activated and its inhibition leads to a considerable delay in symptom development, provides evidence that ethylene acts as a modulator of ToxA-triggered cell death.

ToxA-induced cell death is also correlated with light-dependent accumulation of reactive oxygen species (ROS); in the absence of light and ROS accumulation there is no symptom development (Manning and Ciuffetti 2005; Manning et al. 2009). ToxA treatment differentially regulates transcript and protein levels of ROS detoxification enzymes with chloroplast-associated genes being down-regulated. There is a general trend for down-regulation of chloroplast-associated transcripts. While some of the cytosolic, ROS-associated genes are up-regulated, this is insufficient to prevent ROS accumulation and cell death (Pandelova et al. 2009; Vincent et al. 2012). Ethylene induction by ToxA may also facilitate ROS accumulation as it has been shown that ethylene can act as an amplifier of ROS (Wi et al. 2010).

The accumulation of ROS in ToxA-treated plants predominantly occurs in chloroplasts and is associated with the disruption of protein homeostasis in both photosystems (PS) (Pandelova et al. 2009; Manning et al. 2009; Vincent et al. 2012). Perturbations in PS complexes also occur in the absence of ROS and may be due to direct ToxA interaction with proteins involved in PS function. One of the proteins that interacts with ToxA is a chloroplast-localized protein named ToxA binding protein 1 (ToxABP1) (Manning et al. 2007), which is orthologous to the product of the *A. thaliana Thf1* (thylakoid formation1) gene implicated in biogenesis/degradation of photosystem II (Keren et al. 2005). Silencing of *ToxABP1* only partially reduces ToxA-induced necrosis (Manning et al. 2010), therefore, additional factors likely contribute to cell death. Plastocyanin, another chloroplast protein, is involved in electron transfer between PSII and PSI, and reported to have a direct interaction with ToxA. However, the contribution of this interaction to ToxA-induced necrosis has not yet been validated (Tai et al. 2007). Also, transcription of PS-associated genes is down-regulated in response to ToxA, which may prevent PS repair and assembly (Pandelova et al. 2009). Therefore, disruptions in PS homeostasis are likely the consequence of both direct and indirect actions of ToxA. In response to diminished photosynthetic capacity, ToxA-treated cells appear to attempt to replenish reducing equivalents and ATP by activation of carbon metabolism (Vincent et al. 2012).

The importance of chloroplasts as major energy providers makes them logical targets to impair cell function. Similar to ToxA, ToxB-induced chlorosis is light-dependent and involves chloroplast damage and inhibition of photosynthesis (Strelkov et al. 1998; Kim et al. 2010). However, cell death occurs only after 24 h of ToxB treatment and, unlike ToxA, does not lead to tissue collapse. The extensive transcriptional changes that precede symptom development are also delayed compared to ToxA (Pandelova et al.

2012). Pandelova et al. 2012 showed that despite the delay in responses, ToxA and ToxB induce similar sets of genes including ones involved in activation of defense-related pathways, which raises the possibility that, like ToxA-induced responses, ToxB-mediated responses may be due to interaction with an R gene-like protein. Differences in perception of ToxB, as well as lower levels of activation of some defense pathways (i.e., PAL and ethylene biosynthesis), may explain the delay in symptom development.

ToxB treatment also leads to ROS accumulation in chloroplasts, and similar to ToxA, down-regulation of PS components and chloroplast ROS detoxification enzymes (Pandelova et al. 2012; Kim et al. 2010). This may be a common response to both biotrophic and necrotrophic effectors, as a study involving comparative analyses of different types of biotic damage suggested that transcriptional responses of several groups of genes, including PS and ROS-detoxifying genes are consistently co-regulated. This study proposed that down-regulation of photosynthetic genes is compensatory by the plant to allow up-regulation of defense-related genes (Bilgen et al. 2010). The commonalities of ToxA and ToxB in triggering responses typical of an incompatible host-biotrophic interaction is consistent with the hypothesis that necrotrophic pathogens exploit the activation of defense-related pathways, and the associated biochemical and physiological outcomes, as a mechanism to promote cell death and host colonization.

1.2 Insights from the *P. tritici-repentis* Genome

Karyotype analyses of isolates of all currently characterized races from diverse geographic regions have shown a range of size and number of chromosomes, both between races and amongst the same race (Aboukhaddour et al. 2009; Lichter et al. 2002; Martinez et al. 2004). Genome sizes were estimated to range from 25.5 to 48 Mb with chromosome numbers between 8 and 11. In some cases, these estimates may be low, given the difficulty of resolving large chromosomes and chromosomes of similar sizes. There is no real correlation between size of the genome and race of the isolate, with the exception that all of the nonpathogenic isolates trended toward smaller genome sizes and chromosome numbers. An important step in understanding the plasticity and variability of the *Ptr* genome has been accomplished in the sequencing and assembly of a reference genome of a race 1 isolate of *Ptr*, Pt-1C-BFP, and hereafter referred to as BFP. Much of the work that is described in this section, unless otherwise noted, was conducted by Manning et al. (2013) and has been presented there in greater detail.

Genome sequencing of BFP was initiated for several reasons. It provides the agricultural community with a complete, publically available genome sequence of the causal agent of tan spot of wheat, an economically and scientifically important plant pathogen. As the first member of its genus to be sequenced, it also provides a basis for comparison with other *Pyrenophora* species, a number of which are agriculturally important pathogens including *P. teres*, which is causal to net blotch of barley (Liu et al. 2011) and *P. bromi*, the most closely related species to *Ptr*, which causes brown leaf spot on the important forage crop, smooth bromegrass (Chamberlain and Allison 1945; Zeiders et al. 1986). In addition, the *Ptr*-wheat pathosystem has become a model for the study of inverse gene-for-gene interactions, where pathogenicity/virulence effectors, or HSTs, interact with the product of host sensitivity genes to confer *Ptr* pathogenicity and wheat susceptibility. Genomic data inform studies that address the underlying molecular mechanisms of pathogenicity (virulence) and disease susceptibility (compatibility), as well as serve as a basis for research and discovery directed toward controlling the important disease caused by this pathogen. The genome sequence data generated has already been valuable in comparative genomic studies of the *Ptr* species (Manning et al. 2013) and the broader fungal research community (Condon et al. 2013; Ohm et al. 2012; Rouxel et al. 2011). A race 1 isolate was chosen for sequencing as this is the predominant race found in tan spot outbreaks in North America (Ali and

Francl 2003; Lamari et al. 1998). In addition, the first proteinaceous HST characterized, ToxA (Ballance et al. 1989; Ciuffetti et al. 1997; Tomas and Bockus 1987; Tomas et al. 1990; Tuori et al. 1995), is produced by this race as is the HST, ToxC, which has yet to be fully characterized (Effertz et al. 2002; Gamba and Lamari 1998; Lamari and Bernier 1991). The isolate that was sequenced has also been demonstrated to produce an additional, uncharacterized HST(s) (Tuori et al. 1995).

1.2.1 Genome Architecture

1.2.1.1 Optical Mapping of the Reference Genome

To derive a comprehensive landscape of the *Ptr* genome structure, an optical genetic map was produced. The process of optical mapping (Dimalanta et al. 2004; Lin et al. 1999; Zhou et al. 2007, 2009) involves restriction enzyme digestion of sheared chromosomal DNA that has been adhered to a special matrix, and length measurements of the resultant fragments, which remain in the order that they occur in the chromosomal DNA. These fragments are aligned and assembled into chromosomes and a catalog of the position of the restriction enzyme sites can be used to anchor sequence scaffolds onto chromosomes. The average physical coverage of the optical map of the isolate BFP was ~98× and the map indicated that the genome consists of 11 chromosomes and is approximately 40 Mb (Fig. 1.3). The chromosomes range in size from 2.1 to 10.2 Mb, consistent with karyotype analysis of this isolate that predicted nine of the smaller chromosomes that range from 2.1 to 3.7 Mb, but was unable to resolve the largest two chromosomes that are 5.2 and 10.2 Mb (Lichter et al. 2002).

1.2.1.2 Genome Assembly and Annotation

A high-quality genome assembly of BFP was generated by whole genome shotgun Sanger sequencing. Paired-end sequences of three libraries with different insert sizes, two plasmid libraries of insert size 4 and 10 kb and one fosmid library of insert size of ~40 kb, were generated and assembled (~7× coverage) with an improved version of Arachne (Jaffe et al. 2003). The assembly consisted of 47 sequence scaffolds with a N50 scaffold length of 1.99 Mb and a total length of 37.8 Mb. Sequence quality was high, as >96 % of the sequence had quality scores of at least 40 (one error for every 10^4 bases). When sequence scaffolds were anchored and positioned onto the optical map the sequences were highly congruent; greater than 96 % of the sequence contained in scaffolds aligned, and >90 % of the optical map was covered (Fig. 1.3). Those scaffolds that did not align were found in subsequent analyses to have a relatively high repetitive DNA content. Overall GC content of the genome is ~51 %. The size of the genome and GC content are within the range of other Dothideomycete plant pathogens (Ohm et al. 2012).

Gene annotation was performed with an evidence-based approach that included a combination of manual curation, EST alignments, multiple ab initio gene predictions, and BLAST searches of known genes available in public databases. This resulted in an initial prediction of 12,141 genes in the nuclear genome. Additionally, 129 transfer RNA (tRNA) genes were predicted in the nuclear genome. Most of the commonly used tRNAs are represented in the nuclear genome with the exception of tRNA's for histidine and tyrosine. There is a large number of tRNAs for serine and of an undetermined amino acid specificity, some of which are components of a larger repetitive unit, and will be discussed later.

1.2.1.3 Mitochondrial Genome

Mitochondria are autonomous organelles within the cells of eukaryotes. Their primary function is to provide energy to cells in the form of ATP molecules generated during the process of respiration. Mitochondria are thought to have been derived from endosymbiotic bacteria that developed a mutualistic relationship with their

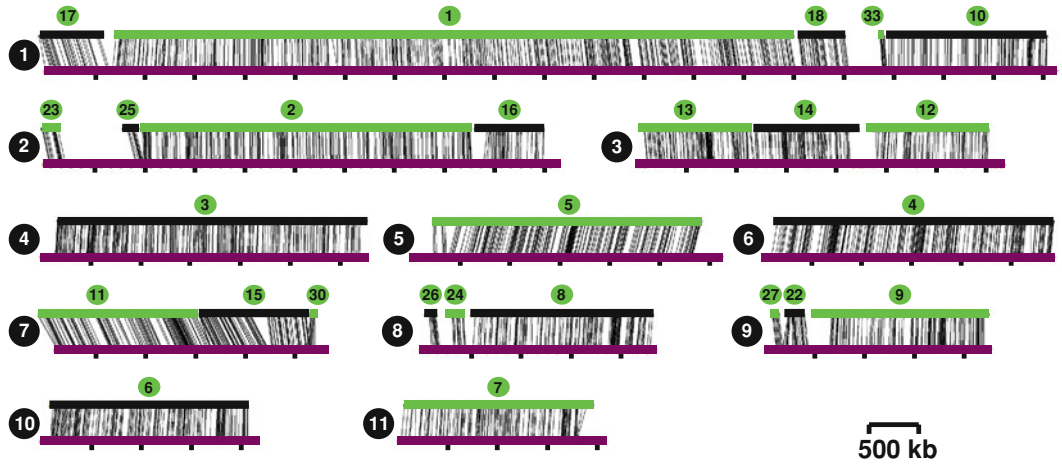


Fig. 1.3 Alignments of the physical and optical maps of the chromosomes of *P. tritici-repentis*. Optical maps (size indicated by *bottom bars*) were generated by randomly shearing genomic DNA, adhering the fragments to a specialized surface, digesting these fragments with the restriction enzyme *Afl*II A, and measuring their lengths. As fragments remain ordered, a landmark map was generated that allowed for anchoring and aligning sequence data onto the optical map. An in silico *Afl*II A restriction map was generated from the *P. tritici-repentis* genome assembly and aligned with the *Afl*II A sites (*vertical lines*) of the optical map; sequence scaffolds (*top bars*) were anchored and aligned and positioned onto the chromosomes. A poster-sized image, similar to this figure, can be downloaded from: http://www.broadinstitute.org/annotation/genome/pyrenophora_tritici_repentis.3/MapsIndex.html

hosts to generate the first eukaryotes. Most of the genes required for mitochondrial function that were present in the original bacterial genomes have been lost or transferred to the nuclear genome, but many of those involved in aerobic respiration have been retained. In a reflection of their bacterial ancestry, mitochondrial genomes in most species are small, circular molecules with genes that lack classical introns and do not produce polyadenylated messenger RNA. Mitochondria typically are inherited from one parent only without recombination, making them particularly useful for reconstructing phylogenetic relationships. Fungal mitochondrial genomes are of particular interest because a specific mutation at position 143 of the cytochrome b (*cob*) gene, which causes a substitution of a glycine to an alanine in the amino acid sequence, makes them resistant to strobilurin fungicides (Sierotzki et al. 2000). Application of fungicides can cause rapid evolution of fungal mitochondrial genomes within populations that are polymorphic for the G143A mutation (Torriani et al. 2009).

Like those in most other organisms, fungal mitochondrial genomes typically are small, circular molecules, except for the yeasts where they often are linear (Fukuhara et al. 1993). They usually are transcribed in a single direction and commonly contain up to 15 protein-coding genes: seven nicotinamide adenine dinucleotide ubiquinone oxidoreductase subunits (*nad1–nad6* and *nad4L*); three ATP synthase subunits (*atp6*, *atp8*, *atp9*); three cytochrome oxidase subunits (*cox1–cox3*); one gene for cytochrome b (*cob*); and a ribosomal protein (*rps3*). However, the number of genes can vary considerably, with some yeasts having as few as seven (Pramateftaki et al. 2006). Reflecting their origins from bacterial endosymbiotes, fungal mitochondrial genes lack introns of the type seen in nuclear genomes. Instead, when introns are present, they usually are very dynamic and result from insertions and deletions of homing endonuclease genes (Ferandon et al. 2013). In addition to the protein-coding genes, fungal mitochondrial genomes usually contain tRNA genes for translation plus genes for large and small ribosomal RNA subunits. Although many fungal mitochondrial genomes have been sequenced, there still is no tool for automated annotation of protein-coding genes so

they must be identified and characterized manually. However, an excellent tool for automated annotation of the tRNA genes is available that works very well for fungal mitochondrial genomes (Lowe and Eddy 1997).

Mitochondrial genomes of two fungi in the Dothideomycetes have been analyzed thoroughly and published, those for *Stagonospora nodorum* in the Pleosporales and *Mycosphaerella graminicola* (synonym: *Zymoseptoria tritici*) in the Capnodiales. Both species had typical fungal mitochondrial genomes composed of small, circular molecules with a full array of tRNA genes with anticodons for all 20 amino acids, both ribosomal RNA genes and a subset of the usual 15 protein-coding genes (Hane et al. 2007; Torriani et al. 2008). Two major differences from other fungal mitochondrial genomes were that those of *S. nodorum* and *M. graminicola* each contained a change of direction so that a large, contiguous block of genes was transcribed from the opposite DNA strand, and neither contained all 15 of the usual protein-coding genes. The mitochondrial genome of *S. nodorum* was missing the *atp8* and *atp9* genes (Hane et al. 2007), while that of *M. graminicola* was missing *rps5* (Torriani et al. 2008). There were no introns in the *M. graminicola* mitochondrial genome (Torriani et al. 2008), while four of the protein-coding genes in the *S. nodorum* mitochondrial genome each contained a single intron (Hane et al. 2007). Total sizes of the mitochondrial genomes were 49.8 and 44.0 kb for *S. nodorum* and *M. graminicola*, respectively. Therefore, both Dothideomycetes mitochondrial genomes were fairly typical for filamentous fungi with small, circular genomes coding for most of the usual complement of ribosomal, tRNA and protein-coding genes with the few minor exceptions noted above.

As the third mitochondrial genome from a fungus in the Dothideomycetes to be analyzed thoroughly, that of *Ptr* was expected to be similar to the other two, particularly *S. nodorum,* which is in the same order and therefore much more closely related than *M. graminicola*. However, the results turned out to be surprising (Goodwin et al. unpublished data). Instead of the 40–50 kb mitochondrial genome expected, that of *Ptr* is very large. The entire molecule did not assemble cleanly from the sequencing reads, but instead appeared as two pieces that were identified by BLAST searches among the pool of total genomic scaffolds. These two pieces together total almost 157 kb, making the *Ptr* mitochondrial genome the largest reported for fungi to date; the next largest is that of *Chaetomium thermophilum* var. *thermophilum* at just over 127 kb (Amlacher et al. 2011).

Manual annotation and preliminary analyses identified more than 90 open reading frames (ORFs) on the two pieces of the *Ptr* mitochondrial genome. These ORFs contained 13 of the usual 15 protein-coding genes; as with *S. nodorum*, the *atp8* and *atp9* genes are missing. The *cob* gene appears to span the break between the two pieces; the 5′ part of the gene is on the first piece while the 3′ part is on the second and an unknown amount in the middle is missing. The *Ptr* mitochondrial genome contained tRNA genes with anticodons for all 20 amino acids plus large- and small-subunit rRNA genes. Some of the other ORFs appear to code for DNA or RNA polymerases, which are often seen in mitochondrial plasmids rather than mitochondrial genomes (Griffiths 1995).

The extremely large size of the *Ptr* mitochondrial genome was mostly due to amplifications of homing endonuclease genes that formed introns in many of the protein-coding genes, sometimes with multiple insertions into the same gene. Homing endonuclease genes are common in fungal mitochondrial genomes where they form self-splicing introns (Mullineux et al. 2010). Those in the *Ptr* mitochondrial genome appear to be of several types and individual protein-coding genes contained from 0 to 5 introns each; 6 of the 13 protein-coding genes contained at least one intron. Therefore, individual genes of *Ptr* contained more introns than there were in the entire mitochondrial genomes of *S. nodorum* and *M. graminicola* combined. Acquisition and amplification of these homing endonuclease genes appears to be the cause of the greatly increased size of the *Ptr* mitochondrial genome. However, the source of these

genes and how they are able to amplify in some species such as *Ptr* are not known.

Overall, the mitochondrial genome of *Ptr* is much more similar to that of *S. nodorum* than *M. graminicola*, reflecting their closer phylogenetic relationships as seen from analyses of nuclear genes (Schoch et al. 2009). Both mitochondrial genomes from species in the Pleosporales lack the *atp8* and *atp9* genes, which most likely have been transferred to the nuclear genome (Hane et al. 2007). Whether this is a common feature of mitochondrial genomes of fungi in the Pleosporales or is specific to these two species remains to be tested. These and many other questions will be answered as more mitochondrial genomes are sequenced, annotated and analyzed.

1.2.2 Protein Coding Regions

All of the currently identified HSTs produced by *Ptr* are secreted proteins with the possible exception of ToxC, which may be a secondary metabolite. Therefore, when mining the genome for information on potential virulence/pathogenicity factors, attention was focused on secreted proteins and potential secondary metabolite clusters, especially those clusters that contain genes that encode polyketide synthetases (PKS) and nonribosomal peptide synthetases (NRPS), the backbone compounds of several characterized HSTs produced by other fungi (Wolpert et al. 2002; Gardiner et al. 2004; Johnson et al. 2000; Walton 2006). Functional annotations of proteins included predictions of conserved functional domain with HMMER searches against the Pfam database (Finn et al. 2010) and gene ontology (GO) term annotation in Blast2GO (Conesa et al. 2005).

1.2.2.1 Secreted Proteins

Two methods were used to predict secreted proteins from the total predicted protein set, SignalP (Dyrløv Bendtsen et al. 2004), whose predictions are based on the idea that the cleavage site position and the amino acid composition of a secretory signal peptide are correlated and WoLF PSORT (Horton et al. 2007), which predicts subcellular localization, including extracellular localization, based on sorting signals, amino acid composition and functional motifs. This dataset was searched for the presence of transmembrane domains (TM) (http://www.cbs.dtu.dk/services/TMHMM/), and all proteins that had >1 TM were removed; because it is sometimes difficult to differentiate TMs from signal peptides, those proteins that were predicted to have one TM that began before amino acid 10 were retained in the data set. Those proteins with a predicted signal peptide or extracellular localization were combined into a set of 1,146 potential secreted proteins. Of these, 317 were shown to be small (<200 amino acids) and 69 are cysteine-rich, characteristics of small proteinaceous fungal effectors (Rep 2005; Stergiopoulos and de Wit 2009). Of the secreted proteins, 110, 95 % of which were <200 amino acids, had no BLAST hits to the nonredundant database at the NCBI and were considered *Ptr* specific.

Of the small secreted proteins encoded by the reference genome, the best described is the HST ToxA. *ToxA* is present in approximately the center of chromosome 6, which consists of a single 2.8-Mb scaffold. The optical map estimates the actual size of the chromosome to be 2.95 Mb, which is consistent with previous analyses that showed *ToxA* resides on a 3-Mb chromosome (Lichter et al. 2002). A recent horizontal gene transfer (HGT) event of *ToxA* between *S. nodorum* and *Ptr* has been proposed due to the presence of a highly similar (98–100 %) 11-kb ToxA-containing region of DNA in the genome of *S. nodorum* and a race 2 isolate of *Ptr* (Friesen et al. 2006). This region is also present in BFP, with similar levels of identity.

While *ToxB* is present in multiple copies in the more virulent isolates that produce ToxB, it is not present in the reference genome. However, there are other small secreted proteins in BFP that are present in multiple copies. These include a family of genes that encode small cysteine-rich proteins that have expanded through tandem duplication followed by diversification (*PTRG_11771*, *11772*, *11773*) on chromosome 1, and another recent duplication event (*PTRG_11346*). The most recent duplication is present on a scaffold that could not be positioned on the optical map

and may or may not be adjacent to the other members of the family, as there is a gap on chromosome 1 near *PTRG_11773*. The proteins encoded by the most recently duplicated genes are 100 % identical at the amino acid level, and even the most diverged member of the family has conserved cysteine residues. Interestingly, homologues are present in several plant pathogenic fungi, including the wheat pathogen *M. graminicola*, and there is also a family of homologues in the wheat pathogen *Colletotrichum* (*Glomerella*) *graminicola*. The presence of homologues in other plant pathogens, and an expansion of the family in another wheat pathogen, as well as evidence of expression of at least one of these genes *in planta*, suggests this family of proteins may play a role in pathogenesis.

Additional small secreted proteins that are interesting candidates for roles in pathogenicity include PTRG_11888 and PTRG_12138, both of which are expressed during infection. PTRG_11888 is cysteine rich and encodes a conserved 2Fe–2S ferredoxin, iron–sulfur binding site. A screen of a panel of pathogenic and nonpathogenic *Ptr* isolates revealed that this gene is pathogen specific. PTRG_12138 has no conserved domains but has high cysteine content, consistent with effectors that must withstand the plant apoplastic proteases to affect the disease process.

While small secreted proteins are likely candidates for proteins that function as effectors, other secreted proteins play a large role in the infection process. A group of proteins that play a major role in infection of plants are the cell wall degrading enzymes (CWDE). *Ptr* contains similar numbers of CWDE as many plant-pathogenic fungi that seem to be specialized toward grass hosts, which must degrade cell walls with relatively high amounts of glucuronoarabinoxylans and mixed-linked glucans, and relatively low amounts of xyloglucans, mannans and glucomannans, and pectin (Vogel 2008). There are fewer genes for pectin-degrading enzymes than in the genomes of the dicot pathogens *Verticillium dahlia* and *Fusarium oxysporum* (Klosterman et al. 2011; Ma et al. 2010), but there are at least three mixed-linkage glucanases. *Ptr* also appears to have fewer proteins of the CBM18 family of carbohydrate enzymes that are predicted to be fungal chitinases; how this might impact pathogenicity is currently unknown.

There are several classes of secreted proteins that may be relevant in the protection of *Ptr* from plant responses during the establishment of disease. Plant responses to fungal pathogens often involve the production of ROS, pathogen-induced proteins and anti-fungal compounds. Consistent with the need to combat these plant responses is the observation that many of the most common functional annotations of secreted proteins in *Ptr* include those that are associated with oxidation-reduction processes and proteolysis, as well as the fore-mentioned carbohydrate metabolic processes. For example, there are a surprisingly large number, 29, of predicted cytochrome p450s in the secreted protein dataset. While this number may be inflated by the inclusion of proteins that have TM domains at their N-termini that cannot be differentiated from signal peptides, there is still a good likelihood that some of these proteins are secreted. Perhaps the best described cytochrome p450 that aids in establishment of disease in plants is pisatin demethylase, which neutralizes the pea phytoalexin pisatin, produced by *Nectria haematococca* (Maloney and VanEtten 1994). It is therefore possible that the secreted p450s of *Ptr* may be important in detection and neutralization of both induced and preformed secondary metabolites produced by plants in response to infection.

Another interesting finding in the *Ptr* secretome is the large number, 21, of berberine bridge-containing flavoproteins (BBEs). This number includes a recent segmental duplication. The function of these proteins in the necrotrophic lifestyle has not yet been explored; however, the abundance of them in the secreted protein set implies their importance. These enzymes are best known for their role in the production of hydrogen peroxide and alkaloids, some of which function as phytoalexins or carbohydrate oxidases (Carter and Thornburg 2004; Custers et al. 2004; Dittrich and Kutchan 1991; Facchini et al. 1996). BBE-containing flavoproteins are also over-represented in the secreted proteins of the oomycete plant

pathogen *Phytophthora infestans* and have been shown to be either weakly or strongly induced during infection (Raffaele et al. 2010). Glucose-methanol-choline (GMC) oxidoreductases, which are known to have multiple catalytic functions (Cavener 1992) are also present at fairly high numbers, 18, in the predicted *Ptr* secretome. Again, the role of these proteins in pathogenesis is unknown, but it has been proposed that a GMC oxidoreductase contributes to pathogenicity of the broad host-range pathogen *Glomerella cingulata* through reduction of fungal-induced, plant-produced anti-fungal quinones and phenoxy-radicals (Sygmund et al. 2011). Proteins with the GMC oxidoreductase domains are also abundant in the necrotrophic plant pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Amselem et al. 2011) and two GMC oxidoreductases were amongst the most abundant proteins in a proteomic analysis of the *Ustilago maydis* secretome (Couturier et al. 2012).

1.2.2.2 Nonribosomal Peptide Synthases and Polyketide Synthetases of *P. tritici-repentis*

Nonribosomal peptide synthetases are large, modular enzymes that can synthesize peptides in the absence of ribosomes (Finking and Marahiel 2004). These proteins may be mono- or multi-modular, with each module responsible for the incorporation of an amino acid into the growing peptide chain. Modules can be divided into two types, initiation and elongation, with the initiation module composed of two domains: an activation or adenylation (A) domain, followed by a thiolation (T) or peptidyl carrier protein (PCP) domain. The elongation module contains the A and T domains and a condensation domain (C) responsible for peptide bond formation in the growing peptide chain. The function of the A domain consumes an ATP and the T domain requires a 4′-phosphopantetheinyl cofactor. The conserved binding sites for these two molecules and the conserved nature of the condensation domains were used to predict genes that encode NRPSs in the *Ptr* genome.

There were 17 NRPS genes predicted in the *Ptr* reference genome, eight that have putative orthologs in other Ascomycota, and another eight that are closely associated with transposable elements (TEs). Two are hybrids that contain both NRPS and PKS modules, with one associated with TEs. Interestingly, TEs have been implicated as a possible mechanism for HGT in fungi (Richards et al. 2011). Several of the NRPS genes are found in biosynthetic clusters. In fact, one cluster contains orthologues of proteins known to be important for production of histone deacetylase inhibitors. These inhibitors include HC-toxin, which functions as an HST in *C. carbonum* (Walton 2006), and apicidin, which is produced by *F. incarnatum* (Jin et al. 2010).

The NRPSs associated with TEs are quite interesting as there is evidence of recent duplication of entire proteins or duplication and recombination of domains within and between proteins leading to unique protein coding regions. One of these proteins, the largest NRPS in *Ptr* PTRG_10433, is composed of six complete modules with an additional A domain at the C-terminus. Within this protein, no one module is identical, yet domains within modules can be >96 % similar at the amino acid level to domains in other modules, which infers a complex pattern of domain duplication and recombination within this one gene. A fragment of this NRPS was used to probe DNA from pathogenic and nonpathogenic isolates of *Ptr* and this NRPS was shown to be pathogen specific (Lichter et al. 2002). It is intriguing that while there may be related NRPS-encoding genes in other pathogenic strains of *Ptr*, they may actually have different combinations of these various domains and modules, and thus produce very different products. In addition to domain duplication and recombination, there have been three recent segmental duplications that have resulted in the replication of an NRPS, cytochrome p450, amino transferase and methyltransferase genes, although these genes have not all been equally maintained during the duplication process.

Polyketide synthetases are also multidomain proteins (Keller et al. 2005). A similar method for prediction of PKSs in the genome was used as for prediction of NRPS where predicted proteins were searched for conserved domains characteristic of PKSs; these include the beta-

ketoacyl synthase N-terminal domain, the acyl transferase domain, and phosphopantetheine attachments sites. Of the 21 putative PKSs identified, all but four appear to have homologs in other Ascomycota. Eleven appear to be in clusters and two are truncated and may not be functional. As with the NRPS-encoding genes, several PKS-encoding genes are associated with TEs. In addition to identifying the PKS and proteins necessary for the production of melanin (Kihara et al. 2008), other PKS-containing clusters were identified that have proteins similar to those necessary for the production of the toxic compounds emodin (Bok et al. 2009), alternapyrone (Fujii et al. 2005), and zealerone (Lysoe et al. 2009; Reeves et al. 2008). *Ptr* has been shown to produce anthroquinone-derived phytotoxins (Bouras et al. 2009; Bouras and Strelkov 2010), and therefore the emodin-like cluster (PTRG_02706-02730) may be responsible for their synthesis. There is also evidence of recent gene duplication in the PKSs, where two PKS share 100 % amino acid identity.

1.2.3 Repetitive DNA

1.2.3.1 Repeat Families and Repeat Induced Point Mutation

Analyses of the repeat content of the *Ptr* genome indicated that ~16 % of the reference genome is repetitive DNA; out of which a remarkably high percentage, 81 %, is 95–100 % similar. This suggests that many of the repeat elements present in the reference genome have undergone recent expansions. Additionally, it appears that mechanisms fungi are known to employ to cope with repeat element expansions in their genomes are not very effective in this isolate of *Ptr*, and possibly the species as a whole. One of these mechanisms is repeat induced point mutation (RIP) (Cambareri et al. 1989; Selker et al. 1987; Galagan and Selker 2004), where a duplication event is detected and some of the cytosine residues adjacent to adenine residues are converted to thymidine residues in both copies, which may lead to cytosine methylation and silencing or to mutations that impact function and eventually lead to degradation of a copy. When the repeat component of the *Ptr* genome was searched for evidence of RIP, ~10 % of the repeat families showed evidence of a CpA to TpA bias; however, not all of the members of the repeat families that showed this bias had these mutations. These data, and another independent analysis for evidence of C to T transitions (Clutterbuck 2011) in the *Ptr* genome, is consistent with the lack of efficient RIP in this organism.

DNA transposons and LTR retrotransposons are the most common repeat elements in the genome at 36.4 and 40.5 % of repetitive DNA, respectively. Other TE types are also present, but constitute a smaller part of the repeat component of the genome. These include miniature inverted-repeat transposable elements (MITES), one of which is present in more than 200 copies and two families of short interspersed nuclear elements (SINEs) that contain a tRNA at the 5′ end of the element. One family contains a tRNA for an "undetermined" amino acid, but the other contains a tRNA for serine. The SINE element has replicated this $tRNA^{ser}$ 25X in the genome; however, there is no evidence that the presence of many copies of the GCT anti-codon present on this $tRNA^{ser}$ have, so far, had an impact on serine codon bias in the genome.

1.2.3.2 Transduplication in *P. tritici-repentis*

Perhaps the most intriguing find when the repeat families were annotated was the presence of "genes" or ORFs that are not typical components of TEs. This included histone H3-like genes and an ORF of unknown function that contains an osmosensory coiled-coil domain. This was the first indication that transduplication may occur in fungi. Transduplication is the process by which DNA TEs that have captured whole genes or gene fragments between their terminal inverted repeats (TIR) will replicate these non-TE gene/gene fragments throughout the genome, every time it replicates itself; in other words, the gene/gene fragments have become a part of the TE. This process was first

documented in rice, where Pack-MULEs (Pack-*Mutator*-like transposable elements) were shown to have amplified genes or gene fragments on a huge scale (Jiang et al. 2004; Juretic et al. 2005; Lisch 2005). The replication of genes/gene fragments by the Pack-MULEs has been shown to impact transcription of the cellular genes from which they were derived (Hanada et al. 2009). Interestingly, as with transduplications identified in *Ptr*, chimeras of genes within the transduplicating transposons are known to occur and a preference for incorporation of DNA-binding proteins has been reported (Hanada et al. 2009; Hoen et al. 2006).

There are two examples of transposons that transduplicate genes in *Ptr*. The first is a *hAT* (*hobo-Activator-Tam3*) superfamily member that has sequestered two ORFs within its short TIRs, a central predicted ORF of unknown function and the histone H3-like gene mentioned above at the opposite end from the transposase (Fig. 1.4a). In addition to this full-length element, there is a smaller element that was produced as a result of a recombination event between the central ORF and the transposase. The larger, likely autonomous element, is present 10 times in almost identical copy in the genome, whereas the smaller, likely nonautonomous element, is present 16 times. From EST mapping to the genome, we know that the chimeric transposase and the histone H3-like protein are being expressed from this element; what we do not yet know is if the expression of the histone H3-like protein impacts expression of the cellular histone H3. The other example of transduplication is the presence of 46 copies of a *Tc1/Mariner element* that has an ORF that contains an osmosensory coiled-coil domain within its imperfect long TIRs (Fig. 1.4b).

1.2.3.3 Importance of Transposable Element Activity on Pathogenicity

It is clear that TEs in *Ptr* are involved in the transformation of the genome landscape. Many of the transposons are transcriptionally active, which suggests they are also mobile. Furthermore, many of the families have members that share a high level of sequence identity, which suggests they have been recently expanded. This influx and movement of TEs creates a highly plastic genome capable of rapid adaptation. Rapid adaptability to changing environments is the hallmark of a successful pathogen.

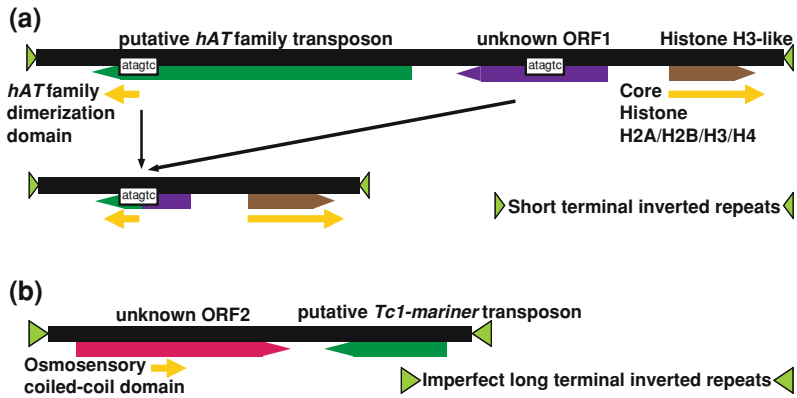


Fig. 1.4 Transduplication in *P. tritici-repentis*. Transduplication occurs when gene or gene fragments are sequestered between the terminal inverted repeats of a DNA transposable element (TE); this leads to replication of these genes/gene fragments each time the transposon is mobilized. **a** A putative ORF (unknown ORF1) with no conserved domains, located in the center of the element, and a histone H3-like protein on the opposite end of the element from the transposase, is transduplicated by a *hAT* (*hobo-Activator-Tam3*)-like transposon; a related TE has been formed through a recombination event within this TE, whereby the central ORF and the transposase join to form a chimeric protein. **b** A *Tc1-mariner*-like TE is responsible for transduplication of an ORF (unknown ORF2) that contains an osmosensory coiled-coil domain. These TEs are delimited by terminal inverted repeats of different sizes and characteristics

Before *Ptr* reference genome analyses, studies on pathogenic and nonpathogenic isolates of *Ptr* had already provided evidence that TEs impact pathogenicity (Lichter et al. 2002; Martinez et al. 2004; Strelkov et al. 2006; Friesen et al. 2006). For example, analyses of *ToxB*-expressing isolates showed a correlation between pathogen virulence and *ToxB* copy number (Amaike et al. 2008; Ciuffetti et al. 2010; Strelkov et al. 2002) and that *ToxB*-containing loci are associated with TEs (Martinez et al. 2004; Strelkov et al. 2006), which led to the hypothesis that TEs are responsible for copy number variation (CNV) of *ToxB* in *ToxB*-containing isolates. Analysis of the reference genome shows that *ToxB* is not the only gene that is present in multiple copies and associated with TEs in this fungus. Other genes present in multiple copies include small secreted proteins that may act as effectors, as well as PKSs, cytochrome p450s and BBE-enzymes that may also play a role in establishment of disease.

It has also been postulated that TEs can facilitate HGT between organisms (Richards et al. 2011). Certainly, the *ToxA* gene, which was postulated to have been recently transferred to *Ptr* from *S. nodorum* (Friesen et al. 2006), is surrounded by TEs. There are other proteins and clusters of proteins that include NRPS and PKS surrounded by TEs; these may be important in the production of secondary metabolites that can increase pathogen virulence and could have arrived in the genome via HGT.

Southern analysis with probes to TEs showed that pathogenic isolates have different repeat elements than nonpathogenic isolates (Lichter et al. 2002). This, and the observation that nonpathogenic isolates have smaller overall genome sizes (Aboukhaddour et al. 2009; Lichter et al. 2002; Martinez et al. 2004), suggests that the TEs of pathogenic isolates contribute to increased genome size and to pathogenicity.

1.3 Genome-Enabled Discoveries

As previously discussed, isolates of *Ptr* are categorized into races based on the complement of characterized HSTs that they produce (or do not produce in the case of nonpathogenic isolates). The current race structure is likely an oversimplification, as there is evidence that other HSTs exist. This has been demonstrated for the isolate from which we derived the reference genome and therefore comparative genome analyses between isolates that do not conform to the current race structure should facilitate discovery of new HSTs. Additionally, nonpathogenic isolates have been shown to have differences from pathogenic isolates beyond the absence of HSTs (Lichter et al. 2002; Aboukhaddour et al. 2011; Leisova-Svobodova et al. 2010; Lepoint et al. 2010; Cao et al. 2009). In these first genome-enabled analyses, attempts were made to discover possible HST-encoding genes in the reference genome and to understand the broader differences between pathogenic and nonpathogenic isolates. To perform these analyses, genome sequences of a pathogenic race 5 and a nonpathogenic race 4 isolate were obtained by high-throughput sequencing methods and compared to the reference genome. Also, transcriptomes of the race 4 and an additional pathogenic isolate that does not conform to the current race structure (Andrie et al. 2007) were compared to the reference genome.

With a blue-print of genes in the reference genome, a large-scale examination of genes expressed under various conditions can be conducted. Of great interest are those genes that are necessary for pathogenicity. Therefore, a preliminary genome-enabled analysis of transcripts produced during infection was undertaken. For a more detailed description of the genome-enabled analyses, please see Manning et al. (2013).

1.3.1 Comparative Analyses

1.3.1.1 Genomes of Pathogenic and Nonpathogenic Isolates of *P. tritici-repentis*

To determine proteins specific to the reference race 1 isolate BFP, genome sequences were generated for two additional *Ptr* isolates, a race 5 isolate (DW7), which produces ToxB, and a nonpathogenic race 4 isolate (SD20), which

produces no known toxins and is nonpathogenic on wheat, and these sequences were compared to the reference genome. Paired-end, 75-bp reads of Illumina-generated sequence were obtained from libraries of genomic DNA randomly sheared into ~200-bp fragments. These libraries yielded 34.2 and 67.2 million (M) paired-end reads for DW7 and SD20, respectively. For comparisons with the reference genome, two methods were used for sequence read alignment and mapping; maq (Li et al. 2008) for initial read mapping to determine coverage statistics and to call single nucleotide polymorphisms (SNPs) and SOAP2 (Li et al. 2009), which allows for reads to map multiple times to the genome in order to capture information on shared repeat elements. In initial read mapping with maq, a higher percentage of reads from DW7 than SD20 mapped to the reference genome, 93 and 85 %, respectively, and 10× more SNP were predicted in SD20 than in DW7 (Fig. 1.5a). These data strongly support the hypothesis that the nonpathogenic race 4 is more diverged from the reference race 1 than the pathogenic race 5. Low stringency mapping with SOAP2, that allows for mapping reads multiple times to the genome and up to four mismatches per mapped read resulted in 2.5× the number of mapping events with input reads from the pathogenic race 5 isolate, but only 0.84× the number of mapping events with input reads from the nonpathogenic race 4 (Fig. 1.5b). These data indicate that the two pathogens likely share a significant number of repeat elements missing from the nonpathogen and that the nonpathogen most likely has a larger number of unique genes compared to the reference. A comparison of the density of reads

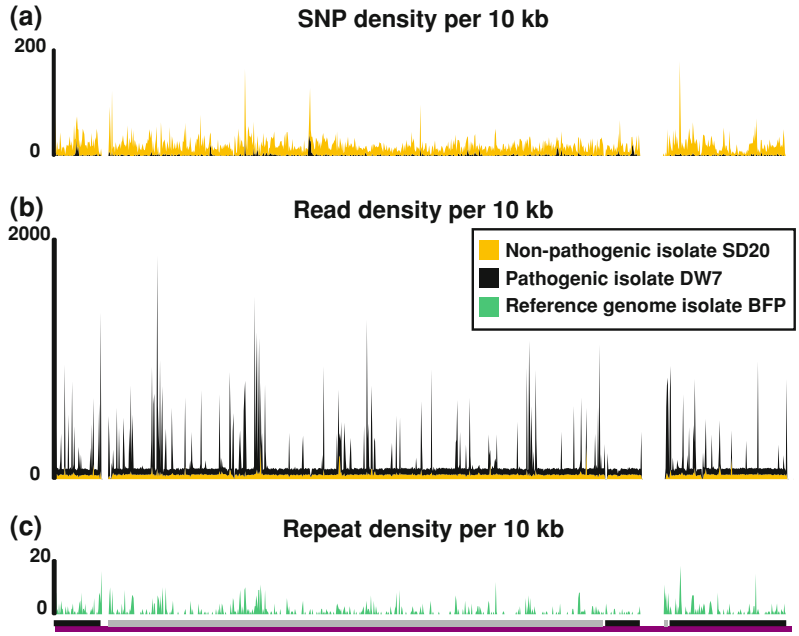


Fig. 1.5 Genome sequence comparisons of a non-pathogenic and pathogenic isolate of *P. tritici-repentis* to the reference genome from the pathogenic isolate, BFP. This figure represents data for chromosome 1; other chromosomes have similar mapping results. Short sequence reads (75 bp) were generated for the non-pathogenic race 4 isolate, SD20, and the pathogenic race 5 isolate, DW7 (**a**) and (**b**), and mapped to the reference genome. The mapping data were used to determine (SNPs) (*panel (b)*) and shared genomic regions (*panel (a)*). SNP density/10 kb for the non-pathogen SD20 is greater than for the pathogen DW7. The number of reads mapped/10 kb of the reference genome was greater for DW7 than for SD20, despite a higher number of SD20 reads available for mapping (29 vs. 67 million, respectively). The high-amplitude peaks in the DW7 mapping histogram (**b**) are congruent with the presence of repeats in the reference genome (**c**). *Bottom and top bars* represent optical map and sequence scaffolds respectively. Adapted from Manning et al. 2013, Genes Genomes Genetics 3: 41–63

mapped under low stringency conditions and the density of repeats present in the reference genome shows that a high density of reads mapped from the pathogenic isolate correlates with a high density of repeats in the reference genome (Fig. 1.5c). A high stringency SOAP2 alignment that does not allow any repeat mapping or mismatches gave similar results as the mapping performed with maq for the pathogenic race 5, ~93 % of the reads mapped, but for the nonpathogenic race 4, 28.5 % of the reads did not map, again suggesting greater divergence of the nonpathogen from the pathogens and a significant number of unique genes in the nonpathogen.

To capture those genes that are specific to BFP or shared by the two pathogens, SOAP2 mapping data for both the pathogenic race 5 and nonpathogenic race 4 isolate was used and reads mapped per kilobase of DNA per million input reads (RPKM) calculated for each gene. Calculating RPKM normalizes the number of reads per feature by the size of the feature and the number of input reads. If no reads map to a reference gene, or there is an RPKM value less than expected for a single copy gene, we considered this as absent in the high-throughput sequenced isolate. These data showed that 96 % of all genes in the reference isolate genome were shared by all three isolates and of those that were not shared by all (~800), 57 % were race 1 specific, 30 % were shared by the two pathogens (race 1 and 5) and 13 % were shared by race 1 and the nonpathogen (race 4). Focusing on those subsets of genes that might be required for HST or other effector function, we found eleven small, secreted proteins that were unique to the pathogenic race 1 and 5 isolates, six of which are unique to the BFP. There are seven NRPSs and four PKSs that are unique to BFP, and one or more may be necessary for ToxC production; of those shared with the nonpathogen, most have significant numbers of SNP that may impact function. We confirmed the RPKM results of the absence of a particular gene by BLAST searches of the coding region of the reference gene of interest to de novo assemblies of the genomes of race 5 and 4 isolates performed in Velvet (Zerbino and Birney 2008). We have not yet identified those genes that are present in the above assemblies that are unique to the race 5 and/or race 4 isolates.

Another striking finding was that both DW7 and SD20 are missing a ~145-kb region of chromosome 6 that contains the 11-kb ToxA-containing fragment that is presumed to have arrived via HGT (Friesen et al. 2006). Comparative analyses of this genomic region between the reference genome, the de novo assembled *Ptr* isolates DW7 and SD20, and another Pleosporalean plant pathogen, *C. heterostrophus*, suggests that this 145-kb region is the result of an insertion into the genome. While it is possible that the entire region was obtained during the HGT event with *S. nodorum*, sequence identity between *S. nodorum* and *Ptr* is high only at the 11-kb ToxA-containing region and we would expect that if the entire region was acquired at the same time, high levels of sequence identity would have been maintained throughout the acquired fragment. The evolution of *ToxA* in *Ptr* seems to be a complicated story that will require the sequencing and comparison of more ToxA-containing *S. nodorum* and *Ptr* isolates.

1.3.1.2 Transcriptomes of Pathogenic and Nonpathogenic Isolates of *P. tritici-repentis*

To aide in reference genome annotation and gene discovery, several transcript libraries of *Ptr* isolates were sequenced by Sanger technology. For the reference isolate, libraries were prepared from transcripts produced both *in planta* and in culture. Transcript libraries were also produced from the nonpathogenic isolate, SD20, and the pathogenic isolate SO3, which does not conform to the current race structure. SO3 produces symptoms similar to a race 2 isolate that produces ToxA, but does not have the *ToxA* gene (Andrie et al. 2007). Five thousand ESTs from each library were sequenced from both ends, producing ~10,000 sequence reads. These reads were then mapped to the reference genome to aid in gene annotation.

Of the ~10,000 sequence reads produced from each of the EST libraries of the pathogens

BFP and SO3 grown in culture, more than 12,000 sequence alignment and mapping events occurred for each library. This is due to the expression of TEs whose sequences are so similar that transcripts map to multiple places in the genome. Some of the most abundant transcripts present in these pathogen EST libraries belong to the two SINE elements described above. Also, the large number of the TEs defined in the reference genome that are transcribed, are also transcribed in SO3. However, only 79 % of the ~10,000 ESTs reads from the nonpathogen transcript library map to the reference genome; and while there are some TE transcripts that are shared between the two, there are far fewer than the number shared between the two pathogens.

1.3.2 *In Planta*-Enriched Transcriptome of *P. tritici-repentis*

Interaction of *Ptr* with its wheat host requires the expression of genes that reflect the need of the pathogen to attach to and penetrate the leaf, withstand the host response to invasion, and establish a necrotrophic lifestyle. Many of these genes are likely to differ from those required for growth in culture. To begin to define those genes that are important during the infection process, an EST library was produced from transcripts derived from leaves that had been harvested 48 h after inoculation with the reference isolate, BFP. This library was enriched for fungal transcripts by subtraction, and of the ~10,000 sequences obtained, ~3,000 mapped to the reference genome.

The most abundant transcripts present in the *in planta* EST library were those of the two SINE TEs identified in the reference genome that encode either a $tRNA^{ser}$ or a $tRNA^{undet}$. This is most likely due to transcription from more than one of the highly similar copies of these elements in each of the two TE families. Additional proteins that are highly expressed include two proteins with ubiquitin domains and several ribosomal proteins, which may reflect the need for rapid production and turnover of proteins during infection, and a putative abhydrolase and exoglucanase, likely important in cell wall degradation. Interestingly, three small secreted proteins also have fairly abundant transcript numbers; these include a putative anti-viral cyanovirin-N family protein (*PTRG_01200*), the HST ToxA (*PTRG_04889*), and a gene that appears to be present only in pathogenic races of *Ptr* (*PTRG_11888*).

Only a subset of the genes that are expressed *in planta* have been functionally annotated, and even fewer have been validated. However, we can gain insight into the types of proteins that are important during infection by comparison of functional annotations of genes expressed in *in planta* versus in culture libraries. Some of the GO terms used for functional annotation that were enriched in the *in planta* library include proteins involved in ribosomal function, ATPase transporter activities, cell wall degradation, oxidative stress management, and mycelial development.

Some transcripts were detected only in the *in planta* library. Of those that have conserved protein domains that we can use to deduce putative function, there are several putative glycosyl hydrolases most likely involved in cell wall degradation, major facilitator superfamily proteins typically involved in transport of solutes across the cell membrane, and peptidases that may contribute to pathogen survival by degradation of proteins expressed by the host plant during the defense response. Interestingly, there is a PKS that is expressed only *in planta* that may be necessary for the production of a secondary metabolite that contributes to disease development. The *in planta* transcripts identified thus far provide insight into a small part of the infection cycle of *Ptr* and the depth of sequencing of the library described above is not likely to represent the total of transcripts produced by the fungus at this time point. It will be important to obtain data from multiple time points during the life cycle and to sequence transcripts to a greater depth to generate a more complete understanding of those proteins necessary for infection.

1.3.3 Genome Resources and Genetic Tools

Reference genome sequencing was performed in collaboration with the Broad Institute (http://www.broadinstitute.org/annotation/genome/pyrenophora_tritici_repentis/). The *Ptr* database maintained by the Broad Institute contains genome information that is separated into seven different categories. The Home page allows you to access each of these categories. Project Information, includes facts on the *Ptr*-wheat pathosystem, strain selection, the specific aims of the sequencing project, and an overview of how the sequencing was performed. The 'Genome' section provides genome and assembly statistics, additional information on *Ptr*, a description of the optical mapping process as well as downloadable maps, and a section that allows the viewer to browse regions of the genome based on chromosome, supercontig, or contig number. The genomic regions can be viewed with an Argo applet downloadable from the site, FeatureMap, or as sequence data. The Argo applet and FeatureMap provide a large amount of assembly and annotation data in a visual format. These data include gene annotations and the information used to produce them such as EST mapping, conserved domains, BLAST hits, and data from multiple ab initio gene calling methods. Also included are repeat and conserved RNA family predictions, and information on the clones that were sequenced and assembled. The viewer can visualize all of these features, or select only those that they are interested in viewing. The 'Genes' section provides information on how the gene set was defined, allows for gene searches based on the gene name, locus, position, or conserved domain, and provides a link to sequence data in FASTA format, a list of all conserved RFAM Family members detected, and a searchable list of conserved domains contained in predicted proteins. The 'Feature Search' provides yet another method for searching the genome based on the FeatureMap categories. There is a 'BLAST' section for similarity searches with genome and putative protein sequences and a Download section where sequence data can be obtained.

Sequencing of additional *Ptr* isolates was performed by the Joint Genome Institute (JGI) (http://genome.jgi-psf.org/Pyrtr1/Pyrtr1.home.html). The JGI also hosts a *Ptr* database as part of a larger MycoCosm site (http://genome.jgi-psf.org/programs/fungi/index.jsf) that houses many diverse fungal genomes. In addition to the sequence and annotation data present on the Broad Institute website, the JGI *Ptr* database has additional information that is intuitively and easily accessible and includes functional annotation by GO terms, KOG classification, and KEGG metabolic pathway categorization. This site is especially useful for comparative genomics as it provides information on best BLAST hits, orthologous protein clusters, and synteny of genomic regions between *Ptr* and other fungi in MycoCosm. In the browser, you can select what you would like to visualize, including sequence similarity with other Dothideomycete plant pathogens, as well as JGI and custom generated feature tracks that for *Ptr* include SNPs and repeats. Unlike the Broad database, the JGI database is not static.

Understanding protein function in the context of the infection process is facilitated by either prevention (gene deletion) or reduction (gene silencing) of expression of the gene that encodes it, or expression of the gene of interest in an isolate that does not contain or express that gene, and monitoring various aspects of the infection process. These studies require the introduction and recombination of heterologous DNA into the fungal genome, either randomly or at specific locations, and both chemical- and *Agrobacterium*-mediated methods have been successfully used in transformation of heterologous DNA into *Ptr* protoplasts (Aboukhaddour et al. 2012; Andrie et al. 2005; Ciuffetti et al. 1997, 2010). Selection of *Ptr* transformants has most often been accomplished by the inclusion of an antibiotic resistance gene, typically the hygromycin resistance (HygR) cassette , present in the transformed DNA, although other antibiotics/

resistance cassettes have been used (Lynda Ciuffetti, personal communication). Gene silencing has been effective in examining the impact of the HSTs ToxA (Lynda Ciuffetti and Genevieve Weber, personal communication) and ToxB (Aboukhaddour et al. 2012) on disease progression. The ability to silence gene expression is particularly important in the *Ptr*-wheat pathosystem, as genes in *Ptr*, i.e. *ToxB*, that we know contribute to disease, and other genes that may, are present in multiple copies that are identical and therefore gene replacement is not an option. Gene deletion of *ToxA* has also been accomplished by replacement of the coding region with a HygR cassette. Three different methods were used to delete the coding region of *ToxA* (Lynda Ciuffetti and Viola Manning unpublished data), with the most efficient being a split-marker approach adapted from Catlett et al. (2003). Promoter regions of HST-encoding genes of *Ptr* have been utilized in the synthesis of a suite of fluorescent vectors (Andrie et al. 2005; Lorang et al. 2001) that have proven useful in the study of the biology of *Ptr* and other filamentous fungi. The *ToxA* promoter has been widely used for expression of GFP in both hyphae and conidia, making it a useful tool to study infection and growth *in planta*. In addition to GFP, the *ToxA* promoter has been used to drive expression of yellow, cyan, and red fluorescent proteins. The promoter of one of the loci that encode *ToxB*, *ToxB1*, has also been used to drive expression of *gfp* (Andrie et al. 2005), and like expression from the *ToxA* promoter, GFP can be visualized in vegetative growth and asexual conidia.

1.4 Future Perspectives

The *Ptr*-wheat pathosystem, with a focus on the production of HSTs, provides both a practical approach to disease control and an experimental model to study necrotrophic effectors and the critical components necessary for disease susceptibility. To date, significant progress has been made both on the pathogen and host side of this interaction; yet additional critical discoveries are needed toward accomplishing the goal of controlling tan spot of wheat worldwide. Current data reveal considerable genetic diversity among *Ptr* isolates with respect to geographical regions, especially those from the host center of diversity. Comparative analyses of *Ptr* races thus far, have indicated that TEs dictate significant changes in the genomic landscape of pathogenic isolates. Genome re-arrangement and expansion has likely contributed to genome flexibility and the creation and diversification of effectors involved in pathogenesis. The discovery of transduplication of genes that resemble cellular genes and/or encode functional domains in *Ptr* may have important implications in both the creation of novel genes and regulation of cellular gene expression, which in turn may impact pathogenicity of *Ptr*.

There is a clear and compelling need to characterize additional HSTs, which can be greatly facilitated by comparative genomics. Sequencing multiple isolates from among currently designated races (and those yet to be determined) will enable a more comprehensive comparison among and between pathogenic and nonpathogenic isolates and thus, the identification of the genes associated with virulence. In conjunction, additional *in planta* transcriptional analyses will further contribute to the clarification of those genes associated with pathogenesis. Such comparative genomic approaches, and gene-expression studies coupled with currently developed functional genomic tools, including the ability to genetically transform *Ptr* (gene insertion, gene replacement, and gene silencing), will enable the unambiguous identification of factors that dictate the pathogenic potential of this important pathogen. It is anticipated that these approaches will confirm the current hypothesis that pathogenicity/virulence of this pathogen is predominantly controlled by multiple HSTs, the majority of which have yet to be identified. Significantly, characterization of these HSTs will not only contribute to our general understanding of how necrotrophs cause disease, but also have direct practical implications. It is apparent that selection of host cultivars insensitive to the HSTs produced by *Ptr* will lead to control of this economically significant disease of worldwide importance.

Acknowledgements The authors would like to acknowledge the funding agencies that provided generous support for the research of their primary work cited in this chapter. Funding from the National Research Initiative of the USDA Cooperative State Research Education and Extension Service Competitive Grants Program, the Agriculture and Food Research Initiative Competitive Grants Program from the USDA National Institute of Food and Agriculture, and the National Science Foundation to LMC; funding from the USDA Agricultural Research Service and Agriculture and Food Research Initiative Competitive Grants Program from the USDA National Institute of Food and Agriculture to JDF; funding from the Natural Sciences and Engineering Research Council of Canada, and the A.W. Henry Endowment Fund (University of Alberta) to SES; funding from the USDA Agricultural Research Service to SBG; funding from a National Science Foundation Minority Postdoctoral Fellowship to MF. We would like to thank Dr. L.-J. Ma and the Broad Institute and Dr. I. Grigoriev and the US Joint Genome Institute for productive collaborations and sequencing of the *Ptr* reference genome (isolate Pt-1C-BFP) and *Ptr* isolates, DW7 and SD20, respectively. We would like to thank Nathan Miller for original artwork included in Fig. 1.1A.

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Comparative Genomics of *Cochliobolus* Phytopathogens

2

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2.1 Introduction

2.1.1 Agricultural Biology of the Genus

Cochliobolus spp. are young, closely related species (<20 MYA, Ohm et al. 2012), which make them ideal for comparative studies (Fig. 2.1, Table 2.1). The genus divides phylogenetically into two groups each associated with a distinct anamorphic stage. The first group, which encompasses the majority of known aggressive pathogenic species with significant impact on host crops, has a *Bipolaris* asexual stage while the second group has a *Curvularia* asexual stage (Sivanesan 1987). To comply with the *International Code of Nomenclature for algae, fungi, and plants* (McNeil et al. 2012), a discussion is underway in the community as to whether the name *Bipolaris/Curvularia* or *Cochliobolus* should be used to align with the "one name one fungus" recommendation. Most contemporary genetic, molecular, and genomic research on virulence determinants and reproductive development of the group has employed the *Cochliobolus* designation. The first group of species includes the necrotrophic corn pathogens, *Cochliobolus heterostrophus* and *Cochliobolus carbonum,* the oat pathogen, *Cochliobolus victoriae,* the rice pathogen, *Cochliobolus miyabeanus*, the sorghum pathogen, *Bipolaris sorghicola*, and the sugarcane pathogen, *Bipolaris sacchari* (Figs. 2.1 and 2.2, Table 2.1). *Cochliobolus lunatus,* also a pathogen of sorghum, falls in the second group (Figs. 2.1 and 2.2). The only species with a known hemibiotrophic lifestyle is the generalized cereal and grass pathogen, *Cochliobolus sativus*, which belongs to the first group. Some of these species, i.e., *C. lunatus*, can act as opportunistic human pathogens.

