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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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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 Subcommittee of *Colletotrichum* Taxonomy (www.fungaltaxonomy.org/ subcommittees) 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

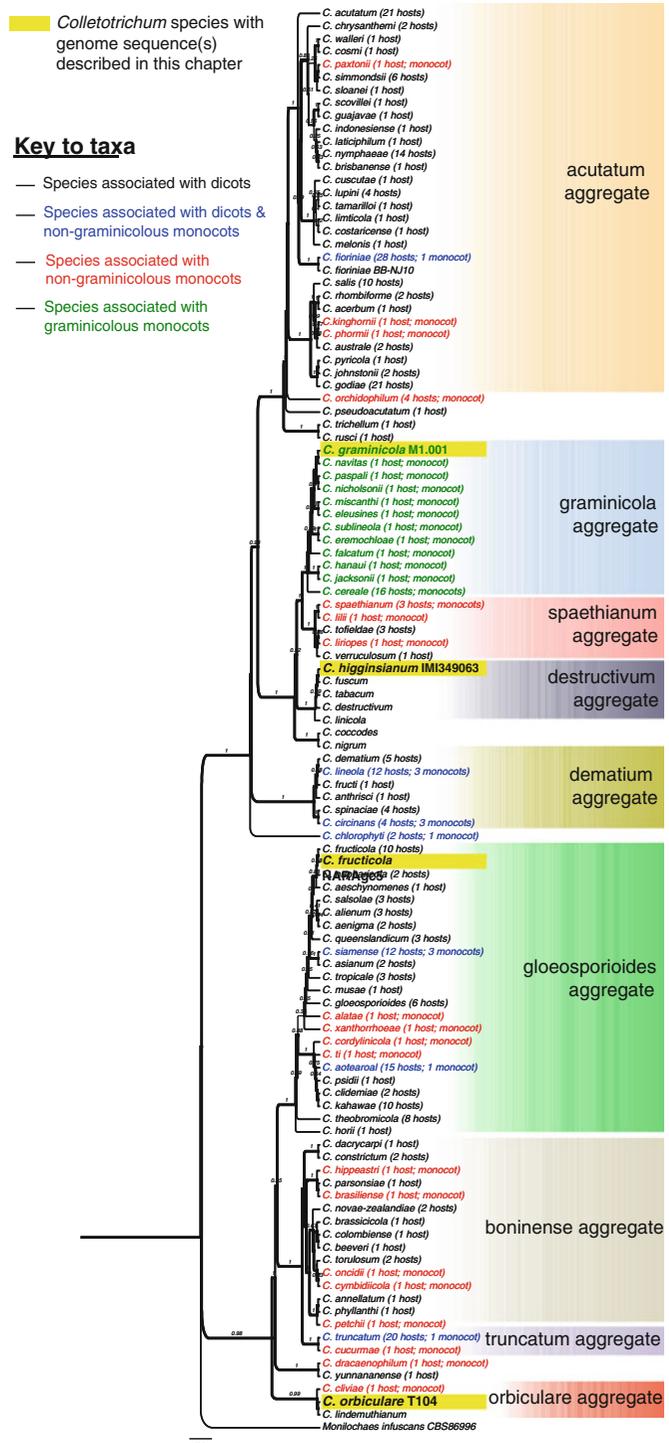
characterized through multilocus phylogenies (Crouch et al. 2009a; Damm et al. 2009; 2012a, b; Weir et al. 2012), yielding a framework for understanding evolutionary relationships across the genus as a whole (Cannon et al. 2012b).

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-

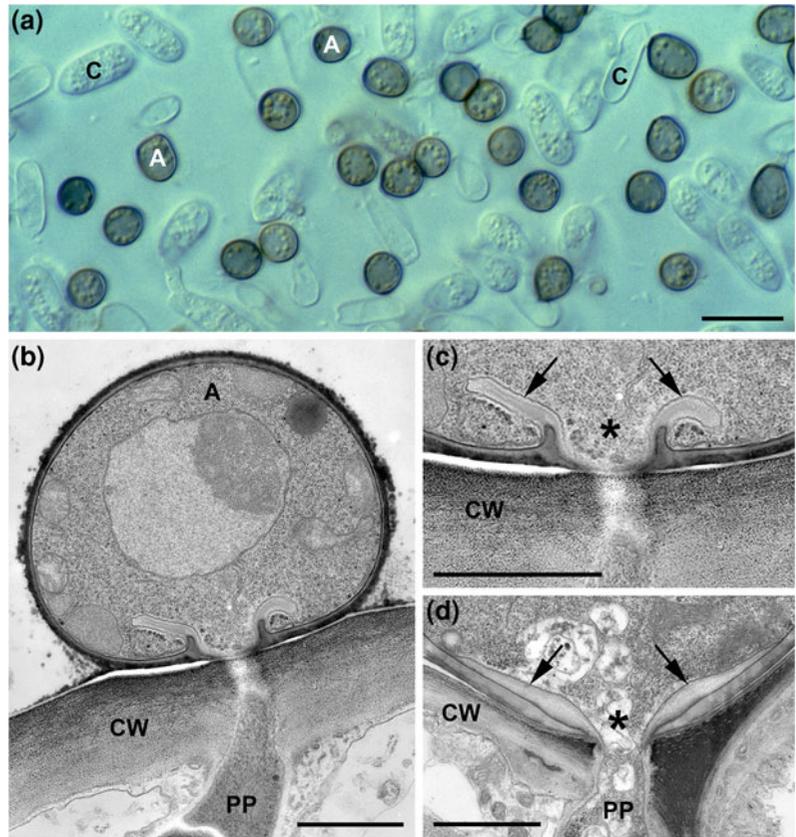
Fig. 3.1 Bayesian phylogenetic tree of the *Colletotrichum* genus, showing the evolutionary relationship between the four *Colletotrichum* species with genome sequences, as described in this chapter. The phylogeny was constructed from DNA sequences of four markers (chitin synthase, actin, β -tubulin 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 *Colletotrichum* 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

