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Cellular and molecular aspects of the interaction betwen maize and the anthracnose pathogen Colletotrichum graminicola

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Cellular and molecular aspects of the interaction between maize and the anthracnose pathogen *Colletotrichum graminicola*

DISSERTATION

A dissertation submitted in partial fulfillment

of the requirements for the degree of Doctor of Philosophy in the

College of Agriculture at the University of Kentucky

By

Maria Fernanda Torres

Lexington, Kentucky

Director: Dr. Lisa J. Vaillancourt, Professor of Plant Pathology

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ABSTRACT OF DISSERTATION

CELLULAR AND MOLECULAR ASPECTS OF THE INTERACTION BETWEEN MAIZE AND THE ANTHRACNOSE PATHOGEN *Colletotrichum graminicola*

Maize anthracnose, caused by the fungus *Colletotrichum graminicola*, is an economically important species contributing to major yield losses*. C. graminicola* is a hemibiotroph; initially it invades its host while it is alive, and then it switches to destructive necrotrophic growth and the host is killed. Establishment of compatible interactions by biotrophic pathogens is usually associated with suppression of host defenses and cell death, while necrotrophic pathogens typically secrete phytotoxic compounds and induce cell death. To understand the relationship of hemibiotrophy in *C. graminicola* to biotrophy and necrotrophy, I compared a compatible and an incompatible interaction, utilizing a non-pathogenic mutant strain that is very similar to the wild type *in vitro*. I developed an assay to visualize in detail living fungal and host cells during pathogenic and nonpathogenic interactions. My results provided evidence that *C. graminicola* produces diffusible substances during colonization that predispose nearby living host cells for fungal invasion. My observations further suggested that the mutant is nonpathogenic because it fails to produce these substances. To explore the possibility that the *C. graminicola* mutant is impaired in the production and/or secretion of one or more secondary metabolites (SM), I characterized the range of SM-associated genes in *C. graminicola. C. graminicola* has a large and diverse repetoire of these genes, indicating significant capacity for the production of SM. I then characterized the global expression of fungal genes during different developmental phases in both compatible and incompatible interactions. I found that SM-associated genes are expressed during early and late stages of maize infection. Secreted proteins and putative effectors were overrepresented among differentially regulated predicted gene products. There were relatively few differences in expression between the mutant and wild type, suggesting that differences between them may relate to post-transcriptional events. The transcriptional analysis indicated that the mutant was defective very early in biotrophy. This study indicates that biotrophy and necrotrophy coexist in this pathosystem in different cells, and that arrays of differentially regulated and locally expressed genes are involved in maintaining this balance. Understanding the nature of induced susceptibility may lead to new therapeutic targets for management of this damaging disease.

KEYWORDS: hemibiotroph, anthracnose, non-pathogenic,secondary metabolite, transcriptome.

Maria Fernanda Torres

May 27, 2013

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A mis papas, que con su ejemplo y sacrificio me enseñaron que no hay nada imposible y con su amor incondicional me han permitido perseguir mis sueños.
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“The brick walls are there for a reason. The brick walls are not there to keep us out. The brick walls are there to give us a chance to show how badly we want something. Because the brick walls are there to stop the people who don’t want it badly enough. They’re there to stop the other people.” Randy Pausch.

Table of Contents

List of Tables

List of figures

Chapter 1

Colletotrichum graminicola as a pathogen of maize (*Zea mays*)