The best-studied necrotrophic *Cochliobolus* spp. are notorious for their ability to evolve novel, highly virulent races producing host-selective toxins (HSTs) associated with the capacity of their producers to cause diseases on cereal crops that were bred, inadvertently, for susceptibility to the HST-producing pathogen (Yoder 1980; Turgeon and Baker 2007) (Table 2.1, Fig. 2.2). For example, in 1970, race T, a novel race of *C. heterostrophus* (*Bipolaris maydis*), caused a major epidemic of Southern Corn Leaf Blight (SCLB) that destroyed more than 15 % of the maize crop on the US eastern seaboard (Ullstrup 1970). Race T is genetically

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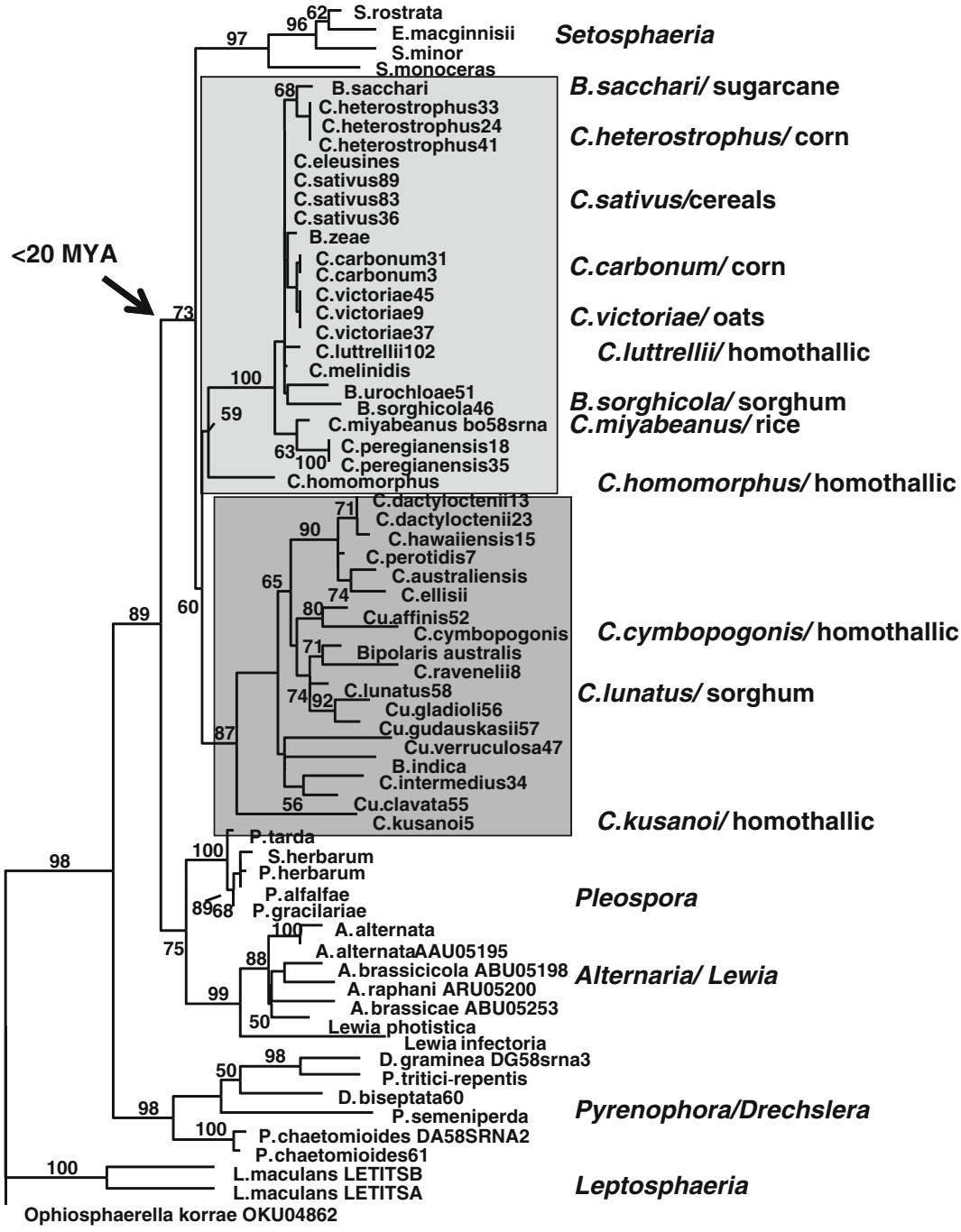


Fig. 2.1 Phylogenetic tree showing distribution of *Cochliobolus* species. *Cochliobolus* species fall into two distinct groups, boxed in two shades of gray. "*C*" *Cochliobolus*, "*B*" *Bipolaris*, "*Cu*" *Curvularia*. Host plants are indicated. All *Cochliobolus* species not designated as homothallic are heterothallic, while those indicated with a "*B*" or "*Cu*" have no known sexual stage. Numbers after species name are isolate designations. Tree constructed by M. Berbee, University of British Columbia, using GPD and ITS sequences. Genera sister to *Cochliobolus* are indicated

Table 2.1 *Cochliobolus*–host interaction biology

Species[a] (strains)	Host/tissue	Disease	HST/ effector?	HST/ effector target	Pathogen lifestyle
Ch race O (C5, Hm540)	Corn/leaves	Southern corn leaf blight	?	–	Necrotroph
Ch race T (C4, Hm338, PR1x412)	Corn with Tcms[b]/ leaves	Southern corn leaf blight	T-toxin	URF13 protein	Necrotroph
Cc race 1 (26-R-13)	*hm1hm1*[c] corn/ leaves	Northern leaf spot	HC-toxin	Histone deacetylase	Necrotroph
Cv (FI3)	*Vb*[d] oats/crown	Victoria blight	Victorin	Thioredoxin	Necrotroph
Cm (WK1C)	Rice/leaves	Brown spot	?	–	Necrotroph
Cs (ND90Pr)	Barley, wheat, cereals/leaves	Spot blotch, common root rot	?	–	Hemibiotroph
Cl (m118)	Sorghum, cereals, humans	Leaf spot, black kernel	?	–	?

[a] *Ch* = *C. heterostrophus, Cc* = *C. carbonum, Cv* = *C. victoriae, Cm* = *C. miyabeanus, Cs* = *C. sativus, Cl* = *C. lunatus*
[b] Tcms = Cytoplasmic male sterility
[c] *hm1hm1* = Homozygous recessive for carbonyl reductase
[d] *Vb* = Presumed to be the same as the *LOV1* (*Pc-2*) gene for resistance to *P. coronata*

distinct from race O, first described in 1925 (Drechsler 1925), in that it uniquely carries genes for biosynthesis of T-toxin, an HST essential for high virulence (Yoder 1980) to Texas male sterile cytoplasm (Tcms) maize (Turgeon and Lu 2000).

Cochliobolus victoriae (*Bipolaris victoriae*), causal agent of Victoria Blight of oats, produces the chlorinated cyclic pentapeptide HST, victorin, rendering it highly virulent to oats carrying the dominant *Vb* allele (Fig. 2.2, Table 2.1) (Litzenberger 1949). The *Vb*-associated trait, susceptibility to *C. victoriae,* and a *Pc-2*-associated trait, resistance to *Puccinia coronata,* cannot be separated genetically (Lorang et al. 2012). Recent work with Arabidopsis revealed an NB-LRR-type resistance protein (LOV1), guarding a thioredoxin protein target (TRX-h5), that when activated confers susceptibility to *C. victoriae* and victorin (Lorang et al. 2004, 2007). Victorin thus acts by co-opting effector triggered defenses against the biotroph, *P. coronata*, to promote susceptibility to a necrotroph.

In contrast to the dominant plant host genes required for susceptibility to *C. heterostrophus* and *C. victoriae,* susceptibility to Northern Corn Leaf Spot caused by *C. carbonum* (*Bipolaris zeicola*) is conferred by a homozygous recessive maize gene(s) (Johal and Briggs 1992; Multani et al. 1998). *C. carbonum* race 1 produces the cyclic-tetrapeptide HST, HC-toxin, which is specifically active, as is the fungus itself, against corn with the naturally occurring or mutant genotype *hmhm* (Fig. 2.2, Table 2.1) (Yoder 1980; Walton 1987, 1996). The site of action of HC-toxin in susceptible corn is histone deacetylase; it is hypothesized that HC-toxin acts to promote infection of maize of genotype *hm1hm1* by inhibiting this enzyme, resulting in the accumulation of hyperacetylated core histones. This then alters expression of genes encoding regulatory proteins involved in plant defense (Ransom and Walton 1997; Walton 2006). *C. carbonum* races 2 and 3 do not produce the toxin.

Cochliobolus sativus (*Bipolaris sorokiniana*), a hemibiotroph and less specialized cereal pathogen, causes diseases of roots (common root rot), leaves (spot blotch), and spikes (black point or kernel blight) of cereals (mainly barley and wheat) (Fig. 2.2, Table 2.1) (Mathre 1997; Weise 1987). Three *C. sativus* pathotypes (0, 1, and 2) have been described (Valjavec-Gratian and Steffenson 1997) based on differential virulence

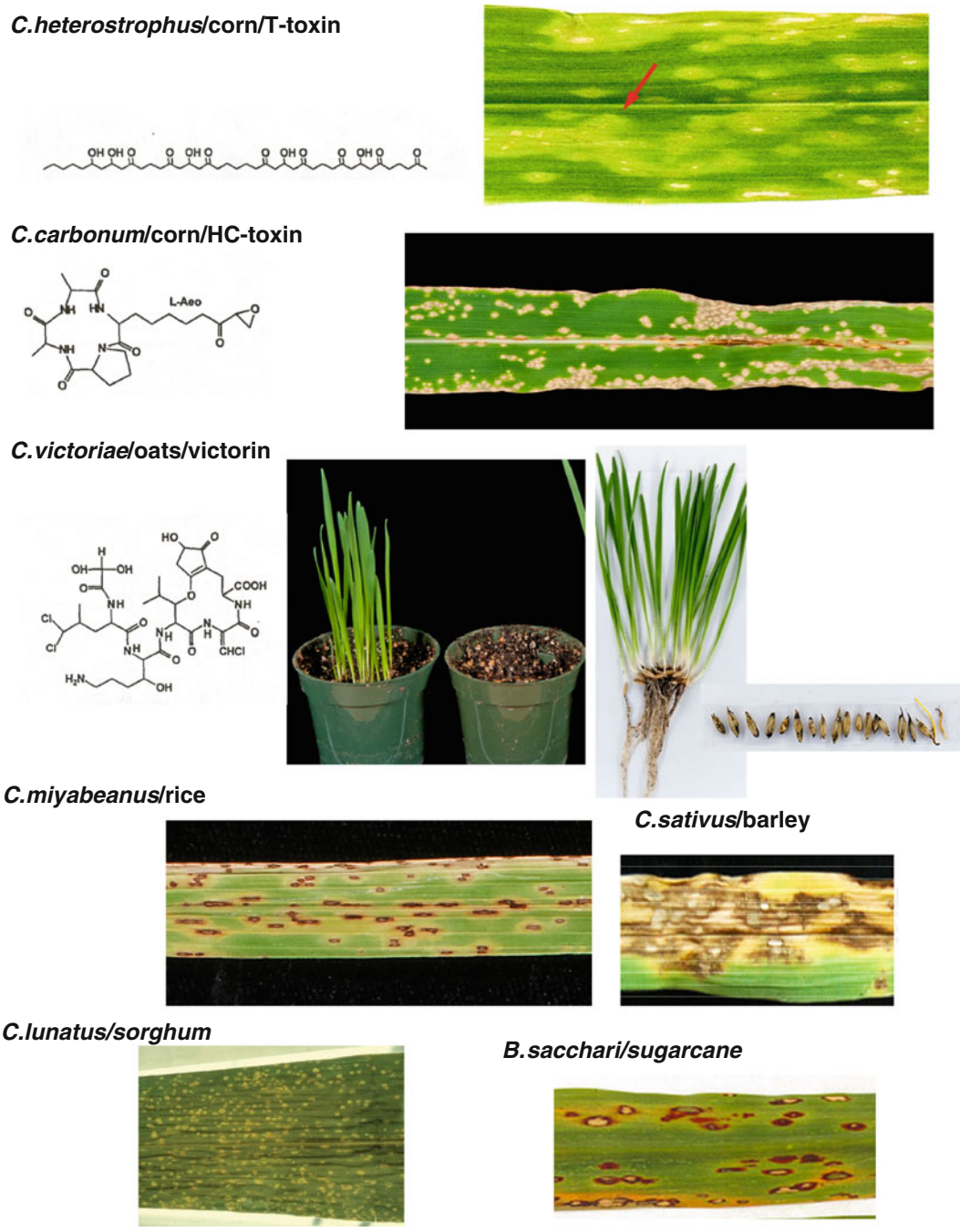


Fig. 2.2 *Cochliobolus* species and their disease phenotypes. All species, except *C. victoriae,* cause lesions on host leaves and in some cases on other plant tissues. *C. heterostrophus* race T produces T-toxin (*arrow*) which increases virulence on Tcms corn; *C. carbonum* race 1 and *C. victoriae* produce the HSTs HC-toxin and victorin, respectively, which are required for pathogenicity on the host. Pots contain resistant (*left*) and susceptible (*right*) oat seeds inoculated with a slurry of *C. victoriae—Note* none of the susceptible oat seeds germinated (*extreme right*). *Image* of *C. lunatus* from maizedoctor.cimmyt.org/index.php?option=com_content&t

Table 2.2 *Cochliobolus* spp. mating type characteristics

Isolate	Mating type	Lifestyle	Comments
Ch C5	*MAT1-1*	Heterothallic	Inbred line
Ch C4	*MAT1-2*	Heterothallic	Inbred line
Ch Hm540	*MAT1-1*	Heterothallic	Field isolate
Ch Hm338	*MAT1-2*	Heterothallic	Field isolate
Ch PR1x412	*MAT1-1*	Heterothallic	Progeny of cross between strain PR1 and strain 412
Cv FI3	*MAT1-2*	Heterothallic	All known isolates are *MAT1-2* and female sterile (Christiansen et al. 1998)
Cc 26-R-13	*MAT1-1*	Heterothallic	(Christiansen et al. 1998)
Cs ND90Pr	*MAT1-2*	Heterothallic	Pathotype 2
Cm WK1C	*MAT1-2*	Heterothallic	(Arie et al. 1997)
Cl m118	*MAT1-2*	Heterothallic	
C. ellisii	*MAT1-2*	Heterothallic	(Yun et al. 1999)
C. luttrellii	*MAT1-1*:*MAT1-2*	Homothallic	−115 aa 3′*MAT1-1*, −49 aa 5′ *MAT1-2* (Yun et al. 1999)
C. homomorphus	*MAT1-2*:*MAT1-1*	Homothallic	−9 aa 3′*MAT1-2*, −7 aa 5′ *MAT1-1* (Yun et al. 1999)
C. kusanoi	*MAT1-1*:*MAT1-2*	Homothallic	(Yun et al. 1999)
C. cymbopogonis	*MAT1-1*, *MAT1-2*	Homothallic	*MAT1-1* and *MAT1-2* are unlinked (Yun et al. 1999)

patterns on three barley genotypes (ND5883, Bowman, and NDB112). Pathotype 0 isolates show low virulence on all three barley genotypes. Pathotype 1 isolates show high virulence on ND5883 but low virulence on other barley genotypes. Pathotype 2 isolates show high virulence on Bowman but low virulence on ND5883 and NDB112. Genetic analysis and molecular mapping indicates that a single locus, *VHv1*, controls high virulence of the pathotype 2 isolate ND90Pr on Bowman (Valjavec Gratian and Steffenson 1997; Zhong et al. 2002). The *VHv1* locus is unique to pathotype 2 and encodes two nonribosomal peptide synthetases (NRPSs), one of which when deleted, drastically reduces virulence of pathotype 2 on cultivar Bowman (Condon et al. 2013).

Cochliobolus miyabeanus (*Bipolaris oryzae*) is the causal agent of brown spot of rice which contributed, along with a cyclone and tidal waves, to the Bengal rice famine of 1942/1943 that resulted in starvation of more than two million people (Dasgupta 1984) (Fig. 2.2, Table 2.1). The interaction between rice and *C. miyabeanus* is inadequately understood from the perspective of genetic and molecular mechanisms and no HST has been correlated with the ability of *C. miyabeanus* to cause disease.

Cochliobolus lunatus (*Curvularia lunata*) is a pathogen of sorghum (Fig. 2.2, Table 2.1) (Thakur et al. 2006) and is also known to be an opportunistic human pathogen (Thakur et al. 2006; Manamgoda et al. 2011, 2012). The sequenced strain (m118, MUCL 38696) was selected originally as a pilot organism for steroid biotransformation (Vitas et al. 1994, 1995) in the laboratories of Friedrich Schiller University, Jena, Germany. This, and another strain, *C. lunata* AT46, have been utilized widely for steroid transformation (Rozman et al. 1996).

2.1.2 Reproductive Biology

Sexual *Cochliobolus* species can be self-sterile (heterothallic, requiring genetically distinct partners) or self-fertile (homothallic, no partner required) (Fig. 2.1, Table 2.2). As in most ascomycetes, a single mating type locus (*MAT*) controls the ability to reproduce sexually and in

Cochliobolus, all heterothallic species have either *MAT1-1* or *MAT1-2* (but never both) in different individuals whereas all homothallic species carry both *MAT1-1* and *MAT1-2* in the same nucleus of an individual (Turgeon et al. 1993). Asexual species (i.e., those with no known sexual cycle), such as *B. sacchari,* also are found in the group. It is well documented that asexual species also carry *MAT* genes (Sharon et al. 1996). Thus, *Cochliobolus* spp. are an excellent choice for comparisons of reproductive mechanisms in asexual, heterothallic and homothallic species within a closely related group of species in the same genus (Fig. 2.3) (Turgeon and Debuchy 2007; Debuchy and Turgeon 2006; Yun et al. 1999).

The coexistence of heterothallic and homothallic species in the same genus is common to many classes of ascomycete and coexistence of both mating type genes in the same nucleus is common to most homothallic species, including *Cochliobolus*. For *Cochliobolus* spp., a self-sterile to self-fertile evolution is well supported since all homothallic *Cochliobolus* spp. are polyphyletic, their *MAT* genes are diverse in structure and arose independently, while all heterothallic *MAT* genes are conserved in structure; plus, molecular evidence exists for recombination mechanisms (Yun et al. 1999; Inderbitzin et al. 2005). Functional analyses (Yun et al. 1999; Lu et al. 2011) indicate that *MAT* genes can be transferred from heterothallic to homothallic species or vice versa and retain function, although there are yet to be understood nuances associated with fertility. Where causes of asexuality have been studied functionally, asexual species such as *B. sacchari* have been found to be asexual for reasons not associated with the *MAT* genes themselves; their *MAT* genes are fully functional in *mat* null strains of *C. heterostrophus* (Sharon et al. 1996).

C. carbonum and *C. victoriae* are capable of crossing to each other (Scheffer et al. 1967; Christiansen et al. 1998). We have hypothesized that *C. victoriae* may have evolved from a *MAT1-2 C. carbonum* strain (Christiansen et al. 1998). This is supported by our finding that all extant strains of *C. victoriae* are *MAT1-2* and female sterile. As noted below, *C. victoriae* and *C. carbonum* share an intermediate number of SNPs at the whole-genome level compared to *C. heterostrophus* inter- and intra-species comparisons, in support of this close relationship. Table 2.2 is a summary of mating attributes for the *Cochliobolus* species with sequenced mating type loci.

2.1.3 Genetic Tools

Cochliobolus spp. are easily grown in culture, produce abundant asexual spores (except *C. lunatus*), and can be stored for long periods of time in glycerol or silica gels (Yoder 1988). They also have an efficient sexual stage readily produced in the laboratory in 3 weeks (Fig. 2.3) (Leach et al. 1982), and are easily transformed (Turgeon et al. 2010). Targeted gene deletion using PCR fragments is highly efficient (Turgeon et al. 2010; Wirsel et al. 1996; Catlett et al. 2003a). Chromosomes can be resolved using pulsed-field gel electrophoresis (Kodama et al. 1999; Tzeng et al. 1992).

In this review, we compare genome similarities and differences among sequenced *Cochliobolus* pathogens, with particular emphasis on strain and species-unique sequences, virulence determinants (secondary metabolites, iron and oxidative stress), mechanisms of reproduction, and signaling.

2.2 Genome Structure

2.2.1 Genome Sequence Comparisons

Five strains of *C. heterostrophus* and one strain each of *C. victoriae, C. carbonum*, *C. miyabeanus, C. sativus,* and *C. lunatus* were sequenced by the Joint Genome Institute (JGI). Two *C. heterostrophus* strains and the *C. sativus* strain were fully sequenced, while the remaining genomes were sequenced using Illumina and assembled de novo using Velvet or AllPathsLG (http://genome.jgi.doe.gov/programs/fungi/index.jsf). The highly

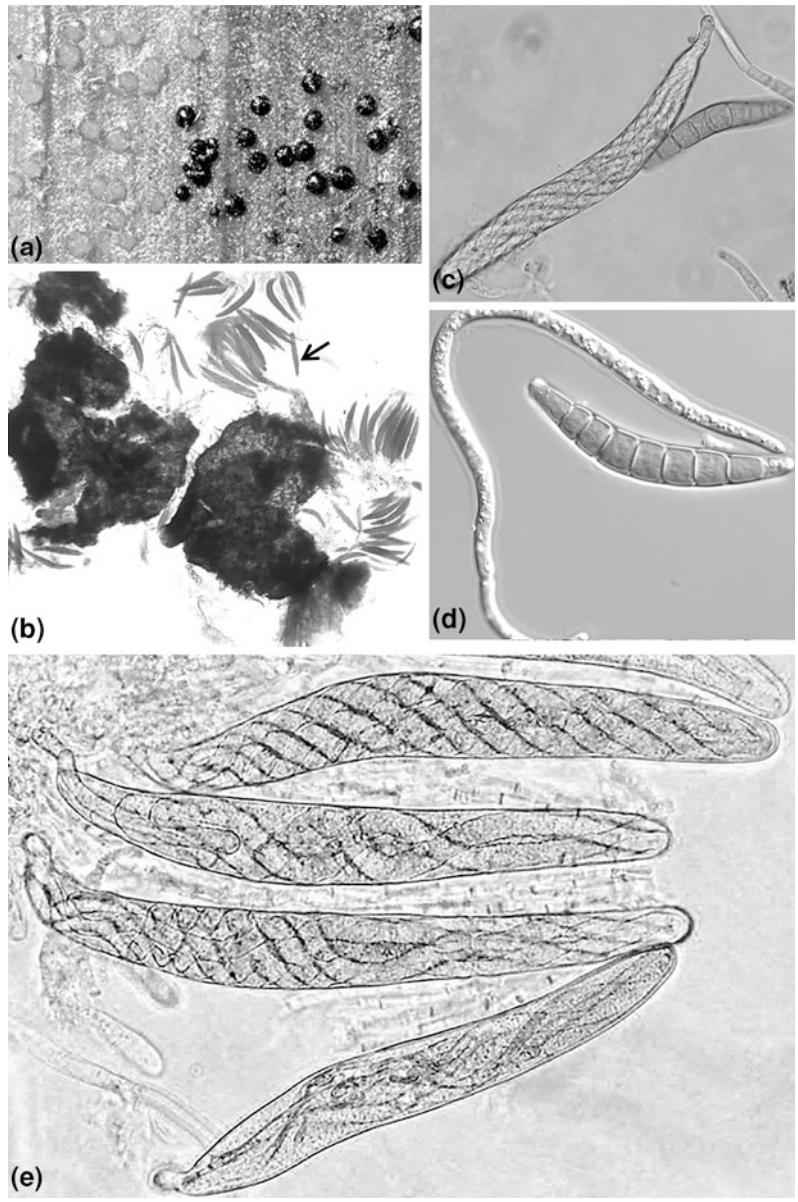


Fig. 2.3 Reproductive stages of *C. heterostrophus*. **a** Portion of a mating plate containing a senescent corn leaf inoculated with a pigmented *MAT1-1* strain and an albino *MAT1-2* strain. Both *black* and *white* pseudothecia are formed indicating both strains are hermaphroditic. **b** Two pseudothecia that have been squeezed to release asci (*arrow*). **c** A single ascus containing ascospores (*tetrad*) and a single conidium. **d** A single ascospore and a single conidium. **e** Several asci containing tetrads with varying numbers of ascospores

inbred *C. heterostrophus* race O lab strain C5 was used as the reference sequence for all comparisons, as it is the most complete, consisting of only 68 scaffolds. Three additional *C. sativus* strains have been sequenced recently, but are not discussed here (Zhong unpublished).

Overall sequence assembly and annotation statistics are presented in Table 2.3. All *Cochliobolus* genomes are in the 31–37 Mb range with an estimated gene content of 12,000–13,300. Gene content and genome organization are highly similar within this group of fungi, although less so for *C. lunatus*. In contrast, comparative analysis of *C. heterostrophus* and *C. sativus* in the context of 15 more distantly related Dothideomycetes genomes (Ohm et al. 2012) revealed significant variation.

The relative scale of conservation at the nucleotide level, compared to *C. heterostrophus* C5, was used as an estimation of similarity.

Table 2.3 Genome statistics

Species (strain)[a]	Genome characteristics							
	Assembly size (Mb)	Scaffold #	Scaffold N50/L50 (Mb)	# Predicted genes	# NPS genes	# PKS genes	# P450 genes	# SSP genes
Ch (C5)	36.46	68	7/1.84	13,336	14	23	156	180
Ch (C4)	32.93	207	13/0.96	12,720	14	25	149	171
Cv (F13)	32.83	676	47/0.23	12,894	18	21	138	160
Cc (26-R-13)	31.27	844	82/0.11	12,857	20	27	143	153
Cm (WK1C)	31.36	619	68/0.13	12,007	11	21	124	143
Cs (ND90Pr)	34.42	157	7/1.79	12,250	25	18	127	289
Cl (m118)	31.17	171	10/1.53	12,131	9	15	106	230

[a] For species designations, *see* footnotes, Table 2.1

Most of the *C. heterostrophus* race T strain C4 assembly could be aligned and only 1,584 SNPs were found between the two strains (Fig. 2.4). This remarkable level of conservation is molecular verification of the highly inbred nature of the two strains, achieved through generations of successive backcrossing. Note, since race O strain C5 is the reference to which all sequences are aligned, alignment of C4–C5 excludes the 1.2 Mb of *Tox1* DNA that is unique to race T. In contrast to results with the inbred strains, comparison of each *C. heterostrophus* field strain to C5 revealed roughly 10 times more SNPs than the same comparison with C4 and comparison of each *Cochliobolus* species revealed roughly 10 times more SNPs than did any *C. heterostrophus* field strain. Thus, there is a clear diminishing gradation of similarity at the whole-genome level as comparisons move from inbred strains to field strains within a species, to across species. As expected, based on phylogenetic distance (Fig. 2.1), *C. lunatus* appears to be the most diverged species, as only 20 % of its genome could be aligned to reference C5, compared to ~75 % for other *Cochliobolus* species (Fig. 2.4).

Most significantly, at the species level, a total of 11.76 Mb present in all *C. heterostrophus* genomes was missing from *C. victoriae, C. carbonum, C. sativus,* and *C. miyabeanus* (*C. lunatus* was excluded from this analysis). Only 1.6 Mb of this was in segments larger than 5 kb in the alignment to C5. Most of the sequence that separates *C. heterostrophus* from other species, therefore, is not the result of large wholesale insertions or deletions of DNA, but from a more piecewise gain and loss. We and others (Hane et al. 2011; Goodwin et al. 2011; Rouxel et al. 2011) have recently coined the term mesosynteny (Ohm et al. 2012) to describe organizational conservation between species. Genetic content is conserved across chromosomes, but not colinearly. It seems possible that our findings with *Cochliobolus*, showing that many small, scattered differences sum to significant quantitative differences (i.e., 25 % dissimilar), could be the product of the same mechanisms.

Pathogens of the same host (e.g., *C. carbonum* and *C. heterostrophus* on maize) were not more similar to each other than those with different hosts. Instead, overarching genetic patterns followed phylogenetic lines. A telling example of this is our finding that *C. carbonum* and *C. victoriae* have fewer SNPs between them than revealed in comparisons between other pairs of *Cochliobolus* species. These comparisons support our previously reported hypothesis that *C. victoriae* arose from a *MAT1-2* strain of a non-HC-toxin-producing strain of *C. carbonum* and is expected therefore to be more closely related to it than to other species (Christiansen et al. 1998). Given that the Pleosporaceae arose as a group less than 23–17 MYA (*see* Fig. 2.1 in Ohm et al.

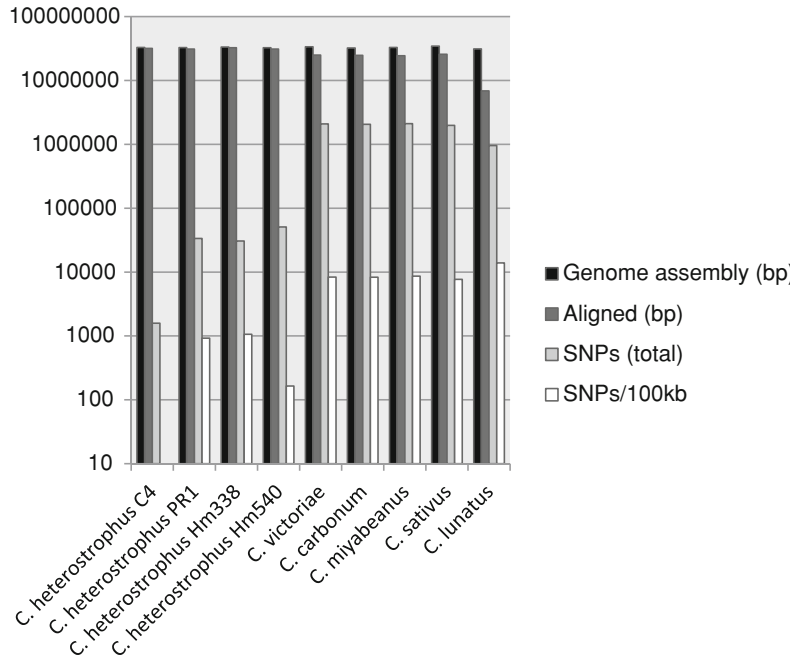


Fig. 2.4 Relative conservation of *Cochliobolus* species and *C. heterostrophus* strains to *C. heterostrophus* C5 reference. Each genome in this study was aligned, pairwise, to the *C. heterostrophus* C5 assembly using the MUMmer DNAdiff tool (Kurtz et al. 2004), and data were plotted logarithmically. The majority of each *Cochliobolus* species genome could be aligned (*dark gray bars*) to *C. heterostrophus* C5, except for *C. lunatus.* SNPs called between aligned regions (*light gray bars*) demonstrate that the inbred *C. heterostrophus* C5 and C4 strains are highly similar and *C. heterostrophus* field strains are more similar to *C. heterostrophus* strain C5 than to any other *Cochliobolus* species. SNPs/100 kb of aligned sequence (*white bars*) support this trend and show *C. lunatus* is the most dissimilar to *C. heterostrophus* of the *Cochliobolus* species, which fits with phylogenetic placement (Fig. 2.1). Data are displayed relative to the total query assembly size (*black bar*)

(2012)) and the genus *Cochliobolus* is young in the Pleosporaceae group, genome comparisons provide us with an overall picture of a timeline of how genome diversity varies with speciation.

Less than 1 year after the comprehensive analyses of 18 genomes in Ohm et al. (2012) and Condon et al. (2013) were published, the number of sequenced Dothideomycete genomes has doubled in the JGI Mycocosm (http://genome.jgi.doe.gov/programs/fungi/index.jsf). As more genera are sequenced to the same depth as *Cochliobolus*, the close similarity seen among *Cochliobolus* species can be compared to relationships among suites of species taxa within other genera. Attempting to align separate *Aspergillus* species, using our methodologies, for example, would yield poor alignments, as they are much more distantly related to each other. Indeed—studies that identify syntenic genomic regions between *Aspergillus* species do so with a much lower threshold for similarity and conservation (Fedorova et al. 2008).

2.2.2 Insights from Genome Comparisons

2.2.2.1 Secondary Metabolism

Armed with the knowledge that most of the best known aggressive *Cochliobolus* pathogens are necrotrophs and that high virulence/pathogenicity of the most devastating of these is associated with secondary metabolite production in the form of HSTs biosynthesized by NRPS and PKSs, we extracted all NRPS and PKS encoding genes from all 6 species (10 strains). Number of *NPS*s per genome, ranged

from 9–25, while number of *PKS*s ranged from 15–27 (Table 2.3). Comparative analyses revealed that the suites of these genes are astoundingly diverse among species but remarkably conserved among isolates of the same species, whether inbred or field strains, except for defining examples that generally map to unique genomic regions. Functional analysis of several of these strain-unique *PKSs* and *NPSs* reveals a strong correlation with a role in virulence as hinted at decades earlier with, e.g., the *PKS* genes for T-toxin production in *C. heterostrophus* race T and the genes for HC-toxin production by race 1 of *C. carbonum,* which are not found in any other *Cochliobolus* species.

Comparing the inventories of secondary metabolism genes across several closely related species yields key insights (Figs. 2.5 and 2.6). The first insight is that broadly conserved NRPSs or PKSs are most likely to produce metabolites of biological function central to the fungal cell itself. *NPS2*, *NPS6*, *NPS4, NPS10,* and *PKS18* are *C. heterostrophus NPS* and *PKS* genes conserved across all *Cochliobolus* species (Figs. 2.5, 2.6 and 2.7). Functional studies of *C. heterostrophus* mutants deleted for these genes demonstrate that the metabolites produced by the conserved biosynthetic enzymes affect developmental processes such as sexual and asexual development, morphology, hydrophobicity of colony surfaces, as well as stress (oxidative, iron, etc.) management (Figs. 2.6 and 2.7). Properly defining the scope of inclusion for this inference is essential—across the 18 Dothideomycetes examined in Ohm et al. (2012), only *NPS10* is conserved in all, despite the importance of these metabolites in *Cochliobolus* species. This finding is in agreement with the earlier hypotheses (Bushley and Turgeon 2010) that *NPS10* is among the more ancestral NRPSs. The product of NPS10 is not known, however, *C. heterostrophus* mutants are sensitive to oxidative stress. *C. heterostrophus* NPS2 is responsible for siderophore biosynthesis and intracellular iron storage and is conserved in 17 out of the 18 Dothideomycetes examined in Ohm et al. (2012, Table S19) (Fig. 2.6). NPS6 is present in 11 of the 18 genomes and is responsible for extracellular siderophore biosynthesis and thus competition for iron in the plant–fungal interaction (Fig. 2.6). NPS6 has been shown to be involved in virulence of *C. heterostrophus* to corn, of *C. miyabeanus* to rice, of *A. brassicicola* to *Arabidopsis thaliana,* and of *Fusarium graminearum* to wheat. It is also required for in vitro oxidative stress management (Oide et al. 2006). NPS4 makes an unknown product, but is present in 10 of the 18 genomes. *C. heterostrophus*, *A. brassicicola*, and *F. graminearum nps4* mutant colony surfaces are hydrophilic, rather than hydrophobic like wild type (Fig. 2.7) (Turgeon et al. 2008). PKS18, responsible for melanin biosynthesis, is conserved in all *Cochliobolus* species and was reported as conserved in 17 of 18 genomes in the study of Ohm et al. (2012). We have subsequently observed that *A. brassicicola,* the species missing PKS18, does in fact posses the gene (Fig. 2.6).

The second key insight is that genes encoded by genes "unique" to a particular species or strain of a species, encode enzymes that are likely biosynthesizing secondary metabolites involved in virulence (Fig. 2.8). A canonical example is the identification of a group of *C. sativus* pathotype 2-specific AMP domains (Fig. 2.5), one of which (ID 115356) when deleted, drastically reduces virulence on cultivar Bowman (Fig. 2.8). Another example is the *C. heterostrophus* race T- specific *PKS1* and *PKS2* genes (Fig. 2.6). These two polyketide synthases are responsible for production of T-toxin in race T and high virulence to Tcms maize and have long been described as unique to race T based on DNA–DNA hybridization blots. Phylogenetic analyses of PKS KS domains confirmed that they are not found in any other *Cochliobolus* species (Fig. 2.6).

A third example is the genes encoding *C. carbonum* HC-toxin (Fig. 2.5). *C. carbonum* race 1 is the only *Cochliobolus* species to possess HTS1, the NRPS (4 AMP domains) responsible for producing HC-toxin (Fig. 2.8). Wider genome resources, however, uncover candidate orthologs for all 4 AMP domains plus other genes associated with biosynthesis of HC-toxin, in

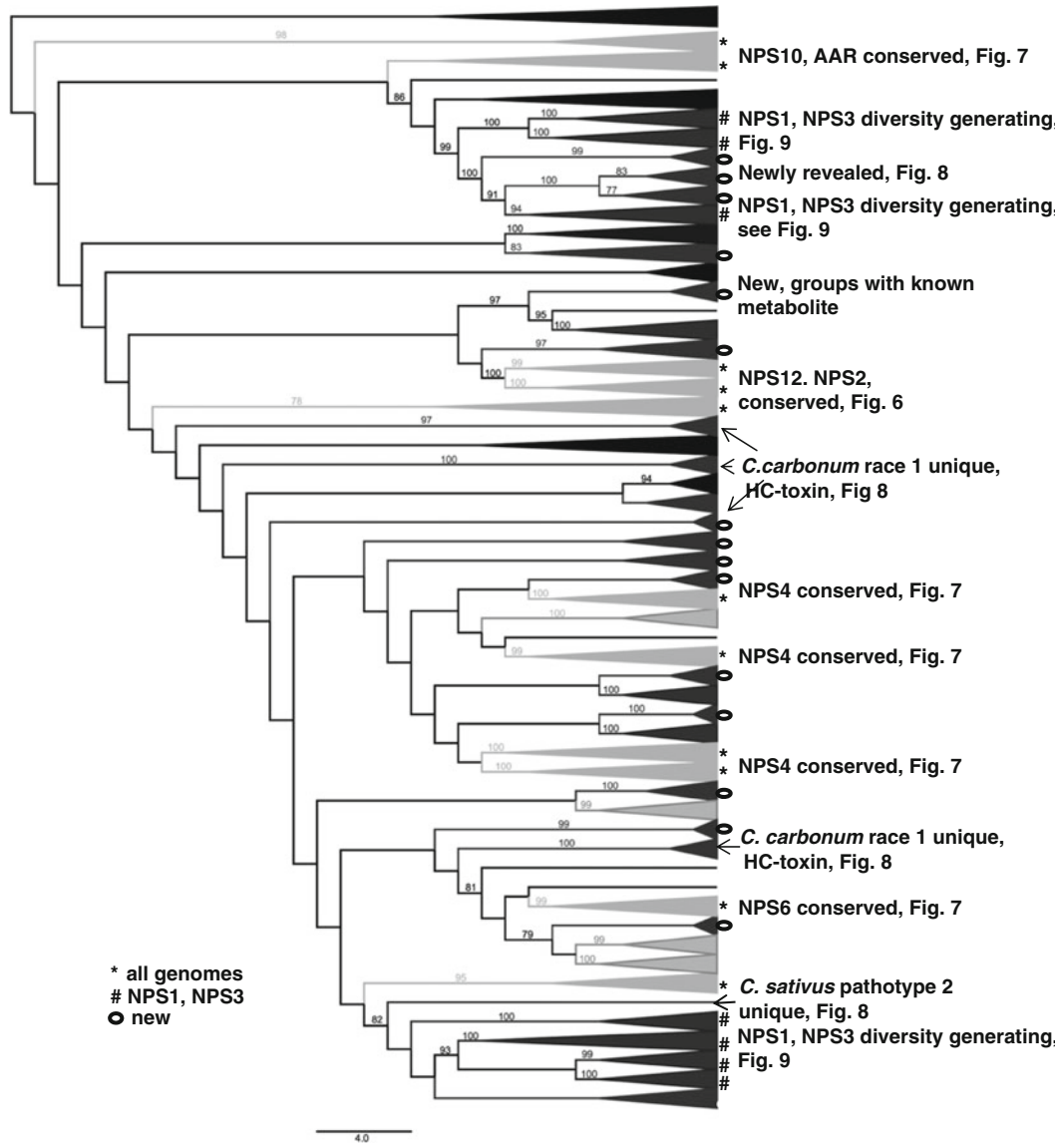


Fig. 2.5 Cartoon of cross-species phylogenomic analyses of individual AMP binding domains from NRPS proteins. NRPS AMP domains were extracted from all five *C. heterostrophus* and from the *C. victoriae, C. carbonum, C. miyabeanus*, *C. sativus,*, and *Setosphaeria turcica* genomes. Members of the reference set of previously annotated *C. heterostrophus* NRPS AMP domains (Lee et al. 2005; Bushley and Turgeon 2010) were used as benchmarks for branches. Branches of the full phylogenetic tree are collapsed according to clustering with the reference set of *C. heterostrophus* AMP domains. Presence in each of the five *C. heterostrophus* strains, *Cochliobolus* species, and *S. turcica* is noted by "*", AMP domains not grouping with the previously annotated *C. heterostrophus* set are labeled as "newly revealed" or "new, groups with known metabolite." NPS1, NPS3 AMPs are labeled as diversity generating (Fig. 2.9). *C. carbonum* HTS1 AMPs are indicated, as is the *C. sativus* pathotype 2 NRPS discussed in text (Fig. 2.8)

Setosphaeria turcica, Alternaria jesenkae, and *Pyrenophora tritici-repentis* and *Fusarium semitectum* (Manning et al. 2013; Condon et al. 2013). The metabolites produced by the first three of these *HTS1* orthologous clusters have not been identified and they may not be HC-toxin

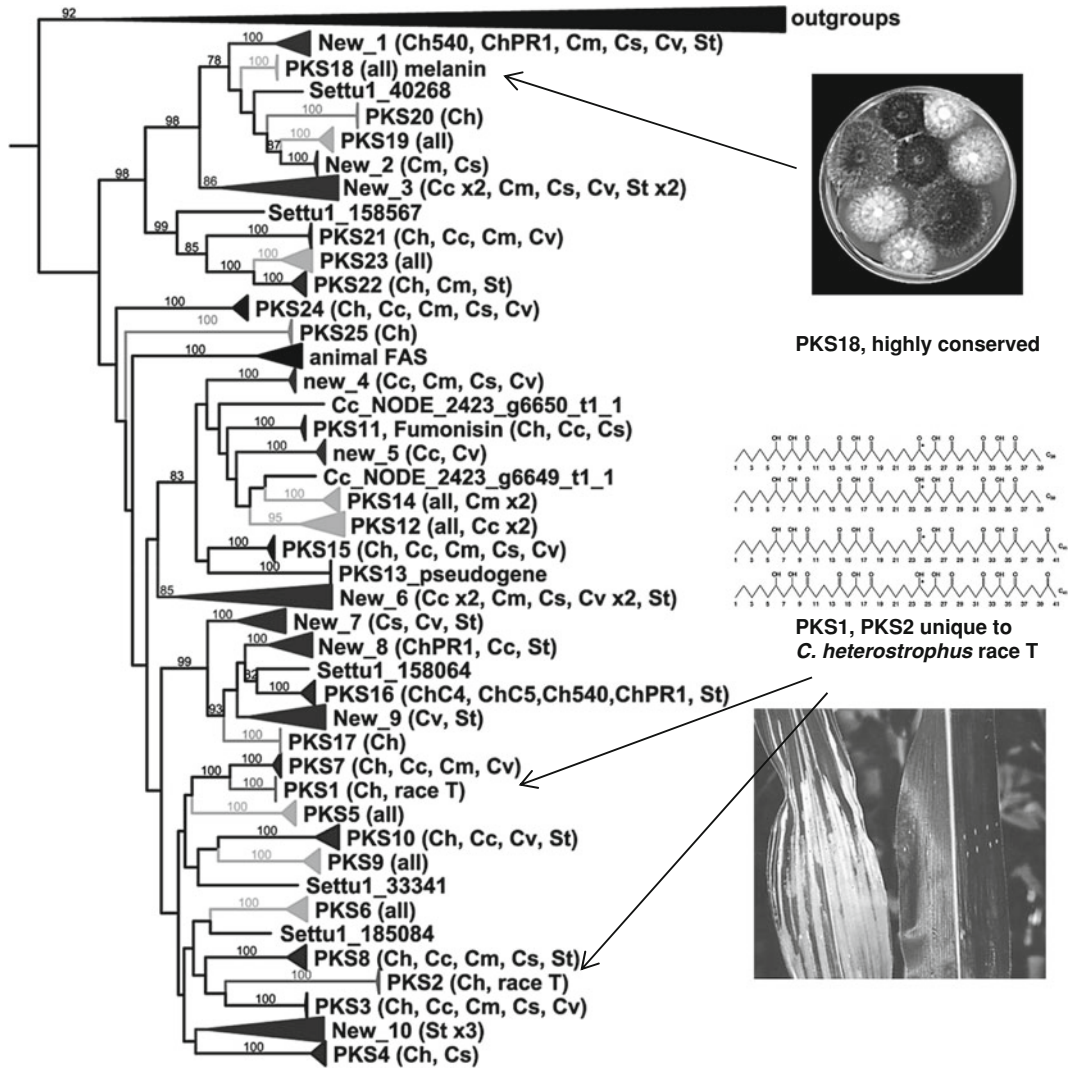
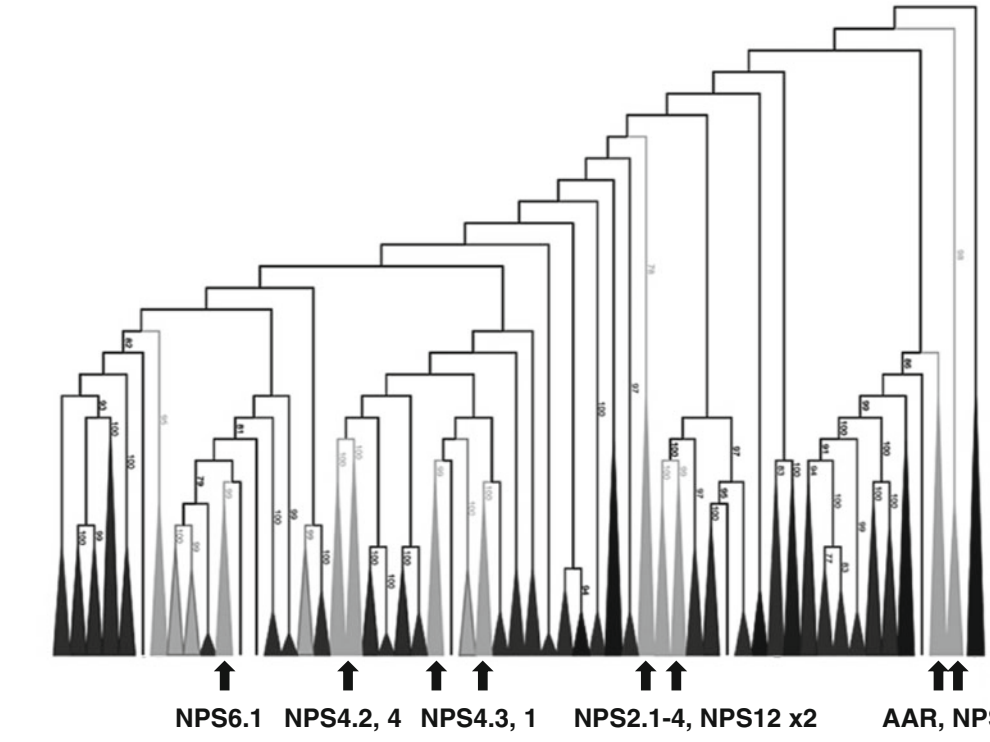


Fig. 2.6 Cartoon of cross-species phylogenomic analyses of individual ketosynthase (KS) domains from PKS proteins. The KS domains were extracted from all five *C. heterostrophus* and from the *C. victoriae, C. carbonum, C. miyabeanus*, *C. sativus* and *S. turcica* genomes. PKS designations match the *C. heterostrophus* set. KS domains not grouping with the previously annotated *C. heterostrophus* set are labeled as "New_1 through _10." Highly conserved PKS18, encoding the PKS for melanin biosynthesis and the unique PKSs (PKS1, PKS2) for *C. heterostrophus* race T T-toxin production are indicated

($C_{22}H_{34}N_4O_6$). *Fusarium semitectum,* for example, has the HTS1 ortholog, APS1, however this is the core NRPS for biosynthesis of apicidin ($C_{34}H_{49}N_5O_6$), a structurally different metabolite with the same biological activity as HC-toxin (both are histone deacetylase inhibitors) (Jin et al. 2010). Whether or not the other species produce HC-toxin, the discovery of these *HTS1* orthologs furthers our understanding of evolution of genes associated with HSTs in the fungal–plant interaction. Like apicidin and HC-toxin, orthologs may have profound medicinal application (Jin et al. 2010; Han et al. 2000). Thus, HST genes that were originally thought to be unique to the producer, like those for HC-toxin in race 1 of *C. carbonum* may prove not to be. As more and more genome sequences become available, it is even likelier that genes, such as *HTS1,* are not


NPS6.1
NPS4.2, 4
NPS4.3, 1
NPS2.1-4, NPS12 x2
AAR, NPS10
- H_2O_2
+ H_2O_2
nps6
WT
WT
nps4
WTx WT
nps2 x nps2
WT
nps2
nps6
nps2nps6
coprogen
ferricrocin

◀ **Fig. 2.7** The NRPS AMP domain tree (Fig. 2.5) and highly conserved AMPs. *See text* NPS2 consists of four AMP domains that group together and produce the hexapeptide intracellular siderophore, ferricrocin, responsible for iron storage within cells. When deleted, sexual reproduction (ascus formation, *right*) is absent. NPS4 consists of four AMP domains, only two of which group together. Product is unknown but lack of NPS4 converts colony surfaces from hydrophobic to hydrophilic (*middle*). NPS6 consists of one complete and one incomplete AMP domain for production of the tripeptide extracellular siderophore, coprogen, which when absent impacts ability to acquire iron, resist oxidative stress (*left*), and reduces wild-type virulence (*bottom*). There are two copies of NPS12 which has no known phenotype. AAR is alpha-aminoadipate reductase responsible for lysine biosynthesis in fungi. For each NPS, the number after the period refers to a particular AMP domain in the protein, starting from the N terminal end

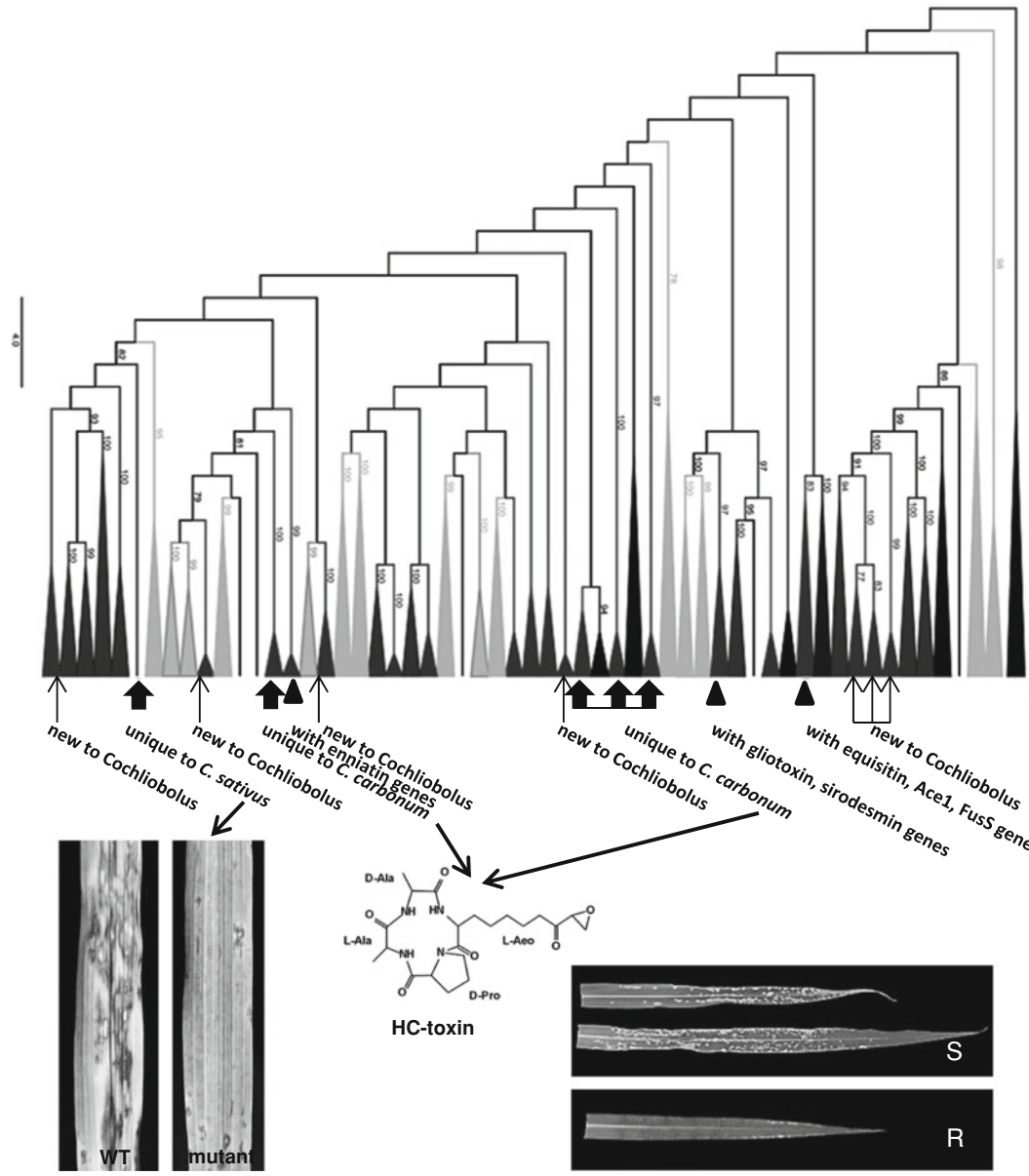


Fig. 2.8 The NRPS AMP domain tree (Fig. 2.5) and unique AMPs. *See* Figs. 2.5 and 2.7 for labeling. An example of a unique NRPS in *C. sativus,* associated with virulence of the strain on a particular cultivar of the host is shown (*left*). Barley cv. Bowman was inoculated with wild type (ND90Pr) and a mutant lacking the gene corresponding to protein ID 115356 shows reduced virulence. *Right* Susceptible (*S*) and resistant (*R*) maize inoculated with *C. carbonum* race 1, which produces the HST HC-toxin

unique but are spottily distributed with candidate orthologs in distant and/or isolated branches of the fungal phylogenetic tree.

As orthologs are discovered in more and more species, horizontal gene transfer may be a less enticing hypothesis—or, it may be the best explanation, depending on the distribution. The alternative hypothesis for rare distribution among species is rapid selective duplication and loss (Kroken et al. 2003; Bushley and Turgeon 2010). By diversifying inventories of HSTs or effectors, pathogens prevent hosts from developing a single resistant genotype. It is possible that uncharacterized members of the pool of uniquely distributed secondary metabolism genes act as HSTs in undiscovered contexts. Their anonymity may relate more to the fact that the corresponding host target, or host itself, is not widely deployed in agriculture, and therefore, the pathogenic potential of these metabolites is not known to us.

The third insight comes from our species inventory of NRPS genes and may weigh on the last point above. The AMP domains comprising *C. heterostrophus* NPS1, NPS3, NPS13 NRPS proteins indicate a complex evolutionary history (Condon et al. 2013) (Fig. 2.5). On the whole protein level, the complete *C. heterostrophus* NPS1 (trimodular) and NPS3 (tetramodular) domain sets are either present or absent in other species (Fig. 2.9). NPS1 is intact in *C. victoriae, C. carbonum,* and *C. lunatus,* while NPS3 is intact in *C. miyabeanus* and *C. sativus,* but absent from the other genomes. Mono-modular *C. heterostrophus* NPS13 is found only in *C. heterostrophus*. NPS1, NPS3, and NPS13 protein AMP domains are expanded discontinuously resulting in a suite of novel proteins which may be mono- or multi-modular (Fig. 2.9). All of the AMP domains that comprise these proteins form two separate clades in the phylogenetic tree of *Cochliobolus* AMP domains (Figs. 2.5 and 2.9).

We speculate that this group of AMP domains is a hotbed of evolutionary activity. Domains are rapidly duplicated, swapped, recombined, and genes are gained and lost. Future studies on the evolutionary signatures of different clades could help support this hypothesis. If the idea holds, it could explain how some NRPS are found in such a patchwork distribution throughout a phylogeny.

2.2.2.2 Iron and Oxidative Stress

Among the NRPSs involved in running the fungal cell itself are those biosynthesizing intracellular and extracellular siderophores for iron chelation. Iron is indispensable for virtually all organisms (Winkelmann 1991) and is involved in many fundamental biochemical reactions (respiration, the TCA cycle). It is also required for success as a pathogen. Iron can occur either in reduced ferrous (Fe^{2+}) or oxidized ferric (Fe^{3+}) form; this capacity to gain or lose electrons makes iron a major redox mediator. Iron has the potential to catalyze the Fenton/Haber Weiss reactions (Fenton 1894) generating highly cytotoxic ROS. Hence, mechanisms that sequester iron in cells are critical for survival. Paradoxically, although iron is essential, bioavailable forms are very limited in aerobic environments (Neilands and Leong 1986; Lesuisse and Labbe 1994; Haas 2003). Therefore, efficient and competitive iron-uptake mechanisms are also critical to survival of all organisms, including fungi during infection of plants. For this, fungi employ a variety of strategies, including two high-affinity uptake mechanisms, siderophore-assisted mobilization, and non-siderophore reductive iron assimilation (RIA) (Schrettl et al. 2004; Oide et al. 2006; Wolpert et al. 2011). As noted, with their strong iron-binding activity, siderophores function both in acquisition and in storage/sequestration of iron (Neubauer et al. 2000; Oide et al. 2006). Fungal (and bacterial) siderophores are biosynthesized by multi-modular NRPSs (encoded by *NPS2* and *NPS6*, previous section) (Fig. 2.7) (Oide et al. 2006). The alternative high-affinity iron-chelating mechanism in fungi, RIA, is a three step process in which ferric iron is reduced by a metalloreductase (*Fre*1p) extracellularly, and then the ferrous iron is oxidized by an iron multicopper oxidase (*Fet*3p) that is coupled to a high-affinity iron permease (*Ftr*1p) for transport

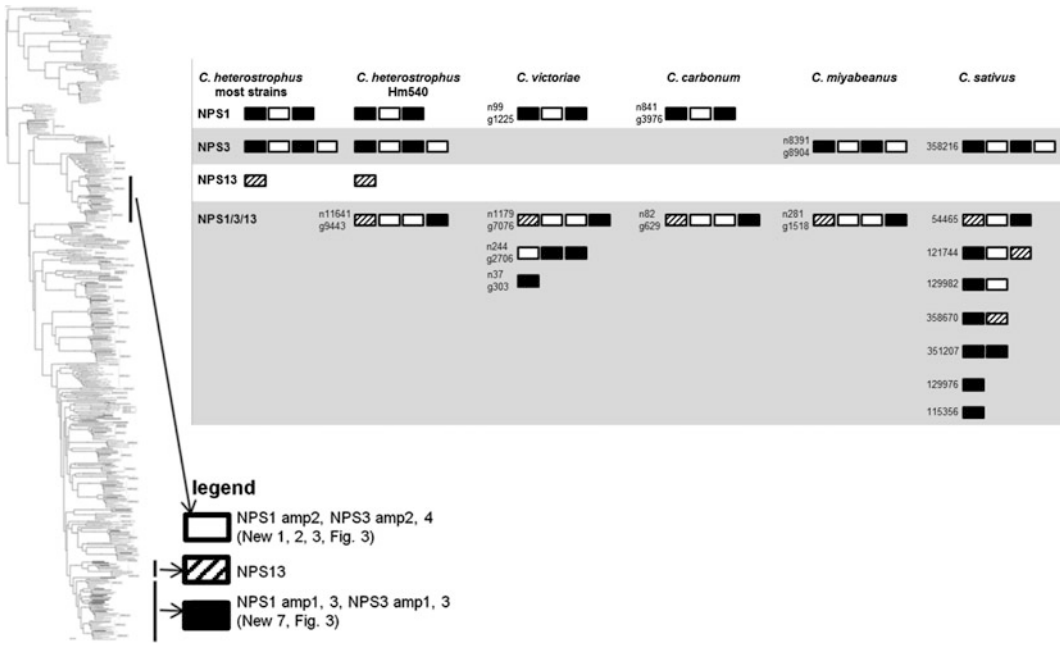


Fig. 2.9 NPS1, NPS3, and NPS13 are examples of NRPS proteins encoded by highly recombinogenic and expanded *NPS* genes. Full AMP domain phylogenetic tree (Condon et al. 2013) is cartooned at *left*. The reference NPS1, NPS3, and NPS13 proteins are cartooned *bottom left*. AMP domains corresponding to these proteins are completely conserved in the five strains of *C. heterostrophus,* but show discontinuous presence in all other *Cochliobolus* species (Fig. 2.5) and *Setosphaeria.* *Note* some AMP domains from NPS1 to NPS3 group at the *top* of the tree (AMPs 2 and 4, *white box*), while the rest group at the *bottom* of the tree (AMPs 1 and 3, *hatched box*); NPS13 AMP1 (*black*) also groups at the *bottom* of the tree. *Branches* correspond to individual AMP domains which group together and the particular corresponding AMP domain is depicted on the *right* of the diagram. *Note* collection of novel NRPSs composed of NPS1, NPS3, and NPS13 AMPs, at *bottom*

across the plasma membrane to the cytosol (Haas 2003). To a first approximation, necrotrophs, such as most *Cochliobolus* species, rely on extracellular siderophores for in planta iron acquisition, while (hemi)biotrophs tend to use the RIA mechanism of iron gathering.

We have generated many *C. heterostrophus* mutants lacking iron or oxidative stress related genes (Fig. 2.7). Associated phenotypes are shown in Table 2.4. NPS6 is a virulence factor for several pathogens (Oide et al. 2006). *nps6* mutants still have the RIA route available and also still produce the intracellular siderophore, ferricrocin, made by the product of the NRPS encoding gene, *NPS2*. Ferricrocin is not required for virulence of *C. heterostrophus,* but is required for sexual reproduction (Fig. 2.7). *nps2nps6* double mutants exhibit a greater reduction in virulence and impairment in sexual development than single *nps6* or *nps2* mutants (Fig. 2.7). Triple iron acquisition and storage mutants (*nps2nps6ftr1*) are almost avirulent, but do attach to and penetrate the host (Condon, Turgeon unpublished). *nps6* mutants are also hypersensitive to oxidative stress (Fig. 2.7) and there is a gradation of sensitivity of the single, double, and triple mutants, with the latter being the most sensitive.