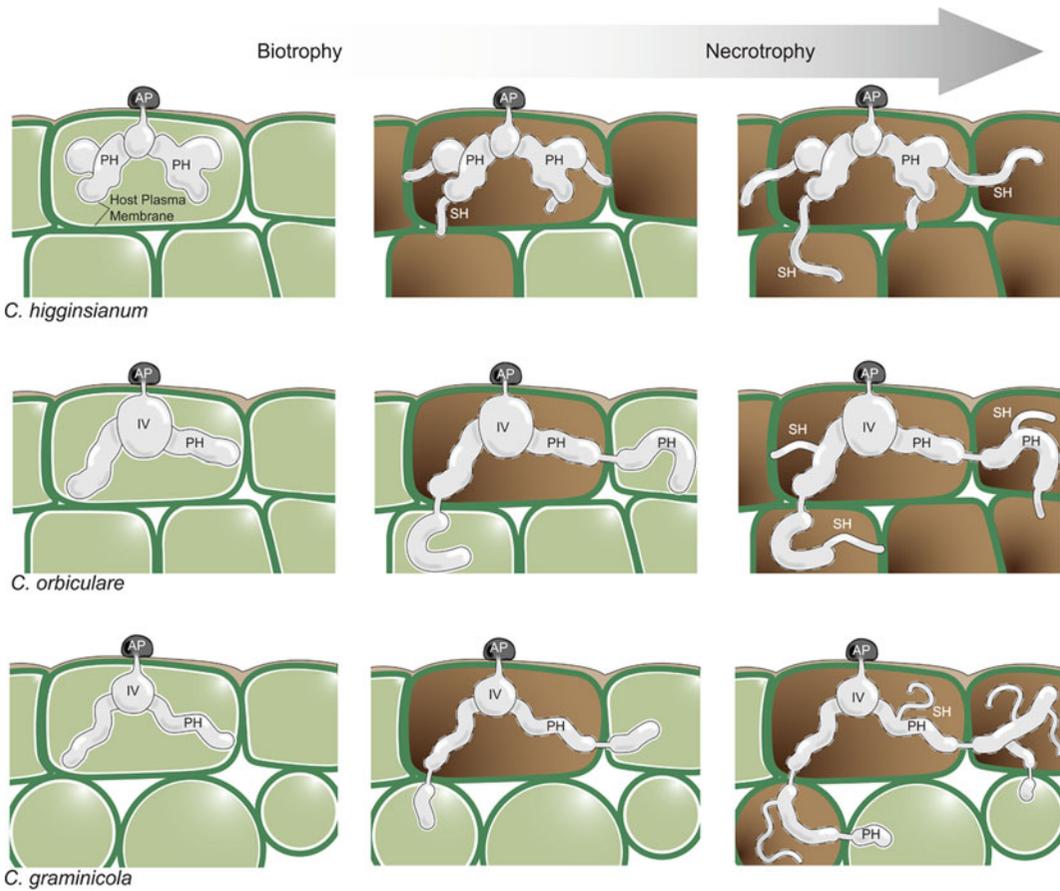


Fig. 3.3 Three variants of intracellular hemibiotrophy. Row 1 *C. destructivum* model (*C. higginsianum*). Biotrophic primary hyphae (PH) colonize only one epidermal cell, followed by a complete switch to necrotrophy, with thinner secondary hyphae (SH) killing host cells ahead of infection. Row 2 *C. 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*