Colletotrichum graminicola is a plant-pathogenic Ascomycete that infects maize roots, stalks, leaves, ears, and kernels(Warren & Nicholson, 1975) . On leaves and stalks, infection results in a disease known as anthracnose, with anthracnose stalk rot (ASR) causing the most damage. The first report of *C. graminicola* causing a severe disease on maize stalks in the United States (U.S.) was in Ohio in 1963 (Williams, 1963). Prior to that time, the pathogen had been encountered only occasionally on maize in the U.S., causing a relatively minor leaf blight (Sprague, 1950). *C. graminicola* was widespread in maize fields in the U.S. by the early 1970s (Poneleit *et al.*, 1972, Nicholson R. L, 1976). The first anthracnose stalk rot epidemic reported in the U.S. occurred on the sweet corn variety Jubilee in Indiana in 1972, where more than 50% of the plants were severely infected (Warren *et al.*, 1973). Severe outbreaks were also reported in dent corn in Maryland and in Kentucky, in 1971 and 1972, respectively (Morgan, 1971, Wheeler *et al.*, 1972). *C. graminicola* is now considered a major pathogen throughout the U.S. corn belt that can reduce yields by up to 40% (Bergstrom & Nicholson, 1999), resulting in annual losses of more than 1 billion dollars in the U.S. alone (Frey *et al.*, 2011). The impact of this pathogen is likely to continue to increase, due to the growing demand for maize for animal feed, food, and ethanol worldwide (Bergstrom & Nicholson, 1999).

Limitations in maize production due to anthracnose leaf blight (ALB) are caused by reduction of the total photosynthetic area, leading to premature leaf senescence and reduced grain fill (Ali *et al.*, 1987). In the ASR phase, the pathogen causes damage to vascular tissues, limiting the amount of water and nutrients that are supplied to the kernels. The most severe symptoms of ASR are premature plant death and lodging, produced by breakage of the lower internodes that can complicate the harvesting process (Cota *et al.*, 2012, Bergstrom & Nicholson, 1999).

Control of maize anthracnose is based on the use of cultivars resistant to ALB and ASR, but the mechanisms that mediate resistance are not known (Muimba-Kankolongo & Bergstrom, 2011, Balint-Kurti, 2009, Cota *et al*., 2012). Yield reduction due to ASR is highly dependent on environmental conditions, which has made genetic studies and identification of reliable traits associated with disease resistance difficult (Callaway *et al.*, 1992, Balint-Kurti, 2009). The anthracnose resistance locus *Rcg1* (resistance to *C. graminicola* 1), was recently cloned and characterized. The locus contains two leucine-rich repeat type R-genes (NBS-LRR), both of which are required for resistance (Broglie, 2006). Maize hybrids carrying this resistance locus showed no difference in yield in the absence of the disease; however yields increased by 13% after infection with *C. graminicola*, compared with near-isogenic maize hybrids lacking the gene (Frey et al., 2011). Commercial hybrids containing the *Rcg1* locus should soon be available to growers from Pioneer Hi-bred. However, it is not known how durable the trait will prove to be in the field. Qualitative resistance to other *Colletotrichum* pathogens (e.g. the sorghum anthracnose pathogen *C. sublineola*) is notoriously unstable (Ali & Warren, 1992, Casela *et al.*, 1992).

Insights into the *Colletotrichum graminicola* lifestyle

Most members of the genus *Colletotrichum* are intracellular hemibiotrophic pathogens. This means that they initially colonize their host biotrophically, with the fungus apparently obtaining nutrients from plant cells that remain alive, and then they switch to necrotrophy, in which the fungus feeds from dead cells and symptoms develop (Mendgen & Hahn, 2002, Wharton *et al.*, 2001, Wharton & Julian, 1996, Latunde-Dada *et al.*, 1996). When *C. graminicola* spores land on the plant surface, they germinate and produce an appressorium that becomes melanized, facilitating the accumulation of turgor pressure that allows the fungus to penetrate the plant tissue (Latunde-Dada, 2001, Takano *et al.*, 1995, Rasmussen & Hanau, 1989). Initial penetration occurs through an appressorial pore from which a penetration peg emerges (Politis & Wheeler, 1973). During penetration, the host plasma membrane becomes expanded and invaginated, and invasive intracellular primary hyphae are formed in the host cells, while the

host cytoplasm remains alive and intact (Bergstrom & Nicholson, 1999). After approximately 48 hours, *C. graminicola* switches to necrotrophy, characterized by extensive colonization and maceration of plant tissue, and development of thin, secondary hyphae that are in direct contact with the host cytoplasm, and which are believed to be responsible for the secretion of large amounts of cell wall degrading enzymes (CWDE) (Venard & Vaillancourt, 2007a, Mims & Vaillancourt, 2002, O'Connell *et al.*, 2012). The mechanisms involved in establishment of a compatible *C. graminicola*-maize interaction, and further progression to necrotrophy, are not understood (Krijger *et al.*, 2008, Horbach *et al.*, 2009, Thon *et al.*, 2002). Current hypotheses are based on more detailed studies performed with biotrophic and necrotrophic fungal pathogens.