Double mutants lacking *ChAP1* (Lev et al. 2005), a gene encoding a redox-regulated transcription factor and *NPS6* (*Chap1nps6*), or lacking *ChAP1* and the iron-sensitive transcription factor *Sre*1 (*Chap1sre1*) have been constructed (Table 2.4) and tested for oxidative stress and virulence. *Chap1nps6* mutants are more sensitive to oxidative stress than either parent, while *Chap1sre1*

Table 2.4 Iron- and ROS-related *C. heterostrophus* genes and mutants available

Gene (Acc.#)	Protein	Function/phenotype of mutant (References)
Iron-related genes deleted singly or in multiples		
NPS2 (77609)	NRPS	Intracellular sid/reduced sex, (Oide et al. 2007)
NPS6 (33171)	NRPS	Extracellular sid/reduced virulence, reduced resistance to ROS, hypersensitive to low iron, (Oide et al. 2006)
NPS2;NPS6	*See* above	Augmented in all above phenotypes
FTR1 (104817)	Iron permease	Reductive Fe assimilation/WT in virulence and sensitivity to ROS, and low iron
FTR1;NPS2	*See* above	nps2 phenotypes; otherwise WT as for ftr1
FTR1;NPS6	*See* above	Greater virulence/sensitivity phenotypes compared to nps6. Requires supplemental iron for growth/ conidiation on CM
FTR1;NPS2;NPS6	*See* above	Decreased virulence, sensitivity to ROS, more severe phenotypes than nps6;ftr1
SRE1 (109473)	Transcription factor	GATA-type Zn-finger/ regulator of iron metabolism, slow radial growth, less resistant to ROS than WT
NPS6;CHAP1	*See* above	Virulence = nps6, very reduced resistance to ROS, > either single mutant
ROS-related genes deleted singly or in multiples		
CHAP1 (130082)	bZIP tf	More sensitive to ROS than nps6 (Lev et al. 2005)
CHAP1;SRE1	*See* above	Virulence = nps6, reduced resistance to ROS, but < Chap1 mutant
CAT1 (115312)	Catalase	Decomposition of H_2O_2 to H_2O and O_2/non secreted, WT (Robbertse 2003)
CAT2 (110605)	Catalase	Decomposition of H_2O_2 to H_2O and O_2/non secreted, WT (Robbertse 2003)
CAT3 (109994)	Catalase	Decomposition of H_2O_2 to H_2O and O_2/secreted, sensitive to ROS (Robbertse 2003)
CAT1;CAT2	*See* above	WT (Robbertse 2003)
CAT1;CAT3	*See* above	Sensitive to ROS, WT virulence (Robbertse 2003)
CAT2;CAT3	*See* above	*See* above
CAT1;2;3	*See* above	*See* above
SOD1 (24548)	sup ox (Cu/ Zn)	Catalyzes dismutation of superoxide into O_2 and H_2O_2/WT
SOD2 (30814)	sup ox (Mn/ Fe)	*See* above, mitochondrial/WT
SOD3 (90570)	sup ox (Mn/ Fe)	*See* above/possibly essential (*see* footnote)
NOXA (95484)	NADPH oxidase	Membrane-bound enzyme complex; generates superoxide/WT in virulence, delayed conidiation, reduced somewhat decrease pigmentation
NOXB (95158)	As above	*See* above/reduced virulence
NOXC (117226)	As above	*See* above/decreased conidiation and somewhat decreased pigmentation
NOXR (28914)	Regulator	*See* above/reduced virulence, decreased conidiation

For oxidative stress and virulence phenotypes *see* Fig. 2.7; *sid* siderophore; *L-orn mono* L-ornithine monooxygenase; *sup ox* superoxide dismutase; *tf* transcription factor; attempts to delete *SOD3* have failed, suggesting it is essential. Acc.# = JGI

mutants partially rescue the *Chap1* oxidant-sensitive phenotype. Double mutant phenotypes are consistent with a model in which sequestering of iron by the NPS6 siderophore defends the fungal pathogen against oxidative stress.

2.2.2.3 The CYPome of *Cochliobolus* spp.

The published *Cochliobolus* genome manuscript (Condon et al. 2013) did not include *C. lunatus*, a species, as indicated in the

Introduction, that has been used as a workhorse for steroid biosynthesis centered on the activity of cytochromes P450 (CYPs). CYPs, a superfamily of heme-containing monooxygenases, are ubiquitously present in all kingdoms of life with fungi having the second largest number after plants. Some are involved in primary metabolism and are indispensable for normal development and homeostasis or in allowing fungi to live on particular carbon sources. Others are involved in xenobiotic metabolism and provide defense against natural products, while still others are associated with genes for secondary metabolite production and the biosynthesis of pigments, antioxidants, defense compounds, and toxins.

Despite the fact that CYPs play roles in hydroxylation and oxidation processes leading to degradation, detoxification, and syntheses of compounds crucial for life or for niche survival, the substrates on which they act are largely unknown. To identify P450s and annotate those associated with secondary metabolite gene clusters across *Cochliobolus* species, we searched gene models for annotations with the PF00067 (P450 superfamily) domain. Almost one thousand predicted P450s (943) were identified across six *Cochliobolus* species, averaging ~135 P450s per species and represents ~1 % of the total gene catalog (Table 2.3). This tally is comparable to the number in *Aspergillus nidulans* (version AN.3, CADRE (Kelly et al. 2009)) and other *Aspergillus* species (~125 P450s per species). The CYPome of the Dothideomycete *Mycosphaerella graminicola* has fewer (82 P450s plus one pseudogene) (Newsome et al. 2013). P450s in close proximity to secondary metabolism backbone genes (such as *NPS* or *PKS* genes) may be involved in secondary metabolite biosynthesis. *NPS* or *PKS* genes were located near 13–17 % of *C. heterostrophus* P450s, slightly lower than when this analysis was done for *A. nidulans*, 29 % (32 of 111 functional P450s) (Kelly et al. 2009). No preference was observed in the association of P450s with mono- or multi-modular *NPS* genes.

It is difficult and in most instances impossible, to predict the specific functions of the CYPs from their sequence similarities or even their association with *PKS*s or *NPS*s in gene clusters, as it is known that a single amino acid change can significantly alter metabolic capabilities. These difficulties, in combination with the abundance of P450s, make phylogenetic analyses an essential first step for studying these crucial genes.

2.2.2.4 Small Secreted Proteins (SSP)

A search for candidate effector proteins that are cysteine rich (>2 % cysteine), small (<200 amino acids), predicted to be secreted (using Phobius (Kall et al. 2007)), and without transmembrane domains revealed between 143 and 289 SSPs per *Cochliobolus* (Table 2.3) (Condon et al. 2013). An all-versus-all BLAST analysis to determine if SSPs were strain or species-unique revealed that few candidate *C. heterostrophus* SSPs were unique to any particular strain within the species. Among species, *C. sativus* had the most isolate-unique SSPs, containing 167 candidates (Condon et al. 2013). As this is the only *Cochliobolus* strain thought to act as a hemibiotroph, it is interesting that it contains more SSPs, and more unique SSPs, than the necrotrophic isolates, although this is only a correlation at this point. As is typical with candidate effectors, functional domain predictions were lacking, with only 37 candidates having some predicted function, generally involved in cell wall or extracellular matrix function. An additional 23 candidates were conserved in other fungi outside of the Dothideomycetes. The remaining 120 predicted candidates were featureless and seemingly unique to the Dothideomycetes (Condon et al. 2013). *Cochliobolus heterostrophus* strain C5 SSP predicted candidates were rich in SNP calls to other *Cochliobolus* genomes: 101 candidate SSPs had SNPs with at least one other *Cochliobolus* genome (Condon et al. 2013).

In our all-versus-all BLAST analysis, only 6 of the 180 *C. heterostrophus* C5 SSPs were found in all 10 strains examined and 14 were unique to strain C5 (Condon et al. 2013). The presence or absence of most SSPs did not fall

into easily categorized bins such as *C. heterostrophus*-specific, or maize-pathogens only. Instead, SSPs were present and absent in no particular pattern across the genomes. 115 SSPs were present in at least one other species (*C. victoriae, C. miyabeanus, C. carbonum*), with seven found in all species, and 27 in all *Cochliobolus* species. Unlike those in some phytopathogens, such as *Leptosphaeria maculans* (Rouxel et al. 2011), SSP-encoding genes did not occur in clusters; candidates seldom were located within 10 kb of each other. It has become clear in recent years that necrotrophs, like (hemi)biotrophs, also use effectors to manipulate specific targets in the host cell for the benefit of the pathogen. Unlike (hemi)biotrophs, however, the aim seems to be to *trigger* host defenses or cell death, rather than circumvent these processes. Two clear examples are victorin produced by the necrotroph *C. victoriae* and ToxA produced by the necrotrophs *Pyrenophora tritici-repentis* (Ciuffetti et al. 1998) and *Stagonospora nodorum* (Friesen et al. 2008). The extent to which necrotrophs employ effectors is an exciting and unknown frontier. Do necrotrophic effector molecules always aim to trip host defenses, or do some act more according to (hemi)biotrophic principles, quelling host defense response and intercepting signaling? As for their metabolic origins, are (presumably) secondarily encoded molecules like victorin the norm, or do necrotrophs utilize small cysteine rich ribosomally encoded effector proteins typical of hemi(biotrophic) interactions? The bioinformatics analysis described above is an earnest attempt to break ground answering these questions. The limitations of this approach, however, cannot be overstated. Bioinformatically predicted SSPs require in planta expression or protein secretion data, or functional knockout data, before they can be considered *bona fide* effectors. SSPs are small, and typically lack predicted functional domains—a trait they share with miscalled ORFs. Secretion prediction is also an imprecise technique with many false-positive and false-negative predictions. That our SSP inventories are larger for known hemibiotrophs than necrotrophs seems to suggest that we are indeed including at least some effectors in our prediction.

2.2.2.5 Signaling

As for P450s, the published *Cochliobolus* genome manuscript (Condon et al. 2013) did not include a comprehensive analyses of genes associated with signaling. Because signaling mechanisms are the centerpiece of interaction biology, we have included a brief summary of annotation of relevant genes in *Cochliobolus* species.

Conserved signaling pathways. If two closely related pathogens infect different hosts, one might conclude that they respond to different signals and hypothesize that comparison of the genomes of *Cochliobolus* pathogens of different hosts would identify critical response differences. There is, however, no simple correlation. Genes encoding heterotrimeric G protein subunits, MAP kinases, and histidine kinase response regulators have been studied in *C. heterostrophus* since the 1990s and these studies are now facilitated by the genome projects (Horwitz et al. 1999; Lev and Horwitz 2003; Lev et al. 2009; Oide et al.; Degani et al. 2004). Two signaling pathways (MAP kinase and heterotrimeric G protein) are shown schematically in Fig. 2.10. As in other pathogens, the *C. heterostrophus* core signaling proteins could be considered virulence factors because mutants are unsuccessful pathogens, but additional developmental alterations, obfuscate how exactly, signaling impacts virulence.

One way that host specificity could be attained is for cell surface receptors in each species to recognize host-specific ligands, which then transmit the signal via a conserved intracellular cascade. *C. heterostrophus,* for example, has 21 genes predicted to encode histidine kinase sensors but only four downstream response regulators (Catlett et al. 2003b; Oide et al. 2010). The two-component pathways initiated by histidine kinase sensors in *C. heterostrophus* have central functions in morphogenesis, stress response, and virulence (Oide et al. 2010). Comparing histidine kinase sensors suites across

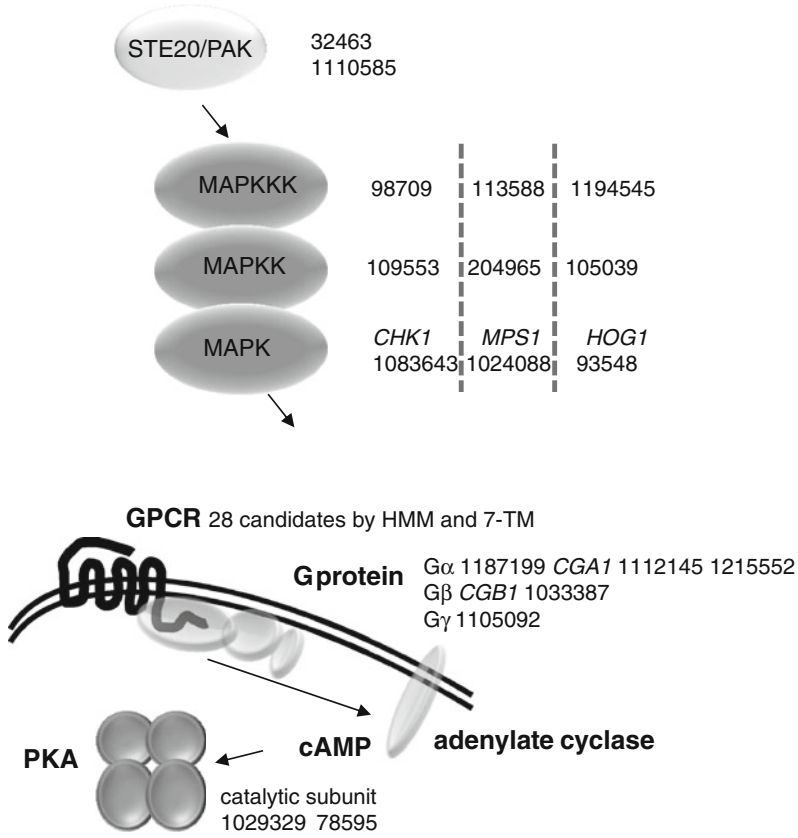


Fig. 2.10 Illustration of two conserved signaling pathways, with corresponding gene models from *C. heterostrophus.* The following genes have been studied by constructing deletion mutants: MAP kinases *ChHK1*, *MPS1*, and *HOG1*; G protein Gα subunit *CGA1*, and G protein G*β* subunit *CGB1*. The gene models identified by reciprocal BLASTP search and/or other methods (*see text*) are indicated as *C. heterostrophus* strain C5 v2.0 protein ID numbers. Two examples of signaling pathways are shown: *above*, MAP kinase cascade; the *vertical dashed lines* indicate a tentative association into MAPK modules, by homology (there is no functional information to support this, as yet) *below*, a model of heterotrimeric G protein signaling in which activation of adenylyl cyclase produces cAMP, which activates protein kinase A (PKA). Heterotrimeric G protein signaling could lower or raise cAMP levels

Cochliobolus pathogens may reveal nuances not readily apparent when core signaling components are compared.

For heterotrimeric G protein pathways, the capacity for signals from multiple receptors to converge on a few downstream transducers may be even greater than for the two-component pathways. G protein-coupled receptors (GPCRs) are more difficult to identify bioinformatically, than the highly conserved signal transducers, but methods are improving (Xue et al. 2008; Lafon et al. 2006; Omann et al. 2012; Kim et al. 2012). To estimate the number of GPCRs in *C. heterostrophus*, an initial analysis was done as part of the annotation effort (Horwitz lab unpublished; Ohm et al. 2012): filtered protein models were searched with an HMM tool designed to identify GPCRs (Wistrand et al. 2006), then those with seven transmembrane segments as predicted by PHOBIUS (Kall et al. 2007) selected. An initial phylogenetic tree was constructed. The candidate sequences were used to query the NCBI database to identify those with convincing homology to transporters, or having a conserved domain indicating that they may be transporters. These sequences, as well as

sequences falling on branches of the initial phylogeny together with annotated transporters or ATPases, were removed and the phylogeny was then recalculated. The analysis indicates orthologs of pheromone receptors *Ste*3 (1203184) and *Ste*2 (1215526), whose function in mating could be tested by gene deletion experiments. 20 candidates group with sequences annotated as related to the CFEM/Pth11 family in other fungi. Of these 20 candidates, three contain CFEM domains detected by Pfam, and in addition, have similarity with annotated CFEM-containing sequences. Three sequences show similarity to annotated Pth11-like sequences. These classes are proposed to be involved in pathogenicity (DeZwaan et al. 1999; Kulkarni et al. 2003). It would be of interest to compare these among *Cochliobolus* species for species-specific associations and, where possible, to test their function by gene deletion. This analysis provided no obvious orthologs of fungal opsins, even though two candidates were recognized previously by homology (C5 protein IDs 1195154 and 1139038, Oide and Turgeon unpublished). No members of the GPCR classes represented by *Neurospora* Gpr1-1, Gpr-5, and Gpr-4 (*see* Xue et al. 2008) were identified either, supporting our statement above that GPCRs are difficult to extract bioinformatically.

Light regulation. Not all signals are transduced from the cell surface to the nucleus by G protein and protein kinase pathways. In particular, dedicated fungal transcription factors relay information about light, pH, oxidants, and hypoxia. Once activated by the primary stimulus, these transcription factors may rely on additional regulators in order to produce the physiological output. The Neurospora circadian clock is a good example: the stress-activated MAPK (Hog1, Fig. 2.10) is activated rhythmically by the circadian oscillator (Vitalini et al. 2007). Circadian rhythmicity has not been studied in detail in any *Cochliobolus* species, but *C. heterostrophus* shows a clear banding pattern when grown under light/dark (L/D) cycles (Wu et al. 2012). A *C. heterostrophus* mutant lacking the ortholog of *N. crassa WC1* shows defective banding with a weaker, residual banding pattern suggesting that additional photoreceptors are active. Initial evidence that the circadian clock controls Hog1 phosphorylation via the response regulator Ssk1 (*N. crassa* RRG-1) in *C. heterostrophus* comes from our finding that the L/D banding pattern is defective in *hog1* and *ssk1* mutants (Oide et al. 2010), similar to that of *wc1* mutants (Turgeon and Horwitz labs unpublished).

Light regulation is of particular interest because it couples environmental sensing and secondary metabolism. *C. heterostrophus* mutants lacking key components of the velvet complex (*VEL1* or *LAE1*), which controls reproduction and secondary metabolism produce much less T-toxin than WT in the dark (Wu et al. 2012). Light conditions could be particularly relevant because light, as the source of energy, is a critical environmental factor for the host plant. Light could synchronize gene expression of the pathogen to match the host; thus, photocontrol of plant–pathogen interactions has received recent attention (Kim et al. 2011a, b; Lee et al. 2006a, b).

Redox signals. As noted above, *ChAp*1, an ortholog of yeast Yap1, senses oxidants in *C. heterostrophus*. Yap1 homolog-mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus *Alternaria alternata* on citrus (Lin et al. 2009; Kim et al. 2009) and *M. oryzae* on rice (Guo et al. 2011). *C. heterostrophus Chap1* mutants, although hypersensitive to oxidants, retain wild-type (Lev et al. 2005) or moderately reduced (Zhang et al. submitted) virulence on maize. The *Botrytis cinerea* Yap1 ortholog is required to resist peroxide stress in vitro, yet, Yap1 is not a virulence factor on bean, Arabidopsis, apple or tomato fruits, and its target genes are not induced on bean although H_2O_2 was detected (Temme and Tudzynski 2009).

There is strong genetic evidence for the involvement of multiple pathways in sensing oxidants. Loss of *C. heterostrophus* Hog1 (Fig. 2.10), its upstream response regulator Ssk1, or the response regulator Skn7, all result in hypersensitivity to oxidants (Oide et al. 2010). Although the oxidative burst is considered key to plant defense, it is worth noting that the ability

to cope with hypoxic stress is important for pathogens of animals. Neutrophils in the mammalian immune system produce ROS, yet *Aspergillus fumigatus* needs the hypoxic stress response for virulence (Blatzer et al. 2011; Grahl and Cramer 2010; Willger et al. 2012). Hypoxic stress has received less attention in plant pathogens.

Signaling pathways cannot be studied in isolation and the study of oxidants provides a good example. Loss of *HOG1*, *ChAP1*, *SKN7* as well as the NRPS responsible for extracellular siderophore production (Fig. 2.7), all diminish the ability of the pathogen to resist oxidative stress. Light signals are directly detected by the white collar transcription factors, but once again, the pathway is not a linear one because the global regulators Vel1 and Lae1 also participate. The light-sensing complex could be similar to that of other fungi but the details, and the genes regulated, likely hold surprises specific to *Cochliobolus*.

2.3 Applications from the Genome and Future Perspectives

The genomic resources available for comparative studies across the *Cochliobolus* genus are legion, thanks to the generous contributions of the JGI, with sequences and resources available for many different *Cochliobolus* species (and, for many species, multiple strains). The long history of using *Cochliobolus* species as model organisms allows an exciting marriage of functional work and in silico comparative genomics. As discussed in the Introduction, *Cochliobolus* taxa vary in their biology, host specificity, and developmental pathways. Whole-genome comparisons were startling vis-*a*-vis the incredible homology between most *Cochliobolus* species. Attempts to characterize "species-unique" sequence, found in all five *C. heterostrophus* strains, but no other *Cochliobolus* species, did not result in identification of large *C. heterostrophus* unique regions, but smaller differences. Uncovering the core identity of each species as it relates to their biology, therefore, is not as easy as identifying and characterizing a large obvious patch of genome. To address questions of differential biology, more refined approaches are necessary, facilitated by the history of molecular-genetic work for each species.

Molecular investigations into virulence factors have run the gamut from discovery of highly specific HSTs, to more general mechanisms involving iron and oxidative stress. Initially, secondary metabolism as a source of HSTs was considered the most compelling type of functional investigation for *Cochliobolus* pathogens (Lee et al. 2005; Turgeon et al. 2008). Extensive bioinformatic analyses of secondary metabolism genes occupy a large share of this chapter and these studies coupled with experimental research support our reasoning in this regard. The observation that phenotypes associated with secondary metabolism gene mutants follow phylogenetic distribution signatures provides a strong hypothesis and platform for further work. Conserved secondary metabolite clusters are likely to biosynthesize metabolites that broker basic cellular metabolism (iron gathering, oxidative stress management, etc.), while discontinuous and severely restricted gene distribution suggests niche-specific/virulence-specific function.

The second major aim of our comparative genomics study was to consider the role small secreted proteins may play in *Cochliobolus* species. Unlike secondary metabolites, this was not done against the backdrop of years of genetic characterization, but rather in the broader context of plant–microbe interactions. It has long been understood that biotrophic pathogens secrete effectors, which are often small and cysteine-rich proteins that elegantly subvert host defenses and prevent cell death. The traditional necrotroph, on the other hand, was thought to use a combination of toxins (including HSTs) and "brute force" methods (cell wall-degrading cellulases, pectinases) to overpower hosts. Recent work suggests that many necrotrophic virulence factors should truly be classified as effectors. An example of this is *C. victoriae*'s HST, victorin, which in the presence of an NB-LRR-type protein results in host susceptibility, instead of resistance. In light of these and similar observations, the obvious question is to what

extent do necrotrophs utilize effectors and do they employ small secreted proteins, as biotrophs do? *Cochliobolus* is a wonderful system to ask these questions, as it contains both hemibiotrophic and necrotrophic pathogens. Bioinformatic searches found SSPs in all species examined, although the number of predicted SSPs, and species-unique SSPs, was higher in hemibiotrophic species. The set of SSPs identified serves as a toehold for identifying candidate SSP effectors in necrotrophs and concomitant functional analysis has the capacity to greatly alter our perception of such pathogens.

We have also sought to discuss the functional work conducted in different *Cochliobolus* species on other topics pertinent to pathogenic and reproductive development, including iron metabolism, oxidative stress management, P450s, signaling components, and mating determinants. Extrapolation of rich molecular work within a single species, such as *C. heterostrophus*, to other closely related species, results in new hypotheses. Genomic differences among species can unearth biological phenomena that might go unnoticed examining one system alone. Each analysis, of course, must be taken with a digital grain of salt, until functional work can support a given hypothesis. Comparative bioinformatics offers us a tentative and highly valuable glimpse into the inner workings of an entire genus.

Acknowledgments We gratefully acknowledge the contribution of Igor Grigoriev and colleagues at the JGI for sequencing the genomes and for their consistent interest in *Cochliobolus* and the Dothideomycete class this fascinating genus belongs to. Work in our labs including some unpublished data shown here was supported by the Agriculture and Food Research Initiative of USDA's National Institute of Food and Agriculture (BGT), the US-Israel Binational Agricultural Research and Development Fund (BARD) (BH, BGT), the National Science Foundation (BGT), and the Slovenian Research Agency (NK).

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The Genomics of *Colletotrichum*

3

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3.1 Introduction

The fungal genus *Colletotrichum* includes more than 100 species responsible for anthracnose foliar blight and rot diseases of nearly every crop grown for food, fiber, and forage worldwide (Cannon et al. 2012b; Hyde et al. 2009). Because of their ubiquity, substantial capacity for destruction, and scientific importance as model pathosystems, fungi in the genus *Colletotrichum* are collectively ranked by the international plant pathology community among the top ten most important fungal phytopathogens (Dean et al. 2012).

Economically important diseases caused by *Colletotrichum* are widespread, occurring on maize, beans, strawberries, coffee, chili peppers, cucurbits, potatoes, and countless other cultivated plants (e.g., Bergstrom and Nicholson 1999; Hyde et al. 2009; Lees and Hilton 2003; Legard 2000; Melotto et al. 2000; Prihastuti et al. 2009; Singh and Schwartz 2010; Than et al. 2008; Ureña-Padilla et al. 2002; Varzea et al. 2002; Waller 1992; Wasilwa et al. 1993; Xie et al. 2010). *Colletotrichum* postharvest fruit rots are responsible for major economic losses, with severe infections resulting in up to 100 % loss during storage (Prusky 1996). *Colletotrichum* diseases also produce substantial damage on important subsistence crops including lentil, cowpea, yam, banana, sorghum, and cassava (Adegbite and Amusa 2008; Chona 1980; Chongo et al. 2002; Finlay and Brown 1993;

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R. A. Dean et al. (eds.), *Genomics of Plant-Associated Fungi: Monocot Pathogens*,
DOI: 10.1007/978-3-662-44053-7_3,

Green and Simons 1994; Moses et al. 1996; Moura-Costa et al. 1993).

Colletotrichum diseases can negatively impact many of the most important monocots targeted as candidate bioenergy crops, including switchgrass, miscanthus, maize, sorghum, indiangrass, and sugarcane (Crouch 2013; Crouch and Beirn 2009; Crouch et al. 2009a, b; Cortese and Bonos 2012; Dahlberg et al. 2011; Hartman et al. 2011; King et al. 2011; Waxman and Bergstrom 2011a, b; Zeiders 1987). Plants in a wide variety of uncultivated terrestrial and aquatic biomes may also be impacted by *Colletotrichum* infections, including forests, grasslands, prairie, shrub land, savannahs, and deserts (Abang et al. 2006; Ammar and El-Naggar 2011; Crouch 2013; Crouch et al. 2009b; Damm et al. 2012a; Dingley and Gilmour 1972; Lubbe et al. 2004; Soares et al. 2009).

Colletotrichum occupies a noteworthy place in the history of plant pathology and mycology. The first description of physiological races and cultivar specificity involved the causal agent of bean anthracnose, *C. lindemuthianum* (Barrus 1911), with that work leading to some of the first resistance breeding efforts using race differentials (reviewed in Geffroy et al. 1999). Subsequent work with the bean anthracnose pathosystem has greatly advanced our understanding of the gene-for-gene system (López et al. 2003; Melotto and Kelly 2001). Work with the teleomorph of *C. gloeosporioides* pioneered early investigations of fungal sexual determination and development (Lucas et al. 1944, Chilton et al. 1945, Chilton and Wheeler 1949a, b; Driver and Wheeler 1955; Edgerton et al. 1945; Lucas 1946; Wheeler 1950, 1954; Wheeler et al. 1948; Wheeler and McGahan 1952). In the 1960s and 1970s, *Colletotrichum* studies were at the cutting edge of our understanding of the nature of systemic induced resistance, the chemistry of host defense, and the importance of phytoalexins in the defense response, and they enabled purification of elicitor molecules from fungal cell walls for the first time (Kuc 1972; Sticher et al. 1997). The development and function of melanized appressoria has been substantially elucidated using *Colletotrichum* (Kubo and Takano 2013). Key components of the cyclic-AMP, MAP kinase, and calcium-mediated signaling pathways have been cloned and characterized from *Colletotrichum* species (e.g., Chen and Dickman 2002, 2004; Dickman and Yarden 1999; Ha et al. 2003; Kim et al. 2000; Takano et al. 2000; Warwar and Dickman 1996; Yang and Dickman 1997, 1999a, b). Today, *Colletotrichum* species continue to serve as important models for studies of the molecular and cellular basis of pathogenicity (Kubo and Takano 2013; O'Connell et al. 2012; O'Connell and Panstruga 2006; Perfect et al. 1999).

3.2 Systematics of *Colletotrichum*

Colletotrichum is an asexual fungus, with the sexual state traditionally classified in the Ascomycete genus *Glomerella* (*Sordariomycetes*; Hypocreomycetidae; Glomerellaceae; Glomerellales) (Réblová et al. 2011). With the adoption of single name nomenclature for pleomorphic fungi established by the 2013 Melbourne Code of the International Code of Nomenclature for algae, fungi, and plants (www.iapt-taxon.org/nomen/main.php), it is unlikely that the *Glomerella* name will continue to be used in the future. Although several species in the genus are known that produce the teleomorph readily (e.g., *G. cingulata*, *G. acutata*), *Colletotrichum* species are predominantly observed in the vegetative or asexual state, with the sexual morph rarely identified for most species (Vaillancourt et al. 2000b). Since plant pathologists and mycologists working with the fungus typically encounter the anamorph, the *Colletotrichum* name more accurately communicates biological information about the organism. Furthermore, *Colletotrichum* is the older of the two genera and has priority (1831 vs. 1903; www.mycobank.org). Final resolution of the sole adopted genus name will go through the formal channels established by the International Subcommission of *Colletotrichum* Taxonomy (www.fungaltaxonomy.org/ subcommissions) to ensure community consensus. In this

chapter we will use *Colletotrichum* to refer both to the anamorphic and the teleomorphic phases.

Colletotrichum is the sole member of the Glomerellaceae, one of three families that collectively make up the order Glomerellales in the Sordariomycete subclass Hypocreomycetidae (Réblová et al. 2011). Earlier reports suggested *Colletotrichum* as a sister group to *Verticillium* (Zhang et al. 2006), but more comprehensive research has shown that this inferred relationship reflected insufficient sampling rather than an actual close phylogenetic association, as *Verticillium* is a member of the Plectosphaerellaceae (Cannon et al. 2012a; Réblová et al. 2011; Zare et al. 2000).

During the past several years, *Colletotrichum* taxonomy has been the subject of several substantive revisions. Species concepts are still in a state of flux, but it is now well-established that the genus consists of several major monophyletic clades that are referred to as species aggregates, described by the name and attributes of the most prominent representative species in the group (O'Connell et al. 2012; Cannon et al. 2012b; Fig. 3.1). To date, nine aggregates have been described based on multilocus molecular phylogenetics, namely acutatum, graminicola, spaethianum, destructivum, dematium, gloeosporioides, boninense, truncatum, and orbiculare (Cannon et al. 2012b). Although the *Colletotrichum* aggregates carry no formal taxonomic rank, they provide a convenient way to connect widely used, but outdated and overly broad species concepts with the revised taxonomy. For example, the gloeosporioides aggregate consists of at least 22 species traditionally referred to as *C. gloeosporioides*, including *C. gloeosporioides* sensu stricto (Weir et al. 2012). Under the new, more accurate molecular-based taxonomy, *C. gloeosporioides* sensu stricto is now known to be much less common in the environment than previously thought (e.g., Phoulivong et al. 2010; Weir et al. 2012). The aggregate terminology is especially useful for disease diagnostics that still rely on ITS sequence similarity and/or morphology to identify causal agents. Several of the aggregate groups have been broadly characterized through multilocus phylogenies (Crouch et al. 2009a; Damm et al. 2009; 2012a, b; Weir et al. 2012), yielding a framework for understanding evolutionary relationships across the genus as a whole (Cannon et al. 2012b).

3.3 *Colletotrichum* Lifestyles and Modes of Infection

Fungi in the genus *Colletotrichum* display a range of nutritional strategies and lifestyles, including plant associations that occupy a continuum from necrotrophy to hemibiotrophy and endophytism. Some species employ a saprotrophic lifestyle to obtain nutrients from soil and organic matter. *Colletotrichum* are also known to colonize organisms outside the plant kingdom, including insects and humans.

Plant-associated *Colletotrichum* species typically use a melanized appressorium to penetrate host tissues (Kubo and Takano 2013) (Fig. 3.2). The melanin is required for appressorial function, permitting the accumulation of significant turgor pressure that facilitates mechanical penetration of the host cell wall (Bechinger et al. 1999; Kubo and Furusawa 1991). The appressorium also secretes pectinases and cell wall-degrading enzymes that are likely to play diverse roles in preparing the infection court, adhesion, signaling, and softening the host cell wall (Kleemann et al. 2008; Mendgen et al. 1996). The appressorium of *Colletotrichum* is morphologically and functionally similar to that formed by *Magnaporthe*, in spite of the evolutionary distance between these two fungal genera (Mendgen et al. 1996). Appressorial ultrastructure is a taxonomically informative trait in *Colletotrichum*. Members of the destructivum and graminicola aggregates have a "pore wall overlay" structure surrounding the penetration pore that is similar to that found in *Magnaporthe* (Howard and Valent 1996), whereas the orbiculare and gloeosporioides aggregates have a distinctive cone-shaped structure associated with the appressorial pore (Fig. 3.2). This cone is surrounded by the appressorial plasma mem-

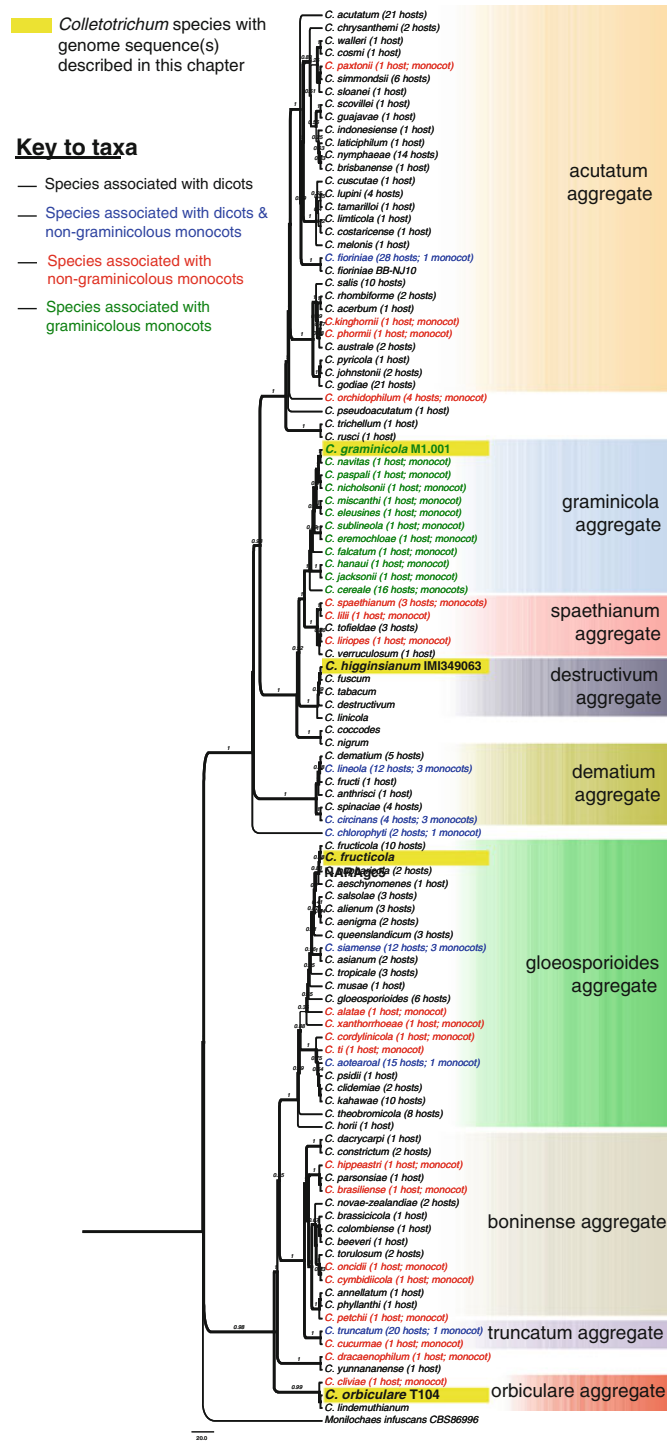

Fig. 3.1 Bayesian phylogenetic tree of the *Colletotrichum* genus, showing the evolutionary relationship between the four *Colletotrichum* species with genome sequences, as described in this chapter. The phylogeny was constructed from DNA sequences of four markers (chitin synthase, actin, β-tubulin 2, rDNA ITS; 1514-bp total). The analysis was performed using BEAST for 20 million generations, under the following model: GTR + i, empirical base frequencies, and yule process mode of speciation. The analysis is summarized as a maximum clade credibility tree from 20,001 trees, with the first 2,000 trees ignored (10 %) as burn-in. Posterior probability support values are listed on branches. Branches with thick lines were highly supported by posterior probability values, relative to branches with thin lines. Datasets for included species were generated from alignments of sequences from type specimens, following Cannon et al. (2012a, b). Number of hosts is given following the species name where known. Hosts are dicots except where otherwise noted

brane, and consists of modified cell wall material lacking chitin that is similar in structure to the pore wall overlay (O'Connell and Ride 1990). The cone and the pore wall overlay are both continuous with the cell wall of the penetration peg (Fig. 3.2). The functions of the cone and

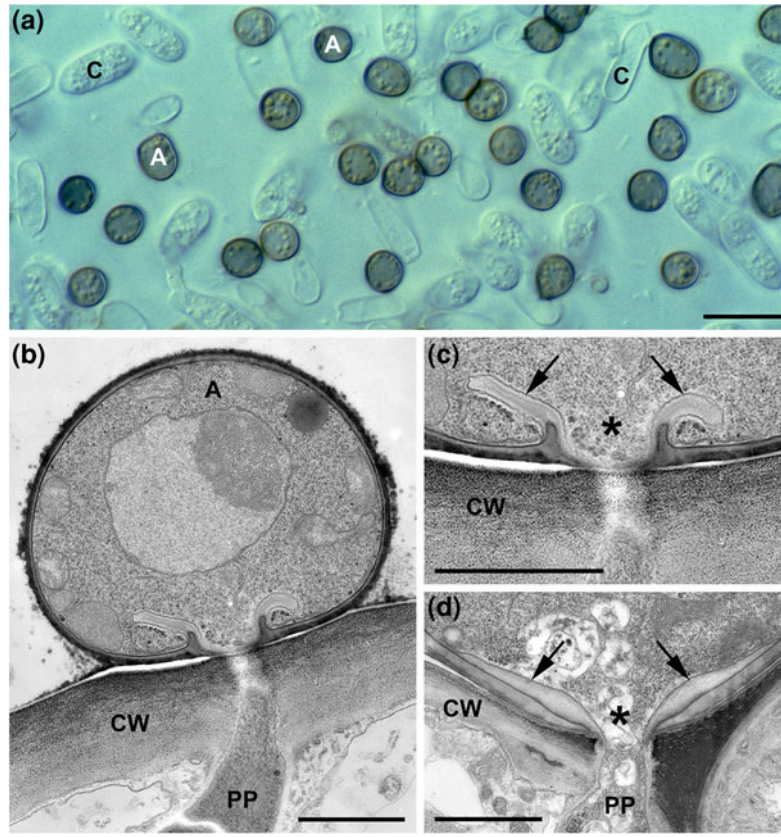

Fig. 3.2 Appressoria. **a** Melanized *C. orbiculare* appressoria (*A*) formed on glass. Bar = 10 μm, C = conidia. **b** Penetration peg (*PP*) emerging from the base of a *C. orbiculare* appressorium (*A*) and entering cucumber epidermal cell wall (*CW*). Bar = 1 μm. **c** In *C. orbiculare*, a cone-shaped elaboration of the appressorial cell wall (*arrows*) surrounds the penetration pore (*asterisk*). Bar = 0.5 μm. **d** In *C. higginsianum*, a pore wall overlay (*arrows*) surrounds the penetration pore (*asterisk*). Bar = 0.5 μm. Photos by Richard O'Connell

pore wall overlay structures are unknown, but they may serve to reinforce the penetration pore, to focus turgor pressure, or to direct secretion of enzymes and other proteins to the host-pathogen interface.

At one extreme of the spectrum of plant-associated lifestyles, some *Colletotrichum* fungi rely on a primarily necrotrophic lifestyle to obtain nutrients. These species include most of the causal agents of fruit rots. Necrotrophic *Colletorichum* do not appear to colonize living host tissue; instead, they first become established either as latent infections confined to unpenetrated appressoria, or as a subcuticular mycelium, before eventually switching to a pathogenic lifestyle, killing host tissue in advance of colonization and inciting significant damage. *Colletotrichum* species from the acutatum and gloeosporioides aggregates are the most common causes of necrotrophic *Colletotrichum* diseases (Liao et al. 2012; Prusky 1996; Walker 1921).

At the opposite end of the plant-associated lifestyle continuum are *Colletotrichum* that colonize host tissue using intracellular hemibiotrophy (IH), a stealthy, highly orchestrated and remarkably effective infection strategy. Intracellular hemibiotrophic *Colletotrichum* produce specialized infection structures called primary hyphae that are used to invade living host cells, with or without the initial formation of an infection vesicle (O'Connell et al. 2000; Perfect et al. 1999; Perfect and Green 2001). Primary hyphae are thickened or bulbous and are surrounded by a host-derived membrane separating the fungal cell wall from the living host cytoplasm (Mims and Vaillancourt 2002; O'Connell et al. 2000; Perfect et al. 1999; Perfect and Green 2001; Wharton and Julian 1996). The host

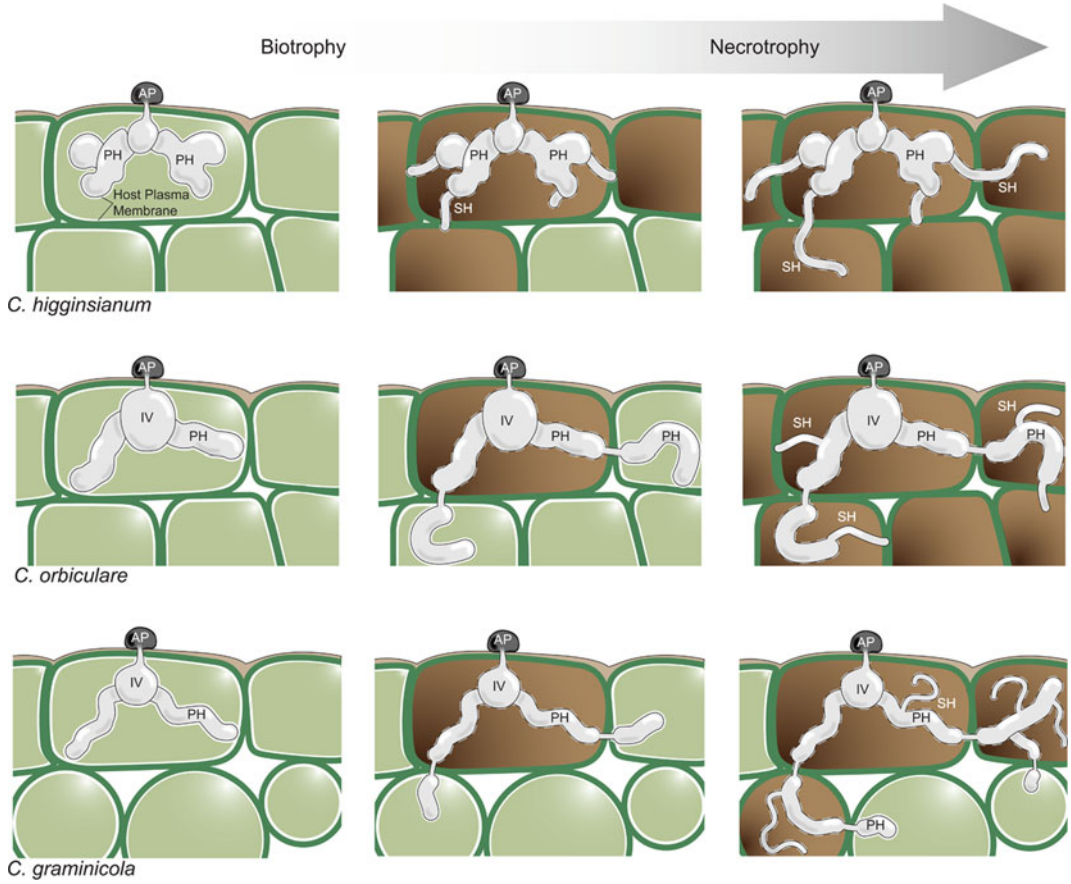

Fig. 3.3 *Three* variants of intracellular hemibiotrophy. *Row 1 C. destructivum* model (*C. higginsianum*). Biotrophic primary hyphae (*PH*) colonize only one epidermal cell, followed by a complete switch to necrotrophy, with thinner secondary hyphae (*SH*) killing host cells ahead of infection. *Row 2 C. orbiculare* model. Biotrophic primary hyphae colonize multiple host cells (first infected cells dead, later infected cells alive), followed by a complete switch to necrotrophy, with secondary hyphae killing host cells ahead of infection. *Row 3 C. graminicola* model. Biotrophic primary hyphae colonize multiple host cells, as with *C. orbiculare*, but biotrophy persists at the advancing colony edge while necrotrophy is confined to the colony center. *AP* = appressorium; *IV* = infection vesicle. Diagrams by Guillaume Robin

cell remains alive for a variable period of time that may last for just a few hours up to several days, depending on the interaction (Mims and Vaillancourt 2002; O'Connell et al. 2000; Perfect et al. 1999; Perfect and Green 2001). During biotrophy, the fungus appears to evade plant defenses (Vargas et al. 2012). The transient symptomless phase is followed by a shift to a destructive form of necrotrophic development, accompanied by production of a distinct hyphal morphology that is thinner, not surrounded by a membrane, and exhibits a different wall composition (Mims and Vaillancourt 2002; O'Connell et al. 1996; Pain et al. 1994; Perfect and Green 2001; Perfect et al. 2001; Wharton et al. 2001). These necrotrophic stage hyphae secrete substantial amounts of lytic enzymes, resulting in host tissue collapse and symptom development (O'Connell et al. 2000).

There are at least three major variants of IH utilized by pathogenic *Colletotrichum* (Fig. 3.3). The first IH strategy is typified by *C. higginsianum* and other members of the destructivum species aggregate. In this model, there is a limited biotrophic phase confined to the first infected epidermal cell, followed by a complete

switch to necrotrophy, marked by development of narrower secondary hyphae, killing of host tissues in advance of fungal colonization, and development of symptoms (Latunde-Dada and Lucas 2007; O'Connell et al. 2004, 2012). The second IH model is employed by members of the orbiculare species aggregate (O'Connell et al. 2000; Perfect et al. 1999; Perfect and Green 2001). Here, the biotrophic phase persists during sequential colonization of several cells. Cells behind the advancing colonization front die gradually, but there is no widespread destruction of cells, and the infection remains symptomless. At some point, the colonizing fungus switches to production of narrow necrotrophic hyphae that kill cells in advance of colonization, and symptoms appear. The third IH strategy is typified by the graminicola species aggregate (Mims and Vaillancourt 2002; Wharton and Julian 1996; Wharton et al. 2001). The graminicola IH model is initially similar to that of orbiculare IH, but differs at the switch to necrotrophy. In the graminicola IH model, narrow secondary necrotrophic hyphae are produced as branches from the thicker primary intercalary hyphae in the cells behind the advancing colony front, which continues to invade new cells biotrophically. Thus, in the graminicola model, biotrophy and necrotrophy exist simultaneously in different parts of the colony (Fig. 3.4). Disease symptoms appear soon after the emergence of necrotrophic hyphae, as the centers of the colonies collapse and die. Relatively few *Colletotrichum* fungi have been subjected to detailed cytological analysis, and so it is not clear if these models are found in other species aggregates, or even if they are typical of all members of a single aggregate, or of all tissues in a single host. Much more work is needed in this area.

Beyond their notoriety as destructive agricultural pathogens, members of the genus *Colletotrichum* are among the most common endophytic fungi associated with plants (e.g., Crouch et al. 2009c; Gazis et al. 2011; Hyde et al. 2009; Rodriguez and Redman 2008; Rojas et al. 2010; Vega et al. 2010). For example, the most common endophytic fungi recovered from asymptomatic leaves of forest trees are *Colletotrichum*

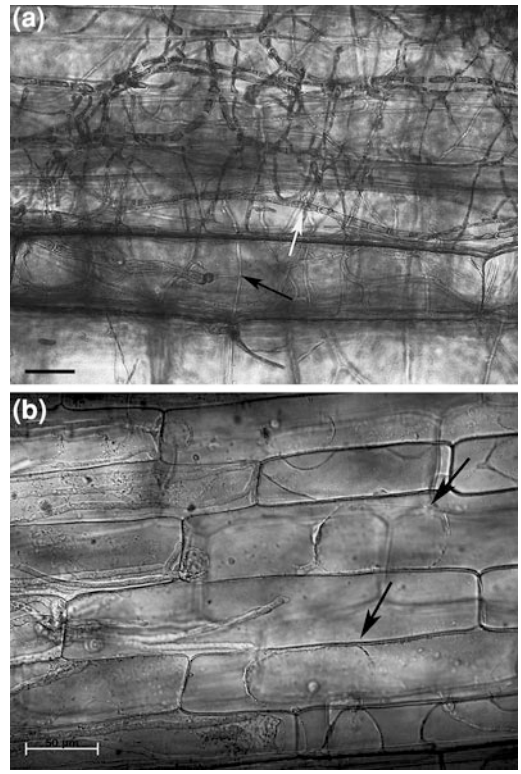

Fig. 3.4 *Trypan-blue* stained hyphae of *C. graminicola* colonizing maize leaf sheath epidermal cells during the necrotrophic phase of development. **a** In the center of the colony, the thicker primary hyphae (*white arrow*) give rise to thinner necrotrophic hyphae (*black arrows*) that define this stage of development. **b** The edge of the same colony is still being colonized biotrophically, evidenced by the ability of the newly invaded and surrounding cells to plasmolyze (*black arrows*). Scale bars = 50 microns. *Photos* by Maria Torres

species, especially members of the gloeosporioides aggregate (Cannon and Simmons 2002; Arnold et al. 2003). Similarly, a recent survey of wild *Arabidopsis thaliana* populations identified five different *Colletotrichum* species as foliar endophytes (García et al. 2013). Asymptomatic colonization of host tissue by *Colletotrichum* endophytes may lead to any of several outcomes for the plant, including enhanced growth, drought and heat tolerance, and/or disease resistance (e.g., Arnold et al. 2003; Prusky et al. 1994; Redman et al. 2001, 2002). Based on environmental cues such as host senescence, wounding, or other factors associated with changes in plant physiology (Rodriguez et al. 2009), endophytic

Colletotrichum may also adopt a saprotrophic lifestyle (Promputtha et al. 2007, 2010), or induce disease symptoms or rots that are only manifested after an extended period of asymptomatic colonization (Freeman et al. 2001; Photita et al. 2004; Rodriguez et al. 2009). Latency/endophytism in association with plants may be an important component of the life cycle of many or most *Colletotrichum* fungi, but this aspect of development is poorly understood. In particular, there are relatively few cytological studies and so the degree of host colonization by *Colletotrichum* endophytes is usually unknown. It could range from unpenetrated appressoria (latency) to extended systemic colonization. Much more work is needed in this area.

Surveys of foliar epiphytes show that *Colletotrichum* fungi are also widespread in the phyllosphere (Alvinidia and Natsuaki 2008; Freeman et al. 2001; Osono 2007, 2008; Santamaría and Bayman 2005). Several *Colletotrichum* species function as saprophytes, surviving in organic matter or soil; however, this free-living lifestyle may be strictly facultative. In general, *Colletotrichum* appears to be ill-equipped for long term survival in soil (e.g., Bergstrom and Nicholson 1999; Ripoche et al. 2008), although there are notable exceptions (e.g., Dillard and Cobb 1998; Freeman et al. 2002), and melanized microsclerotia have been observed in several species including *C. truncatum*, *C. sublineola*, and *C. coccodes* (e.g., Boyette et al. 2007; Dillard and Cobb 1998; Sukno et al. 2008). In addition, members of the genus are occasionally reported as opportunistic pathogens of organisms outside the plant kingdom, including insects, turtles, cats, and humans (Cano et al. 2004; Manire et al. 2002; Marcellino et al. 2008, 2009; O'Quinn et al. 2001; Shivaprakash et al. 2011; Winter et al. 2010).

The functional relationships between *Colletotrichum* lifestyles along the plant-associated continuum are unclear. The mechanisms regulating the developmental switches, and the role of host signals, are also mysterious. It is possible that all of the plant-associated lifestyles are manifestations of a similar underlying interaction, with details of timing dependent on the degree of host resistance. Thus, high levels of resistance may result in latency/endophytism, while reduced resistance could lead to IH, and a further reduction in resistance could trigger a switch to necrotrophy. The duration of the initial biotrophic phase could also partially explain the spectrum of lifestyles. The length of the biotrophic phase may be dependent on the relative ability of the pathogen to mask infection structures from detection. True endophytes that colonize extensively without causing symptoms are likely to have highly developed stealth strategies, for example low expression of lytic enzymes, masking of chitin and other pathogen-associated molecular pattern (PAMP) elicitors, and production of protein and secondary metabolite (SM) effectors to suppress defense.

3.3.1 *Colletotrichum* Genomics

Genome-scale analyses of *Colletotrichum* strains with contrasting lifestyles could help us to identify commonalities and provide conserved targets for the management and control of these fungi. Unfortunately, very few *Colletotrichum* species have been studied in depth at the molecular level, and relatively little is known about those species of the greatest economic importance, or those species that cause the most significant damage to subsistence crops. Individual findings in one pathosystem have only rarely been validated or tested in other systems, so it is not clear to what extent mechanisms of pathogenicity are similar across all lineages. As the genomes of other economically important plant pathogenic fungi were sequenced, beginning with *M. oryzae* in 2005 (Dean et al. 2005), *Colletotrichum* remained in the background of the genomics revolution, primarily because it was difficult to make a case for sequencing any single species to represent all the others, without understanding how the pathogenic models related to one another. Problematic taxonomy, and a woefully inadequate understanding of species boundaries and evolutionary relationships, also

initially limited our ability to identify the most suitable subjects for genome analysis.

As genome sequencing technologies became faster and cheaper, whole genome sequences were finally generated and published in quick succession for four species of *Colletotrichum*: *C. higginsianum, C. graminicola, C. orbiculare,* and *C. fructicola* (formerly *C. gloeosporioides*; Fig. 3.1; Gan et al. 2013; O'Connell et al. 2012). According to our current understanding of *Colletotrichum* taxonomy, these four species are each positioned within distinct monophyletic lineages in the *Colletotrichum* phylogenetic tree (Fig. 3.1; Cannon et al. 2012a, b). *C. graminicola* is a member of the graminicola aggregate. *C. higginsianum* is part of the destructivum aggregate, the sister clade to the graminicola aggregate. *C. orbiculare* is a close relative of the bean anthracnose pathogen *C. lindemuthianum*, and together they occupy the orbiculare species aggregate. *C. fructicola* is a member of the gloeosporioides aggregate, one of the most diverse groups of *Colletotrichum*, encompassing numerous species associated with a huge number of hosts worldwide (Weir et al. 2012). Although the Nara-gc5 strain was considered a member of *C. gloeosporioides* sensu lato at the time the genome sequence was published, recent revisions to the taxonomy of the gloeosporioides aggregate now enable a precise species identification (Weir et al. 2012). As shown in the genus-wide multilocus phylogenetic tree in Fig. 3.1, Nara-gc5 is a member of *C. fructicola*, a globally distributed species within the gloeosporioides aggregate that has been isolated from eight different plant families to date (Weir et al. 2012; Prihastuti et al. 2009). Accordingly, in this review we will refer to Nara-gc5 as *C. fructicola* to reflect the revised, more accurate taxonomy.

C. graminicola strain M1.001 (aka FGSC 10212, CBS 130836, M2; formerly known as *Glomerella graminicola*) was the first species of *Colletotrichum* to have a complete genome sequence available, and is also one of the last of the fungal genomes to be substantially sequenced using Sanger dideoxy technology. *C. graminicola* is among the best characterized and most tractable of the *Colletotrichum* fungi, and is one of very few species in the genus in which sexual crosses can be made (Vaillancourt and Hanau 1991). *C. graminicola* causes one of the most destructive diseases of maize, anthracnose stalk rot, resulting in annual losses of more than $1 billion in the United States (Bergstrom and Nicholson 1999; Frey et al. 2011). Sequences for *C. graminicola* M1.001 (Forgey et al. 1978) and for M5.001 (aka CBS 130839), a strain that is sexually compatible with M1.001 (Vaillancourt and Hanau 1991), are available.

C. higginsianum strain IMI349063 causes anthracnose on *Brassica* and *Raphanus* crops, as well as wild cruciferous species. Although the fungus may occasionally cause significant crop losses, *C. higginsianum* is generally of only minor importance in commercial agricultural production (Horie et al. 1988; Lin and Huang 2002). However, this species is of considerable scientific interest because of its ability to cause disease on certain ecotypes of the model plant Arabidopsis (O'Connell et al. 2004; Narusaka et al. 2004). The sequenced strain of *C. higginsianum* was originally isolated from *Brassica chinensis*, but it also readily infects and causes disease in Arabidopsis (O'Connell et al. 2004, 2012). As such, *C. higginsianum* provides an experimental system in which both host and fungal partners can be genetically manipulated. In particular, the availability of powerful genetic tools and resources on the plant side facilitate the analysis of host resistance and susceptibility (e.g., Birker et al. 2009; Narusaka et al. 2009).

C. orbiculare 104-T (aka CBS 514.97, LARS414; formerly known as *C. lagenarium*) is a common and significant problem on cucurbits, causing anthracnose lesions on vegetative tissue and fruit (Westcott 2001; Kubo and Takano 2013). At one point, anthracnose was among the most common and destructive diseases of cucumbers and melons in the United States (Gardner 1918). Today, losses due to *C. orbiculare* are kept in check through improved crop management strategies, although anthracnose remains a prevalent disease of commercial watermelons grown in regions of high humidity (Maynard and Hopkins 1999). The sequenced strain of *C. orbiculare* also infects the model

plants *Nicotiana benthamiana* and *N. tabacum* (Shen et al. 2001), which are amenable to transient gene expression and silencing assays. Techniques for genetic manipulation of *C. orbiculare* have been established, including gene targeting or random gene insertion. In addition, *C. orbiculare* pathogenesis is stable and is well characterized cytologically, making this pathosystem an attractive platform for experimental studies (Kubo 2012).

C. fructicola strain Nara-gc5 (formerly known as *C. gloeosporioides*) causes crown rot of strawberry (Okayama and Tsujimoto 1994, 2007), a disease responsible for substantial losses for strawberry producers worldwide. Strawberry anthracnose can result in up to 80 % losses for nursery plants, and up to 50 % losses in the field (Howard and Albregts 1983; Xie et al. 2010). Other members of *C. fructicola* are important pathogens of a broad range of commercially grown crops, including coffee, apples, yams, pears, and avocados (Prihastuti et al. 2009; Weir et al. 2012). Comparisons between members of this species, and within the larger gloeosporioides aggregate, promise to be informative in determining factors contributing to adaptation to particular hosts and lifestyles.

3.3.2 *Colletotrichum* Comparative Genomics

Genome assembly statistics for the four sequenced *Colletotrichum* strains are summarized in Table 3.1. Differences in the read lengths generated by the different sequencing methods are reflected in the quality of the resulting genome assemblies, with short read technologies producing more fragmented assemblies than those that incorporated Sanger and Roche-generated data. Despite the differences in sequencing approaches, overall gene coverage was high for all four assemblies (Table 3.1; Gan et al. 2013; O'Connell et al. 2012) when assessed using the CEGMA pipeline (Core Eukaryotic Genes Mapping Approach; Parra et al. 2007). Nonetheless, direct comparisons between these four genomes should be made with caution, given that different sequencing strategies and methodologies were used, and different computational tools were employed to assemble and annotate the genomes.

Three of the four sequenced *Colletotrichum* strains, (*C. graminicola* M1.001, *C. higginsianum* IMI349063, and *C. fructicola* Nara-gc5), yielded genome assemblies with estimated sizes ranging from 53 to 58 Mb, somewhat larger than the average 38 Mb sequenced Pezizomycota genome (Table 3.1; Cuomo and Birren 2010; Gan et al. 2013; Kelkar and Ochman 2012; O'Connell et al. 2012). At 88 Mb, the *C. orbiculare* 104-T assembly is considerably larger than the other three *Colletotrichum* species (Gan et al. 2013). It also dwarfs the genomes of most ascomycetes sequenced to date, surpassed in size only by the biotrophic powdery mildew fungi and *Tuber melanosporum* (Cuomo and Birren 2010; Gan et al. 2013; Spanu et al. 2010). The large genome of *C. orbiculare* strain 104-T is the result of blocks of low-complexity AT-rich sequences dispersed among the coding sequences, accounting for nearly half of the genome assembly (Gan et al. 2013). These AT-rich sequence blocks may have arisen from modification of transposable elements by repeat-induced point mutation (RIP; Galagan and Selker 2004).