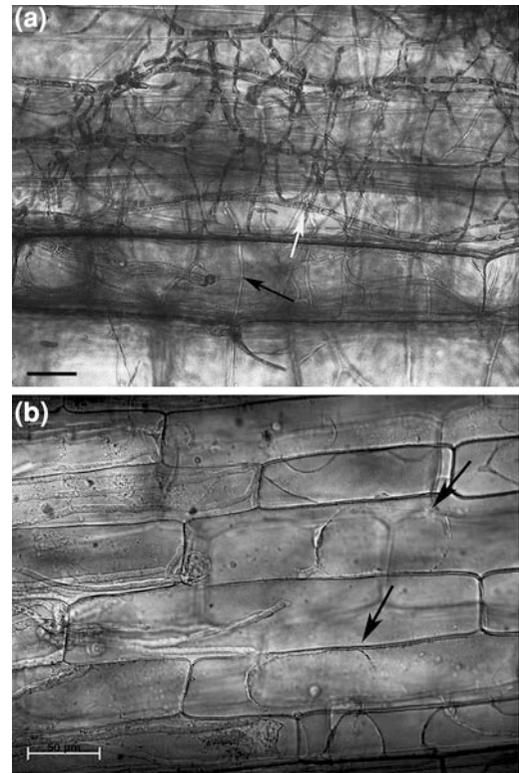


Fig. 3.4 Trypan-blue stained hyphae of *C. graminicola* colonizing maize leaf sheath epidermal cells during the necrotrophic phase of development. **a** In the center of the colony, the thicker primary hyphae (white arrow) give rise to thinner necrotrophic hyphae (black arrows) that define this stage 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; Ripoché 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)

	<i>C. higginsianum</i>	<i>C. graminicola</i>	<i>C. fruticola</i>	<i>C. orbiculare</i>
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. fruticola* 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. fruticola* 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 Cret1, Cret2, Cret3, 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. 2011; 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 minichromosomes, *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).

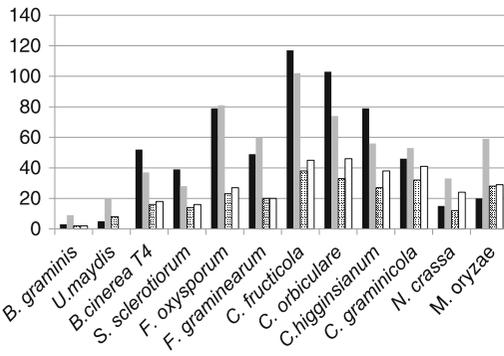


Fig. 3.5 Relative numbers of genes encoding CAZymes targeting different plant cell wall components in *Colletotrichum*, and in other related fungi with various lifestyles. Black bars pectin. Gray bars pectin and hemicellulose. Speckled bars hemicellulose. White bars cellulose. *Blumeria graminis* (biotroph); *Ustilago maydis* (biotroph); *Botrytis cinerea* (necrotroph); *Sclerotinia sclerotiorum* (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

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

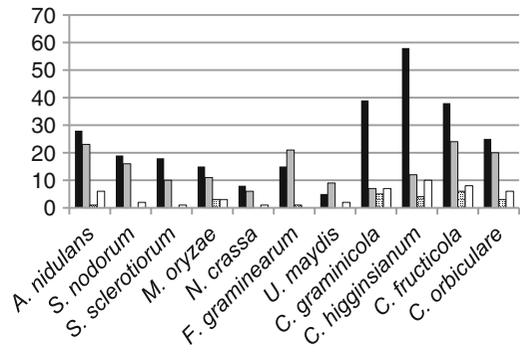


Fig. 3.6 Relative numbers of genes encoding putative SM-related enzymes in *Colletotrichum*, and in other fungi with various lifestyles. Black bars PKS and PKS-like, Gray bars NRPS and NRPS-like, Speckled bars PKS-NRPS hybrids, White bars DMATs. *Aspergillus nidulans*, saprophyte; *Stagonospora nodorum*, 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)

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), colleto-trichins 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 tetra-hydroxylated 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)

	<i>C. orbiculare</i>	<i>C. fructicola</i>	<i>C. higginsianum</i>	<i>C. graminicola</i>
Number of secondary metabolite clusters	42	56	43	44
With homologs in <i>C. orbiculare</i>	–	35	26	26
With homologs in <i>C. fructicola</i>	29	–	26	24
With homologs in <i>C. higginsianum</i>	22	21	–	20
With homologs in <i>C. graminicola</i>	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)

	<i>C. graminicola</i>	<i>C. higginsianum</i>	<i>C. fructicola</i>	<i>C. orbiculare</i>
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.wolfsort.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	<i>C. graminicola</i>	<i>C. higginsianum</i>	<i>C. orbiculare</i>	<i>C. fructicola</i>
<i>CgDN3</i>	AAB92221.1	0	2	1	1
<i>NPP1</i>	EGZ24512.1	3	4	7	7
<i>NEP1</i>	AF036580.1	4	5	7	9
<i>NIS1</i>	BAL70334.1	1	1	2	2
<i>ToxB</i>	AAO49374.1	0	0	1	0
<i>MSP1</i>	AAX07670.1	24	22	23	26
<i>CIH1</i>	AJ271296.1	2	2	3	2
<i>SIX1</i>	ACY39281.1	0	0	5	0
<i>SIX5</i>	ACN87967.1	0	0	1	1
<i>SIX6</i>	ACN69116.1	0	1	2	0
<i>BAS2</i>	ACQ73207.1	2	3	2	1
<i>BAS3</i>	ACQ73208.1	1	1	0	0
<i>Ctmudix</i>	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 *CIHI* 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 *CIHI*.

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 acquisition 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.

Anthraxnose 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 bio-diesel 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.

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