Evidence indicates that biotrophic plant pathogens normally secrete a limited variety and quantity of CWDE and secondary metabolites (Spanu et al., 2010, Kamper *et al.*, 2006), and that they suppress cell death and host defense responses in order to obtain the energy they require from living plant cells (Doehlemann *et al.*, 2009, Djamei *et al.*, 2011, Doehlemann *et al.*, 2008). In contrast, many necrotrophic plant pathogens take advantage of plant defense responses to enhance their pathogenicity, and induce cell death by secreting a wide variety of phytotoxic secondary metabolites (Amselem *et al.*, 2011, Cessna *et al.*, 2000, Baker *et al.*, 2006, Rolke *et al.*, 2004). There are multiple morphological similarities between the early stages of infection of maize by *C. graminicola*, and the interaction of plants with obligate biotrophs, suggesting that they could share some early infection strategies (Münch *et al.*, 2008, O'Connell & Panstruga, 2006b). During penetration by obligate biotrophs like *Uromyces appendiculatus* and *Blumeria graminis*, the host plasma membrane is also invaginated and a specialized structure called a haustorium develops (Hardwick *et al.*, 1971, Bushnell *et al.*, 1967). Haustoria are responsible for the secretion of molecules that suppress host defense responses, reprogram host metabolism, and allow the establishment of a compatible interaction (Lyngkjær *et al.*, 2001, Godfrey *et al.*, 2009). They are also responsible for nutrient uptake from the host, evidenced by the haustorial expression of sugar and amino acid

transporters (O'Connell & Panstruga, 2006a, Jakupović *et al.*, 2006, Struck *et al.*, 2002). An interface that separates the fungal cell wall from the host plasma membrane, called the extrahaustorial matrix, seems to be important for mediation of the exchange of signals and nutrients between pathogen and host (Doehlemann *et al*., 2008, Struck *et al.*, 1998). An interfacial matrix was also observed in the *C. lindemuthianum*-bean interaction, and was found to contain proteins with potential roles in protection from host defense responses (Perfect *et al.*, 1998b). However, an interfacial matrix does not seem to be present in the *C. graminicola*-maize interaction (Mims & Vaillancourt, 2002).

It has been suggested that, as with obligate biotrophs, the host plant does not recognize or respond to *Colletotrichum* during the early, biotrophic stages of infection (Perfect *et al.*, 1999). Because members of this genus can be cultured and transformed (Mathur, 1950, Tu, 1985), this led to the idea that the transient biotrophic phase could be used as an experimentally tractable model to elucidate the events that led to the establishment plant interactions by obligate biotrophs (Perfect *et al*., 1999). However, more recent studies of different members of this genus show that the host plant does recognize and respond to the fungus, sometimes even before penetration occurs (Stephenson *et al.*, 2000, Vargas *et al.*, 2012, O'Connell *et al.*, 2012). The recently sequenced *C. graminicola* and *C. higginsianum*(Hahn *et al.*, 1997) genomes contain a large number of predicted genes associated with secondary metabolism and carbohydrate-active enzymes (CAZy), including CWDE (O'Connell *et al*., 2012). These characteristics are more similar to necrotrophic than to biotrophic plant pathogens (Kamper *et al.*, 2006, Amselem *et al*., 2011, Spanu *et al*., 2010).