Despite the differences observed in overall genome size, predicted gene numbers were similar for the four *Colletotrichum* species, ranging from 12,006 (*C. graminicola*) to 16,172 (*C. higginsianum*); all were larger than the average set of 11,281 genes observed in other sequenced Pezizomycota (Table 3.1; Cuomo and Birren 2010; Gan et al. 2013; O'Connell et al. 2012). The reduced number of genes observed in *C. graminicola*, relative to the other three sequenced *Colletotrichum* genomes, was largely due to the presence of fewer gene paralogs, with the *C. graminicola* genome appearing to have undergone less gene duplication. In particular, more than twice as many multicopy genes were identified in the *C. fructicola* genome as in that of *C. graminicola* (Table 3.1).

A remarkably low level of synteny was observed among the four sequenced *Colletotrichum*

Table 3.1 Genome assembly statistics for the four sequenced *Colletotrichum* species (O'Connell et al. 2012; Gan et al. 2013)

	C. higginsianum	*C. graminicola*	*C. fructicola*	*C. orbiculare*
Assembly size (Mb)	53.4	57.4	55.6	88.3
Coverage	101x	9.1x	37x	55x
Sequencing technology	Roche 454 (25x) Illumina (76x) Sanger (0.2x)	Sanger (7.9x) Roche 454 (1.2x)	Illumina (37x)	Roche 454 (22x) Illumina (34x)
Number of scaffolds	653	367	1241	525
N50 contig length	265.5	579.2	112.8	428.9
Number of contigs	10269	1151	5335	10545
Gene space coverage	95.1 %	99.2 %	96.4 %	98.0 %
Number of predicted genes	16172	12006	15469	13479
Overall GC content	55.1 %	49.1 %	53.6 %	37.5 %
GC content of genes	58.4 %	59.3 %	56.0 %	57.1 %
Number of chromosomes	10 major, 2 "B" chromosomes	10 major, 3 "B" chromosomes	Unknown	10 major, no "B" chromosomes
Multicopy genes	9713	6468	14933	7475
Conserved single copy genes	4725	4767	372[a]	4553
Repeat elements	1.2 %	12.2 %	0.75 %	8.3 %
Public access to genome	NCBI (Accession CACQ02000000); Broad Institute website	NCBI (Accession ACOD01000001); Broad Institute website	NCBI (Bioproject PRJNA171218) or the Dryad Digital Repository (doi:10.5061/dryad.r4026)	NCBI (Bioproject PRJNA171217) or the Dryad Digital Repository (doi:10.5061/dryad.r4026)

The number of multicopy genes was determined by clustering the predicted proteins within each genome using MCL and an inflation value of 2.0. To estimate the numbers of fungal conserved genes, BLASTp was performed against the 11 other fungal genomes in addition to the other three *Colletotrichum* genomes, with a cutoff of 1E-5

[a] The low number of conserved genes in *C. fructicola* is likely to be an artifact resulting from the much shorter average read length for that genome

genomes, much less than that displayed between members of two different genera (*Botrytis* and *Sclerotinia*; Amselem et al. 2011). Synteny between *C. graminicola* and *C. higginsianum* was only 35 %, while the more distantly related *C. orbiculare* and *C. fructicola* shared only 40 % synteny (Gan et al. 2013; O'Connell et al. 2012). These low levels of shared gene order, which appear to be independent of the degree of taxonomic relatedness, suggest that major genome rearrangements have been a common feature during the history of the *Colletotrichum* genus. Unfortunately, this also means that the value of the high quality assembly of the *C. graminicola* M1.001 genome as a reference for assembling other *Colletotrichum* species may be limited.

In marked contrast with the low degree of synteny documented between *Colletotrichum* species, intraspecific chromosomal rearrangements may be rare. Thus, two strains of *C. graminicola*, one isolated in North America in 1972 (M1.001), and a second strain isolated in South America in 1989 (M5.001), appeared to be highly syntenic with relatively few sequence polymorphisms (O'Connell et al. 2012). This indicates that major chromosomal rearrangements within species may be uncommon. This may also suggest that genome rearrangements

play a role in speciation, perhaps by promoting reproductive isolation (Aguileta et al. 2009).

3.4 Repetitive DNA

Prior to the availability of genomic resources, relatively little was known about *Colletotrichum* transposable elements (TEs) and their impact on the host genome. Even with the availability of genome assemblies, *Colletotrichum* TEs have been subject to little detailed analysis, but some general characterizations are possible. All four sequenced *Colletotrichum* genomes contained signatures of common long terminal repeat (LTR) and DNA transposon classes. Summary data showed that the percentages of these repetitive sequences were higher for *C. graminicola* and *C. orbiculare* (12.2 and 8.3 %, respectively) than for *C. higginsianum* (1.2 %) or *C. fructicola* (0.75 %), but this may be an artifact derived from the more complete genome assemblies and sequencing strategies used for *C. graminicola* and *C. orbiculare* (Gan et al. 2013; O'Connell et al. 2012). Several TE sequences in the *Colletotrichum* genomes were similar to the Ccret1, Ccret2, Ccret3, and Cgret Metaviridae family LTR retrotransposons, the non-LTR LINE-like retroelement CgT1, and the Collect1 DNA TE sequences described previously from *C. cereale* and *C. gloeosporioides* (Crouch et al. 2008; He et al. 1996; Zhu and Oudemans 2000).

Clustering of TEs in the context of rapidly evolving genome regions undergoing high rates of duplication is a trait that *Colletotrichum* holds in common with other phytopathogenic ascomycetes, including *M. oryzae*, *Verticillium oxysporum*, and *V. dahlia* (Amyotte et al. 2012; Gan et al. 201; Hua-Van et al. 2000; O'Connell et al. 2012; Thon et al. 2006). In *C. graminicola*, TEs were organized in clusters distributed throughout the genome. *C. graminicola* supernumerary minichromosomes (see below) and unanchored scaffolds were particularly enriched in TEs, relative to the ten primary chromosomes. Almost 23 % of the three minichromosomes and 50 % of the unanchored scaffolds were composed of predicted TE sequences, while the primary chromosome assemblies contained only 5.5 % repetitive DNA. In the *C. graminicola* genome, there was a statistically significant correlation between the location of TEs and paralogous gene families, genes encoding secreted proteins, and genes without orthologues in *C. higginsianum* (O'Connell et al. 2012). Similarly, in the *C. orbiculare* genome, AT-rich blocks, which may represent relics of TEs mutated through repeat-induced point (RIP) mutation, were associated with small unique secreted protein genes (Gan et al. 2013). Unfortunately, the *C. fructicola* and *C. higginsianum* assemblies were too fragmented to perform a similar analysis. The observed transposon clustering in *Colletotrichum* may reflect selection against harmful integration into gene-rich regions, as described for *Saccharomyces cerevisiae* Ty3 LTR elements (Voytas and Boeke 1993). There is no obvious evidence that TE integration has played a role in the duplication of adjacent genes. Regardless of the mechanism(s) responsible for TE clustering, the observed patterns suggest that TEs may play a role in the generation of effector diversity and novel genes in *Colletotrichum*. Further research is needed to investigate these possibilities.

TEs populating the genomes of *C. graminicola*, *C. fructicola*, and *C. orbiculare* showed the signature of widespread RIP mutation, consistent with the mutation patterns documented from elements described previously from *C. cereale* (Crouch et al. 2008). TpA and ApT dinucleotides were both amplified in the TEs of all three genomes, with the corresponding depletion of CpA, CpG, and CpC dinucleotides resulting in the canonical footprint of the RIP process as first described in *Neurospora crassa* (Cambareri et al. 1989). Comparative genome profiles between *C. graminicola* and 48 additional filamentous ascomycetes showed that RIP distortion of dinucleotides exhibited by *C. graminicola* TEs was exceptionally pronounced (Clutterbuck 2011).

3.5 Supernumerary Chromosomes

Supernumerary minichromosomes (aka B-chromosomes) are a common feature in the genus *Colletotrichum*. These small chromosomes are typically conditionally dispensable for growth in fungi, and highly variable from one strain to the next (Covert 1998; Stukenbrock et al. 2010). In some fungi, including *Alternaria alternata*, *Cochliobolus heterostrophus*, *Fusarium oxysporum*, *M. oryzae*, *Mycosphaerella graminicola*, and *Nectria haematococca*, minichromosomes are enriched in secreted genes that encode proteins involved in niche or host adaptation (Chuma et al. 2003; Coleman et al. 2009; Hatta et al. 2002; Ma et al. 2010; Stukenbrock et al. 2010). For several fungi, horizontal transfer of minichromosomes has been demonstrated, often resulting in expanded pathogenicity on new hosts (Mehrabi et al. 2011). In *Colletotrichum* for example, minichromosomes of *C. gloeosporioides* sensu lato infecting *Stylosanthes guianensis* were transferred between different strains of the fungus, conferring novel pathogenicity (Masel et al. 1996). The sequenced strains of *C. higginsianum* and *C. graminicola* are known to possess minichromosomes (Table 3.1; O'Connell et al. 2012). However, the minichromosomes in each case were very poorly assembled due to high levels of repetitive sequences, extensive tracts of AT-rich sequences, and reduced gene density relative to the rest of genome. Although it is not known whether *C. fructicola* has minichromomes, *C. fructicola* sequences shared some similarity with those documented from the minichromosomes of another member of the gloeosporioides aggregate pathogenic to *S. guianensis* (Gan et al. 2013; Masel et al. 1996). In contrast, *C. graminicola* M1.001 minichromosome sequences did not match the genome sequence from a second strain of the same species, M5.001 (O'Connell et al. 2012), confirming earlier hybridization analyses that showed that the minichromosomes were not conserved between the M1.001 and M5.001 strains of *C. graminicola* (Rollins 1996). In both *Colletotrichum* genomes, the minichromosomes possessed such poor quality sequence assemblies that it was not possible to determine if they were enriched for pathogenicity genes.

3.6 Mating Type Genes

Sexual reproduction is rarely documented from the genus *Colletotrichum*. Some species for which the *Glomerella* morph has never been observed in nature, including *C. graminicola*, have been induced to mate in the laboratory (Politis 1975; Vaillancourt and Hanau 1991). The genetics underlying *Colletotrichum* mating are perplexing, in that fungi in this genus do not employ the canonical bipolar mating system characteristic of other ascomycete fungi (Vaillancourt et al. 2000a, b). In the standard bipolar model used by most ascomycetes to regulate sexual compatibility, mating can occur when both idiomorphs of the mating type gene, *Mat1,* are present (*Mat1-1* and *Mat1-2*). This requirement may be met in a single homothallic individual carrying both idiomorphs, or in a combination of two heterothallic individuals, each carrying one of the two different idiomorphs (Ni et al. 2011). *Colletotrichum* does not conform to this system. To date, only the *Mat1-2* idiomorph, with the characteristic conserved high mobility group (HMG) binding domain, is known from any *Colletotrichum* species surveyed, regardless of whether the strains are heterothallic or homothallic (Crouch et al. 2006; Du et al. 2005, Rodríguez-Guerra et al. (2005); García-Serrano et al. (2008); Vaillancourt et al. 2000b). However, genetic evidence does point to at least two unlinked loci acting as mating determinants in crosses involving *C. graminicola* strains M1.001 and M5.001 (Vaillancourt et al. 2000a).

Early attempts to identify the *Mat1-1* gene were made by using Southern hybridizations, degenerate primer pairs, and primer walking in cosmid libraries. These experiments, performed

by multiple laboratories, focused on detection of the highly conserved alpha DNA binding domain that characterizes the *Mat1-1* idiomorph in other ascomycete fungi. None of these approaches provided any evidence for the presence of a *Mat1-1* gene, even from homothallic strains of *Colletotrichum* (Crouch et al. 2006; Du et al. 2005, Rodríguez-Guerra et al. 2005; García-Serrano et al. 2008). BLAST searches of the four *Colletotrichum* genome sequences confirmed the absence of any sequence with significant identity to the *Mat1-1* gene. The *Mat1-1* gene was not found in the genomes of *C. graminicola* strains M1.001 and M5.001, even though these two strains can be mated in vitro to produce fertile progeny (Vaillancourt and Hanau 1991; Vaillancourt et al. 2000a).

Evaluation of 90 genes proximal to the *Mat1-2* gene (~134 Kb) showed that *C. graminicola* strains M1.001 and M5.001 share 99.8 % nucleotide sequence similarity in genic regions (99.6 % overall for the region), and the ordering and orientation of genes in this region were identical between the two strains. The *Mat1-2* gene is highly conserved between M1.001 and M5.001, sharing 99.5 % nucleotide identity. Only four of the 840 nucleotides vary between these two strains of *C. graminicola*, and none of the *Mat1-2* base changes are located within the HMG box DNA binding domain. Three of the four variable *Mat1-2* nucleotides are located in the first intron, while the fourth base change introduces an amino acid change from asparagine in M1.001 to aspartic acid in M5.001 in exon 2. The M5.001 aspartic acid residue at this site is also found in *C. higginsianum* IMI 349063, while the asparagine residue of M1.001 is also found at this site in the *Mat1-2* genes of *C. lindemuthianum* and *C.gloeosporioides* (Du et al. 2005; García-Serrano et al. 2008).

Pairwise comparisons of the *Mat1-2* coding sequence from the four sequenced *Colletotrichum* genomes show that outside of the conserved HMG-box, the coding sequences are quite different among the four species. The *Mat1-2* exons are considerably longer in the *C. higginsianum* genome: 987-bp, relative to the smaller genes encoded by *C. fructicola*, *C. graminicola*, and *C. orbiculare* (726-, 840-, and 750-bp, respectively). *C. fructicola* and *C. orbiculare* share 66 % nucleotide identity at the *Mat1-2* locus, consistent with their closer evolutionary relationship (Fig. 3.1). The more distantly related *C. graminicola* shared 51 % identity with *C. fructicola* and *C. orbiculare* at this locus. However, the *C. higginsianum Mat1-2* coding sequence displays extensive sequence divergence relative to the other three species, sharing only 30–35.5 % identity. Differences between *Mat1-2* encoded by *C. higginsianum* and the other three *Colletotrichum* species is attributable to the presence of numerous insertions throughout the predicted coding sequence—between 193 and 319 nucleotide gaps.

Despite the high level of interspecific differences in the coding sequences of the *Mat1-2* genes, the region surrounding the *Mat1* locus shows a high level of conserved synteny between *C. fructicola*, *C. graminicola*, and *C. orbiculare*. Comparison of the genes proximal to the *Mat1-2* locus shows that this region is 100 % conserved in gene content and gene order in these three *Colletotrichum* species (*Msa1/Cia30/Apc5/Cox13/Apn2/Mat1/Sla2*/L21e 60 s ribosomal protein/S4-9 40 s ribosomal protein /*Slu7/Rev3/Tex2/Ami1*). A similar comparison with the *C. higginsianum* genome could not be completed, as the genome assembly is fragmented, with no more than three genes on a single contig, several genes incomplete/truncated, and some genes predicted that seem unlikely to actually exist.

3.7 Expanded Gene Families

Several gene families are expanded in the genomes of the four sequenced *Colletotrichum* strains, relative to other sequenced ascomycetes. Expansions included genes predicted to encode carbohydrate-active enzymes (CAZymes), secondary metabolism (SM) enzymes, secreted proteases, and putative secreted effectors (Gan et al. 2013; O'Connell et al. 2012).

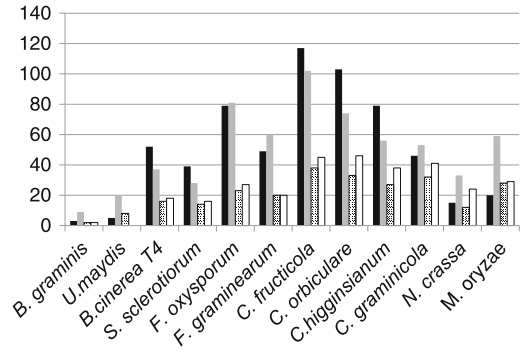

Fig. 3.5 Relative numbers of genes encoding CAZymes targeting different plant cell wall components in *Colletotrichum*, and in other related fungi with various lifestyles. *Black bars* pectin. *Gray bars* pectin and hemicellulose. *Speckled bars* hemicellulose. *White bars* cellulose. *Blumeria graminis* (biotroph); *Ustilago maydis* (biotroph); *Botrytis cinerea* (necrotroph); *Sclerotinia sclerotiorium* (necrotroph); *Fusarium oxysporum* (hemibiotroph); *Fusarium graminearum* (hemibiotroph); *Colletotrichum fructicola* (hemibiotroph); *Colletotrichum orbiculare* (hemibiotroph); *Colletotrichum higginsianum* (hemibiotroph); *Colletotrichum graminicola* (hemibiotroph); *Neurospora crassa* (saprophyte); *Magnaporthe oryzae* (hemibiotroph). *Data* from Gan et al. (2013), and Pamela Gan

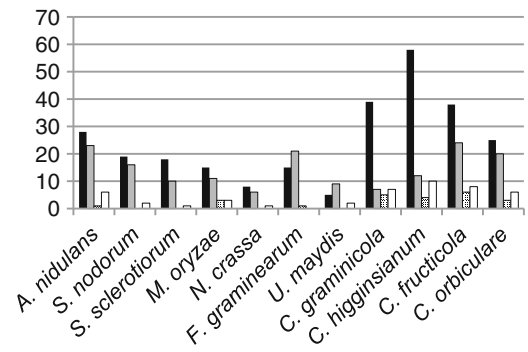

Fig. 3.6 Relative numbers of genes encoding putative SM-related enzymes in *Colletotrichum*, and in other fungi with various lifestyles. *Black bars* PKS and PKS-like, *Gray bars* NRPS and NRPS-like, *Speckled bars* PKS-NRPS hybrids, *White bars* DMATs. *Aspergillus nidulans*, saprophyte; *Stagonospora nodorum*, hemibiotroph; *Sclerotinia sclerotiorum*, necrotroph; *Magnaporthe oryzae*, hemibiotroph; *Neurospora crassa*, saprophyte; *Fusarium graminearum*, hemibiotroph; *Ustilago maydis*, biotroph; *Colletotrichum graminicola*, hemibiotroph; *Colletotrichum higginsianum*, hemibiotroph; *Colletotrichum fructicola*, hemibiotroph; *Colletotrichum orbiculare*, hemibiotroph. *Data* from Gan et al. (2013); O'Connell et al. (2012)

CAZYmes Expanded enzyme arsenals capable of degrading cellulose and other polysaccharides contained within plant cell walls are a common theme for hemibiotrophic and necrotrophic plant pathogens, including *Colletotrichum* species, *M. oryzae* and *Fusarium* species (Cuomo et al. 2007; Dean et al. 2005; Ma et al. 2010). The expansion of cell wall degrading enzymes is a defining feature of the four *Colletotrichum* genomes (Fig. 3.5). The overall abundance of these proteins in *Colletotrichum* is unmatched in any ascomycete sequenced to date, even the destructive necrotrophic gray and white rot fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Fig. 3.5) (Amselem et al. 2011; Gan et al. 2013; O'Connell et al. 2012).

In dicots, pectin comprises approximately 35 % of the cell walls, while the cell walls of monocots such as maize generally contain only 10 % pectin (Vogel 2008). There was an extremely large number of unique pectinases encoded by the genomes of the dicot-infecting *C. higginsianum*, *C. fructicola*, and *C. orbiculare*, likely reflecting an important adaptive trait for these pathogens. In particular, *C. orbiculare* and *C. fructicola* each encode more than 100 different pectinases, greatly surpassing other sequenced fungal genomes (Fig. 3.5). The genome of the maize pathogen, *C. graminicola*, possesses a reduced cohort of pectinase genes compared with the other *Colletotrichum* species: on average, 46 % fewer than the dicot-infecting species. Also consistent with overall pectinase abundance, gene expression profiles during the necrotrophic phase revealed that 51 pectinases were deployed by *C. higginsianum* during necrotrophy, versus only sixteen utilized by *C. graminicola* (O'Connell et al. 2012).

Secondary metabolism genes. Another highly expanded class of genes in the *Colletotrichum* genome encodes putative SM enzymes (Fig. 3.6). SM enzymes are low molecular weight molecules that are not essential for growth and survival of the organism, but may become important for niche adaptation. Production of these metabolites is often associated with successful competition for host resources

through toxic and/or inhibitory effects on other organisms (Bölker et al. 2008; Shwab and Keller 2008). There are numerous examples of SM genes that function in this manner, including the well-known trichothecene gene cluster of *Fusarium graminearum* and the AAL toxin cluster of tomato-infecting *Alternaria alternata* (Akagi et al. 2009; Procter et al. 2009). Large numbers of SM-associated genes are usually found in the genomes of necrotrophic plant pathogens (Amselem et al. 2011), and SM enzymes are often implicated as phytotoxins with direct roles in pathogenicity (e.g., Daub 1982; Gengenbach et al. 1973; Matthews et al. 1979; Scott-Craig et al. 1992). In contrast, a reduced cohort of SM genes is commonly observed in biotrophic fungi such as *Blumeria graminis* (Spanu et al. 2010) and *Ustilago maydis* (Kämper et al. 2006; Bölker et al. 2008).

Relatively little is known about the role of SM in hemibiotrophic plant pathogens (Collemare et al. 2008; Böhnert et al. 2004). *Colletotrichum* species have been reported to produce a variety of SM genes, including flavones, peptides, and terpenes, as well as the polyketide-derived DHN (1,8-dihydroxynaphthalene) melanin, an essential requirement for appressorium-mediated host penetration (Kubo et al. 1991; Singh et al. 2010). Additional examples include the siderophore ferricrocin, isolated from *C. gloeosporioides*, which has phytotoxic activity in grass cotyledons (Ohra et al. 1995), colletotrichins A, B, and C from *C. nicotianae*, which produce symptoms resembling tobacco anthracnose when infiltrated into tobacco leaves (Goddard et al. 1976; Kimura et al. 1977, 1978; García-Pajón and Collado 2003), and a tetrahydroxylated compound with antioxidant properties from *C. gloeosporioides* (Femenía-Ríos et al. 2006). Several secondary metabolites have been characterized from *C. graminicola*, including the antifungal compounds monorden and monicillins I, II, and III (Wicklow et al. 2009), and mycosporine-alanine, a spore germination inhibitor (Leite and Nicholson 1992). It was recently reported that deletion of *PPT1*, a gene encoding a cofactor essential for the enzymatic function of all polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS), resulted in decreased pathogenicity in *C. graminicola,* providing support for the idea that SM genes play an important role in the regulation of pathogenicity to maize (Horbach et al. 2009). Studies have also shown that some *Colletotrichum* fungi, including *C. gloeosporioides*, are able to synthesize the plant growth hormone auxin, which is likely to be important in host manipulation (Chung et al. 2003; Robinson et al. 1998). All four sequenced *Colletotrichum* species contain genes with the potential to encode for production and efflux of auxin (Gan et al. 2013; O'Connell et al. 2012). Genes for synthesis of auxin via an IAM intermediate are present in *C. fructicola* and *C. graminicola*. Auxin synthesis by *C. higginsianum* and *C. orbiculare* may occur via a different intermediate.

Genes encoding putative polyketide synthases (PKS) and PKS-like genes and dimethylallyl transferases (DMATs) are especially abundant in the four sequenced *Colletotrichum* genomes (Gan et al. 2013; O'Connell et al. 2012). *C. higginsianum* and *C. fructicola* possess many more PKS genes, in particular, than any other sequenced fungi (although it is important to point out that these two assemblies had the lowest qualities, and therefore PKS numbers may be somewhat inflated by gene fragmentation). Apart from genes involved in the production of melanin, few of the predicted SM genes have orthologs outside of the *Colletotrichum* genus, and many appear to be specific to individual *Colletotrichum* strains.

In all fungal species, SM genes tend to be organized into clusters; often these clusters include additional enzymes, cytochrome P450 genes, transcription factor genes, and transporter genes (Shwab and Keller 2008). The number of SM clusters predicted from the four sequenced *Colletotrichum* strains is exceptionally high, ranging from 42 clusters in *C. orbiculare* to 56 clusters in *C. fructicola.* This abundance of SM clusters is substantially greater than other plant pathogenic fungi sequenced to date (Gan et al. 2013; O'Connell et al. 2012). Many of the *Colletotrichum* SM clusters are not well conserved among the four species (Table 3.2).

Table 3.2 Secondary metabolite gene clusters in *Colletotrichum* (O'Connell et al. 2012; Gan et al. 2013)

	C. orbiculare	*C. fructicola*	*C. higginsianum*	*C. graminicola*
Number of secondary metabolite clusters	42	56	43	44
With homologs in *C. orbiculare*	–	35	26	26
With homologs in *C. fructicola*	29	–	26	24
With homologs in *C. higginsianum*	22	21	–	20
With homologs in *C. graminicola*	23	26	20	–

Clusters were identified with a combination of SMURF prediction (www.jcvi.org/smurf/index.php) and manual annotation. A cluster was defined as being conserved if the best BLAST hit of at least two members within the cluster belong to a single cluster of another species (except where two genes match a single gene as happens to be the case for some of the hybrid enzymes). It should not be assumed that there is a one-to-one relationship in the number of conserved clusters because of the duplication or split of some of the clusters. For example, there are two noncontiguous *C. higginsianum* ACE1/SYN2 homologous clusters but only one of these clusters was detected in *C. orbiculare* and *C. fructicola*

3.7.1 The *Colletotrichum* Secretome

Fungal secreted proteins are known to have many important roles in plant-fungal interactions, including making plant nutrients accessible to the fungus, or inducing host cell susceptibility or host cell death (Choi et al. 2010; Lowe and Howlett 2012). The predicted secretomes of the four sequenced *Colletotrichum* fungi are large and diverse, ranging in size from 1650 proteins for *C. graminicola* (14 % of the proteome) to 2356 proteins for *C. fructicola* (15 % of the proteome) (Table 3.3). This is comparable to the predicted average of 1798 secreted proteins for Pezizomycotina (Choi et al. 2010). Hemibiotrophic pathogens typically have a larger percentage of their genomes (>10 %) devoted to secreted proteins than other fungi, and *Colletotrichum* fits this pattern (Lowe and Howlett 2012). Approximately 1,500 secreted protein genes were shared by all four *Colletotrichum* species. Many other secreted proteins appeared to be species-specific, including 248 found only in *C. orbiculare*, 225 specific to *C. fructicola*, 227 found only in *C. higginsianum*, and 123 specific to *C. graminicola*.

Compared with other sequenced ascomycetes, the three dicot-infecting species of *Colletotrichum* were highly enriched in genes encoding secreted proteases, particularly the serine proteases known as subtilisins (MEROPS family S8A) that are known in some other pathosystems to function as effectors (Prusky et al. 2001; Olivieri et al. 2002). The relative expansion of these in *C. fructicola, C. higginsianum*, and *C. orbiculare*, but not in *C. graminicola,* may reflect the narrower host range of the latter species. Some of the subtilisins appear to cluster more closely with those of plant origin, suggesting that they could have been acquired by horizontal transfer from their plant hosts (Gan et al. 2013; Jaramillo et al. 2013).

The *Colletotrichum* secretomes contain homologs of genes that encode known fungal pathogenicity effectors, including several necrosis-inducing proteins (Fellbrich et al. 2002; Gijzen and Nuernberger 2006; Kanneganti et al. 2006), and the biotrophy-associated BAS2 and BAS3 proteins from *M. oryzae* (Mosquera et al. 2009) (Table 3.4). Genes encoding members of the necrosis- and ethylene- inducing peptide (NEP) 1-like protein family (Gijzen and Nuernberger 2006) were identified in *C. higginsianum* (Kleemann et al. 2012). Only three of these were able to cause cell death in *N. benthamiana*: the others lacked crucial amino acids and did not function to induce necrosis (Kleemann et al. 2012). Two NEP protein genes in *C. orbiculare* also did not contain necrosis-inducing motifs (Gan et al. 2013). These two had peak expression levels in early biotrophy.

Table 3.3 Summary of *Colletotrichum* secretomes (compiled by Ester Buiate)

	C. graminicola	*C. higginsianum*	*C. fructicola*	*C. obiculare*
Total proteins	12006	16159	15463	13479
Secreted proteins[a]	1650	2142	2356	2149
Percent secreted	14	13	15	16
SSP[b]	687	1173	933	925
Cysteine-rich SSP[c]	204	366	333	373
Species-specific cysteine-rich SSP[d]	32	13	42	88

[a] Secreted proteins, predicted by WoLF PSORT (www.wolfpsort.org)
[b] SSP = small secreted proteins, 300 bp or less
[c] Cysteine-rich SSP, >3 % cysteine
[d] Species-specific: no BLAST hits to the other *Colletotrichum* species, or to the NCBI nr database with a cutoff of 1e-5

Table 3.4 Homologs of conserved some effectors in *Colletotrichum*

Gene	Accession	*C. graminicola*	*C. higginsianum*	*C. orbiculare*	*C. fructicola*
CgDN3	AAB92221.1	0	2	1	1
NPP1	EGZ24512.1	3	4	7	7
NEP1	AF036580.1	4	5	7	9
NIS1	BAL70334.1	1	1	2	2
ToxB	AAO49374.1	0	0	1	0
MSP1	AAX07670.1	24	22	23	26
CIH1	AJ271296.1	2	2	3	2
SIX1	ACY39281.1	0	0	5	0
SIX5	ACN87967.1	0	0	1	1
SIX6	ACN69116.1	0	1	2	0
BAS2	ACQ73207.1	2	3	2	1
BAS3	ACQ73208.1	1	1	0	0
Ctnudix	HO663661.1	1	4	2	2

Conservation was determined by BLASTp searches with a cutoff of 1e-5. (Ester Buiate, and Gan et al. 2013; Kleemann et al. 2012; O'Connell et al. 2012)

A screen for *C. orbiculare* proteins that induced cell death in *N. benthamiana* led to the identification of NIS1, a protein secreted by primary hyphae (Yoshino et al. 2012). Cell death induced by NIS1 is mediated by interaction with the plant heat shock protein 90 (Hsp90), known to be important in R-gene mediated HR-response (Zhang et al. 2010). Homologs of the *NIS1* effector gene are found in all four sequenced *Colletotrichum* species.

Screening of an EST library derived from nitrogen-starved mycelium of *C. gloeosporioides* resulted in the identification of *CgDN3*, a gene predicted to encode a small secreted protein that is required for the successful establishment of this pathogen on *Stylosanthes guianensis* leaves (Stephenson et al. 2000). During infection, the *CgDN3* transcript accumulated in biotrophic infection vesicles. *CgDN3* knockout mutants failed to penetrate or form primary infection hyphae, and they rapidly induced localized cell death. Homologs of *CgDN3* were found in the genomes of *C. fructicola*, and also in *C. orbiculare,* and *C. higginsianum*, but not in *C. graminicola*. The *C. orbiculare* and *C. higginsianum* homologs suppressed cell death induced by the necrosis-inducing effector NIS1 when they were transiently expressed in *N. benthamiana* leaves (Kleemann et al. 2012; Yoshino et al. 2012).

Another conserved secreted effector is the Nudix hydrolase previously identified as an induced gene during the transition to

necrotrophy in the cowpea anthracnose pathogen *C. truncatum* (Bhadauria et al. 2013). Overexpression of CtNudix in *C. truncatum* induced localized host cell death and loss of pathogenicity. Localization studies in *N. benthamiana* indicated that the protein is located in the plant plasma membrane, suggesting that it might alter integrity of host cells by affecting stability of the host plasma membrane. Homologs of the Nudix effector are present in other hemibiotrophic pathogens including *M. oryzae*, and *P. infestans*, but absent in biotrophic and necrotrophic pathogens, suggesting that it might be important specifically for this lifestyle.

Compared with other sequenced ascomycetes, all four *Colletotrichum* genomes contain an expanded family of genes encoding proteins containing CBM50 carbohydrate binding modules, also known as LysM motifs. These genes appear to be highly divergent among the species and thus to be evolving rapidly. They may act as chitin-binding lectins and serve to "mask" the biotrophic hyphae from host recognition by binding to the fungal wall chitin (de Jonge and Thomma 2009). In *C. lindemuthianum*, a LysM protein called *CIH1* was localized to the surface of biotrophic hyphae using a monoclonal antibody (Pain et al. 1994; Perfect et al. 1998). All four sequenced species have homologs of *CIH1*.

Biotrophic plant pathogens are known to produce large numbers of effector candidates, in the form of small secreted proteins (SSP) that act to establish a compatible interaction with the host by suppressing host defenses and reprogramming host cells to accommodate the pathogen (Göhre and Robatzek 2008). These SSP effectors are typically less than 300 amino acids, cysteine-rich, and lineage-specific (Stergiopoulos and de Wit 2009). All four *Colletotrichum* genome annotations include many SSP effector candidates, including a large number that are cysteine-rich and/or unique to each species (Table 3.3). Interestingly, numerous additional candidate effectors were identified after deep 454 pyrosequencing of the *in planta* transcriptome of *C. higginsianum* (Kleeman et al. 2012). It was observed that only about a quarter of these transcripts had been annotated in the initial genome-based analysis, suggesting that the annotated effectors in the four *Colletotrichum* genomes may represent only the tip of the iceberg.

Overall, the genome sequences of the hemibiotrophic *Colletotrichum* fungi are more similar to the genomes of necrotrophic fungi rather than biotrophs, having expanded families of secondary metabolites and CAZymes. Indeed, *Colletotrichum* fungi may have some of the largest and most diverse repertoires of lytic enzymes and secondary metabolites yet found among the pathogenic fungi. In common with necrotrophs, *Colletotrichum* also encode secreted toxin effectors associated with the induction of cell death. At the same time, *Colletotrichum* also encode large and diverse repertoires of putative small, lineage-specific secreted effectors, a hallmark of biotrophic fungal genomes, that may have a similar function, to manipulate host defenses and induce compatibility. We can speculate that this combination of gene arsenals reflects the "schizophrenic" hemibiotrophic existence of *Colletotrichum*, in which they must function almost as two distinct organisms at different stages of their lifecycles.

3.7.2 *Colletotrichum* Transcriptomics During Biotrophy and Necrotrophy

The availability of whole genome sequences enabled genome-wide analysis of the *Colletotrichum* transcriptome at different stages of hemibiotrophic infection. Deep Illumina RNA sequencing was performed for *C. higginsianum* and *C. graminicola* at three stages of development *in planta*: prepenetration (appressoria); biotrophic hyphae; and necrotrophic hyphae (O'Connell et al. 2012). For *C. orbiculare*, whole genome microarrays were produced based on the annotated genome assembly, and were used to investigate gene expression during prepenetration, biotrophic, and necrotrophic growth phases (Gan et al. 2013). In earlier studies, *C. graminicola* biotrophic hyphae were isolated by laser-capture microscopy (LCM) and analyzed using microarrays designed from a limited gene set

based on the preliminary 2X shotgun sequence of strain M5.001 produced by DuPont (Tang et al. 2006). *C. higginsianum* appressoria formed on artificial surfaces, and primary hyphae isolated from the host tissues by fluorescence-activated cell sorting (FACS), were also analyzed by sequencing expressed sequence tags (ESTs), and by Roche 454 sequencing (Kleemann et al. 2008; Kleemann et al. 2012; O'Connell et al. 2012; Takahara et al. 2009).

In interpreting and comparing these various *Colletotrichum* transcriptome datasets, it is important to recall that in *C. higginsianum*, only the first invaded cell contains biotrophic hyphae, followed by a complete switch to necrotrophy. In *C. orbiculare* and *C. graminicola*, the necrotrophic switch is delayed until several cells have been colonized. Thus, the biotrophic phase in these two species consists of a heterogeneous cell population that includes hyphal tip cells advancing into living host cells and intercalary fungal cells occupying dead or dying host cells. Moreover, in *C. graminicola*, the necrotrophic phase is also heterogeneous, composed of necrotrophic colony centers and biotrophic colony margins. This variation in the timing and extent of host cell death caused by *Colletotrichum* species is likely to be reflected in the representation of biotrophy- and necrotrophy-related genes in their transcriptomes.

A common theme that has emerged from studies comparing transcription in vitro to transcription in vivo is that a large number of genes in *Colletotrichum* are plant-induced (Gan et al. 2013; Kleemann et al. 2012; O'Connell et al. 2012; Tang et al. 2006). For example, comparison of gene expression in morphologically identical *C. higginsianum* appressoria produced in vitro versus *in planta* revealed that more than 1,500 genes were significantly induced *in planta* compared with their expression in vitro (O'Connell et al. 2012). Many of these induced genes encoded secreted proteins, including SSP and putative effectors, and many others encoded SM enzymes. Gene Ontology (GO) categories that were over-represented *in planta* included those involved in carbohydrate binding and degradation, protein degradation, and transmembrane transport (Gan et al. 2013). In *C. orbiculare*, most of the genes that were upregulated *in planta* were located in GC-rich, rather than AT-rich, regions (Gan et al. 2013). It is clear that *Colletotrichum* is able to detect and respond to plant signals, although the nature of these signals remains mysterious.

The dome-shaped melanized appressoria of *Colletotrichum* function in penetration of the host cuticle and epidermal cell wall (Fig. 3.2). Cutinases were over-represented in prepenetration appressoria in comparison with later phases of development, as might be expected (O'Connell et al. 2012). Genes involved in cAMP signaling were upregulated in the preinvasion stage of *C. orbiculare,* in agreement with previous reports showing that this is an important signaling pathway in the regulation of germination and appressorium formation (Yang and Dickman 1997, 1999a, b; Yamauchi et al. 2004; Gan et al. 2013). Melanin deposited in the appressorial wall allows accumulation of glycerol and high turgor pressures that facilitate mechanical rupture of the host cell wall (Bechinger et al. 1999; Bastmeyer et al. 2002). Transcriptome analysis of appressorial stages of *C. higginsianum*, *C. graminicola*, and *C. orbiculare* confirmed increased expression of genes of the PKS SM cluster involved in melanin production in all three species (Gan et al. 2013; O'Connell et al. 2012).

A large number of genes encoding putative secreted effector proteins are also expressed in unpenetrated *Colletotrichum* appressoria (Kleemann et al. 2008; 2012; O'Connell et al. 2012; Gan et al. 2013). In *C. graminicola*, homologs of the *M. oryzae* BAS2 and BAS3 effectors were among the most highly expressed genes in appressoria (O'Connell et al. 2012). Lineage-specific SSP effectors were particularly enriched during the early stages of development (appressorial and biotrophic) versus necrotrophy, suggesting they might play a role in establishment of a compatible interaction (Gan et al. 2013; Kleeman et al. 2012; O'Connell et al. 2012). In *C. orbiculare*, 28 of the 100 most highly upregulated genes at the appressorial stage were SSPs (Gan et al. 2013). *C. higginsianum* appressoria were shown to deliver candidate effectors by targeted secretion through pores at the host-

appressorial interface (Kleemann et al. 2012). Numerous SM gene clusters were also expressed during prepenetration and early penetration stages in the appressoria of *C. graminicola*, *C. higginsianum*, and *C. orbiculare* (Gan et al. 2013; O'Connell et al. 2012). These findings suggest that in addition to mechanical breaching of the host cell wall, appressoria play an important role in the secretion of protein and small molecule effectors, which may prepare the infection court for subsequent invasion.

Analysis of genes expressed during biotrophy in the three *Colletotrichum* species identified hundreds of differentially regulated genes, including more than 300 upregulated genes in *C. graminicola*, and more than 700 upregulated genes in *C. higginsianum*. Although the data were generally consistent, shifts in gene expression were not as pronounced in either *C. graminicola* or *C. orbiculare* as they were in *C. higginsianum*, and this is likely because of the more synchronous development of the latter species. A previous LCM-enabled study of *C. graminicola* focused on analysis of biotrophic hyphae, but unfortunately did not provide a full account of the genes that were upregulated in those cells (Tang et al. 2006). Thus analyses of the *C. higginsianum* biotrophic phase, including cells colonizing living host cells, and also biotrophic hyphae isolated by the FACS technique, are likely to be the most informative on the transcriptional status of *Colletotrichum* biotrophic hyphae (Takahara et al. 2009; Kleemann et al. 2012; O'Connell et al. 2012).

As observed in appressoria, many of the genes expressed in *C. higginsianum* biotrophic hyphae encoded secreted effectors and SM enzymes (Kleemann et al. 2012; O'Connell et al. 2012). Several LysM protein genes were expressed specifically in biotrophic primary hyphae, suggesting a role in "masking" of the hyphal wall from host detection (O'Connell et al. 2012). Relatively few genes encoding lytic enzymes were expressed, and in this respect the biotrophic hyphae resemble the haustoria of obligate biotrophs (O'Connell et al. 2012). However, unlike haustoria, there was no specific induction of nutrient uptake transporters at this stage, which would be expected if the biotrophic hyphae of *C. higginsianum* function primarily as organs for nutrient uptake (O'Connell et al. 2012). Consistent with this view, recent evidence indicates that carbohydrate supply by the host is dispensable for the biotrophic growth of *C. higginsianum* (Engelsdorf et al. 2013). Thus, the primary role of biotrophic hyphae seems to be as organs for the secretion of effectors and SM that presumably modulate host responses and suppress host cell death. The SM enzymes and effectors of biotrophic hyphae differ from those expressed in appressoria, with distinct "waves" of these fungal modifiers produced over the course of pathogenic development (Gan et al. 2013; Kleemann et al. 2012; O'Connell et al. 2012).

The switch from biotrophy to necrotrophy is marked by the production of narrow secondary infection hyphae that are not separated from the host cell by a membrane (Fig. 3.4). Analysis of gene expression in all three species during necrotrophy revealed the induction of a large array of genes encoding secreted proteases and CAZymes, producing a cocktail of enzymes that is probably highly efficient for degrading plant cell walls (Gan et al. 2013; O'Connell et al. 2012). Between 23 and 25 % of the 100 most highly expressed genes upregulated by *C. graminicola* and *C. higginsianum* during the necrotrophic phase are CAZymes (O'Connell et al. 2012). However, each species apparently uses a different strategy to deconstruct host cell walls. More pectin-degrading enzymes (51) are induced during necrotrophy in *C. higginsianum*, while *C. graminicola* deploys more cellulases and hemicelluloses at this stage. An example is provided by the GH61 monooxygenases, which act in concert with classical cellulases to enhance lignocellulose hydrolysis (Beeson et al. 2012; Quinlan et al. 2011). Twenty-two of 28 GH61 monooxygenases were induced during *C. graminicola* necrotrophy, with 6 % of the most highly induced genes during necrotrophy belonging to this class (O'Connell et al. 2012). In contrast, only six out of 25 GH61 genes were expressed by *C. higginsianum* during necrotrophy, and none were highly induced (O'Connell et al. 2012).

Numerous nutrient uptake transporters are also induced at the switch to necrotrophy, suggesting that secondary hyphae provide the major

organs for nutrient acquistion for *Colletotrichum* (Gan et al. 2013; O'Connell et al. 2012). In both *C. higginsianum* and *C. orbiculare* there was a general decrease in the number of SM gene clusters expressed during necrotrophy, but in *C. graminicola* a large percentage of the genes induced at this stage were SM genes. Notably, the SM genes expressed by *C. graminicola* during the necrotrophic phase differed from those expressed earlier in the interaction (Gan et al. 2013; O'Connell et al. 2012). Genes encoding secreted effectors, including putative necrosis-inducing proteins, were also induced at the necrotrophic stage of development in all three species (Gan et al. 2013; O'Connell et al. 2012).

3.7.3 How Can Genome Information Help Us to Better Manage and Exploit *Colletotrichum*?

We are now in the postgenomic era for *Colletotrichum* research. Numerous additional *Colletotrichum* genome-sequencing projects are underway as we write, and sequencing technology has advanced to the point that the genome or transcriptome of any strain of interest can be sequenced quickly and cheaply. An important consideration emerging from the comparative transcriptome analyses described here is the need to ensure that future analyses are performed at equivalent infection stages, and under conditions promoting synchronous development, if they are to be useful for a comparative analysis. For finer resolution of transcriptional differences, e.g., within intracellular primary hyphae occupying either living or dead host cells, it may be necessary to use single-cell sampling methods such as FACS or LCM (Kleemann et al. 2012; Takahara et al. 2009; Tang et al. 2006). Careful cytological studies and bioassay development are essential for each new species that is analyzed. Further considerations relate to aspects of bioinformatics and sequencing technologies. In particular, direct comparisons of genomes produced using different sequencing techniques and different assembly and annotation software must be made with extreme caution, and perceived differences must be confirmed by manual annotation before they can be fully accepted.

The genus *Colletotrichum* includes species displaying a broad spectrum of pathogenic lifestyles, providing many exciting opportunities for comparative genomics and transcriptomics to study the molecular and evolutionary basis of these lifestyles. Comparisons of species displaying "extreme" lifestyles, e.g., subcuticular necrotrophy or symptomless endophytism, should reveal how *Colletotrichum* fungi differ from the better-characterized hemibiotrophic species, e.g., in gene repertoires dedicated to host degradation (proteases, carbohydrate-active enzymes) or 'stealth' (protein and secondary metabolite effectors), or in the timing with which those genes are deployed. Likewise, comparisons of species with contrasting lifestyles that infect the same host plant (endophytes vs. pathogens, necrotrophs versus hemibiotrophs, or species preferentially infecting different organs of the same host (shoots or roots) could be especially informative.

Several major conclusions can be drawn from the comparative genome analyses described herein, with implications for both applied and basic research disciplines. Remarkably, considering the extensive repertoire of pathogenicity-related genes encoded in the four sequenced *Colletotrichum* genomes and the patterns with which those 'weapons' are deployed during infection, it is clear that there is far more held in common by these phylogenetically diverse species than there is unique. Thus, one can anticipate that conserved components and mechanisms will be discovered that could provide potential targets for controlling many *Colletotrichum* diseases through chemical intervention or plant breeding. Strong evidence for host specialization was also revealed through genome-scale studies of *Colletotrichum*, including the apparent adaptation of the CAZyme repertoire and its expression to particular host cell wall composition, and the large degree of diversity in SM and secreted protein effectors. Many *Colletotrichum* effectors are species-specific, while others are shared within the genus, or even with other fungal genera, which may indicate conserved functions or

host targets. The priority now will be to identify targets of both conserved and lineage-specific effectors, and to determine the mechanisms by which effectors manipulate host cells to induce compatibility.

Anthracnose diseases of field crops are generally managed by the use of resistant cultivars. But resistance failures occur frequently, due to the ability of pathogen populations to adapt rapidly to traditional R-gene-mediated resistance strategies. Ultimately, these boom-and-bust cycles lead to increased reliance on fungicides (Parlevliet 2002; Thakur 2007). In particular, management of postharvest diseases caused by *Colletotrichum* requires the use of expensive and toxic chemicals (Prusky 1996). Further genome/transcriptome-based insight into the conserved toolbox employed by members of the genus *Colletotrichum* could prove instrumental for the design of durable strategies for disease control through resistance breeding. For example, alternate breeding strategies, including the use of mutant susceptibility (*S*) genes identified using pathogen effectors as molecular probes, show promise in many pathosystems (Gawehns et al. 2013). *S* genes encode host proteins that are co-opted by plant pathogens, resulting in pathogen proliferation and ultimately leading to diseased host tissue. *S* gene inactivation reduces the pathogens' ability to cause disease, providing a durable form of resistance (Pavan et al. 2010). In some cases, *S* gene-based immunity has provided broad spectrum resistance against pathogens for several decades (Gawehns et al. 2013). Although *S* genes provide highly effective sources of resistance, few have been identified to date and even fewer are commercially viable. But with the power of genomics, the discovery of new candidates may be accelerated through *Colletotrichum* effector-target screens.

3.7.4 The Genomics of Hemibiotrophy

Some of the most important results gleaned from genomics-enabled research of *Colletotrichum* are those that provide insight into the hemibiotrophic lifestyle, and the parallels between distinct life stages and the infection strategies of obligately biotrophic or necrotrophic plant pathogens.

The primary hyphae of pathogenic IH *Colletotrichum* are in some ways analogous to the haustoria of obligate intracellular biotrophs (Mendgen and Hahn 2002; O'Connell and Panstruga 2006; Perfect et al. 1999, 2001). These structures share certain morphological similarities, including the presence of a membrane that differs in composition from the normal plant plasma membrane, and serves to separate the fungus from the living host cell (Shimada et al. 2006). Obligate biotrophs cannot be cultured away from their plant hosts, and cannot be easily genetically manipulated, whereas hemibiotrophs are readily cultured and manipulated. This led to the idea that it might be possible to identify pathogen components required for biotrophic growth by studying the more experimentally tractable hemibiotrophs (Mendgen and Hahn 2002). However, genome analysis has suggested that the appressoria and biotrophic hyphae of *Colletotrichum* function primarily as organs for the synthesis and secretion of protein and secondary metabolite effectors to host cells, and not as organs of nutrient uptake like true haustoria.

Transcriptome analysis revealed that *Colletotrichum* fungi are highly responsive to unknown plant signals, and that gene expression can differ remarkably between morphologically similar structures formed in vitro and *in planta*. Understanding the role of plant signals in transcriptional reprogramming and the mechanisms by which those signals are sensed and transduced by the fungus could lead to new opportunities to alter those signals by manipulation of the plant genome or to interfere with their perception by the pathogen. Transcriptomics also demonstrated that massive shifts in gene expression underlie the developmental transitions that occur *in planta*, from spore germination to necrotrophy. Thus, the lifestyle switch to necrotrophy is characterized by a massive shift in fungal gene expression, with the activation of large numbers of genes encoding lytic enzymes and membrane transporters. It will be crucial to understand the signals that trigger this switch and the transcriptional/epigenetic regulators and

signaling pathways that underlie it. It is possible that these differ between hemibiotrophic pathosystems, but comparative transcriptome analyses focused on the key transitional phase will help clarify this.

3.7.5 Genomics Applied to Elucidating the Systematics of *Colletotrichum*

Concurrent with genomics-enabled research of *Colletotrichum* pathology, the genus is undergoing a taxonomic renaissance, enabled by the application of molecular phylogenetic approaches (Crouch et al. 2006, 2009a, b, c; Damm et al. 2009, 2012a, b; Hyde et al. 2009; Rojas et al. 2010; Weir et al. 2012). Continued synergy between genomics, transcriptomics and molecular phylogenetic research is beginning to provide us with a long-awaited glimpse into the forces impacting the evolution of *Colletotrichum* species. Importantly, molecular phylogeny-based diagnosis of species boundaries will enable more accurate predictions about genome evolution, mechanisms of host adaptation, the evolution of key pathogenicity traits and other topics. One illustration of this point involves the host range of *Colletotrichum* species. Molecular phylogenetic studies are confirming long-held suspicions that twentieth century species concepts and host range assumptions made using classical morphology are overly broad and often inaccurate (Sutton 1980; Cannon et al. 2013). Increasingly well-resolved species diagnoses are now incorporated across the entire genus (Cannon et al. 2013). For instance, until recently, *C. graminicola* was recognized as a broad host range generalist pathogen of nearly every grass and cereal in the Poaceae family, despite considerable evidence of physiological specialization and distinctive appressorial structures (Sutton 1968; Sherriff et al. 1995; Hsiang and Goodwin 2001; Du et al. 2005; Crouch et al. 2006). Molecular data showed this broad circumscription as false, with *C. graminicola* limited to maize, while more than sixteen distinct *Colletotrichum* species are now described as pathogens and endophytes of the Poaceae (Crouch et al. 2006, 2009a, b; Crouch and Tomaso-Peterson 2012; Crouch 2013). Similar resolution has resulted from the study of *C. gloeosporioides*, *C. acutatum* and several other taxa (Damm et al. 2009, 2012a, b; Hyde et al. 2009; Weir et al. 2012). With increasingly precise demarcations of species boundaries, and the insight into host association that such data provides, accurate biological, epidemiological, and mechanistic interpretation of genome and transcriptome data become possible.

A very broad pattern of host association is evident across the *Colletotrichum* phylogeny, with older, basal lineages uniquely associated with dicots and non-graminicolous monocots (Fig. 3.1). *Colletotrichum* pathogenic to grasses form a cohesive, monophyletic group, the graminicola aggregate, originating from ancestral lines of non-graminicolous *Colletotrichum*. Thus, the pathogenic association of *Colletotrichum* with grass hosts appears to be a derived trait, of relatively recent origin. Taken together with data from genome and transcriptome analyses, this evolutionary trajectory suggests that the expanded gene cohorts held in common by dicot infecting *Colletotrichum*, particularly genes encoding pectinases, most closely reflect the ancestral state for the genus, and the reduced cohort characterized from *C. graminicola* is potentially due to the loss of these genes as they became unnecessary. Additional research is needed to investigate these possibilities.

Phylogenomic analysis and divergence dating from a sample of *C. graminicola, C. higginsianum* and seventeen other fungi estimated a recent divergence between these two species, just 47 million years ago (O'Connell et al. 2012). Notably, the divergence between *C. graminicola* and *C. higginsianum* occurred approximately 100 million years after the divergence between their respective host groups, monocots and dicots (Chaw et al. 2004). As such, it is unlikely that divergence in this part of the *Colletotrichum* phylogeny was temporally associated with host evolution. However, the divergence of species within the graminicola aggregate may have involved a coevolutionary process with the Poaceae family. The most basal species in the

graminicola aggregate, *C. cereale*, is uniquely associated with cool-season (C3 physiology) Pooideae grasses such as wheat, oats, barley, bluegrass, etc. (Fig. 3.1). *C. cereale* is the progenitor of numerous *Colletotrichum* species adapted to warm-season (C4 physiology) cereals and grasses in the Panicoideae subfamily, including maize, sorghum and sugarcane. The divergence between wheat (Pooideae; C3) and maize (Panicoideae; C4) is estimated at 50–60 million years ago (Chaw et al. 2004), generally compatible with the estimated timing of the *graminicola/higginsianum* divergence, particularly given the margin for error associated with calibration of divergence times (O'Connell et al. 2012). This temporal correspondence suggests that speciation and host adaptation in the graminicola aggregate may have mirrored host diversification, or may even have been driven by the evolutionary radiation of the Poaceae family. Genome and transcriptome assessments of additional *Colletotrichum* species that are either members of, or closely related to, the graminicola and destructivum aggregates, especially those associated with non-graminicolous monocots or members of the Pooideae-infecting *C. cereale*, could be particularly informative.

3.7.6 Genomics Tools for Studying the Population Genetics of *Colletotrichum*

Phylogenetic and population genetic investigations are benefiting from the increased availability of genome-scale datasets. One of the major obstacles facing phylogenetic and population researchers is the identification of appropriate and informative molecular markers to gauge diversity, relationships, and evolutionary traits. Standard methodology uses "borrowed" markers: a handful of conserved loci that are used primarily because primers exist that are capable of amplifying a broad range of organisms with relative ease (e.g., Carbone and Kohn 1999; White et al. 1990). These markers have been in common use for almost two decades in some cases. However it is unknown whether such strategies yield a biased view of evolutionary relationships or gene-specific noise. There is an increasing body of work that shows many of these borrowed markers lack the power to fully resolve distinct organisms or provide incongruous results (Aguileta et al. 2008; Rokas et al. 2003; Townsend 2007; Townsend and Lopez-Giraldez 2010). To overcome these biases, researchers are increasingly developing datasets from larger numbers of orthologous genes, targeted genes or even whole genomes (e.g., Aguileta et al. 2008; Du et al. 2005; Crouch et al. 2009a, b, c; Fitzpatrick et al. 2006; Rokas et al. 2003; Wang et al. 2009). Comparative genome analysis of 52 *Colletotrichum* isolates from the graminicola and acutatum aggregates using Illumina sequenced restriction-associated DNA tagged (RAD-Seq) SNP datasets demonstrated that a subset of commonly used Sanger-sequenced PCR amplicon-derived single locus molecular markers—*Apn2* and *Sod2*—individually provided reliable identification of *Colletotrichum* species comparable to a 1,723 locus genome-wide dataset (Crouch et al. 2013). These findings led to the development of real-time PCR diagnostic assay based on the *Apn2* marker capable of population-specific detection of *C. cereale* from infected host tissue, and herbarium specimens up to 100 years old (Beirn et al. 2013). Similar development and application of genome data for the identification of other economically important *Colletotrichum* could provide pathologists with tools that help mitigate losses due to disease.

3.7.7 Genomics and the Commercial Exploitation of *Colletotrichum*

Colletotrichum have long been utilized for biotechnology applications, with many species yielding a diversity of compounds and secondary metabolites with commercially valuable biological activity (García-Pajón and Collado 2003). Recent notable applications include the purification of large quantities of the alkaloid compound huperzine A used for treatment of Alzheimer's disease from *C. gloeosporioides* (Zhao et al. 2013), the use of lipid-accumulating

Colletotrichum strains as a novel source of biodiesel feedstocks (Dey et al. 2011), and the purification of novel antimicrobial metabolites from *C. gloeosporioides* with effective antibiotic activity against multidrug resistant *Staphylococcus aureus* (Arivudainambi et al. 2011). The potential for identification of novel SM, including those with antimicrobial activity, from *Colletotrichum* appears virtually untapped. *Colletotrichum* may also provide a valuable source of novel enzymes for plant biomass transformation, notably the production of 'second generation' biofuels from lignocellulosic biomass such as residual crop waste or woody crops (Simmons et al. 2008). Genome analysis has revealed that *Colletotrichum* species possess extraordinarily large and complex repertoires of enzymes for lignocellulose degradation and that they tailor these enzymes for particular host substrates. There is thus significant potential for commercial exploitation of these enzyme systems to improve the efficiency of production of lignocellulose-derived biofuels. Transcriptomic information on which enzymes are co-regulated may suggest combinations of enzymes that act synergistically for efficient lignocellulose transformation.

Acknowledgements We are grateful to Guillaume Robin for preparing Fig. 3.3, and to Adnan Ismaiel for the sequencing that contributed to the phylogenetic tree in Fig. 3.1. The work in the Shirasu lab is supported partly by the Programme for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry and Grant-in-Aid for Scientific Research (KAKENHI; 24228008).**List of URLS**Broad Institute *Colletotrichum* Database: (www.broadinstitute.org/annotation/genome/colletotrichum_group/MultiHome.html)Max Planck Institute for Plant Breeding Research Fungal Genomes: (www.mpipz.mpg.de/14157/fungal_genomes).Ensembl-Fungi *G. graminicola* genome(http://fungi.ensembl.org/Glomerella_graminicola/Info/Index)

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Fusarium graminearum Genomics and Beyond

4

Li Guo and Li-Jun Ma

4.1 Introduction

Diseases of cereal crops caused by pathogenic *Fusarium* species pose a great threat to global food production and safety. Head blight of wheat, barley, oats, and many other small grain cereal crops are caused by *Fusarium graminearum*. Ear rot and stalk rot of maize caused by *F. graminearum* and *Fusarium verticillioides* is a worldwide problem threatening both yield and quality of global corn production (White 1999). In addition to losses in yield and quality, infected grains also harbor fungal mycotoxins, many of which are potent carcinogens for humans and livestock and therefore cause for concern (Goswami and Kistler 2004). To understand the fundamental biology of pathogenic *Fusarium* species for the development of more effective disease management strategies, the genome of the major cereal killer, *F. graminearum* (Cuomo et al. 2007) and many other *Fusarium* species including *F. verticillioides, Fusarium oxysporum* (Ma et al. 2010), *Fusarium solani* (Coleman et al. 2009), *F. circinatum* (Wingfield et al. 2012), *F. fujikuroi* (Wiemann et al. 2013), and *F. pseudograminearum* (Gardiner et al. 2012), were sequenced and publicly released (reviewed in Ma et al. 2013). The open availability of these genomes has enabled further studies on fungal biology, genome evolution, pathogenicity, and the host–pathogen interactions using multiple omics approaches. In this chapter, we summarize recent research development and knowledge advancement regarding the *F. graminearum* genome since its public release in May 2003.

4.1.1 *Fusarium graminearum:* Reduces Crop Production and Contaminates Grains

Fusarium graminearum Schwabe (teleomorph *Gibberella zeae* Schwein (Petch)) is a filamentous fungus (Phylum Ascomycota, Subphylum Pezizomycotina, Class Sodariomycetidae, Order Hypocreales, Family Nectriaceae, and Genus *Gibberella*) capable of producing two types of asexual spores, macroconidia and chlamydospores. The ability of the fungus to produce microconidia has also been proposed (Xu, Purdue University, personal communication). The macroconidia are mononucleate and are typically slender, thick-walled, and banana-shaped with 5–6 septa. Similar to a number of other important fungal pathogens, *F. graminearum* is self-fertile and can also exhibit facultative outcrossing (Leslie and Summerell 2006). Sexual reproduction in *F. graminearum* gives rise to perithecia (Fig. 4.1), black and spherical fruiting bodies containing asci of typical eight ascospores. Both ascospores and macroconidia are

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R. A. Dean et al. (eds.), *Genomics of Plant-Associated Fungi: Monocot Pathogens*,
DOI: 10.1007/978-3-662-44053-7_4,

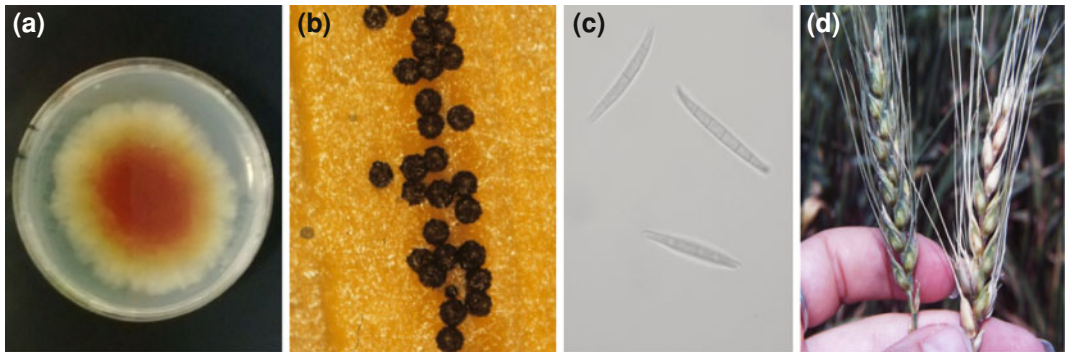

Fig. 4.1 **a** *F. graminearum* growing on potato dextrose agar. **b** peritheica, **c** macroconidia of *F. graminearum*. **d** Pictures of healthy and head blight of wheat infected by *F. graminearum*. (*Photos* **b** and **d** courtesy Dr. H. Corby Kistler at University of Minnesota)

infection propagules for the pathogen. However, ascospores forcibly discharged from perithecia are the primary inoculum for head blight infection of wheat and barley.