In contrast to biotrophs, ultrastructural characterization of the infection process of plants by necrotrophic pathogens is limited. It is believed that for many necrotrophs, e.g. the ascomycete *Botrytis cinerea*, secretion of a large amount of CWDE is more important than turgor pressure to breach the host cuticle (Gourgues *et al.*, 2004, Tenberge, 2007). Shortly after infection of broad bean cells by *B. cinerea*, epidermal cell walls become extensively vacuolated, Golgi vesicles proliferate, the tonoplast disintegrates, and the cytoplasm collapses

(Mansfield & Richardson, 1981). Although extensive colonization is not observed at these early stages of infection, cytoplasm disorganization and proliferation of organelles was observed in cells adjacent to the initial invaded cells, an indication that host cells are killed in advance of fungal penetration.

The complexity of hemibiotrophy

Most of the information that is now available regarding a plant interaction with an intracellular hemibiotrophic fungus comes from studies of the *Magnaporthe oryzae*-rice pathosystem (Kankanala *et al.*, 2007, Dean *et al.*, 2005). *M. oryzae* initially colonizes rice biotrophically, developing a thin primary hypha that enlarges to become a bulbous invasive hypha, then branches to fill the first invaded cell before moving to the adjacent cells (Koga, 2004). Each new invaded cell is initially colonized biotrophically by thin intracellular hyphae, filled, and then dies after the fungus moves to the next cell (Kankanala *et al.*, 2007). Microarray analysis of the invasive biotrophic hyphae determined that they were enriched in transcripts encoding putative secreted proteins, most of which were not expressed *in vitro* or in incompatible interactions, suggesting that these are secreted effector proteins required for the establishment of compatibility in this pathosystem (Mosquera *et al.*, 2009a). Among these predicted effectors, four were further characterized as biotrophy-associated-secreted (BAS) proteins, and were 61- to 100-fold induced in invasive hyphae, when compared with mycelium grown *in vitro*. Fluorescent-labeling experiments indicated that two of these BAS proteins accumulated in the biotrophic interfacial complex (BIC), a pathogen-induced structure that is observed on every invasive hyphae that grows into a new cell (Mosquera *et al.*, 2009a, Khang *et al.*, 2010). Further analysis of these BAS proteins determined that only effector proteins that accumulated in the BIC were later observed in the plant cytoplasm, suggesting that this structure was associated with translocation of rice blast effectors into the plant cytoplasm (Khang *et al.*, 2010). A structure similar to the BIC has not been described in any of the detailed cytological studies involving *Colletotrichum*-plant interactions (Wharton & Julian, 1996, Mims & Vaillancourt, 2002, O'Connell *et al.*, 1985).

However, recent immunolabelling experiments associated putative secreted effectors of *C. higginsianum* with electron-opaque projections that extended from the biotrophic hyphae, between the fungal cell wall and the host plasma membrane (Kleemann *et al.*, 2012). The authors proposed that appressoria and invasive primary hyphae both play important roles in the secretion of effectors required to establish a compatible interaction, and to induce host cell death and produce symptoms.

Factors Involved in Hemibiotrophy in *Colletotrichum*

Screening of an expressed sequence tag (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). Expression of *CgDN3* was strongly induced *in vitro* under nitrogen starvation conditions, but was not detected in spores or appressoria. During infection, the *CgDN3* transcript accumulated in infection vesicles and was also observed to a lesser extent in invasive hyphae. *CgDN3* knockout mutants were not affected in sporulation, germination or appressorium formation, and they grew faster that the wild type *in vitro*. However, they failed to penetrate or form primary infection hyphae, and they rapidly induced localized cell death. The authors suggest that *CgDN3* encodes a putative effector required to overcome plant defense responses, including production of reactive oxygen species.