In addition to morphological characteristics, the species '*F. graminearum*' was defined primarily based on the association with cereal head blight. Members within this species complex were later divided into two groups (Leslie and Summerell 2006) based on their distinctive sexual reproduction strategies. The homothallic strains were grouped together and retained the species name *F. graminearum*, whereas *F. pseudograminearum* was used to delimit heterothallic strains requiring compatible mating types to cross. Further division was proposed based on molecular phylogenetics, resulting in 11 distinct lineages in the *F. graminearum species complex (FGSC)* (O'Donnell et al. 2000, 2004; Starkey et al. 2007). These species are *F. graminearum*, *F. gerlachii*, *F. asiaticum*, *F. vorosii, F. acacia-mearnsii*, *F. boothii*, *F. mesoamericanum*, *F. cortaderiae*, *F. brasilicum, F. austroamericanum, and F. meridionale*. The list of species within *Fg* complex has kept growing (Yli-Mattila et al. 2009; Sarver et al. 2011). However, the fact that these lineages are cross-fertile suggests that they probably still belong to a single biological species.

The most destructive disease caused by *F. graminearum* is Fusarium head blight (FHB) or scab on wheat, barley, oats, rye, rice, and many other small grain crops around the world (Goswami and Kistler 2004), resulting in annual crop losses in the billions of dollars. Although many Fusarium species were known to be associated with FHB, *F. graminearum* is indisputably the most common causal agent of FHB. In 1884, FHB was first reported at England and there have since been countless outbreaks in Europe, Asia, Canada, and the USA. In the USA alone, the direct and indirect economic losses resulting from the FHB epidemics in periods of 1991–1997 (Johnson et al. 1998) and 1998–2000 (Nganje et al. 2004) were estimated at 1.3 and 2.7 billion dollars, respectively. In China, FHB has devastated wheat production of over 7 million hectares and led to yield losses of 1 million tons during severe outbreaks (Bai and Shaner 2004).

The *F. graminearum* disease cycle is well described in the review by Bushnell et al. (2003). Both ascospores and macroconidia can be disseminated by wind, rain, and insects to host plants and deposited on or inside of spike tissues. However, ascospores forcibly discharged from overwintering perithecia under favorable spring conditions are the sources of primary inoculum for the initiation of FHB on new wheat plants. A close association between FHB and wheat anthesis was observed, and extruded anthers are suggested as vulnerable sites for primary infection (Sutton 1982). Once having landed on a suitable host, fungal spores germinate and fungal hyphae enter the stomata. Cell wall degrading enzymes secreted by the

pathogen facilitate the entry of infection hyphae into epidermal cells. Furthermore, infection hyphae can penetrate into the xylem and phloem and move beyond the inoculation site through wheat vascular bundles in rachis and rachilla (Bushnell et al. 2003). As a result, infected florets become blighted and bleached, with pink macroconidia often visible on the outer surface of florets. Overwintering *F. graminearum* can persist on both living plants and dead plant debris (Bushnell et al. 2003). FHB-infected kernels are contaminated with mycotoxins, which include deoxynivalenol (DON), nivalenol (NIV), and acetyldeoxynivalenol (ADON) (more details in the Mycotoxin section). Animals intoxicated with trichothecence mycotoxins exhibit typical symptoms, such as vomiting, dizziness, abdominal pain, and diarrhea (Desjardins et al. 1993). Due to its toxicity, the Food and Drug Administration set strict guidelines on DON levels in grains intended for human (1 ppm) and animal consumption (1–10 ppm).

Head blight or "scab" of rice (HBR) is usually not a major problem in rice production, but could be severe under favorable conditions (Nyvall et al. 1999; Goswami and Kistler 2005). The disease has recently been reported in Asian countries (Carter et al. 2000; Desjardins et al. 2000). Since rice (*Oryza sativa*) is one of the most important cereal crops, there are growing concerns about this disease. Under the new classification, multiple *Fusarium* species are associated with HBR, including *F. graminearum* and *F. asiaticum* (Lee et al. 2009). Even though, no rice scab diseases have been reported in the USA, *F. graminearum* and a few other *Fusarium* spp. were reported to cause head blight on wild rice (*Zizania palustris*) in Minnesota (Nyvall et al. 1999). In addition, Goswami and Kistler (2005) have shown that several *F. graminearum* and *F. asiaticum* isolates pathogenic to wheat were also capable of causing rice scab. However, trichothecenes, an important virulent factor in wheat scab, were not detectable in the infected rice plants (Goswami and Kistler 2005).

Fusarium graminearum also frequently causes ear rot and stalk rot diseases on maize as known as Gibberella stalk rot and Gibberella ear rot, named after *G. zeae,* the teleomorph of *F. graminearum* (White 1999). Typically, ascospores and macroconidia can infect corn through silk channels. Different from Fusarium ear rot caused by *F. verticillioides* and *F. proliferatum,* where infection can spread throughout the ears, Gibberella ear rot develops from the tip of ears with pink or white mycelia colonizing the ears (White 1999). Cool and wet weather during and after the corn silking period favors disease development. The disease cycle is similar as FHB and contaminations of infected ears and stalks with DON and zearalenone (ZEA) are the major concerns with this disease.

Members within FGSC produce a variety of mycotoxins toxic to animals and humans, including two major groups of tricothecenes (Desjardins et al. 1993) and mycoestrogen zearalenone (Leslie and Summerell 2006), and many others (Leslie and Summerell 2006) such as aurofusarin and fusarin C. However, the ability to produce these secondary metabolites differs considerably among members. Given the lack of correlation between certain chemotypes and phylogenetic lineages (O'Donnell et al. 2000), horizontal gene transfer was proposed to be one of the explanations for the metabolic diversity in FGSC and many other fungal species alike (Bömke and Tudzynski 2009; Cambell et al. 2012; Ma et al. 2013)

Tricothecenes are sequisterpenoid fungal secondary metabolites. In addition to *Fusarium spp.,* they can be produced by more than 300 other fungal species, including *Trichoderma* (Nielsen et al. 2005), *Myrothecium* (Fernando and Bean 1986), and *Stachybotrys* (Andersen et al. 2002). Over 150 trichothecene mycotoxins have so far been identified and classified into four groups A, B, C, and D according to their chemical properties and fungal producers. Groups A and B are the most common and best studied trichothecene mycotoxins (Rocha et al. 2005). Type A tricothecenes include T-2 toxin, HT-2 toxin, neosolaniol, and diacetoxyscirpenol (Kimura et al. 2007; Rocha et al. 2005). Type B tricothecenes include DON (also known as vomitoxin), NIV,

and their acetylated derivatives ADON (Kimura et al. 2007; Rocha et al. 2005). *F. graminearum* produces primarily type B trichothecenes during head blight infection of cereal crops. Because of its significance, the trichothecene profiles are used to classify *F. graminearum* strains into different chemotypes, such as DON, NIV, 3-ADON, and 15-ADON (Rocha et al. 2005). The toxicity of trichothecenes comes from the ability to bind to ribosomes and inhibit protein synthesis (Rocha et al. 2005). The toxin can cause severe mycotoxicoses to animals that are fed with contaminated grains even at low concentration (Beasley 1989). The toxic effects include growth retardation, immunocompromization, dizziness, diarrhea, vomiting, and feed refusal (Beasley 1989). Trichothecenes are also phytotoxic and cause necrosis, chlorosis, wilting, and other symptoms in many plants (Cutler Horace 1988). Trichothecenes are virulence factors for FHB disease. *F. graminearum* trichothecene biosynthesis mutant (*ΔTri5*) is unable to produce trichothecenes and has significanlty reduced virulence on wheat (Proctor et al. 1995a).

The best-characterized mycoestrogen toxin is zearalenone (ZEA) (6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactone), and is well reviewed by Zinedine et al. (2007). ZEA is produced by many *Fusarium* species including *F. culmorum, F. cerealis, F. equiseti, F. crookwellense*, *F. semitectum, F. sporotrichioides, and F. venenatum* (Leslie and Summerell 2006). ZEA contaminates primarily corn and several other small grain crops. Gibberella stalk rot and Gibberella ear rot result in significant levels of ZEA on infected corn ears and stalks. Structurally, mycoestrogen resembles the female sex hormone estrogen. Therefore, it can competitively bind to the mammalian estrogen receptor. Humans or animals that consume ZEA-contaminated products are at the risk of many reproductive disorders, such as infertility and abortion. Consumption of ZEA-contaminated food has been associated with human hyperoestrogenic syndromes. ZEA also has been associated with cancer in lab animals (Schoental 1974).

4.1.2 *F. graminearum* Genome: Smaller Genome Size and Ample Potential for Mycotoxin Production

As the first *Fusarium* genome sequenced (strain PH-1 NRRL 31084) (Cuomo et al. 2007), *F. graminearum* has been used as a model for understanding Fusarium mycotoxin production, pathogenicity, signaling transduction, transcription regulation, development, sexual and asexual reproduction, and many other biological processes.

A compartmentalized genome structure was one of the unique features revealed from the *F. graminearum* genome project (Cuomo et al. 2007; Kistler et al. 2013; Ma et al. 2013). As in all genomes, the subtelomeric regions of *F. graminearum* chromosomes are highly polymorphic between different isolates in the *F. graminearum* population. Interestingly, *F. graminearum* chromosomes also contain discrete segments in the middle of the chromosomes that have high single nucleotide polymorphism (SNP) rate and chromosomes that more frequently recombined within these regions. These regions are enriched for genes that are important in plant–fungus interactions, including secreted proteins and genes expressed specifically *in planta* (Cuomo et al. 2007). These segments were proposed to be indicators of where chromosomal fusion occurred, based on the facts that: (1) the discrete segments are subtelomeric like, (2) *F. graminearum* has the smaller number of chromosomes (Table 4.1), and (3) Chromosomal fragments outside these discrete segments are highly conserved with homologous chromosomes in other *Fusarium* species (Ma et al. 2010). These fusion events definitely contributed to the decrease of genome size, even though the significance of chromosomal fusion and genome size reduction is not fully known.

Lacking repetitive sequences is another noticeable feature of the *F. graminearum* genome (Table 4.1), which also contributes to the genome size reduction. The decrease of repeat content was partially attributed to the active

Table 4.1 Statistics of Fusarium genomes

Species	Genes	Strain	Size (Mb)	# og Chr.	Intergenic distance	% coding	% repeat	Ref.
F. verticillioides	14,179	7,600	41.7	11	1,379	42.8	1.76	Ma et al. 2010
F. oxysporum f. sp. *lycopersici*	17,735	4,287	59.9	15	1,977	35.4	27.44	Ma et al. 2010
F. graminearum	13,332	PH-1	36.2	4	1,182	49.0	0.67	Cuomo et al. 2007
F. pseudograminearum	12,488	CS3096	37	ND	−25	49.3	1.6	Gardiner et al. 2012
F. solani f. sp. *pisi*	15,707	77-13-4	54.4	17	−15	−15.0	15.14	Coleman et al. 2009

The 11 chromosomes of *F. verticillioides* reflects the fact that among 12 chromosomes detected in the *Fv* genetic maps, this genome assembly only mapped to 11 chromosomes
ND not detected

process of repeat induced point (RIP) mutation, in which duplicated sequences are subject to extensive mutation (Cuomo et al. 2007). Low repeat contents were also observed in other *Fusarium* genomes, such as *F. verticillioides* (Ma et al. 2010), *F. pseudograminearum* (Gardiner et al. 2012), and *F. fujikuroi* (Wiemann et al. 2013). In contrast, other *Fusarium* genomes, such as *F. oxysporum* (Ma et al. 2010) and *F. solani* (Coleman et al. 2009), have much higher repeat contents.

Genes controlling the biosynthesis of many fungal secondary metabolites, including trichothecenes and ZEA, are clustered together. The biosynthesis gene clusters typically include a terpene synthase (TS), or polyketide synthase (PKS), or nonribosomal peptide synthetase (NRPS) gene that are responsible for a fundamental step in biosynthesis of nonribosomal peptides, polyketides, or terpenes. Additionally, these clusters also include genes responsible for structural modifications of initial metabolites, for metabolite transport, and pathway-specific transcription factors that activate expression of genes in the clusters. Such distinctive features enable the identification of SM biosynthesis clusters and can be used to predict potential SMs synthesized in a genome. The *F. graminearum* genome analysis identified a total of 43 such clusters (Ma et al. 2010), including genes responsible for known secondary metabolites, such as trichothecenes, zearalenones, aurofusarin, fusarins (Desjardins et al. 2006). Importantly, this genome study revealed ample potential to produce many uncharacterized secondary metabolites.

The *F. graminearum* trichothecene biosynthesis core gene cluster (*Tri* cluster, Fig. 4.2) contains 12 genes, including two transcription factors, that are required for the DON production and full virulence of *F. graminearum* on host plants (Baldwin et al. 2010b). The two transcription factor genes, *Tri6* and *Tri10,* have been shown to regulate the expression of the cluster (Seong et al. 2009; Tag et al. 2001). All 12 genes share very similar expression profiles across all public available expression data available at PLEXdb (Guo unpublished data), supporting a tight regulation. Interestingly, these two transcription factors also regulate genes outside of *Tri* cluster, including many genes important for producing precursors essential for DON production (Seong et al. 2009). It is noted that gene cluster responsible for the synthesis of NIV has two extra genes *Tri7* (C-4 acetyltransferase) and *Tri13* (C-4 hydroxylase), which are nonfunctional in DON-producing strains (Lee et al. 2002).

The biosynthesis of ZEA is controlled by a gene cluster including two PKS genes (Gaffoor and Trail 2006; Lysoe et al. 2006), ZEB1 encoding an isoamyl alcohol oxidase (Kim et al. 2005) and ZEB2 encoding a bZIP transcription factor (Kim et al. 2005). Other secondary metabolites are also controlled by gene clusters.

Gene_ID	Tri genes	Gene expression patterns	Annotation	Reference
FGSG_03532	Tri8		trichothecene 3-O-esterase	McCormick and Alexander, 2002
FGSG_03533	Tri7		hypothetical protein	Lee et al., 2002
FGSG_03534	Tri3		trichothecene 15-O-acetyltransferase	McCormick et al., 1996
FGSG_03535	Tri4		cytochrome P450	Hohn et al., 1995
FGSG_03536	Tri6		transcription factor	Hohn et al., 1999, Proctor et al., 1995b
FGSG_03537	Tri5		trichodiene synthase	Proctor et al., 1995a
FGSG_03538	Tri10		transcription factor	Tag et al., 2001
FGSG_03539	Tri9		hypothetical protein	Brown et al., 2001
FGSG_03540	Tri11		isotrichodermin C-15 hydroxylase	Alexander et al., 1998
FGSG_03541	Tri12		trichothecene efflux pump	Alexander et al., 1999
FGSG_03542	Tri13		hypothetical protein	Lee et al., 2002
FGSG_03543	Tri14		hypothetical protein	Brown et al., 2002; Dyer et al., 2005

Fig. 4.2 Schematic summary of trichothecene biosynthetic gene cluster in *Fusarium graminearum*. A diagram of the cluster shows all 12 trichothecene biosynthetic genes (*Tri* genes) modified according to (Rep and Kistler 2010). *Tri6* and *Tri10*, the two transcription factors regulating *Tri* genes are highlighted in *red*. Their functional annotation is summarized in the table together with the gene expression levels (*y-axis*) of all Tri genes in 198 samples representing 62 biological states (*x-axis*)

For instance, biosynthesis of aurofusarin ($C_{30}H_{18}O_{12}$), a secondary metabolite that contributes to the red/yellow pigment in fungal cultures, is controlled by a gene cluster harboring 10 genes, including two PKS genes and two transcription factor genes *aurR1* and *aurR2* (Malz et al. 2005).

4.2 Genetic Tractability

Fusarium graminearum can be easily cultured on a variety of media such as potato dextrose agar, carnation leaf agar, and carrot agar. Tractable genetic manipulation systems with remarkable phenotypic stability have been established for forward genetics screening for a genotype responsible for an observed phenotype, and for reverse genetics discovery of gene function by analyzing the phenotypic effects of manipulating a specific gene sequence.

Forward genetics depends on measurable phenotypic changes. Changing pathogenicity and reduced or lost toxin production are commonly used for screening genes of interest. Collectively, these studies identified and functionally characterized several genes in the trichothecene cluster (McCormick et al. 1996; Proctor et al. 1995a; Hohn et al. 1995, 1999; McCormick and Alexander 2002; Tag et al. 2001; Alexander et al. 1998, 1999; Brown et al. 2002). A gene cluster responsible for aurofusarin biosynthesis was also identified through random mutagenesis (Kim et al. 2005; Malz et al. 2005). The first large scale random mutagenesis study in *F. graminearum* was conducted using restriction enzyme-mediated integration (REMI) approach, which uncovered 11 genes associated with defective pathogenicity among 6,500 mutants (Seong et al. 2005). Subsequently, a high-throughput transposon-tagging mediated mutagenesis approach was developed in *F. graminearum* and identified 19 mutants (5.7 %) with altered phenotypes including novel pathogenicity genes (Dufresne et al. 2008).

Reverse genetics generates knockout mutants through homologous recombination. Applying reverse genetics to functionally characterize *F. graminearum* genes was greatly accelerated with the availability of the complete genome sequence. Many gene replacement vectors, containing some kind of selectable marker gene, the target gene sequence, or its flanking sequences, have been constructed for target gene disruption in protoplasts of filamentous fungi. After the transformation, homologous recombination events occur

between the wild-type target gene and the vector, which results in the replacement of the target gene with the marker gene. Initially, the insertion of the marker gene into the target gene depended on restriction enzyme digestion and subcloning DNA fragments onto plasmids. In recent years, subcloning-independent gene replacement approaches have emerged and triumphed. These methods typically use two or three rounds of PCR amplification, including overlapping PCR (Davidson et al. 2002), double-joint PCR (Yu et al. 2004), and split-marker (Catlett et al. 2003; Fairhead et al. 1996; Goswami 2011). In the split-marker deletion method, two constructs are required per transformation, each containing a flank of the target gene and roughly two-thirds of a selectable marker cassette. Homologous recombination between the overlapping regions of the selectable marker gene and between the flank regions and their genome counterparts results in a targeted gene deletion and replacement with an intact marker gene using only two rounds of PCR (Catlett et al. 2003; Goswami 2011). The ease of this procedure enables almost any molecular biology lab to generate constructs for targeted gene deletion within a few days. This approach is also ideal for high-throughput gene knockout studies and proves to be highly efficient for generating knockouts in a variety of filamentous fungi (de Hoogt et al. 2000; Fu et al. 2006; Li et al. 2011a; Wang et al. 2010; You et al. 2009) including *F. graminearum* (Son et al. 2011b; Wang et al. 2011a).

Fusarium graminearum transformation is usually mediated through either *Agrobacterium tumefaciens* (ATMT) (Malz et al. 2005) or polyethylene glycol (PEG) transformation (Proctor et al. 1995a). ATMT has been used to make knockout mutants in various fungal species with high efficiency. For ATMT, the marker gene and target gene or its flanking region are cloned into the T-DNA and transformed into *Agrobacterium tumefaciens*. Then, the fungal transformation is performed by co-incubation of the *A. tumefaciens* transformants with fungal hyphae or conidia. T-DNA carrying the gene disruption constructs is then introduced via *A. tumefaciens* infection, allowing the disruption of the target genes via homologous recombination. The transformed hyphae or conidia are subject to antibiotic selection and downstream PCR and Southern blot analysis. Alternatively, the gene replacement system can be introduced into *F. graminearum* through protoplasts mediated by polyethylene glycol (Goswami 2011). PEG-mediated transformation has been widely used in mutagenesis of *F. graminearum* with conidia and many other filamentous fungi with high efficiency. Efficiency of PEG transformation depends on the concentration and quality of protoplasts, DNA concentration, and linearity. Overall, comparing to ATMT transformation, procedures for PEG transformation tend to be simpler and more time-effective, while still able to generate large number of transformants.

4.3 Pathogenomics and Omics Approaches in the Postgenomic Era

Fusarium head blight diseases have been intensively investigated for nearly a century. Following the release of whole genome sequence of *F. graminearum*, different omics approaches have been applied to study both pathogen and its host and revealed new insights into infection biology and host resistance. This section will emphasize progress toward the understanding of *F. graminearum* pathogenomics. Research on genomics and genetics of host resistance to FHB has been reviewed previously (Bai and Shaner 2004; Bischof et al. 2011; Buerstmayr et al. 2009) and will not be discussed here.

4.3.1 Transcriptomics

The transcriptome is a collection of all transcripts in a cell or tissue at a given time. As a functional genomics approach, it provides a global view of transcript abundance in given cells or tissues under various environmental stimuli and developmental stages.

Conventionally, transcriptomics was investigated via microarray technology, where mRNAs isolated from biological samples are hybridized to DNA microarrays. A number of arrays have been reported. The first generation Affymetrix Genechip for *F. graminearum* was developed (Guldener et al. 2006) after the release of complete genome sequence of *F. graminearum*. The chip integrated around 14,000 genes of *F. graminearum* based on the genome annotation from the Broad Institute and Munich Information Center for Protein Sequences (MIPS). It was used to profile the fungal transcriptome *in vitro* and *in planta* with high sensitivity (Guldener et al. 2006) and provided a global view of transcriptional regulation during infection of wheat (Bernardo et al. 2007; Golkari et al. 2007; Jia et al. 2009; Lysoe et al. 2011b; Zhang et al. 2012) and barley (Boddu et al. 2006, 2007; Guldener et al. 2006). In the same year, multiple arrays with specific interest were developed to study the gene expression alteration in *mat1-2* mutant strain (Lee et al. 2006), and during perithecium development (Qi et al. 2006). Several other microarray chips based on ESTs or whole genome have been developed for transcriptome analysis of *F. graminearum* during different developmental and plant infection periods (Carapito et al. 2008; Golkari et al. 2007). Most microarray chips are made by Affymetrix using short oligonucleotide probes (25 mers), whereas chips designed by Agilent employing longer probes (60 mers) are also available (Becher et al. 2011). Many of these microarray-based transcriptomic data are available at PLEXdb (www.plexdb.org), a public database currently containing over 200 samples covering 19 different *F. graminearum* microarray experiments of more than 60 data points. With the advancement of array technology, a new Affymetrix exon array that contains nine plant pathogenic fungal genomes and tiling probes for the *F. graminearum* genome was produced, enabling the comparative study across multiple species (www.plexdb.org). The nine selected genomes are *F. graminearum, F. oxysporum* f.sp. *lycopersici, F. verticillioides, F. solani, Ustilago maydis, Puccinia graminis, Magnaporthe oryzae, Pyrenophora tritici-repentis,* and *Verticillium dahliea.*

With rapid technical advancement and drastic cost reduction of various sequencing technologies, sequencing entire transcripts (RNA-seq) has become a fast and cost-effective replacement of microarray technology. Compared to microarrays, RNA-seq enables digitalized quantification of transcripts in almost any given biological sample. It is more sensitive and has no dependency on a reference genome. At the time of this review, two *F. graminearum* transcriptome analysis using RNA-seq have been reported for studying alternate splicing (Zhao et al. 2013) and for comparative studies of perithecium formation in *F. graminearum* and *F. verticillioides* (Sikhakolli et al. 2012). Taking advantage of this new development, many laboratories have generated or are generating RNA-seq data. As will be described in later sections, these transcriptomic analyses have enriched our understanding of *F. graminearum* biology and pathogenesis, providing theoretical guidance for managing disease and controlling mycotoxin production.

4.3.2 Proteomics

Proteomics seeks to capture information on the entire compliment of proteins in an organism. Affected by posttranscriptional regulation or modification, the abundances of a transcript and its protein product are not always well correlated. Complementary with transcriptomic analyses, proteomics provides a more accurate measure of active cellular processes. The method starts with isolation and purification of total proteins, which are then typically separated by liquid chromatography or by 2D electrophoresis gels (e.g., PAGE gel). Protein fractions are then digested and characterized using mass spectrometry (MS) methods, such as Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) and electrospray ionization mass spectrometry (ESI-MS), etc. Peptide mass to charge ratios determined from MS spectra are then searched against predicted proteins from the genome analysis. Several

F. graminearum proteome studies have been reported (Kwon et al. 2009; Lee et al. 2008b; Paper et al. 2007; Phalip et al. 2005; Rampitsch et al. 2010, 2012; Taylor et al. 2008). Most studies focus on research topics, including secretomes/exoproteomes and phosphoproteomes. Secretome studies have identified secreted proteins species, such as cell wall degrading enzymes (CWDEs) and effectors that are likely to play critical roles in fungal virulence (Brown et al. 2012; Paper et al. 2007; Phalip et al. 2005; Yang et al. 2012). Phosphoproteomics of *F. graminearum* revealed important posttranslational modification processes, such as phosphorylation and ubiquitination (Rampitsch et al. 2010, 2012).

4.3.3 Sexual Reproduction

Fusarium graminearum is the only homothallic species in the genus *Fusarium.* Its sexual reproduction plays a pivotal role in disease manifestation of *F. graminearum*. Understanding molecular mechanisms underpinning sexual reproduction, especially perithecia and ascospore development, is an important area in *F. graminearum* research. The *F. graminearum* genome encodes both the *MAT1-1* and the *MAT1-2* loci, whereas all other fertile *Fusarium* species are heterothallic harboring either *MAT1-1* or *MAT1-2* and strains with differing *MAT* genes that are sexually compatible. The homothallic lifestyle likely evolved in the *F. graminearum* lineage from a self-sterile ancestor, potentially providing advantage in establishing infection without the dependency of searching for a mating partner (Ikeda 2010). Interestingly, population surveys have revealed low levels of gametic disequilibrium (Gale et al. 2002), suggesting that outcrossing is a mechanism to maintain population diversity.

Sexual reproduction of *F. graminearum* requires the production of a pheromone and its recognition by a pheromone receptor, a process controlled by the mating-type (MAT) locus and the MAPK cascade, a major signaling pathway orchestrating the sexual reproduction in *F. graminearum* (Kim et al. 2008; Lee et al. 2008a). Upon pheromone binding to receptors, heterometric G proteins are activated, which then activates the MAPK pathway. The importance of the MAPK cascade has long been known in pheromone sensing and mating in yeast and filamentous fungi, such as *M. oryzae* and *N. crassa*.

Microarray analyses of *F. graminearum* during different sexual development stages showed that temporal gene expression changes occur during the development of perithecia (Qi et al. 2006; Hallen et al. 2007). Genes involved in metabolism and cell differentiation are highly expressed in developing immature perithecia, whereas genes responsible for cellular transport such as ion transport genes are highly expressed in mature perithecia (Qi et al. 2006; Hallen et al. 2007). The fact that genes involved in metabolism and cell differentiation are highly transcribed at early perithecial development, but downregulated in the mature perithecia stage indicates that the fungus undergoes substantial physiological and developmental changes and tight regulation of these processes during perithecium formation and ascospore development.

Table 4.2 includes genes known to be involved in *F. graminearum* sexual reproduction, including an F-Box protein *FBP1* (Han et al. 2007), a calcium ion channel protein *CCH1* (Hallen and Trail 2008), a siderophore synthetase gene *NPS2* (Oide et al. 2007), two chitin synthase genes *GzCHS5* and *GzCHS7* (Kim et al. 2009), a protein kinase gene *GzSNF1* (Lee et al. 2009), *ROA* (Min et al. 2010), a cyclin C-like gene *CID1* (Zhou et al. 2010), velvet proteins *FgVeA* (Jiang et al. 2011a) and *FgVelB* (Lee et al. 2012), a histone deacetylase gene *HDF1* (Li et al. 2011b), ATP citrate lyase *ACL* (Son et al. 2011a), transcription factors *MYT1* (Lin et al. 2011), *MYT2* (Lin et al. 2012), *FgStuA* (Lysoe et al. 2011a), and *ZIF1* (Wang et al. 2011b), and phosducin-like protein *BDM1* (Horevaj and Bluhm 2012).

Table 4.2 Summary of *Fusarium graminearum* genes functionally characterized recently

Genes	Annotation	Mutant phenotypes	Disease	Reference
FSR1	WD repeat protein	Female infertility, deterred perithecia formation	◎	(Shim et al. 2006)
RAS2	Ras GTPase	Slow growth, delayed conidia germination	◎	(Bluhm et al. 2007)
SID1	Siderophore synthase	Poor growth at low-iron medium	◎	(Greenshields et al. 2007)
FBP1	F-box protein	Perithecia absent	◎	(Han et al. 2007)
MES1	Hypothetical protein	Reduced conidiation, cell wall deposition	◎	(Rittenour and Harris, 2008)
GzGPA2	G protein α subunit	Increased chitin accumulation	◎	(Yu et al. 2008)
FTL1	Transducin-beta like protein	Reduced conidiation	○	(Ding et al. 2009)
FgTep1	Tensin-like phosphatase 1	Mycelia sensitive to lithium, reduced conidiation and germination	◎	(Zhang et al. 2010)
CID1	Cyclin C-like protein	Reduced growth and conidiation, increase pigmentation Reduced DON, female infertility	◎	(Zhou et al. 2010)
FgVeA	Velvet gene	Reduced aerial hyphae, hydrophobicity and DON production, increased conidiation and delayed germination	◎	(Jiang et al. 2011a)
FgRrg-1	Response regulator protein	Increased sensitivity to osmotic stress and fungicides	◎	(Jiang et al. 2011b)
FgPtc3	Type 2C protein phosphatase	Reduced aerial hyphae and DON, increased conidiation, increased resistance to osmotic stress	–	(Jiang et al. 2010)
HP1	Heterochromatin protein	Increased aurofusarin, decreased DON,	–	(Reyes-Dominguez et al. 2012)
HDF1	Histone deacetylase	Reduced DON, defective sexual reproduction and conidiation	◎	(Li et al. 2011b)
FgATG15	Autophagy-related lipase	Aberrant conidia shapes, reduced storage lipid degradation under N starvation	◎	(Nguyen et al. 2011)
EBR1	Zn_2Cys_6 transcription factor	Reduced radial growth, disrupted hyphal apical dominance	◎	(Zhao et al. 2011)
BDM1	Phosducin-like protein	Abnormal conidia germination and hyphal morphology. Reduced DON	◎	(Horevaj and Bluhm 2012)
FgVelB	Velvet gene	Reduced aerial hyphae and hyphal hydrophobicity, highly increased conidiation, increased resistance to osmotic stress, reduced DON production	◎	(Jiang et al. 2012)
FgERG4	Sterol C-24 reductase	Increased sensitivity to metal cations, increased resistance to cell wall degrading enzymes and sterol biosynthesis inhibitors, reduced DON	◎	(Liu et al. 2012)
FgOS-2	Protein kinase	Reduced *in planta* DON and ZEA production, higher *in vitro* DON production, defective sexual reproduction	◎	(Van Thuat et al. 2012)

(continued)

Table 4.2 (continued)

Genes	Annotation	Mutant phenotypes	Disease	Reference
AMT1	Arginine methyltransferase	Slightly reduced vegetative growth, increased resistance to oxidative stress, reduced DON	◎	(Wang et al. 2012)
FgStuA	Fungal transcription factor	Reduced spore production, perithecia absent, conidiophores and aberrant macroconidia, reduced DON	◎	(Lysoe et al. 2011a)
FGL1	Lipase	Decreased extracellular lipolytic activity	◎	(Voigt et al. 2005)
GPA1	G protein α subunit	Defective sexual reproduction, increased DON and ZEA	●	(Yu et al. 2008)
MAP1	MAP kinase	Defective sexual reproduction Reduced DON	○	(Urban et al. 2003)
GMPK1	MAP kinase	Reduced conidiation, defective sexual production	○	(Jenczmionka et al. 2003)
MGV1	MAP kinase	Defective sexual reproduction, self-incompatible, reduce DON production	○	(Hou et al. 2002)
Fgp1	Wor1 like protein	No DON production, abnormal asexual and sexual spore development	○	(Jonkers et al. 2012)
ZIF1	b-ZIP transcription factor	Reduced DON production, defective sexual reproduction	◎	(Wang et al. 2011b)
Tri12	Major facilitator super family protein	Reduced DON production, reduced radial growth on trichothecene induction medium	◎	(Menke et al. 2012)

○ Nonpathogenic; ◎ reduced virulence; ● full virulence;—not available

4.3.4 Host–Pathogen Interaction

To overcome plant defense, pathogens such as *F. graminearum* probably have evolved an effective and powerful arsenal to establish infection. Like many filamentous fungi, environmental cues are typically sensed by G protein coupled receptors (GPCRs) on the cell membrane, which subsequently activate downstream intracellular signaling pathways. A total of 84 *F. graminearum* GPCRs have been predicted (Ma et al. 2010) and several of them are upregulated in the early stages of wheat infection (Zhang et al. 2012). The signals are then quickly passed to G proteins and the MAPK pathway (Hou et al. 2002). Penetration into the plant tissue is a critical step during early infection. The *F. graminearum* genome is enriched for cutinase genes, which may enable the pathogen to degrade the cuticle and penetrate the plant epidermis. Indeed, these genes are upregulated during infection of barley (Cuomo et al. 2007). *F. graminearum* is also likely to produce several lipases that dismantle the long-chain fatty acids found in cuticle layers to assist cuticle degradation. In fact, Voigt et al. (2005) showed that a secreted lipase *FGL1* by *F. graminearum* is essential for virulence on both wheat and maize. This lipase *FGL1* along with MAPK*GPMK1* is also under transcriptional regulation by the *RAS2* protein in *F. graminearum* (Bluhm et al. 2007). The process is clearly orchestrated by the MAPK cascade and signal RAS proteins.

The mycotoxin DON, is a known virulence factor for *F. graminearum* infection of wheat and barley, as DON nonproducing mutants typically have attenuated disease symptoms (Proctor et al. 1995a). However, DON production is not required for successful penetration, since

Tri5 mutants can still form appressoria-like structures (Boenisch and Schafer 2011) like the wild-type strain. Using a Tri5-GFP-tagged *F. graminearum* strain, the expression level of *Tri5* was monitored to evaluate the induction of trichothecene pathway during *F. graminearum* colonization of wheat heads (Ilgen et al. 2009). It was found that no or very low level of expression of DON biosynthesis gene *Tri5* was observed in infected anthers where the initial colonization occurs. In contrast, *Tri5* was highly expressed in rachis node, where *F. graminearum* uses biosynthesized DON to destroy this major physical barrier, so that the fungal growth is spread throughout the whole spikes and heads (Ilgen et al. 2009). This suggests that DON is produced in a tissue-specific manner during *F. graminearum* infection of wheat heads.

Large-scale functional analyses have been conducted on the entire predicted set of transcription factors (Son et al. 2011b) and protein kinases (PK) (Wang et al. 2011a). The phenotypes of the 657 transcription factor mutants are documented at the *F. graminearum* transcription factor phenotype database (FgTFPD) (http://kropbase.snu.ac.kr/cgi-bin/Fusarium/Fusarium_main.cgi). Among the 657 transcription factors analyzed analyzed, 170 showed phenotypic changes in the deletion mutant, including 73 for mycelial growth, 41 for pigmentation, 105 for sexual reproduction, 69 for ZEA production, 55 for DON production, 41 for conidiation, 62 for virulence, and 49 for stress response. In total, 42 (of 116) PK genes (Wang et al. 2011) are associated with *F. graminearum* virulence and mutants of 22 PK genes, including MAPK genes Gpmk1, Mgv1, and CPKA gene are nonpathogenic or defective in colonizing the plants.

Table 4.2 lists other genes regulating *F. graminearum* pathogenesis reported so far. Most of these genes are essential for full virulence or pathogenicity of *F. graminearum*. Many genes regulate asexual and sexual reproduction besides virulence, indicating normal sporulation and germination required for fungal virulence on plants. Remarkably, there is a strong correlation between defects in DON production and virulence in many of these mutants, confirming the importance of DON in disease symptom development in head blight. In fact, according to the FgTFPD, 35 transcription factor mutants are defective in both virulence and DON production (Sun et al. 2011b). In addition, mutants that have increased sensitivity to environmental stresses, such as oxidation, heavy metals, and antifungal compounds tend to have attenuated virulence as these mutants are likely to be vulnerable to the plant defense actions (Table 4.2).

Fusarium graminearum gene expression is dynamically regulated along the infection course and genes differentially expressed *in planta* are significantly enriched in regions high in single nucleotide polymorphisms (SNPs) in the genome. Over 70 % of them are unique to *F. graminearum* and have unknown functions (Lysoe et al. 2011b). Zhang et al. (2012) investigated *F. graminearum* transcriptomics during infection of wheat coleoptiles at different time points combining laser capture microdissection and microarray analyses, which provided transcriptional regulation at single cell-type resolution (Zhang et al. 2012). The study identified 344 genes differentially expressed during fungal invasive growth *in planta*. Among those, 134 genes encode putative CWDE. These genes exhibited a unique expression profile, depicting increased expression levels at 16 and 64 h after inoculation (HAI), respectively. These two time points correlate to fungal intercellular growth at 16 HAI and at 64 HAI when the infection transits into a necrotrophic stage. Interestingly, the second increase at 64 HAI is much higher suggesting extensive cell wall degradation involved in fungal necrotrophic growth. No DON biosynthesis genes are induced during the coleoptile infection suggesting that DON might not be required for the coleoptile infection. However, an unknown secondary metabolite gene cluster, FG3_54 predicted through genomic study (Ma et al. 2010), was induced at 64 HAI, which may produce an unknown toxin that contributes to the late stage of fungal infection (Zhang et al. 2012).

Transcriptomic studies on knockout mutants provide information on regulatory network rewiring after removing targeted genes (Baldwin

et al. 2010a; Gardiner et al. 2009; Hallen and Trail 2008; Jonkers et al. 2012; Lee et al. 2011; Seong et al. 2009). Transcriptomic analyses of mutants of *tri6* and *tri10*, the two transcription factors encoded in the *Tri* gene cluster, revealed that in addition to genes in the trichothecene biosynthesis gene cluster, these two transcription factors also regulate many other genes, including isoprenoid biosynthesis, disease and virulence, ABC transporter expression, and secondary metabolism (Seong et al. 2009). Jonkers et al. (2012) applied microarray analysis on the deletion mutant of Fgp1 (a Wor1-like protein) and showed that Fgp1 positively regulates *Tri* cluster, butenolide cluster, and NPS8 cluster genes during plant infection (Jonkers et al. 2012).

4.3.5 Molecular Detection and Quantification

Importantly, wheat plants infected with *F. graminearum* may be asymptomatic but still contaminated by mycotoxins (Sinha and Savard 1997). Therefore, it is imperative to be able to detect *F. graminearum* in a timely manner and to quantify mycotoxins in harvested crops beyond morphological identification. For host–pathogen interaction and host resistance studies, it is also valuable to be able to monitor the colonization progression and to quantify fungal biomass. Many molecular detection approaches were developed to quickly and accurately detect *F. graminearum*, including PCR (Bluhm et al. 2002; Li et al. 2005; Niessen et al. 2004; Niessen and Vogel 1998; Wang et al. 2008), real-time PCR (Atoui et al. 2012; Bluhm et al. 2004; Brandfass and Karlovsky 2008; Halstensen et al. 2006; Horevaj et al. 2011; Moradi et al. 2010; Nielsen et al. 2012; Reischer et al. 2004), LAMP (loop-mediated isothermal amplification) (Abd-Elsalam et al. 2011; Niessen and Vogel 2010), and oligonucleotide microarrays (Kristensen et al. 2007b; Nicolaisen et al. 2005). With the available genome sequence, molecular detection can be applied to any given gene of interest. So far genes used for PCR detection include *gaoA* (galactose oxidase) (Wilbert and Kemmelmeier 2003) and trichothecene and zearalenone biosynthesis genes (Bluhm et al. 2002; Horevaj et al. 2011; Nielsen et al. 2012; Niessen and Vogel 1998).

Standard PCR methods are unable to quantify fungal DNA and biomass in plant materials. In the past decade, real-time PCR has been favorably used for both detection and quantification owing to its high accuracy, sensitivity, and speed and it was embraced to detect and quantify *F. graminearum* (Atoui et al. 2012; Bluhm et al. 2004; Burlakoti et al. 2007; Demeke et al. 2010; Dyer et al. 2006; Horevaj et al. 2011; Nielsen et al. 2012; Reischer et al. 2004). There are two major real-time PCR technologies: *TaqMan* and SYBR Green, reviewed previously (Heid et al. 1996; Rebrikov and Trofimov 2006; Wong and Medrano 2005). Similar to conventional PCR, real-time PCR uses gene-specific primers to amplify the target gene. Florescence tags are amplified through every cycle, offering a real-time quantification of the PCR product. Oligonucleotide array (Kristensen et al. 2007b; Nicolaisen et al. 2005) and SNP approaches (Kristensen et al. 2007a) were developed to differentiate *F. graminearum* from other closely related *Fusarium* species. The array hybridization results can be visualized using a colorimetric Silverquant for easy detection (Kristensen et al. 2007b).

4.4 Systems Biology and Future Perspectives

A *F. graminearum* protein–protein interaction (FPPI) database that interconnects 7406 proteins into 223,166 protein–protein interactions was created combining interaction-ortholog and domain–domain interaction (DDI) algorithms (Zhao et al. 2009). Based on this framework and utilizing differential gene expression before and after infection, pathogenicity-related subnetworks were constructed centered on several seed pathogenicity genes in the core FPPI and (Liu et al. 2010).

Fusarium graminearum candidate regulatory motifs were identified by systematically searching for evolutionarily conserved regulatory motifs (Kumar et al. 2010). These studies established a foundation to investigate global regulatory networks in the system.

The next wave of pathogenicity-related studies could focus on identifying and characterizing *F. graminearum* effectors. Comparative proteomic analyses identified secreted proteins during the *F. graminearum* infection process (Paper et al. 2007; Yang et al. 2012), including cell wall and starch degrading enzymes. Secretome studies identified more potential novel effectors (Brown et al. 2012), many of which are *F. graminearum* specific (Brown et al. 2012). Information on these predicted secreted proteins provides a foundation for future experimental studies to verify their function in pathogenesis.

Fusarium graminearum could also be developed into a model system to study genetic mechanisms that control genome stability. Repeat Induced Polymorphisms (RIP) can effectively mutate repeated DNA in the genome. However, RIP, as first discovered in the *N. crassa* genome, only introduces mutation in the repeats with sequence identity above 80 %. The *N. crassa* genome is littered with low sequence identity repeats below 80 %. The phenomenon of almost complete elimination of repeats in the *F. graminearium* genome suggests the existence of additional mechanism(s) for removing repeats. Understanding genetic mechanisms that control the invasion or expansion of repeats will enhance our understanding of genome stability.

In summary, *F. graminearum* genomics has provided detailed genetic information about this destructive fungus. Improved genomic and functional annotations, detailed phenomics and enriched transcriptomics and proteomics data will open up new possibilities for applied systems biology approaches. In the near future, such approaches will enhance investigations of the regulatory networks essential to comprehend cellular functions and pathogenesis.

Acknowledgment This chapter is dedicated to Dr. H. Corby Kistler, an inspirational mentor and colleague, and an excellent *Fusarium* biologist who embraces the power of genomics. The authors would like to thank Dr. Jon Hulvey for critical reading of the manuscript and offering constructive suggestions. LG and LJM are grateful for the support of United States Department of Agriculture, National Institute of Food and Agriculture Grant awards MASR-2009-04374, MAS00441. LJM was also supported by United States Department of Agriculture, National Institute of Food and Agriculture Grant awards 2008-35604-18800 and 2008-35600-04691.

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The Genomes of *Mycosphaerella graminicola* and *M. fijiensis*

5

Stephen B. Goodwin and Gert H. J. Kema

5.1 Introduction

Mycosphaerella graminicola is an economically important pathogen of wheat with a worldwide distribution. It causes Septoria tritici blotch (STB) disease (Fig. 5.1a), named after the anamorph stage of the fungus. Losses to STB can be very high, up to 30–50 % during severe epidemics (Eyal et al. 1987). The disease is particularly prevalent during cool, wet weather, but also occurs in drier climates and is present in most wheat-growing areas every year (reviewed recently in Ponomarenko et al. 2011). Management of the disease is by incorporation of host resistance into wheat cultivars or, when economically feasible, by spraying with fungicides. Although fungicides can be very effective, a major problem is that populations of *M. graminicola* rapidly develop resistance, which leads to loss of control. For example, resistance to the strobilurin fungicides developed within 5 years after they were used commercially (Fraaije et al. 2005a, b) and, in many fields, populations of the pathogen are now nearly 100 % resistant (Torriani et al. 2009; Ware 2006).

Many genes for resistance to STB occur in wheat and can provide an economical form of disease management. So far, 16 major genes (reviewed in Goodwin 2007, 2012; Tabib Ghaffary et al. 2011, 2012) and numerous Quantitative Trait Loci (QTL) with smaller effects (Simon and Cordo 1988; Jlibene et al. 1994; Simón et al. 2004) have been identified and mapped in the wheat genome. However, tightly linked molecular markers have not been available until very recently, so relatively few resistance genes have been used in wheat breeding programs. Those that have been used often break down rapidly (Cowger et al. 2000) and have a limited efficacy, except for *Stb16* (Tabib Ghaffary et al. 2012). Thus, deployment of single, major genes for resistance is not likely to be an effective strategy for long-term disease management. Use of QTL for STB resistance ultimately may be more durable and successful, but progress so far has been slow. High genetic variability within populations of the pathogen, rapid evolution of fungicide resistance, and limited effectiveness of most major resistance genes all contribute to making STB a persistent and economically challenging disease.

Mycosphaerella fijiensis causes black Sigatoka or black leaf streak disease on banana (Fig. 5.1b). This disease was first found in the Sigatoka Valley of Fiji during 1963 (Stover 1976) but probably originated in the New Guinea-

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R. A. Dean et al. (eds.), *Genomics of Plant-Associated Fungi: Monocot Pathogens*,
DOI: 10.1007/978-3-662-44053-7_5, © Springer-Verlag Berlin Heidelberg (outside the USA) 2014

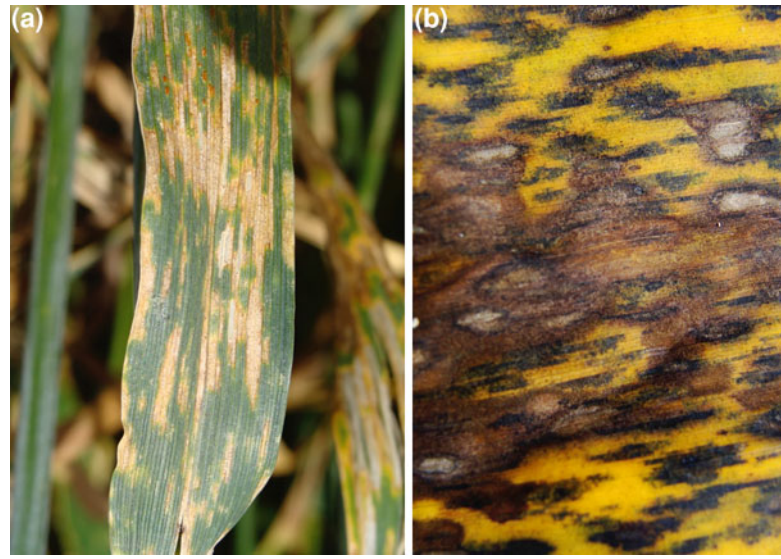

Fig. 5.1 Disease symptoms of **a** Septoria tritici blotch caused by *M. graminicola* (synonym: *Z. tritici*; asexual stage: *S. tritici*) on wheat and **b** Black Sigatoka or leaf streak caused by *M. fijiensis* on banana

Solomon Islands area of Southeast Asia, and has since spread to many banana-producing areas worldwide (Stover 1978). This pathogen is much more aggressive and is rapidly displacing *M. musicola*, the cause of yellow Sigatoka, which had been a major pathogen of banana prior to the emergence of black Sigatoka and still occurs in some areas (Stover 1978; Arzanlou et al. 2007). Damage due to black Sigatoka can be very high resulting in near-complete crop loss if the disease is not managed. Control of this disease is primarily by fungicides, which often must be applied over 60 times per year to provide sufficient control (Ganry et al. 2012). Unfortunately, as with *M. graminicola* on wheat, this heavy spray schedule provides strong selection and resistance develops rapidly leading to loss of fungicide effectiveness (Romero and Sutton 1996).

Genes for host resistance against black Sigatoka occur very rarely in cultivated banana and are of little practical significance due to the difficulty of moving them into new cultivars, which are triploid and sterile. Virtually, all commercial production of bananas worldwide comes from a handful of cultivars, all of which are very susceptible to black Sigatoka and are grown in huge, clonal monocultures. The lack of resistance coupled with clonal propagation of a perennial crop in tropical climates where conditions can be favorable for infection for most of the year make black Sigatoka caused by *M. fijiensis,* one of the two most important diseases of banana globally. Management of this disease can add up to 27 % of the total cost of production due to purchase and frequent application of expensive fungicides (Marín et al. 2003).

Taxonomically, both Mycosphaerella species are in the family Mycosphaerellaceae, order Capnodiales of the class Dothideomycetes. This class is one of the largest in the fungal kingdom, both for number of species and for biological and ecological diversity. The Mycosphaerellaceae appears to be a very old family that is derived from a species with a Mycosphaerella teleomorph. Evolutionary distances between subgroups within this family are very large (Goodwin et al. 2001; Schoch et al. 2009), equivalent to those between orders in other fungal groups and many are now being renamed as other genera. Some of the anamorph genera that are related to *M. graminicola* and *M. fijiensis* include Septoria and Cercospora, both of which are very large with hundreds or thousands of species. In total, over 40 anamorph names have been associated with species in the Mycosphaerellaceae, many of which probably will be elevated to holomorphs under the new rules for fungal nomenclature (Hawksworth 2011).

The branch of the Mycosphaerellaceae phylogenetic tree that leads to *M. graminicola* contains other grass pathogens but no pathogens of dicots (Goodwin et al. 2001; Quaedvlieg et al. 2011), presumably reflecting a long evolutionary history on Poaceous hosts. In contrast, *M. fijiensis* is on a branch that includes many other banana pathogens, such as *M. musicola* and *M. eumusae* (Arzanlou et al. 2008), most likely due to a recent radiation following an initial adaptation to Musaceous hosts. Other well-known plant pathogens in the Dothideomycetes include those in the order Pleosporales, which contains the genera Phaeosphaeria, Leptosphaeria, Alternaria, Cochliobolus, and Pyrenophora, among many others, some of which will be discussed in other chapters.

Due to their ancient origin and separate evolutionary histories, species of Mycosphaerella with Septoria-like anamorphs and related anamorphic species of Septoria were recently renamed in the genus Zymoseptoria (Quaedvlieg et al. 2011). Therefore, *M. graminicola* and its anamorph *S. tritici* are now combined under the single name *Z. tritici*. Since both the mitochondrial (Torriani et al. 2008) and nuclear (Goodwin et al. 2011) genomes were published under the old name, we will continue to use *M. graminicola* here to avoid confusion with the previous publications, although this name is now a synonym for *Z. tritici* (Quaedvlieg et al. 2011). So far *M. fijiensis* remains a Mycosphaerella, although it seems likely that it will be renamed under its anamorph Pseudocercospora at some time in the future.

The life cycles of *M. graminicola* and *M. fijiensis* are similar, even though their hosts and the climactic zones in which they live differ greatly. The sexual stage for both species is very important and ascospores often provide the primary inoculum for epidemics. For *M. graminicola*, survival during the host-free period is on infected wheat residues from the previous season's crop, which occurs either during the summer for winter wheat or over winter for the spring crop. Ascospores are forcibly ejected from the asci and can travel for moderate distances (Shaw and Royle 1989; Fraaije et al. 2005a, b) before encountering and infecting wheat seedlings soon after their emergence in the spring or fall. Primary inoculum also could be asexual pycnidiospores produced on infected wheat stubble but, as these are exuded in a gelatinous matrix and are splash dispersed, their effective dispersal range may be more limited and ascospore inoculum is thought to be the most common for initial infections (Shaw and Royle 1989).

Following initial infection, amplification of the pathogen occurs primarily by rain-splash dispersal of pycnidiospores to new locations on the same or nearby plants. However, when individuals of opposite mating types meet in lesions, additional ascospores can be produced on the living hosts, so spread during epidemics is by a combination of ascospores and pycnidiospores (Kema et al. 1996a). The importance of ascospores and/or pycnidiospores produced on the previous season's wheat residues during the growing season is not known but is believed to be low as the pathogen is not a strong saprobe and will not persist long as those residues decay. The disease cycle of black Sigatoka is similar except there is no host-free period during banana cultivation, and the spread of *M. fijiensis* appears to be primarily by ascospores (Stover 1980). The asexual (Pseudocercospora) stage of *M. fijiensis* is known but is found only rarely, so epidemics are driven primarily by sexual reproduction.

Both *M. graminicola* and *M. fijiensis* infect through stomata without producing appressoria or other specialized structures for penetration (Duncan and Howard 2000; Kema et al. 1996b), although appressorium-like hyphal thickenings can be produced near the stomatal pores. Following entry into and colonization of the substomatal cavities of susceptible hosts, growth is intercellular for days (*M. graminicola*) or weeks (*M. fijiensis*) without triggering effective host-defense responses. During this period, the pathogens survive off of living host cells, so growth is thought to be biotrophic, although there is some disagreement (Keon et al. 2007) and the true mode of nutrition during this growth stage is not known for certain. The end of the biotrophic phase is signaled by rapid cell death in susceptible hosts as the pathogen switches to

necrotrophic growth, presumably obtaining nutrition from the newly killed host tissue. This switch from biotrophic to necrotrophic lifestyle may involve the production of toxins, although their existence has not been proven conclusively for either fungus (Kema et al. 1996a; for *M. fijiensis* reviewed in Churchill (2011)). At the end of the necrotrophic phase, which can last a week or more, both fungi switch into reproductive mode, producing either ascospores if mated with a suitable partner, or pycnidiospores, which continue the disease cycle.

Tools for molecular and genetic analyses of both species exist and, in many ways, *M. graminicola* is becoming a model for the genetics of fungi in the Dothideomycetes. Both species have bipolar, heterothallic mating systems (Kema et al. 1996a; Arzanlou et al. 2010), although *M. fijiensis* also contains two fused mating-type loci that apparently are not functional and are not found in *M. graminicola* (Waalwijk et al. 2002; Arzanlou et al. 2010). For both species, individuals of opposite mating types must fuse to initiate sexual reproduction. Crossing of both species is possible but so far occurs only on the host plants for *M. graminicola*. To make a genetic cross with *M. graminicola*, strains of opposite mating type are co-inoculated onto a susceptible wheat genotype and placed in a greenhouse until symptoms develop (Kema et al. 1996a). The plants containing lesions are then moved outside and monitored for ascospore release, which typically begins within 5–7 weeks after the plants are placed outside. Exactly what triggers mating and ascospore formation—differences in light, temperature, humidity, or other factors—is not known and, so far, crosses cannot be conducted in vitro or solely in a greenhouse. For *M. fijiensis*, crossing is done in vitro by transferring spermatia from one isolate into a culture of a partner of opposite mating type (Mourichon and Zapater 1990). Although this process can be very efficient between fertile isolates leading to a high rate of success generating many progeny isolates (Mourichon and Zapater 1990), *in planta* crossing with a protocol similar to that used for *M. graminicola* also generally works well (Manzo-Sánchez et al. 2008).

Progeny from these in vivo and in vitro crossing protocols have been used to develop genetic maps for both species. The first genetic map for *M. graminicola* was published during 2002. It contained 280 AFLP and RAPD markers plus mating type and virulence in 23 linkage groups covering 1,216 cm (Kema et al. 2002), and was augmented subsequently with 23 microsatellite loci (Goodwin et al. 2007). The most recent update to the map added Diversity Arrays Technology (DArT) markers, to yield a final, high-density map containing 1,793 DArT, 258 AFLP, and 25 microsatellite loci (Wittenberg et al. 2009). This map was extremely useful for understanding the inheritance of pathogenicity and other traits, for assembling the genome sequence, and it confirmed and extended the discovery that eight of the *M. graminicola* chromosomes are dispensable.

The genetic map for *M. fijiensis* consists of 298 AFLP and 16 microsatellite loci plus mating type organized into 23 linkage groups (Manzo-Sánchez et al. 2008). Genetic size of the map was estimated to be 1,879 cm, and it was predicted that the total haploid genome size for *M. fijiensis* would total 4,298 cm. A similar map was used to assist assembly of the genome sequence and now is being used to map pathogenicity and other traits of interest. For both *M. graminicola* and *M. fijiensis*, one of the parents of each respective genetic map was chosen for sequencing of the reference genomes, which greatly aided both the sequencing and the mapping projects. The other parent for each mapping population of both species was later resequenced, giving a complete picture of the parental contributions to each progeny set.

Techniques for genetic transformation of both fungi are available but the frequency of homologous recombination usually is low (Zwiers and De Waard 2001) and strongly depends on

the gene under study (Mehrabi 2006). For *M. graminicola,* this problem has been overcome for the most part by knocking out the *ku70* gene (Bowler et al. 2010); frequencies of homologous recombination of transformants into a *ku70* disruption strain can be as high as 95 % with no obvious effects on wild-type growth. This makes high-throughput transformation for functional analysis of genes in this species feasible. So far, many genes have been knocked out in different laboratories around the world (Choi et al. 2011a, b; Marshall et al. 2011; Mehrabi et al. 2006a, 2009; Motteram et al. 2009) leading to a much better understanding of gene function in *M. graminicola*. Gene-knockout experiments have been less common for *M. fijiensis* (reviewed in Churchill 2011). Knocking out the *ku70* gene in *M. fijiensis* presumably would give the same boost to transformation efficiency as it does in *M. graminicola* but has not yet been accomplished (Churchill 2011).

Due to the long latent periods and specific conditions required for infection, artificial inoculations of both species onto their hosts can be cumbersome but are possible. For *M. graminicola*, inoculum can be prepared from liquid shake cultures or agar plates grown at low temperatures (16 °C) to induce yeast-like rather than filamentous growth (Mehrabi et al. 2006b). The resulting asexual propagules are then sprayed onto plants, which are kept at high humidity (∼95 %) for 2–3 days to allow for host penetration, followed by growth on greenhouse benches for 2–3 weeks until symptoms develop. Depending on the conditions, the first symptoms typically are seen as flecking on the leaves from 8–12 days after inoculation followed by necrosis and asexual sporulation beginning typically 14–21 days after inoculation. Conidia for *M. fijiensis* are much harder to produce and success strongly depends on the isolate under study. Hence, inoculation with fragmented mycelial biomass is an alternative that usually results in good disease development after 6–8 weeks. Methods for plant inoculations and resistance bioassays for *M. fijiensis* were reviewed recently (Churchill 2011), so will not be reiterated here.

5.2 Genome Structure

The genomes of both *M. graminicola* (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html) and *M. fijiensis* (http://genomeportal.jgi-psf.org/Mycfi2/Mycfi2.home.html) were sequenced in successive years through proposals submitted to the Community Sequencing Program (CSP) of the U.S. Department of Energy's Joint Genome Institute (JGI). A recent focus of the CSP has been on the Dothideomycetes, among other groups, and this has led to the sequencing and analysis of numerous fungi in this class in addition to *M. graminicola* and *M. fijiensis* (Ohm et al. 2012).

The basic approach for sequencing of both species was similar. Both species were sequenced with the Sanger method on plasmid libraries with insert sizes of approximately 2–3 and 6–8 kb and fosmid libraries averaging 35–40-kb insert sizes. These reads were then assembled into contigs and scaffolds. For *M. graminicola,* the resulting assembly was then finished through a combination of methods. Small gaps within scaffolds where the approximate size was known but the exact bases were not were filled by automated local alignments to identify the previously missing bases. To assemble the resulting scaffolds into chromosomes, two approaches were used. Sequencing from the ends of BAC clones was used to align and join scaffolds into super scaffolds. Finally, DArT marker sequences that were used to construct the genetic linkage maps were aligned with other mapped and sequenced molecular markers to position the scaffolds in their correct order and orientation. The remaining gaps between the scaffolds were then filled by a combination of automated gap filling and manual sequencing of PCR amplicons produced by primers designed at the ends of each scaffold.

The final result for *M. graminicola* was a finished genome of 21 chromosomes (Goodwin et al. 2011). All chromosomes are complete from telomere to telomere with the exceptions of two small internal gaps of unclonable DNA on chromosome 18 and one telomere of chromosome 21.

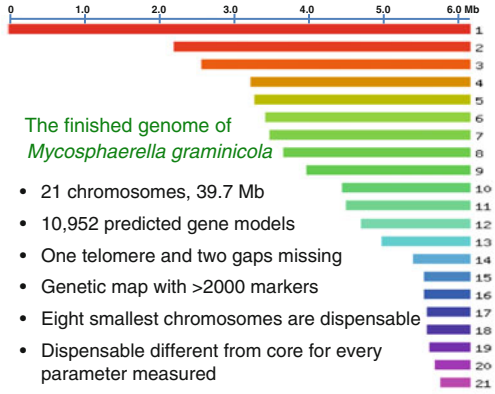

Fig. 5.2 Depiction of the 21 chromosomes by size and summary information about the finished genome of *M. graminicola* (synonym: *Z. tritici*; asexual stage: *S. tritici*). The smallest 8 chromosomes (14–21) are dispensable and collectively comprise the dispensome

These chromosomes range in size from approximately 0.4 to over 6 Mb and together code for over 10,900 genes (Fig. 5.2). The mitochondrial genome of *M. graminicola* also was obtained as a part of this project. It is a circular molecule of 43,960 bp and contains the 14 protein-coding genes that are most commonly associated with fungal mitochondrial genomes plus the large and small ribosomal RNA subunit genes and a full complement of tRNA genes sufficient to translate all 20 amino acids (Torriani et al. 2008).

An unusual feature of the *M. graminicola* nuclear genome is that it contains a large number of dispensable chromosomes (Goodwin et al. 2011). Previous genetic analyses had shown that entire linkage groups sometimes were missing from progeny isolates, presumably due to loss of one or more dispensable chromosomes (Wittenberg et al. 2009). However, the correct number of dispensable chromosomes and how they differed from the core chromosomes were not known. The genome sequence showed that all of the linkage groups that had been shown to be dispensable were present on the eight smallest chromosomes. These chromosomes were different from those of the core set for almost every parameter measured; they were the smallest, had the lowest gene densities, highest proportions of repetitive DNA (Fig. 5.3), lowest GC content (Fig. 5.4) and had different codon usages (Fig. 5.5) among many other statistics analyzed (Goodwin et al. 2011).

Dispensable chromosomes are relatively common in fungi but most species have only one or two, usually facilitating host adaptation or some other aspect of fitness (Covert 1998; Miao et al. 1991; Hatta et al. 2002; Coleman et al. 2009). However, those in *M. graminicola* had no obvious effects on fitness; individuals missing one or more dispensable chromosomes were still pathogenic on wheat, and all QTL for pathogenicity so far have been mapped only to core chromosomes (Ware 2006). Because they are lost so readily during sexual reproduction (Wittenberg et al. 2009), they must provide a selective advantage at some stage during the life cycle to explain their continued presence. Elucidating the function of the numerous dispensable chromosomes in the genome of *M. graminicola* is one of the many questions to emanate from the sequencing project.

The overall amount of repetitive DNA in the *M. graminicola* genome was moderate at around 12 % of the 39.7-Mb total, which puts it at about the middle for fungi in the Dothideomycetes (Ohm et al. 2012). Most of the repetitive fraction was composed of class I transposons, mostly retrotransposons with long terminal repeats (LTRs). Non-LTR retrotransposons also were present along with a lower amount of class II transposons. All of the repetitive sequences showed evidence of repeat induced point mutation (RIP), a mechanism in fungi for mutating and inactivating transposable elements (Galagan and Selker 2004). One interesting result from the RIP process occurred with a DNA methyl transferase gene that is involved with cytosine methylation. The original copy of that gene was on chromosome 6 (Dhillon et al. 2010). This copy somehow became amplified in the genome, most likely through an accidental copy and transfer to a subtelomeric region, which then was amplified through subsequent exchange among telomeres. These multiple copies became visible to the RIP machinery, which mutated and inactivated all copies including the original (Dhillon et al. 2010). This led to a loss of cytosine methylation in *M. graminicola*, which

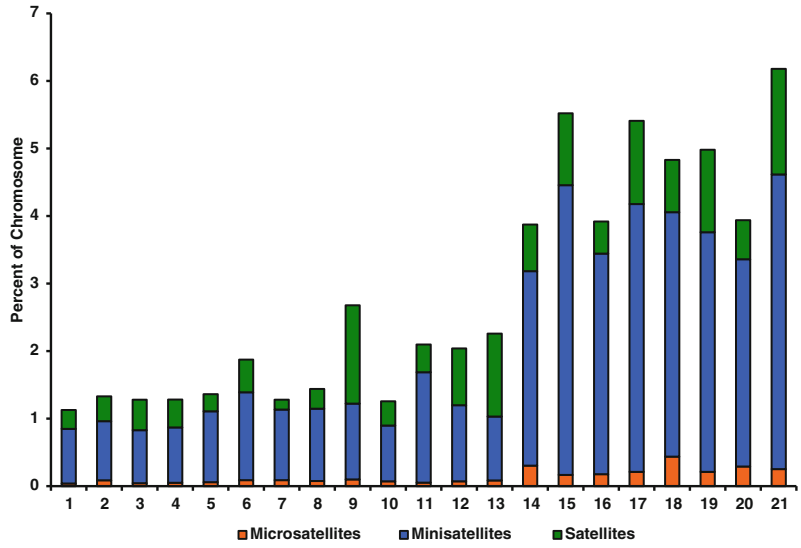

Fig. 5.3 The dispensable chromosomes (numbers 14–21) of *M. graminicola* (synonym: *Z. tritici*; asexual stage: *S. tritici*) contain higher proportions of repetitive DNA compared to the levels seen in the 13 core chromosomes (1–13)

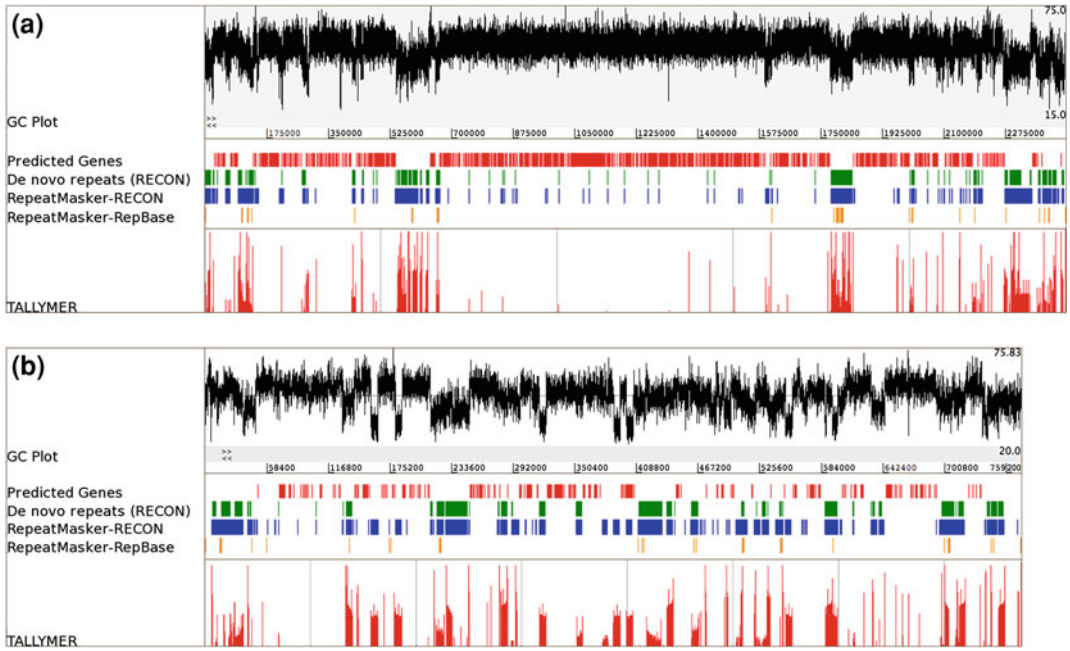

Fig. 5.4 Differences in GC and gene content between typical **a** core and **b** dispensable chromosomes of *M. graminicola* (synonym: *Z. tritici*; asexual stage: *S. tritici*). The dispensable chromosomes contain fewer genes at lower density with a lower GC content

was verified experimentally, and is the first known natural case of RIP leading to inactivation of a previously single-copy gene.

One of the most striking features of the *M. graminicola* genome sequence was a large reduction in the number of genes for cell wall

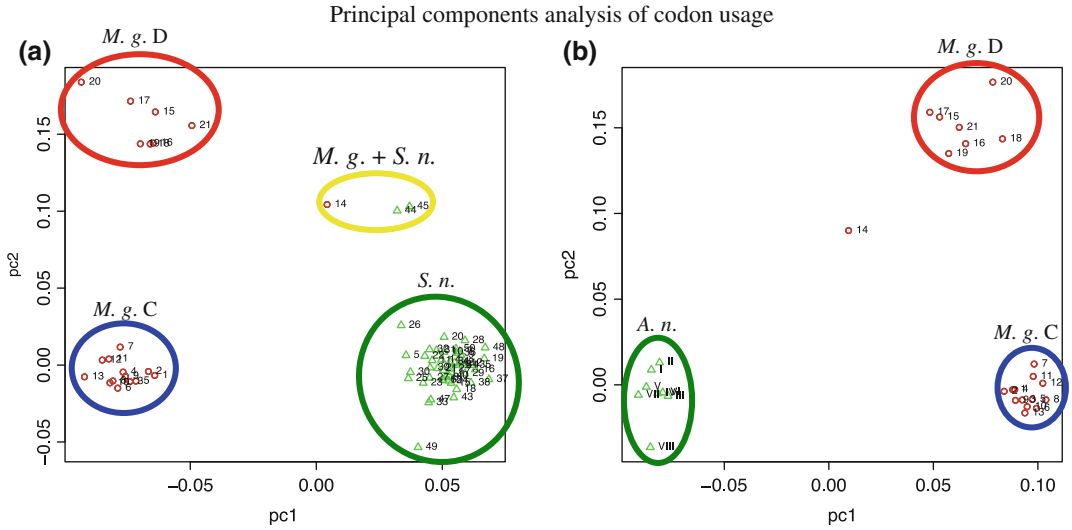

Fig. 5.5 Differences in codon usage between core and dispensable chromosomes of *M. graminicola* (synonym: *Z. tritici*; asexual stage: *S. tritici*) compared to those in related fungi. Combining the genomes of *M. graminicola* with those of **a** *Stagonospora nodorum* or **b** *Aspergillus nidulans* gave the same pattern as between the core and dispensable chromosomes of *M. graminicola*, supporting the hypothesis that the dispensome originated by horizontal transfer from a single donor. *M. g.* C = *M. graminicola* core chromosomes (*circled in blue*); *M. g.* D = *M. graminicola* dispensable chromosomes (*circled in red*); *S. n.* = *Stagonospora nodorum* chromosomes (*circled in green*); *A. n.* = *Aspergillus nidulans* chromosomes (circled in green). The chromosomes *circled in yellow* in (**a**) represent chromosome 14 of *M. graminicola* and two scaffolds of *S. nodorum* that clustered near each other but separated from all other chromosomes in both species

degrading enzymes compared to other fungi with sequenced genomes (Goodwin et al. 2011). Other pathogens of plants, such as the rice blast fungus *Magnaporthe oryzae*, had greatly expanded numbers of genes for cell wall degrading enzymes, presumably as an adaptation to facilitate a plant-pathogenic lifestyle (Dean et al. 2005). The low number of these genes in the genome of *M. graminicola* was most similar to those seen in the genomes of endophytes rather than plant pathogens, and is now known to be common to other members of the Mycosphaerellaceae (Ohm et al. 2012). The reasons for this reduction are not known for certain, but it is thought to facilitate avoidance of the pathogen by its host during infection. Instead of metabolizing carbohydrates during the early, biotrophic phase of infection, *M. graminicola* was hypothesized to obtain nutrition from other materials including proteins, as its number of proteases is not reduced relative to other fungi (Goodwin et al. 2011). Whether the reduced set of genes for cell wall degradation is an adaptation for stealth pathogenicity that was derived from an endophytic ancestor is a subject for future investigation.

The origin of the eight dispensable chromosomes, from here on referred to as the dispensome is not known for certain, but is thought to have been by horizontal transfer from an unknown donor (Goodwin et al. 2011). How this could have occurred also is not known, but presumably resulted from a somatic or sexual fusion between two species, one of which was on the direct lineage that gave rise to *M. graminicola*; the other must have been distantly related because the genes on the dispensome share little sequence identity with genes in other members of the Mycosphaerellaceae. This fusion would have resulted initially in a duplication of almost all genes in both species. Subsequently, genetic material from one species must have been lost preferentially leaving a set of small, remaining chromosomes constituting the dispensome.

The first hypothesis about the origin of the dispensome was that it arose through duplication followed by degeneration and RIPing of individual core chromosomes; duplication of core chromosomes is known to occur during meiosis giving rise to progeny with extra chromosomes (Wittenberg et al. 2009; Goodwin et al. 2011). However, simulation studies showed that this process could not generate the observed differences in codon usage and other parameters (Goodwin et al. 2011). The only logical explanation for the consistent, clear separation between core and dispensable chromosomes for every parameter estimated is that the dispensome most likely arose through a single horizontal transfer event; dispensable chromosomes would not cluster together if they arose through multiple transfers. The timing of this transfer must have been relatively ancient, because content corresponding to most of the dispensome is present in close relatives of *M. graminicola* from wild grasses in Iran (Stukenbrock et al. 2010). These species are thought to have diverged from *M. graminicola* more than 10,000 years ago (Stukenbrock et al. 2007). For the dispensome to have survived through at least one speciation event implies that it must have a positive effect on fitness, at least under some conditions. Identifying those conditions is a promising area for future research.

The genome of *M. fijiensis* is greatly expanded at 74 Mb compared to the 39.7-Mb genome of *M. graminicola*. This expansion appears to be due mostly to a proliferation of class I retrotransposons because the number of genes in both species is similar (Ohm et al. 2012) and there is no evidence for polyploidy or other duplications. As with *M. graminicola*, the genome of *M. fijiensis* contains a reduced number of genes for cell wall degrading enzymes, possibly to help it evade detection by its banana host during the initial, stealth phase of its pathogenesis.

At least 14 of the scaffolds in the *M. fijiensis* genome had similar characteristics to the dispensome of *M. graminicola* and may be dispensable (Ohm et al. 2012) but, if so, they must have had a different origin from those in *M. graminicola*. The genes on the *M. graminicola* dispensome are absent from *M. fijiensis*, while those on the putative dispensable scaffolds of *M. fijiensis* are missing from *M. graminicola*. Therefore, the acquisition of dispensable chromosomes in each species must have occurred after they split from a common ancestor. Whether the scaffolds in the *M. fijiensis* genome that differ from those in the core set really represent dispensable chromosomes is not known for certain and must be tested by analysis of sexual and asexual progeny.

One interesting feature of the *M. fijiensis* genome is that it contains genes for effectors (small, secreted proteins that interact with the host to facilitate infection) that are similar to those seen in other fungi in the Capnodiales. Careful analysis of the *M. fijiensis* genome identified putative homologs of several effectors from the tomato pathogen, *Cladosporium fulvum*(Stergiopoulos et al. 2010). The interaction of *C. fulvum* with tomato has been analyzed in detail. Similar to *C. fulvum*, *M. fijiensis* has an initial biotrophic association with its host. The *C. fulvum* genome contains many transposons giving it an expanded size (de Wit et al. 2012), although not as large as that of *M. fijiensis*. Two of the carefully characterized effectors from *C. fulvum*, Avr4 and Ecp2, had apparent homologs in *M. fijiensis* raising the intriguing possibility that some effectors, which previously were thought to be species specific, might be conserved among species (Stergiopoulos et al. 2010). With the exceptions of those noted above, most other features of the *M. fijiensis* genome were similar to those seen in *M. graminicola*.

5.3 Applications from the Genome

The stealth pathogenicity of *M. graminicola* and *M. fijiensis* is different from what occurs with many other fungal pathogens. In this instance, the biotrophic phase can last more than several days to a few weeks. The observed changes in genome architecture, including the greatly reduced numbers of genes for cell wall degrading enzymes, reflect a different mode of pathogenesis from most other fungal pathogens with sequenced

genomes. The mechanism of pathogenesis for most other fungi is much more aggressive, involving appressoria for direct penetration of host cell walls, expression of many genes for cell wall degrading enzymes to aid penetration and host cell breakdown, and the production of numerous effectors that attack and diminish host-defense responses. These effectors often are recognized by the host to trigger defense responses. To evade host defenses, *M. graminicola* and *M. fijiensis* appear to have evolved a reduced set of genes for cell wall degrading enzymes and others that are expressed early during the infection process of other fungi. Those that are retained in the genome usually are not expressed until the later stages of infection.

Resistance to stealth pathogenesis is likely to be very different from the classical hypersensitive responses that are effective against many other fungi. Wheat lines containing different major genes for resistance against *M. graminicola* show two peaks of gene expression following inoculation with an incompatible isolate of the pathogen (Adhikari et al. 2007). The first occurs from 1–3 days after inoculation (DAI) and mostly involves genes for pathogenesis-related (PR) proteins. This response occurs in both resistant and susceptible hosts but is much stronger in wheat lines with a major gene for resistance. It presumably slows down but does not kill the pathogen or there would not be a late response.

The second major peak of gene expression in resistant wheat lines occurs approximately 12–24 DAI and involves massive upregulation of genes that are very different from those in either classical hypersensitive or nonhost resistance responses, although a few, such as brassinosteroid-6-oxidase and protease inhibitor Bsi1, have been associated with defense responses in other species (Adhikari et al. 2007). An obvious application of the genome sequences will be to identify all of the genes in the pathogens and the hosts that are involved with pathogenicity and resistance, respectively. RNA sequencing experiments of the interactions between *M. graminicola* on wheat and *M. fijiensis* on banana are being performed in several laboratories worldwide and should soon identify promising candidates for subsequent functional analyses. That work will be aided by the genome sequences because they make it very easy to clone and analyze genes and their flanking regions.

A very promising application of the genome sequences comes from functional analyses of the effectors identified in the genome of *M. fijiensis*. The MfAvr4 protein produced by *M. fijiensis* appeared to be a homolog of the Avr4 effector produced by *C. fulvum* based on sequence similarity, and this prediction was confirmed by functional analyses. MfAvr4 binds chitin and can protect fungal cell walls from the action of plant chitinases, so is a functional homolog of the Avr4 protein of *C. fulvum* (Stergiopoulus et al. 2010). More surprisingly, MfAvr4 can be recognized by the *Cf-4* resistance gene of tomato to trigger a defense response. This cross-species recognition of a fungal effector was not expected because most of the previously analyzed fungal effector proteins are species specific and believed to be recognized only by a limited number of hosts (Stergiopoulus et al. 2010).

Similar results were obtained for the Ecp2 effector of *C. fulvum*, which occurred in three possible homologs each in the genomes of *M. fijiensis* and *M. graminicola* (Stergiopoulus et al. 2010). Those in *M. fijiensis* were shown to interact with the *Cf-2* resistance gene in tomato to induce necrosis, a stronger induction than occurs with Ecp2 from *C. fulvum* (Stergiopoulus et al. 2010). The function of Ecp2 is not known for certain, but these cross-species functional tests did prove that effectors from different fungal species could be recognized by hosts as divergent as the dicot tomato and the nongrass monocot banana.

Cross-species functionality opens up the intriguing possible application that these genes may act as effective resistance genes when introduced into other hosts through genetic engineering. Breeding of bananas is extremely difficult because all modern cultivars are sterile triploids, so resistance cannot be introgressed through the usual methods such as backcrossing. Furthermore, very little natural variation for resistance to *M. fijiensis* exists in banana

populations providing only scant material to use in plant improvement programs. However, the MfAvr4 protein of *M. fijiensis* is recognized by the *Cf-4* resistance gene in tomato, and this interaction appears to be dominant (Stergiopoulus et al. 2010). If the *Cf-4* resistance gene of tomato can be incorporated into banana and engineered to induce the same resistance response, then it provides a new source of resistance (Stergiopoulus et al. 2010). The frequency of the *MfAvr4* gene in populations of *M. fijiensis* is not known, but presumably is high as it was present in the isolate that was chosen randomly for sequencing. If its frequency is confirmed to be high or, better yet, if it is required for pathogenesis of *M. fijiensis* on banana, then an engineered resistance using the *Cf-4* resistance gene possibly could be very effective. Resistance genes in other hosts may recognize additional effector proteins produced by *M. graminicola* and *M. fijiensis*, providing a potentially very large pool of cross-species candidate resistance genes. Although many questions remain about whether this type of approach will be successful, the possibilities are very enticing and provide a highly beneficial potential application of the genomic sequences.

The existence of a large dispensome in *M. graminicola* and the possibility of one in *M. fijiensis* mean that fungal genomes are much more plastic than believed previously. This extra genetic material provides a reservoir of variability that could make these pathogens much more adaptable and difficult to control. It seems highly unlikely that the isolates chosen for sequencing have the highest numbers of dispensable chromosomes for their respective species. Therefore, what we know most likely is only a fraction of the total catalog of genes that can be present among individuals of the same biological species. Presence of a variable dispensome makes it hard to define a fungal genome or to know how many individuals must be sequenced to capture most of the genes that are available to a species. The pool of potential genetic variability is greatly expanded when genes, chromosomes, or even genomes can be exchanged among species.

A practical application of this is that species with dispensable chromosomes might be much faster to evolve in response to changed environmental conditions, such as new fungicides, the introduction of new resistance genes or global climate change. Monitoring populations of these pathogens must be done carefully and include markers on the dispensome as well as the core chromosomes for a more complete picture of genome structure and evolution. It is not possible to make markers for the parts of the pan-genome that remain to be discovered, so there will always be a possibility for the sudden appearance of previously unknown genes in these species. For this reason, fungicide targets should always be chosen from among the genes on the core chromosomes rather than the dispensome.

Comparative analyses of genomes can provide a much better understanding of fungal evolution and elucidate differences in lifestyles that may provide clues for better disease management. For example, a comparative analysis of 18 Dothideomycetes genomes revealed a difference in modes of pathogenesis between the two major orders containing plant pathogens in this class, the Pleosporales and the Capnodiales. Genomes of plant pathogens in the Pleosporales were enriched for genes involved in the production of secondary metabolites that often function as toxins, while those in the Capnodiales had far fewer of these genes and also had reduced numbers of genes for cell wall degrading enzymes consistent with a stealth mode of pathogenesis (Ohm et al. 2012). Therefore, breeding for resistance against fungi in the Pleosporales clearly needs to involve resistance against mycotoxins, and purified toxins can be used to select for resistance (Liu et al. 2004). For fungi in the Capnodiales, disrupting the early, biotrophic phase of infection or attacking the transition from biotrophic to necrotrophic growth might be promising approaches for the development of new fungicides. Identification of genes or gene families that are specific to one group also provides the opportunity for development of fungicides that are very specific against the target organisms with little to no effect on other organisms in the environment.

Another discovery that came from comparative analyses of fungal genome sequences that may have some practical applications is the phenomenon of mesosynteny. Most previous analyses of fungal genomes had shown little evidence of synteny between species, i.e., the order and orientation of genes along chromosomes were generally not well conserved from one species to another, with rare exceptions such as around the mating type locus (Cozijnsen and Howlett 2003; Ohm et al. 2012). Comparisons of plant and animal genomes often show macrosynteny, where large blocks of genes in the same order and orientation are conserved among species, often extending to the length of complete chromosomes (Cannon et al. 2006; Shultz et al. 2007). In comparisons among species that are distantly related often only microsynteny is observed, where the numbers of genes in conserved blocks and their frequencies are very low (Irimia et al. 2012). In dot plots where the chromosomes and/or scaffolds of one species are aligned against another, macrosynteny is indicated by long, diagonal lines with a slope that is positive or negative depending on whether the genes align in the same or inverted order. Microsynteny gives the same general pattern, except that the aligned sequences are much smaller and usually occur much less frequently.

Dot-plot analyses between fungi in the Dothideomycetes gave a very different pattern. Instead of the long or short diagonal lines indicating macro- or microsynteny, comparisons between species of Dothideomycetes showed a very high conservation of gene content, but not order or orientation within corresponding scaffolds or chromosomes, giving an almost random pattern of dots in the plot comparisons (Hane et al. 2011). This pattern was first noticed in the comparison between the genomes of *Stagonospora nodorum* and *M. graminicola* (Goodwin et al. 2011), but a thorough analysis showed that it occurs very commonly in the Dothideomycetes and only rarely in other fungal groups (Hane et al. 2011). The cause of mesosynteny is not known but was hypothesized to occur by frequent intra-chromosomal inversions (Hane et al. 2011). Simulation studies and additional comparisons between Dothideomycetes that diverged at different times in the past showed a gradation from macro- to mesosynteny, and demonstrated that inversions alone are sufficient to explain the observed patterns of differences in chromosome structure (Ohm et al. 2012). The mechanism for this apparently increased rate of inversions in the Dothideomycetes is not known, but it has been associated with repeated regions near the breakpoints (Ohm et al. 2012).

A potential application of mesosynteny is for assembly of genome sequences. One problem for assembling fungal genomes is knowing which scaffolds should be joined together and their proper orientation. Mesosynteny relationships can be used to identify potential scaffolds that should be joined when two or more scaffolds in one species show mesosynteny with a single scaffold in another. To test this hypothesis, mesosynteny relationships between the scaffolds in the *M. graminicola* version 1 genome assembly and the previously assembled genome of *S. nodorum* (Hane et al. 2007) were used to predict scaffolds that should be joined or to identify those that might be misjoined (Goodwin et al. 2011). Comparisons of these predictions with the independently generated version 2 assembly confirmed that most were accurate and that knowledge of mesosyntenic relationships could have facilitated the final assembly. This approach is likely to be very helpful for future assemblies of new fungal genome sequences.

5.4 Future Perspectives

The genome sequences of *M. graminicola* and *M. fijiensis* have revealed much about their biology, yet many questions remain unanswered. Some of these questions are about structural genomics and genome architecture, while many others are related to the function of the genes on both core and dispensable chromosomes.

Fortunately, answering questions is now much easier with the genome sequences for reference and the pace of research on both of these pathogens is accelerating rapidly.

The origin of the dispensome of *M. graminicola* appears to have been by horizontal transfer from an unknown donor more than 10,000 years ago (Goodwin et al. 2011). However, we still do not know how the transfer occurred and whether the fusion was sexual or asexual. A sexual fusion seems less likely because it would require mating competence between species. Mating type genes seem to evolve very rapidly in *M. graminicola* and related species (Goodwin et al. 2003), so a sexual fusion between distantly related fungi seems unlikely. This leaves asexual transfer as the most likely mechanism. Transfer of multiple, single genes from one species to another has been reported in the Dothideomycetes (Friesen et al. 2006; Sun et al. 2013) and was hypothesized to be by anastomosis tubes between hyphae (Friesen et al. 2006), which are known to occur in some fungi (Roca et al. 2005) and also occurred commonly in *M. graminicola MgGβ* knock-out strains (Mehrabi et al. 2009). Transfers of single dispensable chromosomes by a similar mechanism have been noted in Fusarium (Ma et al. 2010). Transfer of multiple chromosomes by this mechanism seems unlikely, so a somatic fusion would seem to be a more probable scenario. Presence of elevated numbers of genes in *Nectria haematococca* (Coleman et al. 2009) could have been due to a somatic fusion. A systematic search for horizontally transferred genes among species of Dothideomycetes would reveal much about the frequency of these events, but much more experimental work will be required to elucidate the mechanism of multiple chromosome transfers.

A high frequency of intra-chromosomal inversions can explain the phenomenon of mesosynteny (Ohm et al. 2012), but there is still no proof that it occurs. Presumably, these inversions are generated primarily during meiosis but probably not often enough to be noted in the progeny of a single cross. The best way to test this hypothesis will be by sequencing multiple isolates of closely-related species and comparing the observed rates of inversions to those seen in similar sequencing projects in other fungal groups. This approach probably cannot rely on short-read sequencing technologies, as near-complete assemblies will be required unless most of the inversions are very small. Some of the newest sequencing technologies that promise very long read lengths would be perfect for this application once they become available.

Repetitive regions may facilitate inversions in the Dothideomycetes (Ohm et al. 2012), but we still do not know the origins of repetitive elements in different species or why they are able to proliferate in some species but not others. Some fungi in the Dothideomycetes, such as *Baudoinia compniacensis*, have very little repetitive DNA whereas in others, such as *M. fijiensis* and *C. fulvum*, transposons were able to proliferate to high levels (Ohm et al. 2012) despite evidence that they can still be recognized and inactivated by RIP. We do not know whether the fungi with fewer transposons have a better system for removing them or if differences in their environments or some other aspect of their biology decrease the rate of transposon acquisition. RIP is only active during meiosis (Selker et al. 1987), and another possibility is that fungi with high accumulations of transposons had an extended period of asexual reproduction at some time in their past evolutionary history that prevented RIP from acting and allowed these elements to replicate. Another possibility is that the fungi with fewer transposons have a previously undiscovered mechanism for their identification, inactivation and/or removal. These questions can be addressed to some extent by additional sequencing but most likely will require experimental validation of any conditions that reduce the effectiveness of RIP.

Determining the evolutionary origin of stealth pathogenesis is another major unanswered question that would help to understand the differences between the primarily hemibiotrophic (occasionally biotrophic) lifestyles of fungi in the Capnodiales compared to the primarily toxin-caused pathogenesis of species in the

Pleosporales. It is possible that the ancestor of fungi in the Mycosphaerellaceae was an endophyte and that pathogenicity is an accidental aberration. Additional sequencing of saprobes and endophytes in the Capnodiales is needed to test this hypothesis.

Phylogenetic analyses of Dothideomycetes have revealed several instances of apparently rapid radiations of species either on the same or different hosts. For example, thorough analyses of isolates from banana have identified more than 20 species, at least six of which formed different nodes on the same branch of the evolutionary tree that gave rise to *M. fijiensis* (Arzanlou et al. 2008). In Cercospora, there are numerous closely-related species that infect different hosts. This was hypothesized to have been aided by acquisition of the ability to produce the nonhost-specific toxin cercosporin to facilitate pathogenicity, followed by a rapid radiation onto different hosts (Goodwin et al. 2001). How so many species evolve to infect the same hosts or why these radiations onto different hosts occur are not known but are very important questions for understanding the possible emergence of new pathogens in the future.

Many other questions revolve around the origin and evolution of effectors. We now know that some effectors are widely conserved and probably have a similar function in diverse fungal species (Stergiopoulos et al. 2010). One possibility is that they originated during evolution from a saprobic or endophytic ancestor to a plant pathogen. If they are essential for pathogenicity, then disrupting their function either through fungicides or engineered resistance may facilitate disease control. Comparative genomics of pathogens versus nonpathogens may help to identify how many and which effectors are required for a pathogenic lifestyle.

Future sequencing ideally should include a broader sampling of species from different hosts and substrates, including pathogens, endophytes, saprobes, and extremophiles to help identify the genes and genome architectures that can facilitate adaptation to various environmental niches. We also need a greater sampling of the diversity within species for a better understanding of core and peripheral genomes. Complete assemblies rather than gappy draft genomes will be necessary to accurately identify and analyze changes in genome architecture. Finally, with all of this sequencing the limiting factor will become our ability to store and analyze all of the data. The most pressing future need will be for better bioinformatics tools for comparative analysis of the plethora of soon-to-be-available fungal genomes.

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6 Facilitating the Fungus: Insights from the Genome of the Rice Blast Fungus, *Magnaporthe Oryzae*

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6.1 Introduction: The Past, Present, and Future Importance of the Rice Blast Fungus

Magnaporthe oryzae is the causal agent of rice blast disease and one of the most devastating plant pathogenic fungi, worldwide. Rice blast has caused and continues to cause epidemics wherever rice is grown. A few examples of its sordid history on rice include a 1953 epidemic in Japan, with a 700,000 million ton yield loss, a panicle blast epidemic in Korea in 1978, resulting in 40 % yield loss, a 2006 epidemic in China where in one province alone, 1.3 million metric tons of yield was destroyed, and in 2009 where Arkansas rice farmers lost up to 80 % of their yield due to a blast outbreak (McBeath and McBeath 2010; http://deltafarmpress.com/rice/rice-blast-increasing). History, therefore, dictates that the next epidemic is a matter of "where, when, and how bad", rather than "if". Since 1979, when *M. oryzae* (then *Magnaporthe grisea*) was first defined as a genetically useful fungal "model" by Barbara Valent et al. , research has been intensively focused on characterizing fungal pathogenicity mechanisms, and plant host resistance mechanisms. The last decade of rice blast research has been defined by the age of "omics", or the ability to look at large sets of genes and biological processes simultaneously. Our review herein attempts to summarize these "omics-age" results and to highlight their importance in the elucidation of fungal virulence. We conclude our review with future perspectives on the importance of pushing this research forward, as the threat of a new wheat-infecting strain of *M. oryzae* looms menacingly for U.S. wheat varieties.

Over a decade ago, Couch and Kohn (2002) used molecular taxonomic markers to split what was once known as *M. grisea* into two species; *M. grisea* isolates grouped together into one clade associated with infection of grass (*Digitaria*) species, while *M. oryzae* isolates grouped into another clade associated with rice infection. Currently, there is an ongoing discussion about nomenclature for the rice blast pathogen, *M. oryzae* or *Pyricularia oryzae*, the teleomorph and anamorph, respectively. *Magnaporthe oryzae* has been widely adopted by the community and appears in numerous published studies; while the anamorph (asexual) form is commonly found in the field and associated with infection, *M. oryzae* is used by a large proportion of the scientists who study rice blast and is the term recognized most

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R. A. Dean et al. (eds.), *Genomics of Plant-Associated Fungi: Monocot Pathogens*,
DOI: 10.1007/978-3-662-44053-7_6,

widely by those outside the rice blast research community. As we move toward a "one fungus one name" system, phylogenetic studies like the one from Luo and Zhang 2013, may help clarify taxonomic distinction of current and future isolates, and together with input from the rice blast research community, will ultimately determine the most appropriate name for the rice blast fungus. For the purposes of this chapter, we will utilize the widely recognizable *M. oryzae*.

Magnaporthe oryzae subdivides further into a strain isolate that infects (wheat) *Triticum* species, which was first described in Brazil in 1985 (Igarashi et al. 1986). Since then, wheat blast has caused major epidemics in South America; should it spread into the United States, an epidemic will likely occur as current U.S. wheat cultivars are susceptible to the disease (Marangoni et al. 2013). We are just beginning to scratch the surface in terms of understanding this particular isolate and how to control it. In the next section, we describe current comparative genomics projects of numerous isolates and what this information can tell us about virulence mechanisms and host adaptation.

Apart from its past, present, and potentially strong future concerns as a major pathogen of small grain crops, *M. oryzae* is also a genetically tractable organism with a well-defined and fascinating infective life cycle. Valent et al. began defining this fungus' utility as a model for genetics with their studies on the vegetative diploid phase, and development of genetic mutants to examine specific traits, such as melanin production (Crawford et al. 1986; Chumley and Valent 1990). Since then numerous studies and reviews have chronicled the *M. oryzae* infective life cycle, hence we need not provide substantial detail here (reviewed in Li et al. 2012). In brief, conidiospores, the asexual and arguably the most important stage of the fungus for disease, will germinate on a hydrophobic leaf surface. Given sufficient moisture levels, the spore will undergo autophagy to allow proper formation of the penetration structure called the appressorium. This dome-shaped, melanized structure utilizes mechanical pressure to breach the leaf surface, growing invasively into the first epidermal cells by means of invasive hyphae. This brief phase is rapidly followed by a lengthier biotrophic phase whereby bulbous hyphae grow within epidermal cells, producing "biotrophy-interfacial complexes" or "BICs" (Kankanala et al. 2007; Mosquera et al. 2009; Fig. 6.1). BICs are, in effect, factories for the production and release of fungal effectors into plant cells, as beautifully detailed by Khang et al. (2010). By 48 h post-inoculation, the fungus has become necrotrophic, producing thin, invasive hyphae followed by eventual development of lesions and production of more conidiophores (Fig. 6.1).

The vast majority of recent studies on *M. oryzae*'s infective life cycle were greatly facilitated through the completion of genome sequencing in 2005 (Dean et al. 2005), funded by both USDA and NSF via the jointly administered USDA/NSF Microbial Sequencing Program. This is evidenced by the fact that to date, this seminal paper has been cited 741 times. Part of the Broad Institute's Fungal Genome Initiative, the *M. oryzae* genome has been publicly housed on its website for the last 8 years. The genome is robust, having been through eight iterations of annotation, and within the last year alone was visited by 18,000 individuals (personal communication from web developers at Genome Sequencing and Analysis Program, Broad Institute), indicating an extremely high impact. In the next section, we explore how, as more isolates are sequenced, comparative genomics helps us unravel how particular isolates came to infect important small grain crops such as rice and wheat. There are more than 200 rice blast researchers world-wide, and while we attempted to be comprehensive, due to space considerations, it is not possible to include all recent published works enabled by the genome sequences.

6.2 The Genomic Era: Genome Structure and Variation Informs Host Adaptation

Over the past decade, the collective rice blast research community has been developing extensive resources for whole genome studies.

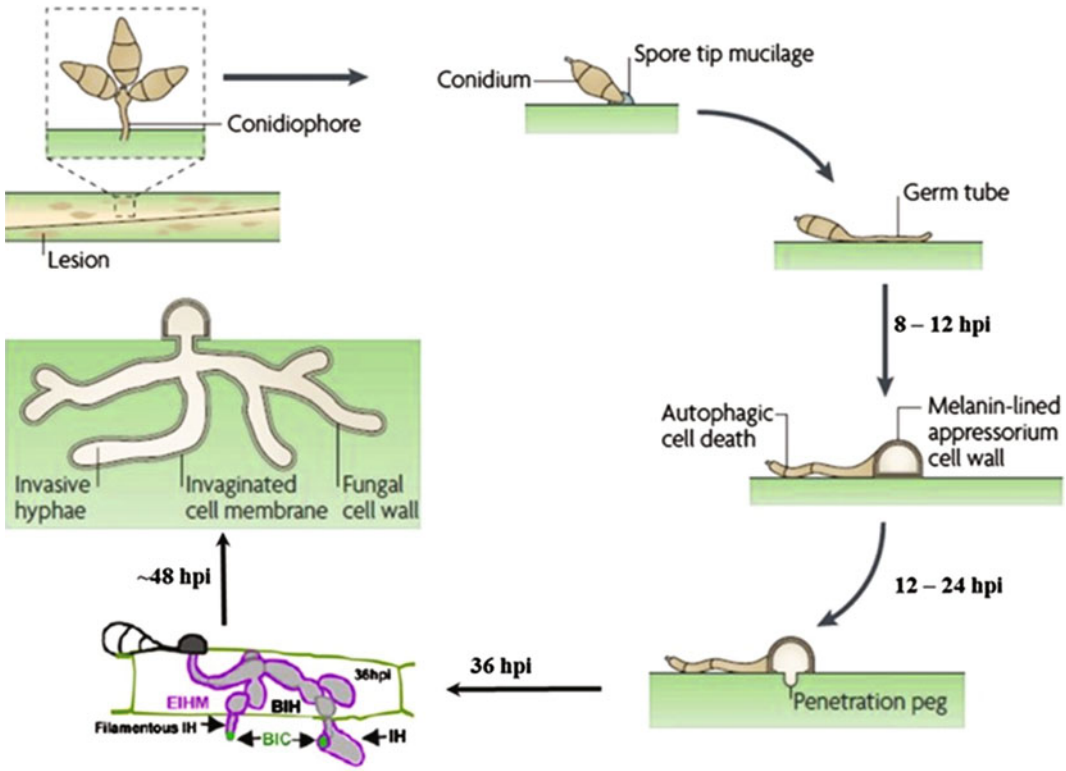

Fig. 6.1 Infective life cycle of *M. oryzae* adapted from Wilson and Talbot (2009) and Mosquera et al. (2009). The figure has been adjusted to include the biotrophic portion of the life cycle, which includes production of bulbous invasive hyphae (*IH*), biotrophic-interfacial complexes (*BICs*) and an extra-invasive hyphal membrane (*EIHM*)

The first funded effort to perform a whole genome sequence assembly was initiated by R. Dean's group in 2000 and took several years to complete with via a coordinated collaborative effort betweenacademic institutes and the Broad Institute using first generation sequencing technologies (Dean et al. 2005). During that time, a parallel effort to generate a complete assembly of the smallest chromosome, chromosome 7, was completed using a BAC-by-BAC approach (Thon et al. 2006). Since the initial release of the 70–15 *M. oryzae* genome in 2002, several annotated versions of the genome have been completed, with the most recent being V8 (Broad Institute 2010) released in April, 2011. 70–15 is not a naturally occurring isolate; it was derived from a fertile cross between isolate GUY11 (rice-infecting isolate from the French Guyana) and an isolate from weeping lovegrass. Progeny from that cross were backcrossed to GUY11 for several generations to produce 70–15 (Leung et al. 1988). This isolate was chosen as the model to be sequenced as it was the most widely used laboratory strain at the time, and its host range, physiology, and gene content were better understood than in other isolates.

6.2.1 The Flagship Sequence Strain 70–15 Launches Additional Efforts

Sequencing of 70–15 was a major accomplishment. It was the first (publically available) filamentous fungal pathogen sequenced, paving the way for others to come. The genome assembly became a powerful tool and a launching pad for the development of a wealth of genome-based

tools providing new insights into the evolution of fungal pathogenesis of plants. Notable, initial discoveries from studies of 70–15 include: (1) identification of a large suite of secreted proteins, many of which likely function as effectors; (2) dramatic invasion of active transposable elements; (3) expanded families of PKS and NRPS genes that function in secondary metabolism; (4) sets of predicted virulence associated genes; (5) localization of known avirulence (*Avr*) genes to telomeric regions and (6) evidence for ancient RIP (repeat-induced point mutation) mechanisms suggesting sexual recombination existed in nature at one point (Ikeda et al. 2002; Dean et al. 2005; Farman et al. 2007). These discoveries fueled an explosion of research, particularly in the area of functional genomics.

With the advent of next generation sequencing, researchers across the globe initiated projects to generate genome assemblies of other lab strains, authentic field isolates, and mapping lines for either interspecific or intraspecific crosses. Additional efforts resulted in sequences being generated and made available for related species including *M. grisea*, *Magnaporthe poae* (summer patch) and *Gaeumannomyces graminis var. tritici* (*G.g.t.*; causal agent of take all disease) (http://www.broadinstitute.org). A survey of the community reveals that a minimum of over 40 sequenced genomes have been generated for fungi in the Magnaporthaceae. These include isolates representing *M. oryzae* (>22 genomes), wheat blast (4 genomes), foxtail millet blast (2 genomes), finger millet blast (2 genomes), *Digitaria* blast (1 genome), *G.g.t.* (1 genome), *M. poae* (1 genome), and perennial ryegrass blast (1 genome). The only other fungal genome with more sequenced isolates is *Saccharomyces cerevisiae*, which has genomes available for as many as 52 isolates. The power of having numerous genomes of different isolates is just being realized with multiple laboratories worldwide currently initiating projects to sequence the genomes of hundreds of isolates from a large diversity of rice growing regions. These genomes represent one of the richest collections of genome sequences for any specific lineage of fungi. However, the current paradigm is that most of these are sequestered in the databases of individual researchers. As comparative genome studies are completed and published, many are expected to be released and the possibilities for comparative genomic queries, including adaptation strategies to new hosts, will be endless.

6.2.2 Location, Location, Location: Genome-Assisted Identification of Avirulence Genes

While many Resistance (R) genes in rice have been identified and cloned, the identification of *Avr* genes in *M. oryzae* has been limited. There are three *Avr* genes involved in host species specificity: PWL1 (Kang et al. 1995), PWL2 (Sweigard et al. 1995), and Avr-CO39 (Farman and Leong 1998), and at least six involved in rice cultivar specificity, including Avr-Pita (Orbach et al. 2000), ACE1 (Bohnert et al. 2004), AvrPiz-t (Li et al. 2009), AvrPia (Miki et al. 2009; Yoshida et al. 2009), Avr-Pii (Yoshida et al. 2009), and Avr-Pik/km/kp have been cloned to date (Yoshida et al. 2009). While R proteins are reported to contain some of the canonical, conserved functional domains—such as NBS-LRR—no obvious sequence or structural patterns can be assigned to *Avr* genes, which make their identification difficult. However, "genome-wide association analysis" (GWAS) can now help in identifying novel *Avr* genes based on the association between *Avr* genes and the cultivar specific virulence. In one study focusing on Avr-Pita family including AvrPita1, AvrPita2, and Avr-Pita3, it was found that AvrPita1 and AvrPita2 were associated with transposon elements and that they localized to different chromosomes in various field isolates (Chuma et al. 2011). This location variability, as well as high diversifying selection, makes it almost impossible to identify them without genome sequencing and association analysis.

The first identification of novel *Avr* genes resulting from a whole genome comparison was

reported in 2009 (Yoshida et al. 2009). An attempt to associate polymorphisms in secreted proteins in 70–15 and different field isolates with pathogenicity resulted in little success. However, comparison of the sequence of a field isolate "Ina168", which is known to contain nine *Avr* genes, with 70–15 identified a 1.68 Mb unique region in 70–15. Through an association study for genes located on this isolate-specific region, three new *Avr* genes were identified. The project demonstrated the value of the 70–15 reference genome for identifying novel *Avr* genes in field isolates.

One of the first published whole genome comparison studies of *M. oryzae* isolates was published in 2012 (Xue et al. 2012). Two field isolates of *M. oryzae*—isolate Y34 from China and isolate P131 from Japan were sequenced and their genomes compared with the reference strain 70–15 (version 6). Sequencing was performed using a mix of traditional Sanger technology and next-generation 454 sequencing. The de novo assembly of the two field isolates showed a slightly smaller genome size than 70–15. One significant finding was the large amount of isolate-specific sequence; 1.69 Mb identified in P131 and 2.56 Mb in Y34. Confirmation of isolate-specific sequences was completed by CHEF gel and Southern hybridization using unique sequences as the probes. Genes were predicted de novo in Y34 and P131, with the result showing a similar total number of genes as compared to 70–15. While all three genomes shared a "core" gene set, comparisons among the three genomes showed that there are about 300 field isolate-specific genes absent from the 70–15 genome, with most of them annotated with an "unknown" function. To determine the biological function of these, nine Y34-specific genes and three P131-specific genes were selected for functional characterization. While most of these deletion mutants produced no obvious phenotypic changes, four mutants showed altered virulence. Multiple duplicated genomic sequences were also identified in the analysis, which were enriched in chromosome II, IV, V, and VII. The non-synonymous to synonymous nucleotide substitution rate (Ka/Ks) analysis showed 697 genes with only non-synonymous nucleotide substitutions and six genes with a Ka/Ks >1. Six known virulence factors were among these. With regard to repetitive sequences, a similar percentage ($\sim$10 %) was identified in all three genomes, while the two field isolates showed them to have a more similar distribution pattern. A total of roughly 200 genes were disrupted in the three genomes by transposable elements, with many associating with isolate-specific or duplicated sequences. This study revealed that gain or loss of unique genes, DNA duplication, gene family expansion, and frequent translocation of transposon-like elements are all important factors contributing to genome variation of *M. oryzae*.

It is interesting to note that these first two studies using whole genome sequencing analysis confirmed by CHEF gel analysis that *M. oryzae* contains supernumerary chromosomes that vary in size, and that *Avr* genes may localize to them. Supernumerary chromosomes, sometimes called "conditionally dispensable chromosomes", were first discovered and gained attention in *Alternaria* species (Johnson et al. 2001; Hatta et al. 2002); however, through whole genome sequencing studies they were reported in different fungi including *Fusarium oxysporum* (Ma et al. 2010), *Nectria haematococca* (Han et al. 2001; Coleman et al. 2009), *Mycosphaerella graminicola* (Stukenbrock et al. 2010), *Cochliobolus heterostrophus* (Tzeng et al. 1992), and *Leptosphaeria maculans* (Leclair et al. 1996).

6.2.3 Is Bigger Always Better? Genome Size Comparisons Across Fungi

Since before the application of next generation sequencing, it was widely known that fungal genomes are variable with regard to size, but the degree of variability is only now being confirmed. Fungal phytopathogens and oomycetes

have extreme variability in genome size. For example, the genome size difference between smut fungi *Ustilago maydis* (~19–21 Mb) and *Phytophthora infestans* (~220–280 Mb) is nearly 15-fold (Kamper et al. 2006; Haas et al. 2009; Raffaele et al. 2010; Schirawski et al. 2010). Generally, filamentous Ascomycete phytopathogens have larger genomes than their yeast type relatives (Haas et al. 2009; Spanu et al. 2010; Duplessis et al. 2011). For example, the powdery mildew *Golovinomyces orontii* has the largest sequenced genome in that phylum at approximately 160 Mb (Spanu et al. 2010). For Basidiomycetes, the rust fungus *Melampsora larci-populina* has the largest genome at over 89 Mb, although the coffee rust fungus is predicted to be close to 500 Mb (Duplessis et al. 2011; personal communication, N. Donofrio). Compared with pathogens, genomes of non-pathogenic fungi sequenced so far are typically ~40 Mb, such as *Aspergillus oryzae* at 37 Mb (Machida et al. 2005), *Neurospora crassa* at 41 Mb (Galagan et al. 2003) and *Schizophyllum commune* at 39 Mb (Ohm et al. 2010). However, the trend of fungal phytopathogens toward larger genomes is not absolute, and some filamentous pathogens actually have relatively small genomes, possibly due to gene loss such as in *Albugo laibachii* (Kemen et al. 2011), intron loss as in *U. maydis* (Kamper et al. 2006), or reduced transposon content in *Sclerotinia sclerotiorum* (Amselem et al. 2011). Given the large variability and plasticity in the size of fungal genomes, it is perhaps not surprising the published and unpublished results of sequencing additional *M. oryzae* isolates shows the genome size to vary dramatically with different isolates having up to 2 MB differences in genome size.

Unpublished reports on single nucleotide polymorphism (SNP) variation show that about 10,000–15,000 SNPs and 3,000–10,000 Indels exist between three field isolates and the lab strain, 70–15. Among the SNPs, 355 were expected to introduce premature stop codons, 752 were expected to alter initiation methionine residues, and 132 were expected to disrupt splicing donor or acceptor sites. Large effect insertion/deletion polymorphisms (IDPs) were identified as the cause of frame shifts. There were 47,083 IDPs identified for all strains and 910 among them were large effect IDPs. It was reported in previous studies that SNPs and IDPs were not distributed evenly along the genome, but were in fact found enriched in some regions and thus make sequences in the regions highly variable (Wei et al. 2007).

It is clear that the genomes of filamentous fungi are plastic and continue to evolve rapidly. Ongoing re-sequencing projects for *M. oryzae* from around the globe support the fact that the genome of this fungus is highly variable between field populations. The potential consequence of this level of variability is the fungus being able to rapidly adapt to changing environmental conditions and new hosts as well as the ability to overcome host resistance mechanisms.

6.3 Transcriptomics: How Genomics Has Enabled Progress from Microarray to RNA-Seq

Since at least 2003, researchers have been using large-scale approaches to study gene expression in the rice blast fungus; topics explored have ranged from gene expression during different developmental stages, to gene expression during various abiotic stresses, to examining the impact on gene expression of losing one specific gene. Pre-genome the methodology of cDNA library sequencing was employed (Takano et al. 2003), but after its public release researchers were able to capitalize on genome-wide approaches. The first whole genome microarray chip was built in 2004 as a coordinated effort among public universities, the Broad Institute, and Agilent Technologies.

6.3.1 Coordinated Global Gene Regulation of Developmental Programs

Given the importance of the specialized infection structure for rice blast disease, much research has focused on characterizing genes

thought to be involved in appressorial development. However, pre-genomics, this had to be performed gene-by-gene. The year 2005 saw the publication of the rice blast genome, along with the first global gene study on appressorial development using the first generation of the Agilent microarray (Dean et al. 2005). This experiment yielded families of genes, including membrane-anchored CFEM domain proteins that play a role in appressorial formation, supporting the genetic CFEM mutant *pth11* identified in 1996 by Sweigard et al. (1998). The genome has enabled studies on global expression during certain developmental regimens, or in response to stress. In 2008, researchers again used the Agilent microarrays to perform a more in depth analysis of appressorial development, and how this process is affected by addition of cyclic AMP (Oh et al. 2008). A comparison of *M. oryzae* spores germinating on hydrophobic (appressoria-inducing), hydrophilic (appressoria-inhibiting), and hydrophilic plus cAMP (appressoria-inducing) yielded the previously unknown finding that appressorium formation requires coordinated down-regulation of genes involved in protein synthesis and associated translational machinery, and up-regulation of genes involved in protein degradation and lipid metabolism (Oh et al. 2008). Putatively secreted genes were also found to be enriched in the upregulated group. In 2012, a microarray-based study examined gene expression during the important process of conidiation, the formation of asexual spores (Kim and Lee 2012). In the rice blast fungus, these are the most important spores in terms of the fungus' ability to infect. Prior to this study, conidiation in fungi had been studied in depth in the model ascomycetes *Aspergillus nidulans* and *N. crassa*, but only a small handful of conidiation-related genes were known in *M. oryzae*. Their study found 603 and 557 genes to be induced and repressed during conidiation, respectively, with the majority of them being involved in catalytic activity.

6.3.2 May-Day! Gene Expression Under Stress

There is a rich history of studies on the relationship between nitrogen stress and fungal infection, from plant to human pathogens (reviewed in Solomon et al. 2003; Hartmann et al. 2011). In the 1990s, studies revealed that nitrogen limitation (NL) in the rice blast fungus was a cue for induction of pathogenicity genes such as the hydrophobin *MPG1* and the regulatory loci *NPR1* and *NPR2*, supporting the idea that NL conditions prevail within the host (Talbot et al. 1997). This pivotal research laid the ground-work for examinations into whether in vitro NL mimicked host colonization. Again, the *M. oryzae* microarray was put to work examining global gene expression during NL (Donofrio et al. 2006). Five known pathogenicity genes, including *MPG1*, were upregulated in response to a shift to nitrogen limitation, and genes for nitrogen catabolite repression were also induced. This study also revealed a new role for a serine protease in both nitrogen utilization and virulence. In 2011, global gene expression during nitrogen stress was re-visited in a larger experiment; gene expression profiles of *M. oryzae* undergoing various types of in vitro and in planta stresses revealed that overall, fungal gene expression in planta most closely resembled that of fungal gene expression during NL (Mathioni et al. 2011). Stresses including carbon and nitrogen, oxidative-inducing paraquat, temperature upshift and invasive growth in rice and barley were then investigated. Results exposed a group of 55 and 129 "stress consensus" genes that were increased or decreased in expression during every condition, respectively. The elevated genes appeared to be involved in carbon metabolism, membrane function or oxidoreduction reactions, while the down-regulated genes were largely involved in molecular transport, signal transduction, and nitrogen metabolism. The ability to analyze how groups of genes behave in concert provides the evidence that

acquisition and/or breakdown of nutrient sources is an important function for the rice blast fungus during substantially different stresses.

Most recently, a 2013 study by Park et al. (2013) used bioinformatics approaches to identify 495 predicted transcription factor genes in *M. oryzae*, 206 of which were examined for expression changes during different stress conditions. Stress conditions studied included several in planta conditions, and 26 in vitro stresses; the magnitude of this study, and the new discoveries on how this fungus regulates stress responses, could only be made manifest by a robust genome sequence.

6.3.3 Global Gene Expression Analyses of Mutants: One Gene Can Impact Many

Global gene expression has allowed us to characterize genetic fungal mutants in new ways; not only can we collect physiologic and phenotypic data, but also obtain a molecular profile of a how fungal genes collectively behave in a single-gene deletion mutant. Table 6.1 provides examples of single-mutant microarray studies that have been performed in *M. oryzae* to date. Some single gene deletions, like the high-affinity phosphodiesterase *PDEH*, impact both pathogenicity and expression of virulence-related genes (Zhang et al. 2011). Others, like the class VII chitinase, *Con7*, and the conidiation regulator, *MoHOX2*, affect cell wall-associated genes and conidial regulation (Odenbach et al. 2007; Kim and Lee 2012). *Con7* was identified in an earlier study, and the mutant was found to be completely defective in its ability to cause disease (Shi and Leung 1995). Global gene expression studies on the *Δcon7* mutant revealed a suite of cell wall-related genes whose expression was dependent upon this putative transcription factor (Odenbach et al. 2007). One in particular, the *CHS7* gene encoding a class VII chitin synthase, was also defective in its ability to cause disease via misshapen appressoria.

The RNA-binding protein RBP35 is fascinating, as its deletion impacts both full virulence and preprocessing of mRNAs via polyadenylation (Franceschetti et al. 2011). Preprocessing of messages, especially at the 3' end, is essential for proper expression, as well as regulation (Millevoi and Vagner 2010). Hence, it is no surprise that the targets of RBP35 would be subjects of interest. Microarray results identified 159 genes with differential expression. A subset of five were down-regulated only in the 3' UTRs and not in their coding sequences. Furthermore, these genes showed alternative splicing, and several additional experiments revealed that *RBP35* was likely involved in processing them with longer 3' UTRs. Interestingly, one of these genes was 14-3-3, a family of proteins that integrates signals through regulation of signaling cascades (Morrison 2009). *MoHOX2* is a homeobox transcription factor; when deleted, fungal mutants produce the spore-bearing stalks, but cannot produce conidia and hence are deficient in disease (Kim et al. 2009). In 2012, microarrays were employed to obtain the fungal profile of this mutant (Kim and Lee 2012). The authors compared their whole-genome expression results with microarray results from an earlier conidiation study in wild type, and determined a "core set" of genes involved in this important developmental process. They identified 137 significantly down-regulated genes that were increased during conidiation in the wild type fungus, and repressed in the *ΔMohox2* mutant. Among this gene set were those involved in regulation, such as transcription factors and kinases, metabolic genes like peptidases and synthases, and cell wall associated genes like chitin-binding proteins. Here, the ability to access the entire genome coupled with genetic mutation, provided much-needed insight into the processes regulating conidiation. Understanding what the rice blast fungus requires in order to form asexual spores provides opportunities to block these pathways, thus control the disease.

Table 6.1 Single mutant whole-genome expression studies

Mutant	Function	Genome Impacts	References
Microarray approach			
Δcon7	Transcription factor	Cell wall-associated proteins	Odenbach et al. (2007)
Δmgwc-1	Blue light receptor	Melanin biosynthetic genes	Kim et al. (2011)
ΔpdeL	Low-affinity phosphodiesterase	Altered expression of many genes	Zhang et al. (2011)
ΔpdeH	High-affinity phosphodiesterase	Required for expression of path genes	Zhang et al. (2011)
Δrbp35	Gene-specific polyadenylation factor	Alternative splicing in pre-mRNA	Franceschetti et al. (2011)
Next-gen. approach			
Δdcl1	Dicer 1	NDE[a]	Raman et al. (2013)
Δdcl2	Dicer 2	Specific size classes of sRNAs	Raman et al. (2013)
Δdcl1/ Δdcl2	Double knock-out	Specific size classes of sRNAs	Raman et al. (2013)
Δdcl2/ Δdcl1	Double knock-out	Specific size classes of sRNAs	Raman et al. (2013)
Δago1	Argonaute	In progress[b]	Raman et al. (2013)
Δago2	Argonaute	In progress	Raman et al. (2013)
Δago3	Argonaute	Conidiation	Raman et al. (2013)
Δrdrp1	RNA-dependent RNA polymerase	Conidiation	Raman et al. (2013)
Δrdrp2	RNA-dependent RNA polymerase	In progress	Raman et al. (2013)
Δrdrp3	RNA-dependent RNA polymerase	Specific size classes of sRNAs	Raman et al. (2013)

[a] *NDE* no discernable effect

[b] In progress indicates that the mutants were used as a tool for the first publication (Raman et al. 2013) and are being more thoroughly examined for additional work

6.4 Turned Down and Turned Off: Genome-Enabled, High-Throughput, Single Gene Analyses

Analyses of the genome sequence resulted in a plethora of data on predicted genes and their function. However, predictions, at some point, must be elevated to the status of "confirmed" in order to truly understand the function of the roughly 11,000 genes in *M. oryzae*. To this end, several high-throughput mutant collections were generated to allow for forward genetic phenotypic screens. In 2007, Betts et al. and Meng et al. detailed the generation and utilization of about 60,000 randomly tagged insertion lines. The latter study in part provided important information on the distribution of T-DNA insertions with respect to gene locations, while the former in part examined phenotypic assays as well as recovery of flanking sequences (Betts et al. 2007; Meng et al. 2007). In the same year, Lee et al. published on their collection of approximately 21,000 random insertion lines, generated exclusively through Agrobacterium-mediated transformation (Choi et al. 2007). While development of constructs for these random insertions studies could not have easily been performed without genome knowledge, the abundant information about virulence mechanisms in this fungus gleaned from reverse genetics, would not exist without the genome sequence.