Recently, homologs of *CgDN3* were identified in the genomes of *C. orbiculare* (Yoshino *et al.*, 2012) and *C. higginsianum* (Kleemann *et al*., 2012). Both suppressed cell death induced by necrosis-inducing effectors when they were transiently expressed in *Nicotiana benthamiana* leaves. A screen for *C. orbiculare* proteins that induced cell death in *N. benthamiana* led to the identification of NIS1, a secreted protein that is produced by primary hyphae. The authors concluded that cell death induced by NIS1 was 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). Interestingly, the *CgDN3* homolog in *C. orbiculare*, *CoDN3*, suppressed cell death induced by NIS1, supporting a role for this effector as a suppressor of HR. Transcriptome profiling of *C. higginsianum* infecting *Arabidopsis thaliana* revealed “waves” of putative effector genes expressed at different times during plant infection (Kleemann *et al*., 2012). A homolog of *CgDN3*, *ChEC3*, was expressed in the appressorial pore prior to penetration and in early invasive hyphae (Kleemann *et al*., 2012). Similar to observations with *C. orbiculare*, *ChEC3* suppressed cell death induced by necrosis-inducing proteins in *N. benthamiana.* Additionally, a group of genes encoding putative necrosis-inducing effectors were identified, and analysis of some of these genes suggests that they are expressed during the switch to necrotrophy in *C. higginsianum*.

Interestingly, *ChEC3* was not detected in appressoria formed on synthetic surfaces. Although *in vitro* and *in planta* appressoria are morphologically identical, plant signals must be involved in triggering the expression of this pathogenicity factor. Several recent studies have compared fungal gene expression *in vitro* versus *in vivo*, and they confirm that *in vitro* conditions induce different subsets of genes from those induced *in planta* (Mosquera *et al.*, 2009b, O'Connell *et al.,* 2012). Thus, some of the genes identified in previous screenings using *in vitro* conditions might not be relevant during growth *in planta*. Effectors, in particular, frequently appear to be plant-induced.

A few factors that are required for the establishment of progression of intracellular infection hyphae from cell to cell, and formation of necrotrophic hyphae, in *Colletotrichum*-plant infections have also been identified. Several studies have tested the hypothesis that nitrogen starvation mimics *in planta* conditions and induces expression of fungal genes that are involved in different stages of pathogenicity (Talbot *et al.*, 1993, Ackerveken *et al.*, 1994). In the absence of the preferred sources of nitrogen (glutamine and ammonium), the major positive regulator *AreA* mediates activation of genes involved in utilization of secondary sources of nitrogen (Kudla *et al.*, 1990). Deletion of the *AreA* gene in *C. lindemuthianum* resulted in a mutant that was unable to produce symptoms

(Pellier *et al.*, 2003). A closer examination of the infection process of the mutant determined that it was comparable to the wild type during initial penetration and colonization stages, but rarely produced secondary hyphae. The authors proposed that *C. lindemuthianum* encounters nitrogen starvation conditions very early during the infection of bean leaves and might be unable to utilize secondary sources of nitrogen to sustain the extensive growth that occurs during the necrotrophic phase. Additionally, it has been suggested that nitrogen starvation might act as a cue to trigger the expression of pathogenicity-associated genes, as has been reported for *avr9* from *C. fulvum* (Pérez-García *et al.*, 2001) and *mpg1* from *M. oryzae* (Talbot et al., 1993), and *AreA* could be involved in sensing the nitrogen signal and activating these genes.

Deletion of the *AreA* ortholog in *C. coccodes* produced mutants that were impaired in ammonium secretion and significantly less virulent on tomato fruits (Alkan *et al.*, 2008). Secretion of ammonium by *C. coccodes* during the colonization of tomato fruits is associated with modulation of salicylic acid and jasmonic acid-mediated defense pathways, production of reactive oxygen species, and induction of programmed cell death during symptom development (Alkan *et al.*, 2011, Alkan *et al.*, 2009). Production of ammonium is also important for modulation of pH and establishment of an alkaline environment suitable for secretion and function of the extracellular CWDE pectate lyase, required for tissue maceration and symptom development by *C. gloeosporioides* in avocado fruits (Kramer-Haimovich *et al.*, 2006).