6.4.1 Turned Down: RNA Interference Goes to Work in Magnaporthe oryzae

RNA interference (RNAi) is a powerful genetic tool, especially for organisms recalcitrant to other gene targeting approaches. Although the homology-based recombination approaches in the rice blast fungus show acceptable efficiency, RNAi provides value especially for essential genes that cannot be fully deleted and for gene families where members have redundant function (Fire et al. 1998). The very first evaluation of RNAi applied in *M. oryzae* as a genetic modification tool, was reported in 2003 (Kadotani et al. 2003), where authors first tested the ability of three different forms of RNA to induce RNA silencing by introducing plasmid constructs expressing sense, antisense, and hairpin RNAs. Similar to the results found in other organisms, hairpin RNAs were found to have the highest efficiency. Additionally, this study showed at least three different sizes of small interfering RNAs (siRNAs) with function. Although being demonstrated as an acceptable method for knocking down the expression of genes, the construction of a hairpin RNA vector requires two steps of oriented cloning, and thus, the application was limited to a small or moderate scale. In 2008, the same research group reported an improved method: a dual promoter system of RNA silencing in filamentous fungi, allowing a high-throughput application (Nguyen et al. 2008). The silencing vector, pSilent-Dual (pSD1) carries two convergent opposing RNA polymerase II promoters and multicloning sites between them. Using this novel system, 37 calcium signaling-related genes were targeted and silenced, with the resultant 26 (70.2 %) genes examined being involved in hyphal growth, 35 (94.6 %) involved in sporulation, and 15 (40.5 %) involved in pathogenicity. Another RNAi study focusing on the function of xylanase enzymes was reported in 2011, where a combination of 40 bp sequences from each of the 10 endoxylanase genes were used to make an artificial RNAi trigger, which was coined as the "building blocks method" (Nguyen et al. 2011). After the functionally redundant xylanase genes were significantly reduced in mutants, their pathogenicity was reduced resulting from a lowered number of lesions, rate of penetration, and extent of infected cells. The level of pathogenicity reduction was associated with the degree of silencing of xylanase mRNA. Overall, the RNAi approach has been demonstrated to operate in *M. oryzae* and has been adapted for high-throughput functional genomics projects.

6.4.2 Turned Off: Forward or Reverse, Genetic Analyses Have Provided Many Answers

In 2007, Lee et al. performed functional analysis of the *M. oryzae* genome by designing and executing an efficient screening process, looking for mutants defective in different life cycle stages (Choi et al. 2007). They utilized Agrobacterium-mediated transformation to generate and then screen approximately 21,000 mutants, from which they recovered about 200 mutants defective in various aspects of the fungus' infective life cycle. This screen proved bountiful, stimulating at least five in-depth studies of fungal genes involved in various aspects of pathogenicity, some of which, like *MoDES1*, would have likely never have been found via reverse genetic means. Among the more striking examples is the discovery of *MoMCK1*, a MAP kinase kinase kinase that when deleted, causes autolysis of mycelia and reduced production of aerial hyphae (Jeon et al. 2008). *MoDES1* is another excellent example; this serine-rich protein surprisingly has a role in tolerance to oxidative stress (Chi et al. 2009). When deleted, mutants inoculated onto plants elicit a stronger suite of defenses from the host, such as defense gene expression and production of reactive oxygen species. The *MoDES1* gene sequence was not reminiscent of ROS-scavenging genes, and without a forward genetic approach, its important function might not otherwise have been discovered. Additionally, the T-DNA

screen unearthed *MoSDR1*, a short chain dehydrogenase/reductase, which, to the best of our knowledge, had not yet been characterized in any way in this fungus. Upon deletion, the fungus is rendered substantially defective in many developmental aspects, including conidial formation and germination, appressorial formation, penetration, and invasive growth (Kwon et al. 2010).

Reverse genetic strategies have also contributed immensely to the determination of gene function, most of which could not be accomplished, without knowledge of the genome sequence. In 2009, Kershaw and Talbot used the genome sequence to design primers to amplify and fuse gene-of-interest flanking regions to selectable markers for high throughput deletion of all the autophagy genes (Kershaw and Talbot 2009). As mentioned previously, this family of genes is involved in development of a fully mature and functional appressorium. In 2010, Kim et al. compared fungal genomes for homeobox domain-containing proteins. They found eight such genes in the *M. oryzae* genome, two of which, *MoHOX2* (mentioned in the preceding section) and *MoHOX7* are required for conidiation and appressorial formation. The discovery of *MoHYR1*, likewise, was via reverse genetics, revealing a gene that, like the aforementioned *DES1*, is involved in regulating ROS scavenging in the fungus and required for successfully coping with the plant's defensive oxidative burst (Huang et al. 2011). In 2013, genome-wide comparisons and analyses allowed for the identification of ABC transporters in *M. oryzae*, known to have important roles in toxin tolerance and resistance. Fifty such genes were identified in *M. oryzae* from which three were selected for further examination. Upon deletion, *MoABC6* and *7* were reduced in conidiation but not virulence, while *MoABC5* was highly reduced in virulence (Kim et al. 2013a, b). Together, these studies based on genome sequence comparisons, set the stage for a better understanding of transcriptional control during pathogenesis, response to oxidative stress, and the role of transporters during invasive growth.

These genetic approaches provide the foundation for determining the mechanism behind how each of these genes, likely in conjunction with other genes, control stages of pathogenicity, and importantly, how the fungus adapts to changing conditions.

6.5 Genome to Protein to Pathway: The Importance of Biochemistry in Rice Blast Infection

Understanding the biochemical processes that allow *M. oryzae* to infect and destroy plants, and how these pathogenic pathways are controlled, is important in understanding the underlying biology of rice blast disease. Prior to the availability of the genome, several studies had success in identifying and characterizing biochemical pathways necessary for host infection. For example, Thompson et al. (2000) contributed to the elucidation of melanin biosynthesis by characterizing a second naphthol reductase gene cloned from *M. oryzae* by identification of cDNA fragments with weak homology to the cDNA of trihydroxynaphthalene reductase. Other biochemical pathways associated with infection that were characterized at this time include the glyoxylate cycle, where the gene encoding isocitrate lyase, *ICL1*, was isolated from an appressorial specific EST library (Wang et al. 2003). Subsequent disruption showed the glyoxylate cycle is required for full virulence; and glycogen and lipid turnover were shown to be pathways important for turgor generation (Thines et al. 2000). Several large-scale efforts to characterize biochemical pathways were also initiated. Hamer et al. (2001) coupled high throughput gene deletion strategies with extensive phenotyping to validate pathways, such as tryptophan and phenylalanine catabolism, which might be developed as new fungicide targets (Hamer et al. 2001). Tanzer et al. (2003) studied the response of *M. oryzae* (and other model and phytopathogenic fungi) to the toxic glutamine synthetase inhibitor glufosinate and the toxic

alcohol analogue allyl alcohol during growth on a wide range of nitrogen and carbon sources, respectively. This provided an outline of the likely metabolic pathways operating in these fungi, and how they could be controlled. Their results suggested that the regulation of nutrient utilization by *M. oryzae* might provide insights into the nutrients typically encountered by the fungus, i.e., during infection.

6.5.1 Genome-Enabled New Insights on Nutrient Requirements

Following the sequencing of the *M. oryzae* genome, progress in elucidating important biochemical pathways has accelerated. Availability of the genome has allowed biochemical pathways to be deduced using post-genomic, high-throughput technologies, such as transcriptomics, proteomics and metabolomics, which rely on an available genome for their utility. For example, in the aforementioned study by Mathioni et al. (2011), they compared transcript abundances under different stress conditions and in planta growth to determine that carbon metabolism and oxidoreduction reactions were likely important processes for infection. Soanes et al. (2012) recently used next generation sequencing to identify transcripts associated with appressorium development. By mapping the transcripts to the genome, and using the genomic sequence to reveal homologous genes with known functions, the authors were able to highlight the role of autophagy, melanin biosynthesis, and lipid metabolism in appressorium function. In addition, the observation that quinate permeases and the quinate utilization cluster are upregulated in appressoria, suggested that quinate produced in rice cells might be an important carbon source for *M. oryzae*. Proteomics studies have also started to reveal biochemical processes associated with infection. Gokce et al. (2012) and Franck et al. (2013) have studied the conidial and appressorium proteomes and identified proteins associated with melanin biosynthesis, lipid metabolism, glycogen metabolism, and anti-oxidation. Advances in metabolomics also promise to reveal how metabolite identity and amount changes during infection, thus giving a glimpse of the underlying biochemical pathways active during infection. Most success to date in this regard has been achieved by monitoring metabolite changes in the host cell following infection. Using metabolic fingerprinting, Parker et al. (2009) have demonstrated changes to metabolism in the cells of rice and *Brachypodium distachyon* following challenges with *M. oryzae*. They demonstrated that in infected cells, the generation of mannitol and glycerol appear to drive hyphal growth. Moreover, while some metabolite changes during infection might be consistent with ameliorating ROS activities, elevated levels of glutamate, aspartate, and GABA suggested a major source of nitrogen for protein synthesis in growing hyphae. Therefore, although metabolite measurements of infected leaves have so far been unable to ascertain changes occurring in the fungus, documenting metabolite changes in the host during infection generates information regarding which sources of nitrogen, for example, the fungus appears to manipulate the plant into producing, thus indicating which biochemical pathways are active to assimilate nutrients from the host.

6.5.2 Gene to Pathway: Comparative Biochemistry Reveals Infection-Related Pathways

Availability of the genome has also enabled genes encoding structural enzymes to be mapped by sequence homology to known biochemical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG). Comparing biochemical pathways for *M. oryzae* predicted in KEGG with those from either other plant pathogenic fungi, or nonplant pathogenic fungi, may indicate what pathways might be important for a pathogenic lifestyle. Also, KEGG facilitates the identification of genes involved in specific biosynthetic pathways, such that those pathways that can be dismantled using homologous gene replacement. One recent example is the disruption of

methionine biosynthesis in *M. oryzae*. Using KEGG, the methionine biosynthetic pathway was identified and the function of one member, cystathionine beta-lyase, was disrupted (Wilson et al. 2012). The resulting mutant strains could form appressoria and penetrate host cuticles, but failed to grow in the cell, suggesting the ability to make methionine is critical to in planta growth. Therefore, combining genomic and KEGG analyses can provide new insights into the biology of infection by *M. oryzae*.

Other biochemical pathways have been targeted since the publication of the genome. These include eight lipase genes and a gene encoding fatty acid beta-oxidation enzyme, identified from the genome, which were shown to have roles in plant infection via the metabolism of fatty acids and the subsequent generation of acetyl CoA (Wang et al. 2007). In addition, mitochondrial β-oxidation (Patkar et al. 2012), and lipid mobilization via the action of peroxisomal alanine: glyoxylate aminotransferase (Bhadauria et al. 2012) are biochemical processes recently demonstrated to be necessary for rice blast disease.

The availability of the *M. oryzae* genome has facilitated the further understanding of biochemical pathways important for disease. Two other studies of note, on a larger scale than those discussed earlier, have used the genome to facilitate biochemical understanding. First, Kim et al. (2010) utilized advanced ChIP-chip and microarray gene expression technologies to locate the genes that are regulated by the Ca^{2+}/calcineurin responsive transcription factor *MoCRZ1*. Increased intracellular Ca^{2+} and calmodulin levels leads to dephosphorylation of CRZ1 by calcineurin, resulting in its nuclear localization. Loss of MoCRZ1 abolished the ability of *M. oryzae* to cause disease (Choi et al. 2009). The study by Kim et al. (2010) aimed to identify the genes directly activated by MoCRZ1. Three hundred and forty-six genes under MoCRZ1 were identified by ChIP-chip and after validation by microarrays, 140 were found to be directly regulated by MoCRZ1. Not only did this study reveal some known virulence factors such as Apt2 to be under MoCRZ1 control, but also revealed feedback regulation of the calcium signaling network by MoCRZ1. Thus, this work provided new insights into calcium signaling, and its outcome, in filamentous fungi.

A second study of note used the available genome sequence to undertake a genome-wide characterization of the components of autophagy. Autophagy is a biochemical process that results in the turnover of bulk cell cytoplasm and cellular components during starvation. Autophagy was known to be necessary for appressorium function (Veneault-Fourrey et al. 2006), and here, Kershaw and Talbot (2009) identified all 22 genes with identities to known autophagy components and disrupted them in the *M. oryzae* genome. In this manner, they demonstrated that loss of any of the 16 genes necessary for non-selective macroautophagy abolished pathogenicity due to loss of appressorium maturation, while the six genes necessary for selective forms of autophagy, such as pexophagy and mitophagy, were dispensable for plant infection. This genome-wide analysis, therefore, characterized nonselective autophagy as being a biochemical process essential for the establishment of rice blast disease.

6.5.3 Proteomic-Based Insight Into Infection

Genome-wide proteomics studies, which are facilitated by the availability of the *M. oryzae* genome, have recently been employed to shed light on the cellular processes associated with infection-related development. Proteomic changes occurring during germination and appressorial formation in response to cAMP treatment were recently determined using nanoLC-MS/MS and spectral counting-based, label-free quantification (Frank et al. 2013). Altered protein abundances during appressorial development revealed the involvement of a wide range of cellular activities during infection-related

development. These included enzymes involved in cell wall biosynthesis and remodeling (including six of the seven *M. oryzae* chitin synthases and other proteins involved in chitin metabolism, and proteins involved in melanin biosynthesis); changes in putative transporter abundances (including altered abundances of ion transporting ATPases and the down-regulation of two putative maltose transporters following conidial germination); altered levels of ribosomal proteins following germination; changes in proteins associated with secondary metabolism; and up-regulation of secreted proteins during appressorium formation. Thus, this work provides important insights into the cellular processes involved in the early stages of rice infection (Frank et al. 2013).

Additional proteomic studies have leveraged the *M. oryzae* genome to analyze the identity of apoplastically secreted proteins during early infection (Kim et al. 2013a, b), and to compare the proteome of wild type conidia and those of a mutant defective in the conidial regulator *COM1* (Bhadauria et al. 2010). Moreover, proteomic studies contributed to the elucidation of metabolic processes altered in a glucose-sensing mutant compared to wild type (Fernandez et al. 2012), thereby providing insights into glucose metabolism in *M. oryzae*, and led to novel mechanistic insights into the processes governing appressorium formation through the identification of ubiquitination, mediated by polyubiquitin, as an essential process in the formation of appressoria (Oh et al. 2012).

Taken together, the work described here demonstrates how basic knowledge of biochemical pathways underpinning rice blast infection has improved with the advent of the rice blast genome. Future challenges remain, particularly in planta, where studies are starting to reveal what biotrophic-specific biochemical pathways might be involved in infection (Wilson et al. 2012; Fernandez et al. 2013). Both of these gene functional studies support earlier, genome-enabled microarray work of Donofrio et al. (2006), which indicated early infection occurred in a nitrogen-poor environment.

6.6 Creating a "Home": Fungal Effector Biology Gets a Boost from the Genome

The importance of pathogen produced effectors during the infection process has been well-studied in the infamous oomycete pathogen, *P. infestans* (reviewed in Kamoun 2006). However, effector biology has lagged behind in pathogenic filamentous fungi. In order to truly comprehend infection, we must learn how the fungus creates an appropriate environment for itself. In preceding sections, we have described genes contributing to its pre-penetration, penetration, and invasion stages. We are also beginning to understand the gene regulation and biochemistry underlying how *M. oryzae* adapts to a changing nutrient environment in the plant host. For the final section of this review, we delve into current efforts to understand how the rice blast fungus generates a hospitable environment for itself. Through the efforts of Valent, Talbot, and others, we begin to understand what these effectors are, where they are "built" and how they are delivered into the host cell.

6.6.1 Re-visiting Gene Expression Analyses: What Are the Effectors?

In 2009, Mosquera et al. developed a technique to enrich for *M. oryzae* effectors. A common limitation in examining fungal genes during invasive, in planta growth is a preponderance of plant RNA compared to fungal RNA, and more specifically, fungal RNA that is from noninvasive hyphae or spores. They overcame this limitation by combining knowledge of the timing of the biotrophic stage of *M. oryzae* (i.e., most hyphae are synchronously invading epidermal cells by 36 h post-inoculation; Fig. 6.1) and using an YFP-labeled fungal strain, in order to visualize groups of highly-infected cells. These areas were harvested for RNA preparation, thus highly enriching for RNAs potentially encoding effectors. Global gene expression patterns were

obtained using microarrays, comparing the enriched IH RNA, with plate-grown mycelial RNA. Overall, 1,120 genes were up-regulated in the IH RNA of which 262 were highly expressed and contained known *Avr* genes such as *Avr-PITA* (Jia et al. 2000). Four genes were characterized and defined as biotrophy-associated secreted (BAS) proteins. These are small, cysteine-rich proteins with secretion signals and no other discernible domains, based on homology searches. This study was seminal in opening the door into the world of pathogenic, filamentous fungal effectors. But the questions remained, how are they being delivered and how do they function?

6.6.2 How Are Effectors Moving, and Where Are They Going?

The Mosquera et al. (2009) study went on to determine that some of the BAS proteins, such as BAS1, are generated in the BICs, the aforementioned blast interfacial complexes that form when bulbous hyphae have invaded host cells. The BIC-generated BAS proteins were found to be cytoplasmically located in their plant hosts, while other small, secreted proteins found in the 2009 study were apoplastically localized (Khang et al. 2010). Research then turned to how effectors were being delivered into host cells. Part of the story comes from the deletion of an endoplasmic reticulum (ER) associated chaperone called LHS1, involved in proper protein import and folding in the ER. When this protein is deleted in *M. oryzae*, the fungus shows strongly attenuated virulence, specific to an inability to conidiate, penetrate, and grow biotrophically in a susceptible host (Yi et al. 2009). This study contributed to the effector story by revealing that without proper folding fungal proteins, among them effectors, lose the ability to function and can lead to impaired virulence.

Building upon this work, the labs of Valent and Talbot collaborated to determine how effectors were secreted into their plant hosts. Initially, they examined the *M. oryzae* genome for evidence of homologs to genes known for roles in polarization and secretion. Genome analysis identified six genes, including several involved in exocytosis, the polarisome and the Spitzenkörper functions (Giraldo et al. 2013). Using genetic deletions coupled with informed use of fluorescent marker constructs, they determined that each piece of the secretion machinery localized at growing hyphal tips. Importantly, they also demonstrated that effectors known to be secreted from BICs into host cells (Khang et al. 2010) were retained in the BICs in a Δ*exo7* mutant and a Δ*sec5* mutant. Importantly, both genes are part of the eight-protein exocyst, involved in docking vesicles to exocytosis sites. Both mutants were defective in secretion of particular effectors such as effector *Pwl2*, and both were reduced in pathogenicity, presumably as a result of inability to secrete specific effectors into host cell's, rendering a favorable environment for further infection.

6.7 Future Prospects and Potential Cures?

The previous section on effector biology in *M. oryzae* is the culmination, but not yet the finale, of where information from the genome can take us. Questions remain as to localization of the entire suite of fungal effectors, and what their host targets might be. Furthermore, it is now up to the research community at large to utilize knowledge gleaned from the genome-enabled comparative genomics, transcriptomics, proteomics, and genetics experiments (only some of which were described here), to find preventative therapies and/or cures for rice blast disease. For example, once we know host targets of these effectors, we might be able to block the interaction, or to manipulate the host target through transgenic approaches, making it unavailable to the fungal effector. Another strategy would be to utilize knowledge from the nutrient studies obtained both through biochemical and transcriptomic approaches, and again manipulate the host environment to reduce the amount of a

potential nutrient source, or sequester it to make it unavailable to the pathogen. This leads directly to an important area that we are just beginning to understand—the role of transporters in *M. oryzae*. Two recent studies suggest that transporters play important roles in pathogenicity, and may as yet represent another way the rice blast fungus, and perhaps fungi in general, manipulates or takes advantage of a host environment. One study comes from the anthracnose pathogen, *Colletotrichum gloeosporioides* and genetic deletion of its ammonia transporters (Shnaiderman et al. 2013). When the methyl ammonia permease *MEPB* was deleted, ammonium was not secreted. As a consequence, appressorial formation was reduced, as well as levels of cyclic AMP (cAMP), the secondary messenger required for proper appressorial formation. The aforementioned 2013 study on ABC transporters in *M. oryzae* (Kim et al. 2013a, b) revealed that three out of the 50 identified had roles in virulence, however the remaining 47 still need to be examined. Recently, Fernandez et al. (2012) identified a MATE transporter to play an important role in virulence. This citrate transporter is required for glucose assimilation, sporulation and pathogenicity and is the first study on this type of transporter in filamentous fungi. *M. oryzae* has two additional putative MATE transporters. Together, transporter studies in filamentous, pathogenic fungi are in an early discovery stage, and can provide valuable clues as to what the fungus needs to import and export in order for successful infection to occur. Transporters could indeed represent an excellent strategy for targeted inhibition.

The power of comparative genomics is just being realized. While we have learned a great deal about the plasticity of the *M. oryzae* genome from the few dozen genome sequences available for this fungus, what remains to be fully explored is the forces and mechanisms at work that drive these changes. Clearly, fungal genomes like that of *M. oryzae* are changing constantly, understanding rate, means, and type of changes will be critical for predicting disease control. For example, several scientists studying rice blast all over the globe are initiating associative genetic projects looking at the genomes of hundreds of *M. oryzae* field isolates within and across regions to develop markers associated with pathogenicity and virulence. These studies, when combined with whole genome-based genotyping of the rice host, will be powerful in predicting the most appropriate rice lines to cultivate, and will result in sustainable and ecofriendly approaches. These types of studies bring us closer to expertly deploying resistance genes and predicting when and how resistance will break down, so that major epidemics can be averted.

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The Genomes of the Cereal Powdery Mildew Fungi, *Blumeria graminis*

7

Pietro D. Spanu

7.1 Introduction

7.1.1 Agricultural Relevance

Powdery mildews are regarded as some of the most common plant diseases. They are caused by fungi that infect a wide variety of hosts including food crops (cereals, fruit, and vegetables) and ornamentals. The disease is easily recognized because of the abundant white/off white conidia on the surface of infected leaves, stems, and flowers (Fig. 7.1a). The airborne conidia form a dry "powder"—hence their name (Glawe 2008). Their importance in agriculture is due to their ubiquity and the impact they have on productivity. Although they do not kill their host, or produce toxic metabolites, they reduce yields and the quality of produce to levels that render the crops economically unviable. In extreme cases, rapid epidemics can result in total crop loss.

The powdery mildews of cereals including wheat, barley, oats, and rye are caused by *Blumeria graminis*. All of these are agriculturally relevant, but the global importance of wheat and barley and their susceptibility to mildews are the reasons why *B. graminis* is regarded as one of the top fungal diseases of plants (Dean et al. 2012), and is why this fungus was the first powdery mildew whose genome was fully sequenced and annotated. *B. graminis* is currently the reference and model for research in the biology of the powdery mildews fungi that infect other hosts such as grapevine, cucurbits, strawberries, roses, and plantain.

In temperate regions with relatively high rainfall, powdery mildews are endemic and ubiquitous. In wheat and barley, protection from this disease is achieved by the combined use of fungicides and resistant host varieties. Genetic resistance in wheat and barley can be determined by "classical", dominant, resistance (R) genes that conform to typical gene-for-gene interactions. Typically, deployment of varieties carrying such R genes is accompanied by rapid evolution of new virulent powdery mildew strains, which breaks down the conferred protection (Wolfe and McDermott 1994). One notable exception is resistance controlled by the *mlo* genes in barley (Jørgensen 1992). *Mlo* genes are different; resistance is determined by recessive alleles and, importantly, so far has turned out to be essentially durable in the field. It has been proposed that developing resistance based on *mlo* homologs in other plants may be a useful strategy for crop improvement and prevention against additional diseases (Acevedo-Garcia et al. 2014).

7.1.2 Taxonomic Position

Powdery mildew fungi are Ascomycetes and all belong to the order Erysiphales of the class

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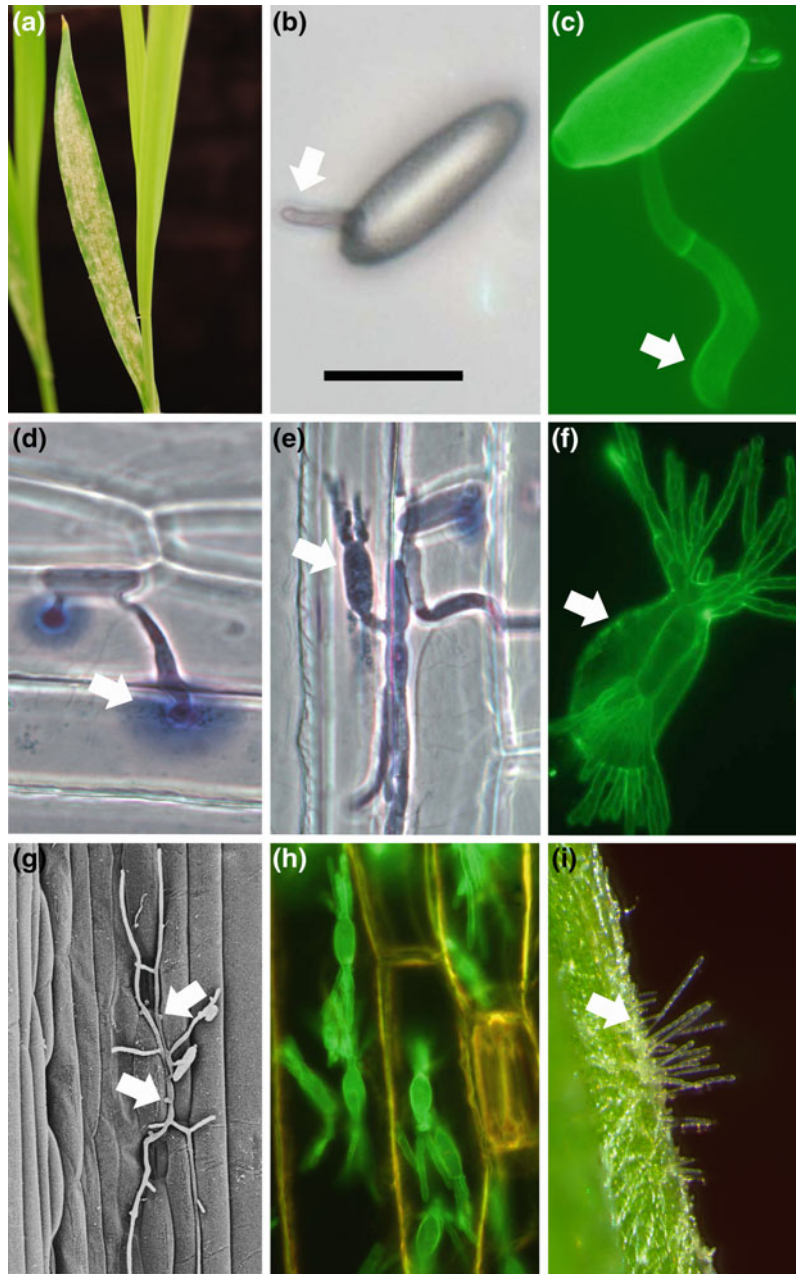
R. A. Dean et al. (eds.), *Genomics of Plant-Associated Fungi: Monocot Pathogens*,
DOI: 10.1007/978-3-662-44053-7_7,



Leotiomycetes. As such, they are closely allied to *Botrytis* and *Sclerotinia*. This affinity is amply confirmed by the similarities between sequences of the protein coding genes. Surprisingly, the obligate biotrophic life cycles and infection strategies of the Erysiphales are in some ways diametrically opposite of those of the necrotrophs *Botrytis* and *Sclerotinia*. Comparative genomics between groups that are at the extremes of the trophic spectrum, but are taxonomically proximal, reveal fundamental insights into the genetic basis for the various life strategies (Spanu 2012).

The powdery mildews are thought to have originated over a 100 million years (Myr) ago,

◀ **Fig. 7.1** Life cycle of the barley powdery mildew fungus, *Blumeria graminis* f. sp. *hordei*. **a** Heavily infected barley displaying masses of asexual spores that are produced on all leaf surfaces and appear as an *off-white* powder. The dry powder is easily dispersed by air. **b** Asexual spore (conidium) 30 min after germination on a solid surface (glass in this case); bar: 20 µm. The primary germ tube (*arrow*) is a structure with a determinate growth. **c** On a leaf, the secondary germ tube develops a slightly swollen and hooked appressorium (visible here 8 h after inoculation, *arrow*). The appressorium is separated from the secondary germ tube by a septum. The fungal structures are stained here with a lectin (wheat germ agglutinin, WGA) coupled to a fluorescent dye (Alexa 488). **d** The host plant responds to the activity of both the primary tubes and appressoria by producing a papilla (*arrow*), visible here as a trypan-blue positive apposition below the fungal structures, 24 h after inoculation. **e** Two days after inoculation, some of the attempted infections will succeed and a haustorium will develop inside the plant's epidermal cell (*arrow*). This feeding structure takes up nutrients from the plant and enables the fungal hyphae to start expanding on the outer surface of the leaves. Note the absence of a clear papilla in correspondence of the successful penetration, whereas a papilla is still visible below the primary germ tube. **f** An isolated haustorium (stained with WGA-Alexa 488). The perihaustorial membrane (*arrow*) is a plant-derived membrane continuous with, but distinct from the plasmalemma. **g** Three days after inoculation, the epiphytic hyphae are visible on the leaf surface (low magnification scanning electron micrograph). Secondary appressoria (*arrows*) enable the fungus to penetrate further cells and develop more haustoria. **h** Epidermal cells that subtend a mature colony may accommodate more than one haustorium. Note that the plant cells remain alive at this time. It is assumed that the host immune system is very effectively suppressed at this stage. **i** Four days after inoculation, foot cells (*arrow*) develop and grow at about right angles to the surface hyphae. These foot cells are the base of the conidiophores that produce chains of conidia acropetally

in the Cretaceous, about the same time as the expansion and the explosive diversification of flowering plants. Within this group, the genus *Blumeria* diverged as a monophyletic line around 60 Myr ago (Takamatsu 2004). More recent diversification and evolution of *B. graminis* into host-specialized *formae speciales* is likely to have occurred over the last 10 Myr, coincidentally with the diversification and expansion of the host cereal plants (Oberhaensli et al. 2011). Data from genome sequencing contributes toward understanding these evolutionary events. This is particularly so with regard to the most recent evolution of virulence associated with agriculture in prehistoric times (Wicker et al. 2013; Hacquard et al. 2013).

7.1.3 Life Cycle

Like all powdery mildews, *B. graminis* is an obligate biotroph. This means that it requires a living host to grow, develop, and complete a life cycle. The life-cycle is simple when compared to some other fungal pathogens.

The most commonly observed cycles are asexual, which represent the principle driver of epidemics. They begin when an ascospore or a conidiospore (conidium) lands on an appropriate host. Germination is rapid and, unusually for plant pathogenic fungi, happens in the absence of liquid water. A primary germ tube emerges from the conidium a few minutes after touching a solid surface (Fig. 7.1b). On a plant, growth of the primary germ tube stops and is limited to a few µm in length. It is thought that the main role of this structure is surface sensing (Carver et al. 1995; Yamaoka et al. 2006). Shortly after the primary germ tube stops elongating, a second germ tube emerges. On a host plant, the secondary germ tube differentiates an elongated, slightly swollen, and often hooked appressorium, which is clearly visible 4–8 h after germination (Fig. 7.1c). Many studies have investigated the role played by surface molecules in determining development in *B. graminis* (reviewed in Both and Spanu 2004). In the absence of a plant or appropriate artificial signals, the conidia produces abortive and unusual developmental programs. For example, on glass many short germ tubes similar to primary germ tubes are formed; on water agar, the germ tubes continue to grow into untypically long hyphae, which eventually stop growing, possibly because of the inability to process nutrients. In the presence of a plant, the appressorium develops a penetration peg. This peg, utilizes a combination of turgor pressure and plant cell-wall degrading

enzymes to facilitate penetration through the cell wall of the epidermis (Pryce-Jones et al. 1999). Commonly, the plant produces a papilla (Fig. 7.1d) and further penetration is restricted. Even on fully compatible host plants, only a small percentage of the penetrations are successful and lead to an established infection.

Once the hypha penetrates through the cell wall, the fungus differentiates a haustorium (Fig. 7.1e). The haustorium is surrounded by a membrane (Fig. 7.1f, arrow) that is continuous with the plasmamembrane, but has distinct properties (Hückelhoven and Panstruga 2011). The haustorium is the only structure likely to take up nutrients in any significant way. It is clear that there are processes that actively suppress host defense at this stage, and it is not unusual to observe multiple penetrations of a single host epidermis cell (Fig. 7.1h).

After a functional haustorium is established, the fungus continues to develop hyphae on the epidermal surface. These hyphae are usually visible 2 days after inoculation and develop small secondary appressoria (Fig. 7.1g, arrow) from which further penetration pegs and haustoria are formed. About 3–5 days from inoculation, "foot cells" develop on the epiphytic structures, from which the asexual conidiophores grow perpendicularly to the surface of the epidermis (Fig. 7.1i). Asexual conidia are produced acropetally on the conidiophores. At this stage, the colonies become visible to the naked eye, then expand and start producing abundant conidia. The conidia are dispersed by air currents and no liquid water is necessary. The asexual cycles are therefore short and may be repeated many times throughout the season of the host's growth, and thus, can support epidemics that spread extremely fast. The capacity to produce masses of conidia to drive swift, airborne epidemics cannot be overstated: one calculation estimated that up to 10^{19} conidia were produced per month during one such epidemic in Europe (Wolfe and McDermott 1994).

The sexual cycle usually occurs at the end of the host's life, when the leaves senesce and dry. Sexual compatibility in *B. graminis* is determined by one of two alleles at a relatively simple mating-type locus (Brewer et al. 2011). When two individuals of opposite mating types grow in proximity to one another, hyphae fuse and a chasmothecium (fruiting body) develops. Karyogamy, meiosis and ascogenesis take place inside these structures. Sexual recombination takes place at this stage. The chasmothecia can act as long-lived resting structures, capable of surviving for long periods in inclement conditions (overwintering or "oversummering"). In mild, humid weather, they break open and the ascospores are liberated, disperse, and can go on to infect a new host.

In some environments, it is possible for *B. graminis* to persist in conditions when the main crop is not grown, by infecting volunteer plants that act as "green bridges" (Liu et al. 2012). In these conditions, the sexual cycle is not necessary for survival and propagation of infection. It is probable that in the agricultural settings of temperate regions (such as Europe) where hosts are grown both in spring and winter, most of the propagation of powdery mildews is actually asexual. This is now confirmed by the first comparative genomic re-sequencing studies (see below) (Wicker et al. 2013; Hacquard et al. 2013).

7.1.4 Host Range

Some powdery mildew fungi are polyphagous generalists and are capable of infecting a wide variety of dicotyledonous hosts (Jones et al. 2001). Unlike these, *B. graminis* displays very narrow host range: it infects only some Pooideae, a sub family of the Poaceae (the true grasses). Moreover, within the *B. graminis* species, eight *formae speciales,* which only infect one host species, have been observed (Hiura 1978). For example *B. graminis* f. sp. *hordei* only infects barley, whereas *B. graminis* f. sp. *tritici* grows exclusively on wheat. These narrow host specificities are genetically determined and, although it has been possible to cross some of these in the laboratory, the progeny do not appear to be very viable and these events do not occur commonly in the wild (Walker et al. 2011). Genetic and cytological analyses of the

events that follow infection indicate that adapted *formae speciales* induce a form of short range susceptibility (Olesen et al. 2003) in the host cells and those in the immediate vicinity (Olesen et al. 2003).

7.1.5 Genetic Tractability and Functional Genomics

The fact that it is possible to obtain crosses between individuals with appropriate mating types, has enabled some significant application of classical genetics. This, however, is very laborious and requires long-term experiments given the constraints imposed by obligate biotrophy and the fact that the sexual cycle is much longer than the asexual one (months, as opposed to days). These analyses have been employed with some degree of success to create genetic maps (Pedersen et al. 2002a) and to identify avirulence genes in *B. graminis* f. sp. *hordei* (Skamnioti et al. 2008).

At present, powdery mildews are not readily transformable. In spite of some reports of successful expression of GUS reporter genes in *B. graminis* (Christiansen et al. 1995; Chaure et al. 2000), these findings could not be reproduced reliably enough to be used effectively in practice. It is not clear exactly what the limiting factors that cause these difficulties are. The frequency of transformation is low and, although expression of heterologous reporter genes such as GFP is possible (James K Brown and Alejandro Perez-Garcia, personal communication), the transgenes appear to be relatively unstable and they are lost after many rounds of subculture. These difficulties are clearly a stumbling block and have imposed some limits on the application of functional genomic techniques to powdery mildews.

Alternative approaches to manipulate gene expression for the functional analysis of genes in *B. graminis* have been more successful. The first well-established method is host-induced gene silencing (HIGS) (Douchkov et al. 2005; Dong et al. 2006; Nowara and Schweizer 2007; Nowara et al. 2010). In HIGS, fungal genes are targeted by the expression in the host of an inverted repeat RNA (separated by an intron) that interferes with mRNA function (RNAi). Typically, leaves are bombarded with particles coated with plasmid DNA that drive the production of RNAi. The production of fungal RNAi in the epidermal cells results in the down-regulation of target RNA in the fungus. Although the precise mechanisms for RNA transfer for host to pathogen are not clear, HIGS can be applied to various other plant-microbe interactions as well (Nunes and Dean 2012). A derivative of HIGS has even been used to modulate parasite gene expression in the plant-plant parasite systems (Bandaranayake and Yoder 2013). HIGS has now been applied with success to validate, experimentally, candidate effector genes first identified in studies of the proteome (Zhang et al. 2012b; Pliego et al. 2013).

Further, approaches to investigate the functionality of powdery mildew genes include virus induced gene silencing (VIGS), virus gene overexpression (VOX), and the delivery of effectors by appropriate bacteria such as *Xanthomonas* spp. (Wise et al., in preparation).

It has been noted that all these methodologies rely on transient expression or delivery of genes and proteins in infected plants and that this may result in artifacts that are difficult to control. It may therefore be desirable to complement and confirm these studies using stable transgenic plants (Spanu and Panstruga 2012). Achieving stable transformation of the powdery mildew fungi in a reliable, reproducible manner at sufficient efficiency and frequency is still a highly desirable goal.

7.2 The Genome

Sequencing the genome of an obligate biotrophic pathogen posed various challenges. Some of these challenges were predictable, others were not; some but not all were mastered. The first evident difficulty was obtaining sufficient DNA for sequencing and free of DNA from non-*B. graminis* sources. The quality and quantity of DNA

required depended on the method eventually used for sequencing. For example, the first stages of the original *B. graminis* f. sp. *hordei* sequencing project relied on dideoxynucleotide (Sanger) sequencing, and therefore on the preparation of appropriate libraries in fosmid vectors. With the advent of "next generation" sequencing platforms (454 Pyrosequencing, Illumina, SOLiD), a reduce quantity and quality was adequate. In the case of *B. graminis* f. sp. *tritici*, a BAC tiling approach was used, which required relatively large DNA fragments as the starting material. Fortunately, sufficient material of adequate size could be obtained from conidia collected from infected leaves. For *B. graminis*, this material is relatively abundant, can be isolated in relatively pure form, with little or no DNA from host cells, or other microbes. Care is needed to remove unknown contaminating substances, which co-purify with DNA (and RNA) and inhibit the activity of many enzymes such as *Taq* polymerase, reverse transcriptase, restriction enzymes used in the down-stream molecular biological processes.

The next challenge, which was not predicted, was related to the genome size. At the start of the project, it was not known exactly how large the *B. graminis* genomes were. Assumptions were made, based on the values obtained for related ascomycetes, that envisaged values of 30–40 Mb; these very optimistic predictions assumed that, being an obligate pathogen, the *B. graminis* genome was likely to be smaller because of probable gene loss associated with obligate parasitism. The genomes are now known to be in excess of 120 Mb. Fortunately, the advent of next generation sequence platforms delivered very high coverage at relatively low cost.

The third hurdle was the exceptionally high proportion of repetitive DNA present in all powdery mildew genomes analyzed to date. Although it was known that *B. graminis* genomes contained repetitive DNA that originated from retrotransposons (Pedersen et al. 2002b), the scale of this and the difficulties posed to assembly were underestimated. As discussed later, this problem has not been solved yet.

In spite of the challenges, a number of strains of *B. graminis* from both *formae speciales*. *hordei* and *tritici* have now been sequenced, partially assembled and deeply annotated thanks to the joint efforts of the international community researching powdery mildews (Spanu et al. 2010; Hacquard et al. 2013; Wicker et al. 2013). The salient findings from these first projects are summarized here.

7.2.1 Genome Structure

It is quite remarkable that we are still uncertain about the overall structure of powdery mildews genomes. There are few published cytogenetic studies. Based on a combination of gel electrophoresis and microscopy of metaphase chromosomes, *B. graminis* f. sp. *hordei* was estimated to have at least seven chromosomes (Borbye et al. 1992), although this may be an underestimate. Genetic analyses have detected a number of linkage groups: the most detailed published map to date identifies 34 linkage groups (Pedersen et al. 2002a). Sequencing the genomes of *B. graminis* has not helped in this matter, because of the difficulties in assembling highly repetitive-rich sequences. The original sequence of *B. graminis* f. sp. *hordei* (Spanu et al. 2010) is highly fragmented into 6898 supercontigs. More recent attempts at assembling *B. graminis* f. sp. *tritici* genome have been facilitated by the use of tiled BAC libraries in addition to conventional "shot gun" sequencing (Wicker et al. 2013); however, even in this case there are still 250 BAC contigs representing 82 % of the genome. Ten sequences resembling conserved telomere ends (tandem repeats of TTAGGG motifs) were identified suggesting a minimum number of five chromosomes. However, the authors cautioned that this is likely an underestimate due to the difficulty in cloning telomeric end sequences. This issue currently remains unsolved and awaits improvements in the strategies for sequencing and assembling repetitive DNA.

Two recent comparative resequencing studies of various strains of both the *B. graminis* f. sp.

hordei (Hacquard et al. 2013) and *tritici* (Wicker et al. 2013) genomes found that these genomes are made up of "mosaics" of monomorphic and polymorphic sequences. These findings support the view that extant populations of cereal mildews are the result of mainly clonal (asexual) propagation interspersed by rare sexual cycles that allow outbreeding. This is an important finding, because it underscores the likely importance of (retro)transposition and expansion of repetitive DNA in the generation of variation in these mildews.

7.2.2 Repetitive DNA

All the powdery mildew fungal genomes analyzed so far are exceptionally rich in repetitive DNA. Estimates vary depending on the methods used to calculate this and on the actual size of the genomes; in the wheat-infecting *B. graminis* f. sp. *tritici*, more than 90 % of genome is made of repetitive DNA (Wicker et al. 2013). In fact, powdery mildews are considered the genomes with the most repetitive DNA, to date.

Repetitive DNA is almost entirely derived from Class I (retro)transposons (Wicker et al. 2013; Spanu et al. 2010). The majority of these transposons are Long Interspersed Nuclear Elements (LINEs), followed by Long Terminal Repeat (LTR) retrotransposons. There are smaller numbers of Short Interspersed Nuclear Elements (SINEs) and only very few DNA based (Class II) transposons (Spanu et al. 2010; Parlange et al. 2011). The extraordinary wealth of repeats therefore appears to be largely due to retrotransposon activity. There is currently no indication of heightened retrotransposition activity per se in the mildew genomes. However, it is notable that Repeat Induced Point mutation (RIP) is absent in the mildews. RIP is one of the mechanisms that is known to be involved in controlling the proliferation of repetitive DNA in filamentous ascomycetes. None of the powdery mildew genomes analyzed so far appear to have any genes that encode key enzymes that promote RIP (Spanu et al. 2010); moreover, a systematic analysis of all repeats showed that there is very little evidence that RIP has ever happened except in very few instances within some unclassified, and possibly very old, repeat sequences (Joelle Amselem, *in preparation*). It is therefore possible that loss of RIP led to the accumulation of the repetitive DNA that is the product of transposon activity.

We have found that the repetitive DNA in the *B. graminis* genomes is homogeneously distributed throughout the genome, which does not have distinct regions with significantly different proportions of repetitive DNA/protein coding genes. This is in contrast with the *Leptosphaeria maculans* genome, where there are clear isochores of repetitive DNA interspersed with isochores of gene-rich DNA (Parlange et al. 2009). It also differs notably from the peculiar situation in *Fusarium oxysporum*, where repeat-rich "lineage specific" chromosomes are found (Ma et al. 2010).

A detailed comparative analysis of a few selected, but representative, loci in the *B. graminis* f. sp. *hordei* and *B. graminis* f. sp. *tritici* highlighted some noteworthy features (Oberhaensli et al. 2011). The protein coding genes are highly conserved and syntenic between the two *formae specialis*. In contrast, the repetitive rich interspersed regions (Fig. 7.2) are extremely diversified. The picture that emerges is that of "two-speed genomes", with DNA that is relatively stable (protein coding genes) embedded into areas that are much more dynamic (the retrotransposons) and subject to relatively fast reshuffling, deletions, and additions.

In other plant pathogens, there are many cases in which repeat- and transponson-rich regions are associated with effectors, i.e., genes responsible for modulating virulence through interactions with the host immunity and metabolism. This is true in the oomycetes (Raffaele and Kamoun 2012; Raffaele et al. 2010; Haas et al. 2009) and in fungi such as *Fusarium* (Ma et al. 2010) and *Leptosphaeria* (Parlange et al. 2009). In *B. graminis* f. sp. *hordei*, candidate effector genes appear to be commonly linked to certain retrotranspons (Pedersen et al. 2012). Direct association between a specific transposable element and a set of effector gene has been

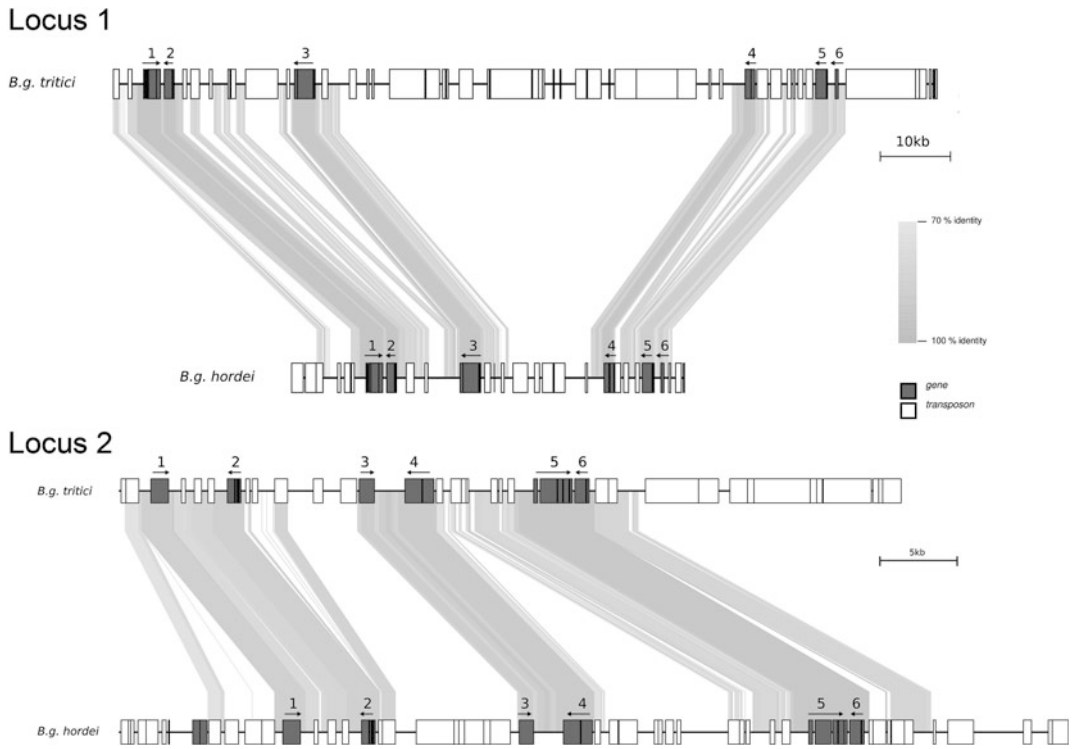


Fig. 7.2 Details of the synteny in two genomic loci in the *B. graminis* f. sp. *tritici* and *B. graminis* f. sp. *hordei* genomes. The protein coding genes (*gray*) are numbered and their direction of transcription is given by the *arrows*. Sequence conservation is shown by the *gray* connectors and the levels of sequence similarity indicated by the intensity of the shading. The retrotransposons are shown as *white* blocks. From these images, it is clear how the protein coding genes are highly conserved, whereas the vast majority of the transposons differ. These events result in genomes that appear to be made up of portions of DNA that evolves at two different speeds: the high-speed retro-elements, which form much of the repetitive DNA, and protein coding genes evolving at a lower speed (Courtesy of Simone Oberhaensli, modified from Oberhaensli, Parlange et al. (2011)

recently found in *F. oxysporum* where this association was used successfully to identify new effector genes (Schmidt et al. 2013). In all these examples, it has been suggested that the physical proximity of effector genes and transposable elements influences effector gene plasticity. The dynamic nature of the transposons enhances evolution of effectors. This, in turn, increases the ability of the pathogens to evolve in response to the evolution of host immunity. In the powdery mildews, these observations may help to explain the terms of the trade-off inherent with increasing genome size. That is, the cost of large genomes full of active retrotransponsons is paid with the "evolutionary currency" of more swiftly adaptable effectors (Spanu 2012). And the cost of large genomes with many active retrotransposons includes gene loss—as we see in the next section.

7.2.3 Protein-Coding Genes

The genome of *B. graminis* f. sp. *hordei* was annotated by a combination of automated prediction and manual curation (Spanu et al. 2010). The latter was the result of a community-based activity, which produced a highly curated set of protein coding genes, supported by multiple lines of evidence. At the time of writing this chapter, 6470 and 6540 genes are identified in the barley powdery mildew (http://www.blugen.org/) and wheat powdery mildew genomes (Wicker et al. 2013), respectively. It is clear that the overall

number of genes in these fungi is about half of those found in closely related ascomycetes, and more akin to the numbers in the hemiascomycete yeasts. In fact, if the respective genome sizes of related ascomycetes are taken into account, the overall protein-coding gene density of both sequenced *B. graminis* formae specialis is very much lower than the average of both eu- and hemiascomycetes (Fig. 7.3).

Detailed analysis of the missing genes noted that this reduction can be attributed to: (1) the near absence of paralogs; (2) the reduction in the size of particular gene families; (3) the absence of genes encoding enzymes in some primary and secondary metabolic pathways. For example, the complement of polysaccharide degrading enzymes is drastically reduced in comparison to other ascomycetes (O'Connell et al. 2012), and there are only two genes encoding polyketide synthases and non-ribosomal peptide synthases (Spanu et al. 2010). A systematic analysis identified 99 conserved "ascomycete core genes" missing in *B. graminis*. Interestingly, where tested, these genes were present and fully expressed during the biotrophic phase of the true hemibiotroph, *Colletotrichum higginsianum*: this led to the suggestion that the genes are not missing because they are detrimental for biotrophy (Spanu 2012).

An alternative explanation is that the missing genes are simply not needed for the powdery mildew lifestyle. For example, hydrophobins are ubiquitous in filamentous fungi (Wösten 2001) and are considered important in water-mediated dissemination of conidia (Whiteford and Spanu 2001) and traversing the challenging water-air barrier (Wösten and Willey 2000). Powdery mildews are unusual fungi, as they do not require liquid water for dissemination or germination of their spore/conidia, and this may explain the absence of hydrophobin genes. Genes encoding alcohol dehydrogenases are also absent from powdery mildews. Since powdery mildew fungi inhabit exclusively the aerial surfaces of plants and are consistently exposed to aerobic environments, they may have dispensed with genes that are essential for anaerobic metabolism.

7.2.4 Gene Loss Convergence in Biotrophs

When the first group of biotrophic plant pathogen genomes were sequenced and annotated, one aspect that caught our attention was the extraordinary similarity in the genes and pathways that were either lost or reduced when compared to hemibiotroph or non-pathogenic related species. For instance, similar reductions in polysaccharide degrading enzymes were also observed in the rusts (basidiomycetes, obligate biotrophic pathogens) (Duplessis et al. 2011) and downy mildew (oomycetes, obligate biotrophic pathogen) (Baxter et al. 2010); carbohydrate transporters are fewer in both *B. graminis* (Wicker et al. 2013) and *P. graminis* (basidiomycetes, obligate biotrophic pathogen) (Duplessis et al. 2011); lower numbers of genes encoding enzymes devoted to secondary metabolite synthesis was observed in *B. graminis*, *Tuber melanosporum* (ascomycete, non-obligate biotroph mutualistic symbiont) and *Ustilago maydis* (basidiomycete, non-obligate biotrophic pathogen) (Spanu et al. 2010).

The most striking instances of convergence are probably those related to the loss of entire metabolic pathways. The genes that encode enzymes needed for inorganic nitrate and inorganic sulfate assimilation are missing in *B. graminis* (Wicker et al. 2013; Spanu et al. 2010), the rusts (Duplessis et al. 2011), and the oomycete downy mildews (Baxter et al. 2010) and white rusts (Kemen et al. 2011). Both *B. graminis* and rusts also lack the same genes encoding enzymes involved aromatic amino acids synthesis and degradation (Fig. 7.4) (Wicker et al. 2013).

The data suggest that some pathways, in particular those involved with primary metabolism, are not active in these biotrophs. The evident corollary for this is that rust and powdery mildew fungi obtain the respective nitrogen, sulfur and amino acids from their plant hosts. This raises the question of whether these microbes are obligate because they are auxotrophic for the metabolites in question. In fact, this interpretation is probably too simple; if this were true, it would be possible

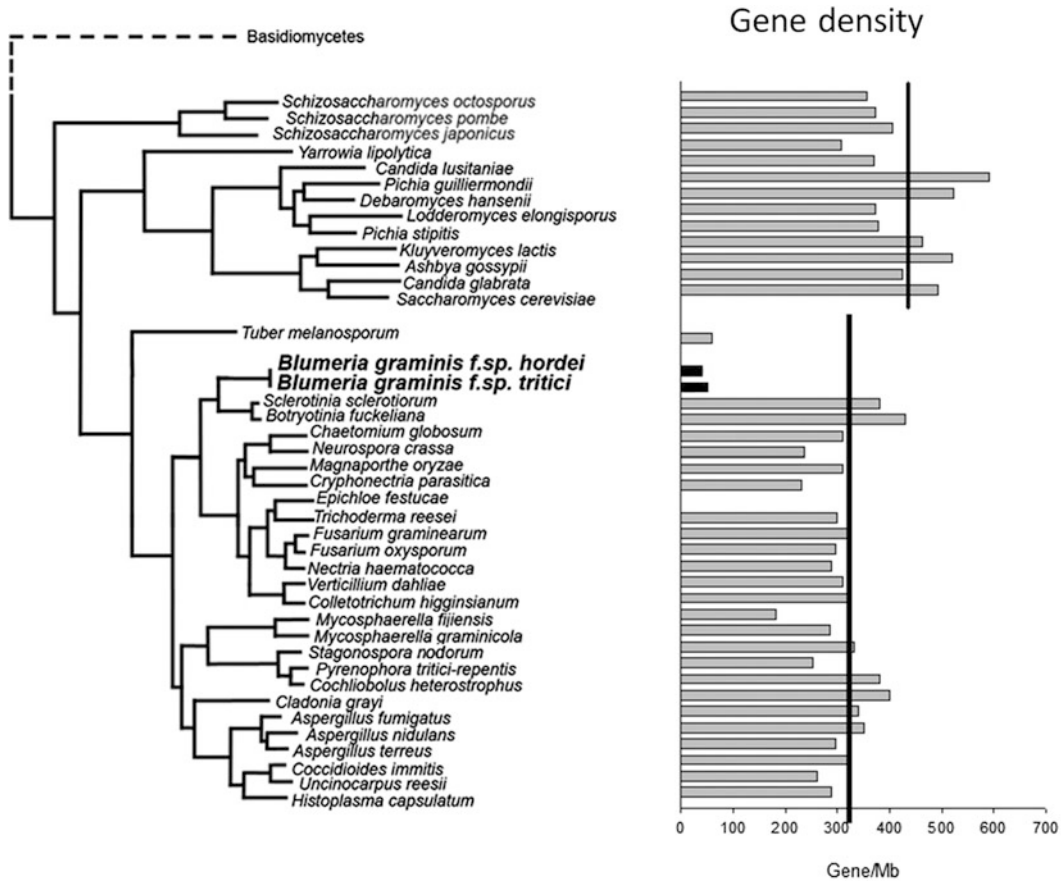


Fig. 7.3 Density of the protein coding genes in the genomes of selected ascomycete fungi. On the *left*, a tree illustrates the relative taxonomic positions of the species (note the length of the tree branches have no significance in relation to taxonomic distance). The histogram on the *left* illustrates the gene density of the sequenced genomes. The average gene densities of the hemiascomycete genomes (*black*) and euascomycete genomes (*green*) are shown here. The gene density of the sequenced cereal powdery mildews (*red*) and of the truffle fungus, are clear outliers. This is the result both of their larger genomes and lower numbers of gene, as described in the text

to culture the powdery mildew fungi in vitro simply by supplying complex media that contain aromatic aminoacids, and sources of organic nitrogen and sulfur. An alternative explanation is that obligate biotrophs may require complex signaling cues to regulate their residual metabolic pathways (Spanu 2006), and these cues are only obtainable from appropriate living host plants.

It is not clear why the same sets of genes are missing from unrelated biotrophs: clearly this is a striking example of convergent evolution. It could be that the costs of these particular metabolic steps are high enough that, given an abundant source of ready alternatives, there was an advantage to losing exactly these genes/proteins. This hypothesis remains to be tested.

7.2.5 Gene Amplification: Effector-like Proteins

Plant pathogens deploy effector proteins to manipulate host immunity and metabolism (Göhre and Robatzek 2008). The importance of genes encoding effectors and effector-like proteins in *B. graminis* is highlighted by the fact that, unlike most genes described in the previous sections, effector gene families are expanded, and

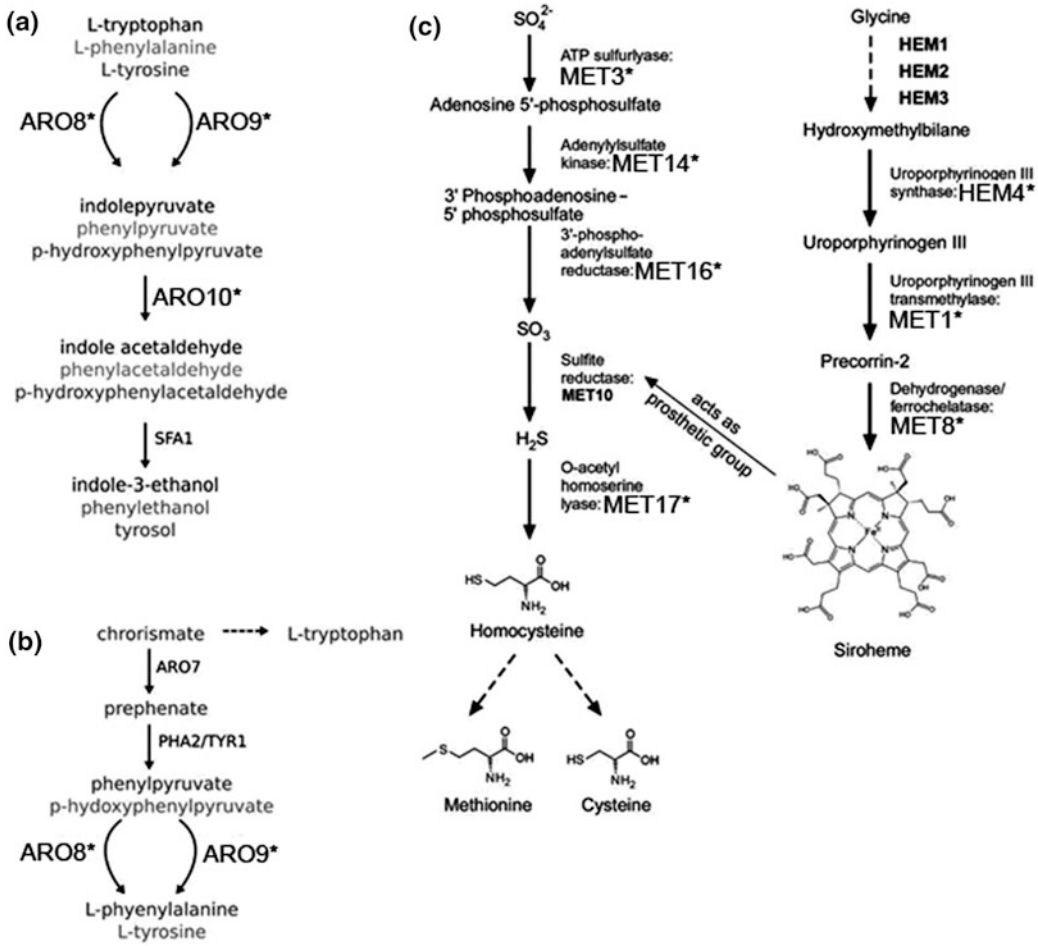


Fig. 7.4 Examples of primary metabolic pathways missing in the cereal powdery mildews. a Aromatic amino acid catabolism/degradation. **b** Aromatic amino acid biosynthesis. **c** Organic sulfur metabolism, and biosynthesis of the key co-factor for sulfite reductase. The genes missing from *B. graminis* that encode the metabolic enzymes are shown as capital/large typeface. All the genes marked with an asterisk (*) are also missing in the rust genomes *Puccinia graminis* and *P. triticina* (modified from Wicker, Oberhaensli et al. (submitted))

some have expanded to a remarkable extent. For example, in *B. graminis* f. sp. *hordei*, 491 Candidate Secreted Effector Proteins (CSEPs) were identified and characterized (Pedersen et al. 2012). Overall, in barley powdery mildew the CSEPs account for over 7 % of the predicted protein coding genes. This number may actually represent an underestimate, because in that work we restricted the assignment to genes encoding proteins, which do not have evident orthologs in non-mildew fungi (as determined by absence of significant hits in BLAST searches). The CSEPs are all predicted to be secreted. In other studies, we have identified effector-like proteins by functional screening candidates that are specifically abundant in the haustoria (Pliego et al. 2013); these effectors include proteins that do have orthologs in other species; for example the BEC1019 effector is similar to proteases commonly found in other fungi. Furthermore, one of the features of many CSEPs is that they have been subject to very intense diversifying selection during the evolution of the mildews; this is evident in the very high non-synonymous/synonymous changes observed between paralogs (Pedersen et al. 2012). In *B. graminis* f. sp. *tritici*,

this observation was used to identify a further 165 Candidate effector Proteins (CEPs) that are actually devoid of a conventional secretion signal, in addition to 437 CSEPs (Wicker et al. 2013).

The absence of evident sequence-determined orthologs notwithstanding, one subset of CSEP families includes proteins that are predicted to have a structural "fold" reminiscent of fungal RNAses (Pedersen et al. 2012). Most of the proteins are not likely to be enzymatically active RNAses because they lack conserved amino acid residues known to be required for hydrolase activity. Remarkably, they all appear to be descended from a single ancestral "proto-effector" gene. This gene was subjected to multiple rounds of duplication followed by very strong diversification. Only the predicted RNAse-like structural motifs are conserved overall. Two of the eight barley powdery mildew effectors verified functionally (BEC1011 and BEC1054) are part of this group (Pliego et al. 2013). Most of the CSEPs that are conserved in the powdery mildew pathogens of non-cereals are members of the RNAse-like families (Spanu et al. 2010; Tollenaere, personal communication). It therefore appears that these effectors proteins have been of central importance for the evolution of the whole Erysiphales clade.

The sheer number and diversity of *CSEP* and *CEP* genes, their specific expression in haustoria, and their very existence in the face of widespread gene loss elsewhere, underscores their centrality in the powdery mildews genome. Effector diversity is driven by coevolution with plant hosts that are themselves under pressure to detect effectors and use them as alarm triggers (Takken and Rep 2010). If this is true, though, the question remains why so many are actually maintained in the powdery mildew genomes, rather than simply jettisoned when the plant evolves to recognizing them. An illuminating insight into this conundrum is given by an exhaustive and in depth transcriptome-wide analysis of gene expression in *B. graminis* f. sp. *hordei* during early stages of infection (Hacquard et al. 2013). This study shows that there are specific groups of *CSEP* genes that are activated at specific stages of the early infection. Intriguingly, the effectors genes activated at the earliest stages, are also those that appear to have been under the strongest diversifying selection; in various cases, CSEPs belonging to the same families are expressed specifically at different times. This suggests that CSEPs expressed early in infection are devoted to challenging the plant's immune system, and are under pressure to diversify by coevolutionary pressure. The CSEPs expressed later, in mature haustoria, may play roles in the maintenance of biotrophy (Hacquard et al. 2013).

7.3 Perspectives

The most pressing challenge posed by the *B. graminis* genomes is that of their fragmentation: assembly of the sequences has clearly failed to deliver anything that even approaches a truly finished status where all, or practically all, of the DNA in the genome is accounted for. This is important for a number of reasons.

We have seen here that, like other biotrophic plant pathogens, the powdery mildew fungi appear to encode fewer protein coding genes than related fungi. Some of these losses may be the result of convergent evolution and simply reflect functions that are no longer necessary in life restricted to a living plant. Other losses may be important in understanding fundamental principles underlying biotrophy or obligate biotrophy. The robustness of these conclusions in this area will be limited until we are certain beyond reasonable doubt that all the genome sequences are accounted for.

Without an accurate, detailed, and complete assembly, the map of the large scale genome structure is uncertain. The finished assembly of other fungal genomes has had significant impact on our understanding of the evolution of those plant pathogens. For example, in *Fusarium* it allowed the discovery of the effector-rich lineage specific chromosomes that play key roles in disease and host range (Ma et al. 2010).

The repetitive DNA in the powdery mildew genomes is particularly affected by partial assembly. It may be argued that this just represents the subpopulation of retrotransposons,

transposons, and the genomic "trash" associated with their activity. However, the many observations that effector genes are often associated with repetitive DNA (Sacristán et al. 2009; Ridout et al. 2006; Schmidt et al. 2013), and that effectors are likely to be key to understanding important aspects of pathogen biology, means that resolving the structure of these parts of the genome accurately, at high resolution, could yield unexpected and invaluable insights.

The difficulties faced in the assembly of genomes with large proportions of long stretches of repetitive DNA sequence will be overcome by new developments in sequencing technology, or by combinations of different techniques. For example, single molecule ultra-long reads exceeding 10 kb have been reported (Niedringhaus et al. 2011). These methodologies may enable the spanning of the repetitive regions unequivocally between non-repetitive elements; at present, their principal drawback is the relative inaccuracy due to elevated error rates. Therefore, it may be useful to couple these long reads with very deep sequencing with current short read techniques (e.g., Illumina). An additional strategy could be the application of optical mapping (Zhang et al. 2012a) of the *B. graminis* genomes. In principle, the single molecule restriction analysis has the potential of resolving the true structure of very long repetitive DNA. In combination with the sequencing techniques described above, current difficulties may be overcome (Lin et al. 2012). Finally, we observed that the *B. graminis* f. sp. *hordei* genome sequence was mostly congruent with the existing genetic maps (Pedersen et al. 2002a). At present, the density of molecular/physical/sequence markers on these maps is too low to improve the genome assemblies. The creation of high density genetic maps based on sequence markers may provide a useful orthogonal cross-check to corroborate the assemblies obtained with sequencing.

Population genomics data holds many promises to our understanding powdery mildews, particularly when the tools available for direct genetic manipulation are restricted. Arguably, "Nature has carried out lots of experiments out there": recent advances in understanding the evolution of pathogens in plant disease demonstrate the power of these approaches (Stukenbrock and Bataillon 2012). In my view, similar studies in powdery mildews promise to explain much with regard to the evolutionary dynamics of mildews in relation to their hosts in both agriculture and the natural environment. Ultimately, this understanding will contribute toward mitigating the impact of powdery mildews on our food and other crops resources.

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Puccinia graminis

8

Les J. Szabo, Christina A. Cuomo, and Robert F. Park

8.1 Introduction

8.1.1 Economic Importance

8.1.1.1 History

The earliest accounts of rust diseases appear as references to epidemics in cereals from the Bible and from Greek and Roman literatures (McIntosh et al. 1995). Various scholars, including Aristotle (384–322 B.C.), Theophrastus (*ca.* 372–287 B.C.) and Pliny (23–79 A.D.), associated rust outbreaks with environmental conditions such as dew and sunshine (Chester 1946). Although not understood, a relationship between rusted grain crops and barberry bushes was recognized as early as the 1600s, at times leading to the destruction of barberries growing near grain crops, a practice that was enforced by law in some regions (e.g. Rouen, France, 1660; Connecticut, USA, 1726).

A year after a severe rust epidemic in Italy in 1766, independent publications by Fontana and Targioni Tozzetti for the first time reported that rust was caused by a parasite (Schafer 1984). The species *Puccinia graminis* was described later from infected wheat by Persoon in 1797 (McAlpine 1906). *P. graminis* causes the disease stem (black) rust on many grasses, including the economically important cereals wheat, barley and oats. While it is not known which rust pathogen species was responsible for the losses referred to in ancient texts, it seems plausible that many would have been due to outbreaks of stem rust of wheat. Indeed, Kislev (1982) reported the detection of this pathogen on two lemma fragments of wheat dating back to the late Bronze Age (*ca.* 1300 B.C.) that were found in a storage jar recovered from archaeological excavations in Israel.

8.1.1.2 Economic Impact

Stem rust has reached epidemic levels in forage ryegrass crops and the cereal crops barley, oats, rye, wheat and the man-made cereal triticale, which have at times resulted in complete crop failure. Of these, the most damaging and concerning have been in wheat crops, caused by the pathogen *P. graminis* f. sp. *tritici* (*Pgt*). An overview of global crop losses caused by the three wheat rusts indicated varying regional significances, with stem rust being important in

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R. A. Dean et al. (eds.), *Genomics of Plant-Associated Fungi: Monocot Pathogens*,
DOI: 10.1007/978-3-662-44053-7_8,

Australasia, the USA and Canada, northern Africa, South Africa and, to some extent, Europe (Saari and Prescott 1985). Within some of these regions, stem rust epidemics were so severe that controlling the disease was the greatest impediment to establishing viable wheat production systems. The economic contribution of resistance breeding to these control efforts was estimated in Canada to be in the order of $C217 million annually (Green and Campbell 1979), and in Australia $A124 million (Brennan and Murray 1988).

8.1.1.3 Current Situation

Apart from epidemics in Ethiopia in 1993 and 1994 (Shank 1994), the incidence of stem rust in wheat crops around the world remained low from the early 1970s until the late 1990s, when it re-emerged as a serious problem in east Africa. Severe stem rust infection was observed in wheat nurseries in Uganda in 1998, and greenhouse assays of a single sample of stem rust collected from these nurseries (accession 'Ug99') in South Africa identified the presence of a new pathotype (Pretorius et al. 2000), which has since become known widely as 'Ug99' and has the North American race designation TTKSK (Jin et al. 2008). This pathotype (race) has the ability to overcome many of the known resistance genes, including *Sr31*, a gene for which virulence had not previously been detected (Singh et al. 2011). Analyses carried out on samples of stem rusted wheat collected from across a wide area have since shown that pathotype TTKSK is a member of a family of closely related pathotypes that is now known as the 'Ug99' lineage. In addition to Uganda, one or more of these pathotypes are present in Eritrea, Ethiopia, Iran, Kenya, Mozambique, Rwanda, South Africa, Sudan, Tanzania, Yemen and Zimbabwe (http://www.RustTracker.cimmyt.org). The 'Ug99' lineage comprises at least seven pathotypes that differ for virulence on resistance genes *Sr21*, *Sr24*, *Sr31* and *Sr36* (Table 8.1; Jin et al. 2008, 2009, Pretorius et al. 2010). Studies using microsatellite markers showed that many of these pathotypes have identical fingerprints, consistent with them being recently derived from a common ancestor via single-step mutation (Pretorius et al. 2010). Significantly, surveys in Turkey, Egypt, Pakistan and India over recent years have failed to detect any of these pathotypes, and three *Pgt* isolates collected from Pakistan in 2009 were clearly shown to differ from pathotype TTKSK in their SSR fingerprint (Karaoglu and Park unpublished) and virulence profile (Fetch unpublished).

8.1.2 Taxonomic Position

8.1.2.1 Taxonomy

The rust fungi comprise the order Pucciniales (formerly Uredinales) of the Phylum Basidiomycota (Basidiomycota, Pucciniomycotina, Puccinales) (Aime et al. 2006). They are one of the largest groups of the Basidiomycota with over 7,000 species, and although they are very distinct as a group, classification above the level of genus is still subject to considerable debate. Currently, 13 families are recognized (Cummins and Hiratsuka 2003). The genus *Puccinia* belongs to the family Pucciniaceae. This genus was first described by Persoon (1801), and later Cunningham (1931) nominated *P. graminis* as the lectotype species for the genus.

8.1.2.2 Phylogenetics

The Puccinales have been regarded as an ancient group of fungi (Leppick 1953) but recent studies have indicated that they are derived from lineages that include parasites of insects and non-vascular plants (Aime et al. 2006). A study of evolutionary relationships among species of *Puccinia* and *Uromyces* based on sequences of the translation elongation factor 1α gene and the β-tubulin 1 gene indicated that sensu lato, neither was monophyletic (van der Merwe et al. 2007). A similar conclusion was made from studies of rDNA sequences in other isolates of the Pucciniaceae (Maier et al. 2007).

Studies of rDNA sequence data also confirmed that *P. graminis* is a genetically variable

Table 8.1 Wheat stem rust pathotypes identified within the 'Ug99' lineage

Pathotype[a]	Differs from 'Ug99' (TTKSK)		Known geographic distribution (year of first detection)
	Virulence	Avirulence	
TTKSK	–	–	Uganda (1998), Kenya (2001), Ethiopia (2003), Sudan (2006), Yemen (2006), Iran (2007), Tanzania (2009)
PTKSK		*Sr21*	Ethiopia (2007), Kenya (2009)
PTKST	*Sr24*	*Sr21*	Ethiopia (2007), Kenya (2008), South Africa (2009), Mozambique (2010), Zimbabwe (2010), Eritrea (2010)
TTKSF	–	*Sr31*	South Africa (2000), Zimbabwe (2009)
TTKSP	*Sr24*	*Sr31*	South Africa (2007)
TTKST	*Sr24*	–	Kenya (2006), Tanzania (2009), Eritrea (2010)
TTTSK	*Sr36*	–	Kenya (2007), Tanzania (2009)

Updated from Park et al. (2011)

[a] According to the North American system for pathotype designation in *P. graminis* f. sp. *tritici* (Jin et al. 2008)

complex species, which is nonetheless likely monophyletic (Zambino and Szabo 1993; Abbasi et al. 2005). *P. graminis* has a wide host range, including telial hosts from some 77 genera of the Poaceae (Cummins 1971) and more than 70 species of *Berberis* and *Mahonia* as aecial hosts (Anikster and Wahl 1979; Roelfs 1985).

8.1.2.3 Subdivisions of *P. graminis*

Subspecific classification of *P. graminis* has been based on either host specificity (formae speciales), or small morphological differences (subspecies, varieties, forms). Formae speciales ('special forms'; f. spp.) are usually named according to the host with which the dikaryon is most commonly associated. In *P. graminis*, those that are most widely recognized include: *P. graminis* f. sp. *agrostidis* (on *Agrostidis* spp.); *P. graminis* f. sp. *avenae* (on oats, *Avena sativa*); *P. graminis* f. sp. *dactylidis* (on *Dactylis glomerata*); *P. graminis* f. sp. *epigaei* (on Calamagrostis spp.); *P. graminis* f. sp. *lolii* (on ryegrass, *Lolium*); *P. graminis* f. sp. *poeae* (on *Poa pratensis*); *P. graminis* f. sp. *secalis* (*Pgs*; on cereal rye, *Secale cereale*); *P. graminis* f. sp. *tritici* (*Pgt*; on wheat, *Triticum aestivum*) (Anikster and Wahl 1979; Anikster 1984; Leonard and Szabo 2005). *Puccinia phlei-pratensis* has been considered by some authors as a specialized form of *P. graminis*, but differs from it in having somewhat smaller spores and a more slender mycelium (Leppik 1961).

Urban (1967) examined urediniospore morphology of a range of specimens of *P. graminis* from Europe and proposed two subspecies, ssp. *graminis* and ssp. *graminicola*. *P. graminis* ssp. *graminis* (*Pg-graminis*) comprises samples from cereals, and was typified by having urediniospores that are long-ellipsoidal to sub-cylindrical and larger than those of the grass attacking *P. graminis* ssp. *graminicola* (*Pg-graminicola*), which were described as being broadly ellipsoidal to ovoid in shape. *Pg-graminis* was further divided into two varieties, var. *stakmanii* and var. *graminis*, small differences in the size of urediniospores and teliospores (for more details see Savile 1984).

The system for grouping isolates of *P. graminis* proposed by Urban (1967) does not agree with the f. spp. system based on host specialization. This is especially so when the inter-fertility of different f. spp. is considered (Zambino and Szabo 1993; Park and Wellings 2012). Based on results from rDNA sequencing, Abbasi et al. (2005) concluded that neither morphological features nor f. spp. host specificity were reliable taxonomic criteria for *P. graminis*. Acknowledging the limitations in the data presented, they further suggested that *P. graminis* should be split into at least two species (Abbasi et al. 2005).

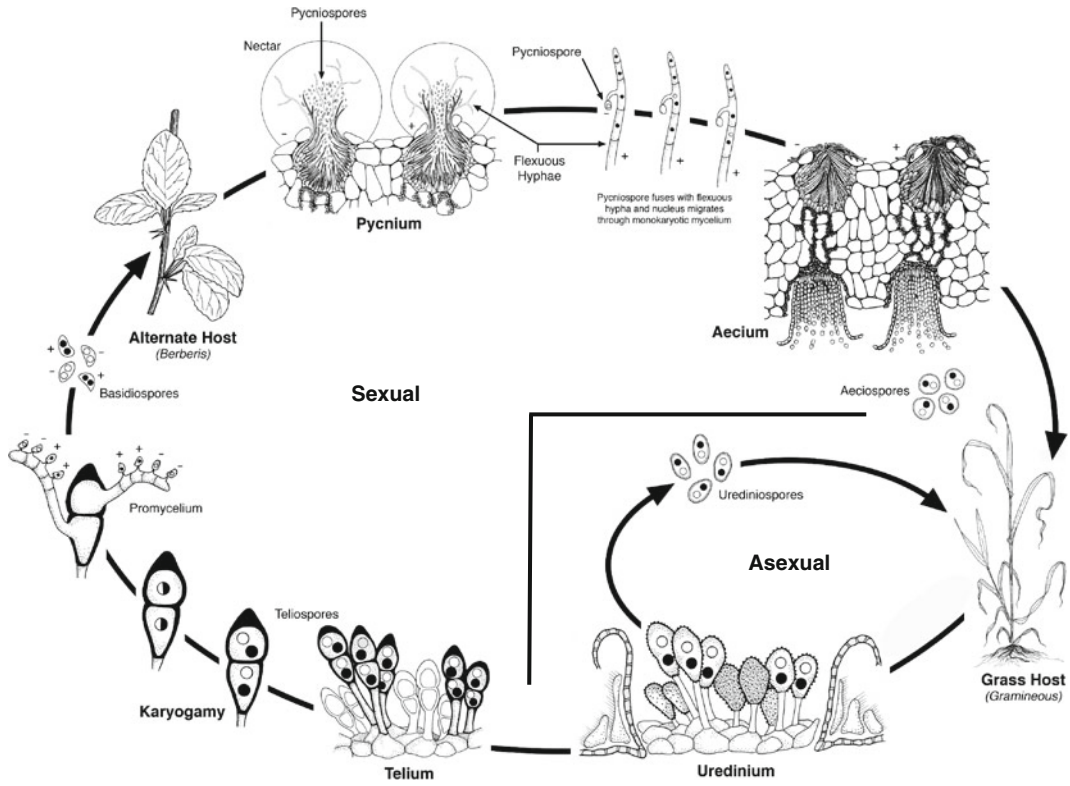

Fig. 8.1 *P. graminis* life cycle. The asexual uredinal stage infects cereal crops and grasses. The sexual stage begins with the production of telia at the end of the growing season of the uredinal host and is completed on an alternate host (*Berberis*). This is adapted from Leonard and Szabo (2005)

8.1.3 Life Cycle

The life cycle of *P. graminis*, as typified by *Pgt*, has been studied in great detail and has been reviewed by many authors (Walker 1976; Leonard and Szabo 2005, Fig. 8.1). Towards the end of the growing season of the telial (grass) host, dikaryotic black teliospores are formed in place of urediniospores. The teliospore is a two-celled resting spore that provides a mechanism for survival during 'winter'. Initially, both cells contain two haploid nuclei that fuse to form a single diploid nucleus as the teliospore matures. Teliospores germinate under favourable conditions after overwintering with one or both cells producing a basidium on which four basidiospores are formed. The basidiospores become airborne after being discharged forcibly from the basidia. Following infection of the alternate host (*Berberis* spp. or *Mahonia* spp.), pycnia are formed on the upper leaf surface and open 1–2 weeks after infection to release pycniospores (spermatia) in a viscous liquid (nectar). The pycnia also contain 'flexuous hyphae', which function as female gametes. Fusion between compatible pycniospores and flexuous hyphae results in the development of the dikaryotic aecium on the underside of barberry leaves. Dikaryotic aeciospores are produced in chains inside the aecium and are air disseminated to infect nearby wheat or grasses. Infections on wheat/grass plant develop into uredinia and thus complete the life cycle.

In many wheat growing areas like Australia, the alternate host is not present. In such regions, *Pgt* reproduces almost exclusively through the asexual urediniospore cycle on the crop itself, on volunteer plants, or on unrelated plant species (Park 2007). In other regions like North America, barberry eradication programmes have

eliminated the alternate host from the major wheat growing areas (Roelfs 1982).

8.1.4 Infection Processes

The germination of urediniospores occurs when they contact a film of water on the host (Leonard and Szabo 2005). In *Pgt*, the infection process is also influenced by environmental conditions such as temperature and light (Rowell 1984). Typically, urediniospores produce an adhesion pad to maintain contact with the host cuticle, and a germ-tube grows perpendicular to the long axis of epidermal cells to form an appressorium over a stomatal opening, from which penetration peg and substomatal vesicle develop (Staples and Macko 1984). Infection hyphae develop from the vesicle and ramify intercellularly throughout leaf tissues to produce a dikaryotic mycelium, on which small terminal branches form (Harder 1984). In turn, the branches form haustorial mother cells, from which a penetration peg extends through the host cell wall to produce a haustorial neck and haustorium. The host plasmalemma becomes invaginated and surrounds the entire haustorium, which extracts nutrients from the host (Staples and Macko 1984).

8.1.5 Axenic Culture

The growth of organisms of a single species in the absence of living organisms or cells of any other species (i.e. growth on non-living substrata) is known as axenic culture. In nature, rust fungi are obligate biotrophs, meaning that they are completely dependent upon a living host for nutrition. Many unsuccessful attempts were made to grow rust fungi on artificial media in isolation from their hosts. The first documented success was obtained with the cedar- apple rust fungus, *Gymnosporangium juniperi-virginianae*, which was cultured on agar medium by subculturing mycelium that grew from cultured sections of telial galls from *Juniperus* (Cutter 1959). *Pgt* was first cultured by Australian scientists (Williams et al. 1966). In this case, the primary inoculum comprised aseptically produced urediniospores that were seeded densely onto media. Mycelia developed slowly on media containing Czapek's nutrients, 0.1 % yeast extract and 3.0 % sucrose. Later studies showed that addition of 0.1 % Evan's peptone resulted in increased vegetative growth and the formation of small amounts of urediniospores and teliospores (Williams et al. 1967). These results led to extensive work refining culture methods and metabolic studies over the next two decades (see reviews by Maclean 1982; Williams 1984). Establishing primary cultures was found to be highly isolate specific with the Australian isolate (126-ANZ-6, 7) used by Williams et al. (1966) to be one of the best. In addition, reduced forms of sulphur and nitrogen are required. In general, primary cultures are determinate and growth terminates in a 'reproductive stage' or becomes necrotic ('staling'). In a few cases, primary culture variants were indeterminate and could be maintained by serial subculturing. In some cases, urediniospores produced from primary cultures were capable of establishing successful infections on a susceptible host under greenhouse conditions. Very little work has been done with axenic cultures since the mid-1980s.

8.1.6 Host Range

The rust fungi have co-evolved with their hosts as a consequence of their obligate parasitism. The narrow host ranges of older rust species and broader host ranges of newer rust species are regarded to have resulted from this process over many years (Anikster and Wahl 1979). The genus *Puccinia* is considered to be of more recent origin, and *P. graminis* has a wide host range that is even more extensive than many other *Puccinia* species (Leonard and Szabo 2005). Leppick (1961) considered *Berberis* to be the primary host of *P. graminis*, and it has been reported that more than 70 species of *Berberis* and *Mahonia* are infected by it. It is thought that *P. graminis* originated in Asia, and that it then

spread to many other parts of the world (Anikster and Wahl 1979). *P. graminis* infects at least 365 cereal and grass species in 54 genera (Anikster 1984).

Studies on the host range of *Pgt* in controlled artificial inoculations have reported wider host ranges than from natural field observations (Anikster 1984). The pathogen was shown to infect 74 species in 34 genera in artificial inoculations of seedlings, but only 28 of those species belonging to eight genera were known to be natural hosts of the fungus. Other formae speciales of *P. graminis* have narrower host ranges than *Pgt*.

Despite the host specificity of the f. spp. of *P. graminis*, there are some host species, or genotypes of host species, which are susceptible to two or more f. spp., and these are referred to as common hosts. Some genotypes of wheat and rye are common hosts for f. spp. *tritici* and *secalis*. These genotypes have been used to examine the genetic basis of resistance of wheat to f. sp. *secalis*. For example, Sanghi and Luig (1971) found that several wheat cultivars lacking effective resistance genes to f. sp. *tritici* possessed from one to four genes for resistance to f. sp. *secalis*. It has been speculated that the difference between *Pgt* and *Pgs* is that they contain slightly different sets of *Avr* genes.

8.1.7 Control

8.1.7.1 Cultural Approaches

The first attempts to control rust fungi included the removal of morning dews by drawing a rope over wheat crops, which is known to have occurred from the 1600s onwards (Zadoks and Bouwman 1985). At about the same time, barberry eradication was being practiced in regions such as Rouen, France, to control *Pgt*. In both cases, factors associated with epidemics (dew, barberry) were recognized well before their biological significance was fully appreciated.

The control of barberry in countries included an extensive eradication programme in the USA in the early years of the twentieth century following a disastrous stem rust epidemic in 1916 (Roelfs 1982; Christensen 1984). Other attempts to break the life cycle of *Pgt* have included the eradication of season volunteer plants (the 'green bridge') for pathogen overwintering or oversummering (Park et al. 2011).

In the late 1800s in Australia, W. J. Farrer bred wheat cultivars that matured earlier and often escaped the threat of stem rust. These cultivars were also adapted to more arid regions that were less favourable for rust development (McIntosh and Wellings 1986). Regulating the crop sowing season was also used by farmers in Mexico, who sowed early to avoid stem rust (Borlaug 1954).

8.1.7.2 Chemical Control

The best known and most common means of chemical control of rust diseases has been the use of fungicides, which act directly by killing fungal structures. While these compounds have been used as either foliar sprays or seed dressings in controlling stripe rust in wheat (e.g. Chen 2005), much less is known about their efficacies in controlling stem rust and the economic value of their use. Studies by Mayfield (1985), Loughman et al. (2005) and Wanyera et al. (2009) all demonstrated significant control of stem rust in wheat with a range of chemicals.

8.1.7.3 Genetic Resistance

The most economical means of controlling rust fungi is the development and cultivation of plants with genetic resistance. The first demonstration of Mendelian inheritance of resistance in wheat to a pathogen in a plant was made nearly a century ago by R. H. Biffin, in studies of resistance in wheat to *Puccinia striiformis* (Biffin 1905). Loci conferring resistance to *P. graminis* have been characterized and used in breeding resistant cultivars of wheat, barley and oats.

To date, some 55 loci conferring resistance to *Pgt* in wheat have been catalogued (McIntosh et al. 1995; Ghazvini et al. 2013). Only three of these loci are known to comprise allelic series, viz. *Sr7* (alleles *Sr7a* and *Sr7b*), *Sr8* (*Sr8a*, *Sr8b*)

and *Sr9* (*Sr9a*, *Sr9b*, *Sr9d*, *Sr9e*, *Sr9f*, *Sr9g*) (McIntosh et al. 1995). Three of the six alleles at the *Sr9* locus (*Sr9a*, *Sr9b*, *Sr9f*) were found in common hexaploid wheat (*T. aestivum*), and three in the tetraploid species *Triticum turgidum* (*Sr9d*, *Sr9e*, *Sr9g*) (McIntosh et al. 1995). Of the remaining 54 loci, 24 were found in *T. aestivum*, and 30 were introgressed into *T. aestivum* from related species: *Aegilops geniculata* (*Sr53*), *Aegilops searsii* (*Sr51*), *Aegilops tauschii* (*Sr33*, *Sr45*, *Sr46*), *Dasypyrum villosum* (*Sr52*), *Thinopyrum intermedium* (*Sr43*, *Sr44*), *Thinopyrum ponticum* (*Sr24*, *Sr25*, *Sr26*), *Triticum comosum* (*Sr34*), *Triticum monococcum* (*Sr21*, *Sr22*, *Sr35*), *T. speltoides* (*Sr32*, *Sr39*, *Sr47*), *Triticum timopheevi* (*Sr36*, *Sr37*, *Sr40*), *T. turgidum* (*Sr2*, *Sr12*, *Sr13*, *Sr14*, *Sr17*), *Triticum ventricosum* (*Sr38*) and *Secale cereale* (*Sr27*, *Sr31*, *Sr50*).

Of the 55 catalogued loci conferring resistance to *Pgt* in wheat, 50 are expressed at all growth stages (often referred to as seedling resistance), and five are expressed only at post-seedling growth stages (adult plant resistance; APR). While virulence matching many of the all-stage resistance genes has been detected, virulence has not been detected on any of the five APR genes. Four of these genes either provide or are thought to provide resistance to other rust pathogens, and are associated with morphological traits: *Sr2*/*Yr30*, pseudo black chaff (Yang et al. 2013); *Sr55*/*Lr67*/*Yr46*, leaf tip necrosis (LTN) (Herrera-Foessel et al. 2011), *Sr57*/*Lr34*/*Yr18*, LTN (Singh 1992) and *Sr58*/*Lr46*/Yr29, LTN (Rosewarne et al. 2006).

Eight genes conferring resistance to stem rust have been catalogued in barley. Those that confer resistance to *Pgt* are *Rpg1* (the 'T'-gene, in cultivars Peatland, Chevron and Kindred; Powers and Hines 1933; Shands 1939; Steffenson 1992), *Rpg2* (Hietpas-5; Patterson et al. 1957); *Rpg3* (PI382313; Jedel 1990; Jedel et al. 1989), *rpg4* (Q21861; Jin et al. 1994), *RpgU* (Peatland; Fox and Harder 1995) and *rpg6* (line 212Y1, a *Hordeum vulgare* line with introgressions from *Hordeum bulbosum* (Fetch et al. 2009). Genes conferring resistance to *Pgs* include *rpgBH* (known formerly as *rpgS*), found in Black Hulless, which is a single recessive gene that confers APR (Steffenson et al. 1984); and *Rpg5* (temporarily designated *RpgQ*) reported in Q21861 (PI 584766) (Sun et al. 1996). Resistance to stem rust in barley was achieved largely by the widespread use of *Rpg1* (Steffenson 1992). *Rpg1* is dominant and considered durable (Johnson 1984), because it is effective against many pathotypes of *Pgt* and it remained effective for a long time in cultivars grown widely across the stem rust-prone Northern Great Plains in the USA (Steffenson 1992). Apart from a minor epidemic caused by *Pgt* pathotype QCC in 1989–1990 (Jin et al. 1994; Steffenson 1992), there have been no significant losses as a result of stem rust in barley since barley cultivars with *Rpg1* were first released in 1938 (USA) and 1942 (Canada).

A total of 18 genes, plus a complementary gene pair referred to as *Pg-a*, have been characterized as conferring resistance to *P. graminis* f. sp. *avenae* in oat (Adhikari et al. 2000; Fetch and Jin 2007). Most (10) of these are from the hexaploid species *Avena sativa* (*Pg-1*, *Pg-2*, *Pg-3*, *Pg-4*, *Pg-5*, *Pg-8*, *Pg-9*, *Pg-11*, *Pg-12* and *Pg-14*), the hexaploid species *Avena sterilis* (*Pg-13*, *Pg-15*, *Pg-17*) and *A. nuda* (*Pg-10*), the tetraploid species *Avena barbata* (*Pg-16*) and the diploid species *Avena strigosa* (*Pg-Sa*, *Pg-6* and *Pg-7*). Two of these genes confer adult plant resistance: *Pg-11*, which is associated with pale green colour and weak straw (McKenzie and Martens 1968), and *Pg-17* (Harder et al. 1992). Most, but not all of the genes been deployed in commercial oat cultivars are *Pg-1*, *Pg-2*, *Pg-3*, *Pg-4*, *Pg-8*, *Pg-9*, *Pg-13*, *Pg-a* and *Pg-Sa* (Adhikari et al. 2000). *Pg-a* maintained a high level of field resistance to the Australian flora of *P. graminis* f. sp. *avenae* until 1997 when it also was overcome by a mutational change in the pathogen (Adhikari et al. 2000).

8.1.7.4 Transgenic Approaches and the Cloning of Stem Rust Resistance Genes

Three of the catalogued genes conferring resistance to stem rust in wheat have been cloned. Gene *Lr34*/*Yr18* also confers resistance to

powdery mildew, caused by *Blumeria graminis* f. sp. *tritici* (gene *Pm38*) and stem rust (gene *Sr57*). It was isolated from wheat by Krattinger et al. (2009), who found that it encoded a protein that resembled adenosine triphosphate-binding (ATP) cassette transporters of the pleiotropic drug resistance subfamily. Time course expression studies showed that the gene is more strongly expressed at later growth stages, consistent with its action as an APR gene. The studies conducted indicated that the *Lr34*/*Yr18*/*Pm38* resistance results from either a senescence-like process that begins at the leaf tips (causing the LTN phenotype), or from a more direct role in exporting metabolites that affect fungal growth (Krattinger et al. 2009). The all-stage resistance gene *Sr33*, introduced into *T. aestivum* from the diploid *A. tauschii*, was cloned and found to encode a coiled-coil, nucleotide-binding, leucine-rich repeat (*CNL*) protein (Periyannan et al. 2013). *Sr33* was shown to be orthologous to the mildew resistance locus *Mla* that confers resistance to *B. graminis* f. sp. *hordei* in barley, showing 80 % similarity to the MLA34 allele. A second all-stage resistance gene, *Sr35* (derived from the diploid *T. monococcum*), was similarly found to encode a *CNL* protein (Saintenac et al. 2013). *CNL* proteins control the recognition of molecules produced during pathogen infection, and are involved in triggering host signal transduction pathways that result in host defence.

Three genes conferring resistance to stem rust in barley have been cloned. The durable gene *Rpg1* was isolated from barley by Brueggeman et al. (2002), who found that it encoded a receptor kinase-like protein that differed from other cloned plant disease resistance genes in having two tandem protein kinase domains. Based on structural similarity to the tomato *Pto* protein, it was suggested that the *Rpg1* protein could be involved in the recognition a matching gene product in avirulent isolates of *Pgt* and function in a signal transduction pathway. The genes *rpg4* (conferring resistance to *Pgt*) and *Rpg5* (conferring resistance to *P. graminis* f. sp. *secalis*) were located to a 70-kb region of chromosome 5H and cloned by Brueggeman et al. (2008). *Rpg5* was found to encode a protein with nucleotide-binding site (NBS), leucine-rich (LRR) and protein kinase (PK) domains, and it was suggested that the LRR domain may reside outside the cell and act in pathogen detection, and the NBS and PK domains are intracellular and trigger resistance signalling. Allele and recombinant sequencing suggested that the probable *rpg4* gene encoded an actin depolymerizing factor-like protein. Such proteins have been shown to be involved in cytoskeleton organization and in non-host resistance.

The recognition of mutation as a major source of variability in wheat rust pathogens led to the strategy of using combinations of resistance genes (Watson and Singh 1952; aka 'gene stacking' or 'gene pyramiding'). This strategy assumes that mutation events are independent, and therefore that the frequency of simultaneous mutations for virulence to more than one resistance gene will be extremely low. In a dikaryotic organism such as *P. graminis*, in which mutation to virulence may have to occur in two nuclei, the probability is even lower. The development of molecular markers linked to genes conferring resistance to *P. graminis* has added to the precision of selecting lines with multiple resistance genes, the cloning and isolation of the actual genes raises the possibility of combining multiple resistance genes in a single construct and transforming them into a breeding line. While such a strategy would accelerate efforts to breed stem rust resistant cereals, it will depend upon public acceptance of GM technologies in these crops.

8.1.8 Genetic Studies of *P. Graminis*

8.1.8.1 Classical Genetics

Genetic studies with rust pathogens are technically demanding and for this reason relatively few have been made. Two common problems are the induction of germination in dormant teliospores, and maintaining purity of the progeny from crosses.

Several genetic studies have shown limited inter-fertility between formae speciales of *P. graminis*. For example, inter-crosses have been established between *Pgt* and *Pgs* (Green 1971; Stakman et al. 1930), and *Pgt* and *P. graminis* f. sp. *agrostidis* (Stakman et al. 1930). In general, the progeny of these crosses had host ranges wider than the parents, but they were less virulent on each of the hosts infected by the parental f. spp. Green (1971) speculated that these features resembled the ancestral form of *P. graminis* from which these f. spp. evolved, and furthermore, stated that while not potentially dangerous to cereal crops, such hybrids could be of evolutionary significance.

Studies of the inheritance of pathogenicity (avirulence/virulence) have usually shown that single loci are involved, with avirulence being dominant to virulence (e.g. Johnson and Newton 1946); however, exceptions to dominance relationships are known. In reciprocal crosses of two isolates of *P. graminis* f. sp. *avenae*, maternal inheritance of avirulence for the resistance gene *Pg-3* was demonstrated (Green and McKenzie 1967), implying that the corresponding avirulence gene *avr-3* is not located on the nuclear genome. Sock et al. (1993) hypothesized that *avr-3* was most likely located on the mitochondrial genome, but were unable to identify it in studies of in mitochondrial DNA from two isolates that differed for pathogenicity on *Pg-3*.

To date, few studies have examined the inheritance of biochemical (e.g. allozyme) or DNA-based markers (e.g. random amplified polymorphic DNAs, RAPDs; amplified fragment length polymorphisms, AFLPs) in rust fungi. The pattern of segregation of five isozyme loci observed in F_2 progeny derived from a cross between two North American isolates of *Pgt* suggested Mendelian inheritance of nuclear genes, and provided evidence of linkage between the loci (Burdon et al. 1986). Zambino et al. (2000) conducted genetic studies on another F_2 population, derived from a cross between two isolates of *Pgt* that differed in pathogenicity on *Sr5*, *Sr8a*, *Sr8b*, *Sr9a*, *Sr9d*, *Sr10*, *Sr21*, *Sr35*, *SrTt-3* and *SrU*. Analyses of the pathogenicity of the parental isolates, F_1 and F_2 progeny indicated that in most cases where segregating progeny were observed, the avirulence phenotype was dominant. In two cases, *Sr9d* and *SrTt-3*, the results suggested the presence of a dominant gene that suppressed the expression of avirulence. Linkage analysis of eight single dominant avirulence genes (*AvrT6*, *AvrT8a*, *AvrT9a*, *AvrT10*, *AvrT21*, *AvrT28*, *AvrT30* and *AvrTU*) and 970 DNA markers generated a partial genetic map with 56 linkage groups, and identified DNA markers linked to each of these avirulence genes. The closest linkages were between *Avr*T6 and the RAPD marker crl34-155 (6 centimorgans [cM]) *Avr*T8a and the AFLP marker eAC/mCT-197 (6 cM) and between *Avr*T9a and the AFLP marker eAC/mCT-184 (6 cM). *AvrT*10 and *AvrT*U are linked at distance of 9 cM (Zambino et al. 2000).

8.1.8.2 Status of Transformation and Transient Expression

Efforts to understand gene function and regulation in fungi such as *Pgt* require the ability to transform modified or foreign genes into the genome of viable isolates, either as integrated copies or stably maintained plasmids, and observe their expression in the transformed isolate. Schillberg et al. (2000) achieved transient transformation of germinated urediniospores of *P. graminis* f. sp. *tritici* using particle bombardment to introduce a construct comprising the *P. graminis* f. sp. *tritici* translation elongation factor 1α gene (*EF-1* α) as a promoter fused to the bacterial marker gene hygromycin B phosphotransferase (*hpt*), *EF-1* α fused to the reporter gene β-glucuronidase (*GUS*), and a promoter from the bean rust pathogen *Uromyces appendiculatus* INF24 fused to GUS. In all three cases, transformation was successful, but transient, lasting up to 5 days for *hpt* transformants, and 4–16 h for *GUS* transformants. To date, the only stable transformation system for rust fungi used Agrobacterium-mediated transformation of *Melampsora lini* infected flax (*Linum usitatissimum*) stems (Lawrence et al. 2010). Transformants were selected via RNA silencing of the avirluence gene *AvrL567*. However, the use of

this transformation system is limited and underscores the need for robust selectable markers, which is the primary impediment in developing an efficient transformation system for rust fungi.

8.2 Genome Structure and Insights

8.2.1 Genome Sequence and Structure

The genome of *Pgt* strain CDL 75-36-700-3, race SCCLC was selected as the initial target for whole genome sequencing. This strain was selected to leverage existing resources and data; it was one of the parents in a mapping population used to generate a partial genetic map from AFLP and RFLP markers including 7 linkage groups and 8 avirulence loci (Zambino et al. 2000). This isolate was collected in the US and has been characterized on 46 wheat stem rust differential cultivars and is avirulent to 25 well-characterized stem rust resistance genes; *Sr6, Sr7b, Sr8a, Sr8b, Sr9b, Sr9d, Sr10, Sr11, Sr13, Sr14, Sr22, Sr24, Sr25, Sr26, Sr27, Sr28, Sr29, Sr30, Sr31, Sr32, Sr33, Sr34, Sr35, Sr36* and *Sr37*. Genomic DNA for sequencing was prepared from germinated dikaryotic urediniospores.

The genome was sequenced to 11-fold depth using a whole genome shotgun approach with Sanger technology (Duplessis et al. 2011). Three libraries were constructed and end-sequenced, two plasmid and one Fosmid, and the resulting reads assembled with Arachne. This produced a 88.6 Mb assembly consisting of 392 scaffolds, where the average base is in a scaffold of 0.96 Mb, the N50 number. Within scaffolds there are 4,557 contigs; the total sequence of contigs is 81.5 Mb. The high gap content (8 %) and lack of higher order linkage between scaffolds is due to the high repetitive content and rate of polymorphism. A fingerprint map constructed from the end-sequenced Fosmids validated the assembly continuity, but did not provide any higher order and orientation to the scaffolds relative to each other.

Karyotypic analysis suggests that there are 18 chromosomes per haploid nucleus (Boehm et al. 1992). Pulse-field gel electrophoresis indicated that the size of the chromosomes range from approximately 2 to 10 Mb, in agreement with cytological length measurements (Backlund 1991; Boehm 1992). Anchoring the sequenced genome to chromosomes will require either a higher order assembly, leveraging new data such as long reads, or the anchoring of sequenced markers to a genetic map.

The genome of *Pgt,* like the poplar rust *Melampsora larici-populina*, is larger than most other fungal genomes due to an expanded population of repetitive elements (Duplessis et al. 2011). A total of 44 % of the *Pgt* sequence was classified as repetitive elements, and a similar amount in *M. larici-populina*. Over half the repetitive sequence could be classified as transposable elements (TEs), either Type I (31 % of total) or Type II (27 % of total). While LTR and TIR elements dominate the Type I and II classes respectively, the most frequent classes of other elements include MITEs, LINEs and Helitrons. While some highly repetitive genomes exhibit clustering of repetitive elements, the TEs found in *Pgt* appear distributed randomly throughout the genome and are not associated with any particular class of genes.

8.2.2 Genome-Wide Comparisons to Other Fungi

An initial set of 17,773 predicted genes was compared to other fungal genomes to characterize the unique features of this genome (Duplessis et al. 2011). The total gene count is larger than most other Basidiomycetes, but similar to that for *M. larici-populina* (16,339) as well as the symbiont *Laccaria bicolor* (19,036). The conservation of protein families was examined by building gene families from a diverse set of Basidiomycetes, as well as two Ascomycetes. A total of 7,959 *Pgt* proteins are found in shared gene families with *M. larici-populina*. These include shared expansions of genes involved in

cell wall modification (glycosyl hydrolases, lipases), transport (oligopeptide transporters) and antioxidant defence (copper/zinc superoxide dismutase). The higher numbers of these gene families could allow for amplification of the protein levels and therefore functional capacity of these groups, or alternatively diversification and specialization of individual family members. Many genes in expanded families are highly upregulated during wheat infection (Duplessis et al. 2011), supporting the hypothesis that they play an important role during the infection process.

Comparison of the carbohydrate-active enzymes (CAZymes) involved in cell wall modification helped to correlate the complement of these genes with the phytopathogenic lifestyle. Both *Pgt* and *M. larici-populina* contain a reduced set of CAZymes that act on the plant cell wall, in particular polysaccharide lyases and glycoside hydrolases (Duplessis et al. 2011). The numbers of these enzymes are larger than those previously characterized for *Ustilago maydis* (Kamper et al. 2006), which shares a biotropic lifestyle with these species. Part of the expanded number in the rust fungi includes moderate expansion of glycoside hydrolases involved in cellulose and hemicellulose cleavage. Some of these enzymes could play a role during initial stages of infection and host penetration.

Analysis of the genome also revealed gene loss events in metabolic pathways, which explain in part the dependency of *Pgt* on its host for growth. Genes involved in nitrate assimilation are found in a cluster in other fungi; both the nitrate/nitrite porter and the nitrite reductase are missing from both *Pgt* and *M. larici-populina*. By contrast, different genes involved in sulphate assimilation are missing from the two rusts; *Pgt* lacks both subunits of the sulphite reductase, whereas in *M. larici-populina* one subunit is missing a key domain. Similar loss of these same metabolic pathways has also been observed in other biotrophs, including the ascomyetes and oomycetes (Spanu et al. 2010; Baxter et al. 2010), suggesting that diverse hosts can provide these metabolites to invading biotrophic pathogens.

8.2.3 Transcriptional Analysis of Infection and Prediction of Effectors

To compare transcript levels between different biological stages, RNA was prepared from four sampled stages and hybridized to microarrays designed from the gene set. The sampled conditions included urediniospores, germinated urediniospores and infected wheat and barley, harvested 8 days after infection when macroscopic flecking was visible. In addition, EST sequences generated from urediniospores, germinated urediniospores, teliospores and isolated haustoria were used for gene structure prediction and for comparison. These data enabled the first genome-wide transcriptional analysis of *Pgt*, and found that the most highly induced genes during infection included those from expanded gene families (transporters, cell wall modification enzymes) and secreted proteins.

Multiple transporters were highly induced during infection, consistent with a role in nutrient acquisition. In *Pgt*, major facilitator transporters involved in sugar and amino acid transport are highly upregulated during infection. This includes a HXT1-like hexose transporter, previously shown in bean rust (*Uromyces fabae*) to be highly upregulated during infection (Voegele et al. 2001). This is consistent with the need to acquire specific nutrients due to the biotrophic nature of rust fungi.

The genome encodes a large set of candidate effectors, which could directly mediate interactions with the host plant. Computational prediction of effectors has utilized a set of signatures, including the presence of a N-terminal secretion signal, small protein size, lack of conservation to other species with different hosts, high family copy number within a species, cysteine-rich proteins and evidence of transcription during infection. Initial analysis of the predicted proteins identified 1,106 small

secreted proteins (SSPs), with a secretion signal and size less than 300 amino acids (Duplessis et al. 2011). In comparison with *M. larici-populina* and other fungi, 84 % of these SSPs show no significant similarity, and so appear species specific. The largest family identified among this set contained a conserved motif of 8 cysteines, which could contribute to secondary structure; of this family, 4 showed evidence of expression in a haustorial EST data set, supporting a potential role as effectors. Overall, transcripts for 40 % of the SSPs were detected in infected wheat leaves; SSPs are highly represented, with 29 of the top 100 for example, among the most induced transcripts during wheat infection.

Additional analysis of candidate effectors has utilized additional signatures or methods to identify candidates for functional analysis. By incorporating the above and additional criteria (such as the presence of internal repeats), candidate effectors were identified and then classified using clustering of protein families (Saunders et al. 2012). Additional N-terminal motifs found in other fungi or oomycetes appear conserved in some rust proteins; an RXLR-like motif, characteristic of *Phytophthora* effectors, ([K/R]RLTG), was characterized for a stripe rust protein (Gu et al. 2011). An alternative [Y/F/W]xC motif was found in a large number of powdery mildew and wheat stem rust proteins (Godfrey et al. 2010). Testing and validation of these candidates will require the establishment of new experimental systems.

8.2.4 Genomic Tools

The genome assembly and gene set for *Pgt* are available for search, analysis and download on websites hosted by the Broad Institute and by EBI. The Broad site (http://www.broadinstitute.org/annotation/genome/puccinia_group) includes the genomes of three wheat rust fungi (*Pgt*, *P. striiformis* and *P. triticina*). In addition to analysis tools, this site provides supplementary data utilized in the first analysis of the genome (Duplessis et al. 2011), including EST data sets and simple sequence repeats. The EBI website (http://fungi.ensembl.org/Puccinia_graminis/Info/Index/) also hosts the genome data in the Ensembl platform, and includes polymorphisms identified from short read sequencing.

Multiple data sets and resources are available for analysis of gene expression. EST sequences generated from urediniospores, germinated urediniospores, teliospores and isolated haustoria can be used to identify genes expressed during these stages. A 70-mer custom expression oligoarray (Roche NimbleGen) was designed from both high and low confidence gene calls; this included the initial set of 20,567 predicted genes, 578 ESTs representing potential missed gene calls and 41 wheat and barley sequences (Duplessis et al. 2011). The array design and data set is available in the GEO database (NCBI) as series GSE25050.

8.2.5 Applications

8.2.5.1 Hunt for Effectors

The ability to isolate highly enriched haustorial fractions from infected leaves provided an important step enabling current molecular characterization of haustoria and identification of effectors. Hahn and Mendgen (1997) constructed the first cDNA library enriched for haustorial-expressed transcripts from bean leaves infected with *U. fabae*. This work led to the identification of the first rust pathogen effector protein (Rust Transferred Protein 1, RTP1p), which is localized not only in the haustoria but also in the cytoplasm of infected plant host cells (Kemen et al. 2005). Analysis of 28 *RTP* homologues from 13 different rust species, including eight from *Pgt*, indicated that this represents a large family of effectors within the Pucciniales (Pretsch et al. 2013). The structure of RTP1p contains a N-terminal region with a signal peptide, post-translational processing site, followed by a highly variable region. The C-terminal region is more conserved and is composed of a globular domain with seven beta strands and

four conserved cysteine residues. Functional analysis indicates that RTPps may have a dual role: as a protease inhibitor (Pretsch et al. 2013), and in stabilizing haustoria by forming filamentous structure in the extra-haustorial matrix (Kemen et al. 2013).

Several avirulence genes have been identified and characterized from the flax rust pathogen, *M. lini*. *AvrL567* was identified using a map-based cloning approach (Dodds 2004), and *AvrM, AvrP123* and *AvrP4* were identified by screening a cDNA library made from RNA isolated from haustoria (Catanzariti et al. 2006). Based on the cytosolic location of the R genes in the host plant (*Linum usitatissiumum*), it was postulated that *AvrL567*, *AvrM*, *AvrP123* and *AvrP4* proteins were secreted from the haustorium and translocated into the plant cell. Confirmation of this was demonstrated through immunolocalization of *AvrM* in plant cells (Rafiqi et al. 2010). Direct interactions between R proteins and cognate Avr proteins were demonstrated (Dodds et al. 2006). Homologues of these effector genes were found in *M. laricipopulina* but not in *Pgt*.

Recently, the first avirulence proteins from *Pgt* were identified using a biochemical approach (Nirmala et al. 2011). It was observed that the RPG1 protein is rapidly phosphorylated upon inoculation of barley leaves containing *Rpg1* with urediniospores of an avirulent pathotype (Nirmala et al. 2010). Phosphorylation is the first step in RPG1 mediated resistance. This rapid response (less than an hour) indicated that the interaction occurs during initial stages of spore germination prior to the development of germ tubes and appressoria, and entry into the host. Earlier work with *U. appendiculatus* demonstrated that treatment of urediniospores with synthetic RGD peptides blocked the formation of adhesion pads and spore germination (Correa et al. 1996). Treatment of *Pgt* urediniospores prior to inoculation of barley leaves prevented the formation of adhesion pads, spore germination, as well as phosphorylation of RPG1. RGD affinity chromatography of urediniospore extracts led to the isolation and identification of two proteins, an RGD-binding protein (PGTG_10537.2) and a vacuolar sorting associated protein 9 (VSP9, PGTG_16791.4). The RGD-binding and VSP9 proteins are predicted to be 818 and 744 amino acids in size, respectively. Infiltration of leaves of a barley genotype carrying the *Rpg1* resistance gene with purified RGD-binding and VPS9 proteins led to a typical HR response. Infiltration with either of these proteins alone or in combination with a carrier protein failed to induce an HR response. When barley lines containing non-functional mutants of *Rpg1* or lacking *Rpg1* were used, HR was not observed. Protein alleles from virulent isolates of *Pgt* failed to produce a HR. Yeast two-hybrid assays demonstrated that the RGD-binding and VPS9 proteins interacted with each other, as well as with the RPG1 protein.

A primary focus of current research in genomics of rust fungi is the identification and characterization of effectors. As described above, extensive work has been done mining genomic and transcript expression data to identify *Pgt* candidate effector genes. This approach has identified hundreds of candidates. In addition, the availability of current genomic resources allows for more efficient map-based strategies. Development of a high-density genetic map by sequencing representative F_2 isolates of a mapping population developed by Zambino et al. (2000) is currently underway (Szabo and Cuomo, unpublished). In addition, as more isolates of *Pgt* are sequenced, genome-wide association mapping will become a powerful tool to identify avirulence genes, as well as effectors that have phenotypes that can be scored reliably.

With the availability of sequenced genomes of rust fungi, there is a renewed interest in developing functional assays for rust fungi. Biolistic methods have been further developed for transient transformation of *U. fabae* (Djulic et al. 2011) and *P. triticina* (Webb et al. 2006). Several systems have been developed using RNAi technology. Barley stripe mosaic virus was used to develop host-induced gene silencing system (HIGS) for *P. striiformis* (Yin et al. 2011;

Zhang et al. 2012) and *P. triticina* (Panwar et al. 2013b). *Agrobacterium tumefaciens* infection of wheat has also been used for in planta-induced transient gene silencing (PITGS) in *P. triticina* (Panwar et al. 2013a). In three of these studies, reduction in fungal development and endogenous targeted transcript levels were demonstrated (Zhang et al. 2012; Panwar et al. 2013a, b).

An alternative to RNAi-mediated loss of function assays is gain of function assays such as infusion of heterologous expressed proteins and bacterial mediated protein secretion systems. Heterologous expression in yeast has been shown to be useful for functional analysis of fungal effector proteins (Nirmala et al. 2011). However, a high-throughput system is needed for the screening of large numbers of effectors identified through genomic analysis. To this end, work is being done to adapt the bacterial type III secretion system. Effector-to-Host Analyzer (EtHAn) was developed by integrating type III secretion system (*hrp/hrc* gene cluster) from *Pseudomonas syringae* into the non-pathogenic bacteria *Pseudomonas fluorescens* (Thomas et al. 2009). Preliminary experiments demonstrated that this system could deliver bacterial effectors into wheat (Yin and Hulbert 2010). An optimized set of expression vectors was developed using a calmodulin-dependent adenylate cyclase (Cya) reporter fused to a modified *AvrRpm1* type III secretion signal (Upadhyaya et al. 2014). Cya activity was observed in both wheat and barley leaves when infiltrated with the *P. fluorescens* EtHAn system. In order to further test this system, alleles of *M. lini AvrM* tested in a transgenic tobacco line expressing flax M resistance protein. Strong HR was observed when the functional allele of *AvrM* was infiltrated into tobacco leaves while no HR was observed when virulent allele (*avrM*) was used. To further test this system, a preliminary screen of *Pgt* candidate effectors resulted in a strong HR that was wheat genotype specific for one of the candidates. This represents a promising new development that needs further testing with rust pathogen effectors.

8.2.5.2 Population Genetics

The primary method for characterization of *Pgt* isolates is the determination of avirulence/virulence phenotypes on standard sets of wheat lines or differentials. This method has been use to study population structure extensively in North America and Australia. In general, this phenotypic method has worked well largely due to the asexual clonal nature of *Pgt* in the major wheat growing areas and the focus on local or regional populations. With the renewed interest in global populations of *Pgt* caused by Ug99, robust molecular markers are need. The availability of *Pgt* genomic resources has enhanced the development of SSR markers, which have been used to examine *Pgt* population structure in North America (Stoxen 2012) and South Africa (Visser et al. 2011a, b). However, improved genotyping methods are made available with the sequenced genome. Re-sequencing of additional isolates has become a powerful tool to examine genome variation and population structure. A preliminary study with 70 *Pgt* isolates identified seven distinct clusters based on a set of 261,991 SNP loci (Szabo and Cuomo, unpublished data). In addition, having sequence data from multiple isolates enabled the development a Pgt SNP Chip (Illumina GoldenGate) containing 1,532 SNP (Szabo, unpublished data).

8.2.6 Molecular Diagnostics

The emergence of Ug99 has underscored the importance of having rapid methods to identify and track new variants of *Pgt*. The current pathotyping method requires live samples and takes several weeks to a month to complete. In addition, current quarantine restrictions limit the movement of live samples and therefore only a few laboratories in the world are able to characterize *Pgt* samples using the standard wheat differentials. The availability of genomic sequence data for several different members of the Ug99 lineage, as well as non-Ug99 isolates, has facilitated the development of a PCR-based method (Szabo 2012). The assay is composed of two

stages: stage-1 determines if the sample belongs to the Ug99 lineage using a set of four PCR assays; stage-2 predicts the pathoype based on a set of specific SNP markers. The current assay is highly specific to the Ug99 lineage and is able to discriminate between six of the known pathotypes (TTKSK, TTKST, TTTSK, TTKSF, TTKSP and PTKST, see Table 8.1). This assay has been shown to work with a range of different sample types, including fresh urediniospores, infected wheat tissue (stems and leaves) and ethanol killed infected wheat tissue. The ability to use non-living collections allows the global transport of samples from farmers' fields to diagnostic laboratories. An unexpected outcome of this work has been the demonstration that individual phenotypic pathotypes often contain several different genotypes.

The development of a DNA-based rapid diagnostic assay for the Ug99 lineage demonstrates that this approach will work for developing assays for other lineages of *Pgt*. Expansion of this assay to include the represented strains from across the globe would allow a diagnostic assay system to rapidly track movement of *Pgt* across continents and around the world. Furthermore, this approach can be used to develop specific assays for differentiating subclasses of *P. graminis*. Several of the formae speciales have broad host ranges that overlap, making it difficult to determine the correct classification without performing host-range studies. This issue is even more acute when trying to determine if *P. graminis* is cycling through the sexual cycle on an alternate host. The aeciospores are not as well characterized as the urediniospores and teliospores. In addition, there are several different species of rust fungi that have pycnial/aecial stages on *Berberis* spp. and *Mahonia* spp.

8.2.7 Future Perspectives

Genomics of rust fungi is still in its infancy; it has been just over 5 years since the first public release of the *Pgt* genome. Since then, several additional genomes of rust fungi have been sequenced and these include: *M. larici-populina,* popular leaf rust pathogen (Duplessis et al. 2011); *M. lini*, flax rust pathogen (Nemri et al. 2014); *P. striiformis* f. sp. *tritici* (*Pst*), wheat stripe rust pathogen (Cantu et al. 2013; Zheng et al. 2013); *P. triticina*, wheat leaf rust pathogen (http://www.broadinstitute.org/annotation/genome/puccinia_group). In addition, there are several more being worked on. However, this represents a small fraction of the 7,000 species of rust fungi known.

A primary emphasis of the current genome projects is the identification of effectors with a focus on small secreted proteins that are abundant in haustoria. This approach has been successful in identifying effectors in several systems and there is no doubt that it will be fruitful with *Pgt* and other related rust fungi. Because this approach selects candidate effectors based on physical rather than functional criteria, other classes of effectors will be missed. Increased effort in genetic analysis of *Pgt* is needed to identify and characterize these other classes.

Elucidation of avirulence genes (effectors corresponding to specific stem rust resistance genes) will allow for the development of more efficient methods of identifying *Sr* genes in wheat lines and cultivars. Currently, there does not exist a set of *Pgt* tester races that contain single avirulence genes and therefore determination of the composition of *Sr* genes requires using multiple *Pgt* strains and often backcrossing to susceptible wheat lines. In addition, these tools will also help facilitate the development of durable complex 'stacks' of *Sr* genes.

P. graminis, like many other rust fungi, has a complex life cycle that includes two very different hosts. The asexual phase of the life cycle occurs on cereal crops and grasses (monocots), while the sexual phase occurs on the herbaceous dicots, *Berberis* and *Mahonia* species. Two different mechanisms are used for host penetration; the asexual dikaryotic phase (uredinial) enters via stomates, while the haploid sexual stage (basidial) enters by direct penetration of the

epidermis. Transcriptome analysis coupled with microscopy will allow a detailed comparison of the infection process of these two hosts, identifying commonalities and differences. It will be interesting to determine what role the different nuclear states (haploid vs. diploid) plays in these processes.

In contrast to the uredinial stage on cereal crops, very little is known about the resistance to *P. graminis* in *Berberis*. Anecdotal evidence indicates that resistance is controlled by single gene(s) and is not race specific. Detailed genetic studies are needed to define stem rust resistance in *Berberis* and to determine whether or not this resistance is race specific. If not, then transferring resistance from *Berberis* to cereal crops may provide a more stable form of resistance.

Phylogenetic analysis indicates that the three wheat rust pathogens (*Pgt*, *Pst* and *P. triticina*) have each adapted independently to infect wheat. Comparative genomics of these three genomes is now possible and should provide insight into this process. Expansion of genomic studies to the different 'forms' of *P. graminis*, as well as close relatives, will greatly enhance studies on the genetic determinants of host range.

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R. A. Dean et al. (eds.), *Genomics of Plant-Associated Fungi: Monocot Pathogens*,
DOI: 10.1007/978-3-662-44053-7, © Springer-Verlag Berlin Heidelberg 2014