A *Colletotrichum* pathogenicity related (*Cpr1*) gene was identified by random mutagenesis using a restriction enzyme-mediated integration approach in *C. graminicola* (Thon *et al.*, 2000) . This mutation was characterized as an insertion in the 3'UTR of a gene similar to *Spc3*, which encodes one of the four essential components of the signal peptidase complex in *S. cerevisiae*, involved in processing of signal peptides from polypeptides across the endoplasmic reticulum (ER) membrane (Fang *et al.*, 1996, Meyer & Hartmann, 1997, Fang *et al.*, 1997). The *cpr1* mutant was comparable to the wild type in various conditions *in vitro* that were tested, but it was completely non-pathogenic to maize stalks

and leaves (Thon *et al*., 2002). A cytological characterization of this mutant in intact maize leaves indicated that it was indistinguishable from the wild type during spore germination, appressorium formation, and penetration. However, similar to the *AreA* mutant of *C. lindemuthianum* mentioned above, the *cpr1* mutant remained confined to the first colonized cells, did not develop secondary hyphae, and never caused symptoms (Mims & Vaillancourt, 2002). The authors proposed that the *cpr1* mutant might be impaired in the secretion of one or more compounds required for establishment of biotrophy and the switch to necrotrophy (Thon *et al.*, 2002).

A GAL-4-like transcription factor required for pathogenicity of *C. lindemuthianum* was identified by random mutagenesis (Dufresne *et al.*, 2000). A mutation in the *CLTA1* (*C. lindemuthianum* transcriptional activator 1) gene produced mutants that were able to penetrate bean leaves, but then rapidly induced localized cell death and were unable to switch to necrotrophy. CLTA1 is predicted to be a member of the zinc cluster family of transcriptional activators, which are associated with control of acetate and nitrate utilization pathways in fungi (Todd *et al.*, 1997, Yuan *et al.*, 1991). It is proposed that CLTA1 is important in the regulation of nutritional changes that the fungus undergoes in the transition from biotrophy to necrotrophy.

Agrobaterium tumefaciens-mediated transformation mutagenesis was used to identify *C. higginsianum* genes required for compatibility with *Arabidopsis* (Huser *et al.*, 2009). The authors identified 40 mutants with defects in pathogenicity, including five that were comparable to the wild type in penetration, infection and induction of host defense responses, but did not cause symptoms and rarely colonized beyond the first epidermal cell. Interestingly, in all five cases, the first invaded cell retained its ability to plasmolyze even 7 days after inoculation, and the authors suggested that these mutants were able to establish and sustain a biotrophic interaction, but were unable to switch to necrotrophy. Two of these mutations were in a gene predicted to encode an importin-β2 subunit-containing protein. In yeast and mammals, this family of proteins act as chaperones mediating the translocation of ribosomal proteins, and of nuclear localization

signal (NLS)-containing proteins into the nucleus (Nachury, 2001, Harel, 2004). This protein family plays an important role in gene regulation in response to extracellular signals (Yashiroda, 2003).

To identify putative effectors involved in the switch to necrotrophy, a cDNA library was constructed from Canadian lentil (*Lens culinaris*), 48 to 56 hours after infection (hpi) with *C. truncatum,* when the first secondary hyphae were observed (Bhadauria *et al.*, 2011). Of the 121 putative secreted protein genes that were identified, sixty-three were predicted to encode extracellular hydrolytic enzymes (CWDE), involved in host cell wall and protein degradation and fungal access to nutrients. Thirty-six had predicted transmembrane domains targeting them to the host plasma membrane or cell wall. This group included MFS-transporters, a superoxide dismutase, an HSP70, three glycoproteins similar to *CIH1* from *C. lindemuthianum* (Perfect *et al.*, 1998a), two lysine-motif (LysM)-domain containing proteins, and a chitin deacetylase. LysM domain proteins and chitin deacetylase are proposed to be involved in protection from host chitinases (Bolton *et al.*, 2008, El Gueddari *et al.*, 2002a). Eleven genes were predicted to encode small, cysteine-rich, secreted proteins with similarities to other predicted fungal effectors, but no known function. Another group of 11 genes with similarities to other known fungal proteins with various functions, including proteins involved in ROS detoxification, nitrogen metabolism and a Nudix hydrolase.

The Nudix hydrolase, CtNUDIX, is the most recently characterized secreted protein from *C. truncatum* (Bhadauria *et al.*, 2012). This protein contains a Nudix domain, associated with “housecleaning functions” involving hydrolysis of different substrates including nucleoside triphosphates damaged as a result of oxidative stress in *Saccharomyces cerevisiae* (McLennan, 2006, Bessman *et al.*, 1996). Although the role of these enzymes is not known in plant pathogenic fungi, one of the best characterized Nudix hydrolases in *S. cerevisiae*, *Ysa1*, is differentially expressed in response to environmental stress (Gasch *et al.*, 2000). *Ysa1* is involved in regulation of levels of ADP-ribose, and it is proposed to play a regulatory role in metabolism and the ability of cells to protect against oxidative

stress (Gasch et al., 2000, Tong *et al.*, 2009). Recently, Avr3b, a nudix-containing secreted effector from *Phytophthora sojae,* was transiently expressed in *N. benthamiana*, where it increased susceptibility to *P. sojae* and *P. parasitica*. It was proposed that Avr3b is involved in suppression of pathogen-triggered ROS accumulation (Dong *et al.*, 2011).

Expression of CtNUDIX peaked at 44 hpi during late biotrophic growth. It was proposed that *C. truncatum* specifically uses it to induce cell death during the switch to necrotrophy. Overexpression of CtNudix in *C. truncatum* induced localized host cell death and loss of pathogenicity, and the authors propose this could be the result of a premature induction of cell death during biotrophy. 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. The authors also determined that orthologs of this Nudix effector were present in other hemibiotrophic pathogens including *C. higginsianum, M. oryzae, C. graminicola* and *P. infestans*, but absent in biotrophic and necrotrophic pathogens, suggesting that it might be important specifically for this lifestyle.

Various factors seem to affect the ability of hemibiotrophic *Colletotrichum* species to establish biotrophic infections and to transition to necrotrophy. A number of proteins involved in fungal responses to environmental signals and metabolism, as well as regulation, synthesis and secretion of compounds potentially involved in suppression of host defense responses and cell death have been characterized. Hemibiotrophy in *Colletotrichum* species is associated with the secretion of different groups of plant-induced effectors. Some produced before penetration and during biotrophy appear to suppress plant defense responses and cell death and allow for establishment of biotrophic hyphae, while others induced during and after the transition to necrotrophy apparently induce host cell death. It appears that fungal metabolism must be reprogrammed by various regulators during the transition. Hemibiotrophy is a complex developmental process that does not depend on any single factor, but rather requires the

interaction of many fungal and plant components, controlled by a variety of host and pathogen regulatory pathways.

Other *C. graminicola* genes required for pathogenicity

C. graminicola, like other *Colletotrichum* fungi, produces melanized appressoria that are required for efficient penetration of the host epidermal cell walls. Ninety percent of the cell wall in most fungi is composed of polysaccharides, mostly cellulose, consisting of polymers of D-glucose, and chitin, comprised of N-acetylglucosamine polymers (Bartnicki-Garcia, 1968). Chitin is synthesized by the enzyme chitin synthase, and inhibitors of this enzyme are used as treatments for fungal diseases in humans (Debono & Gordee, 1994). Deletion of the *CgChsV* chitin synthase gene in *C. graminicola* resulted in mutants with multiple morphological defects, including unusual vegetative growth and distorted appressoria. Mutants were unable to penetrate or grow inside plant cells. Appressorial morphology and functionality also depend on the ability of the fungus to synthesize melanin, a polyketide derived from tetrahydroxyaphtalene (Finch *et al.*, 2012). Melanin confers the ability of the appressorium to accumulate and focus the high turgor pressures that are necessary for initial penetration of the host surface (Howard *et al.*, 1991, Money & Howard, 1996). *C. graminicola* melanin-deficient mutants initiated appressorial formation normally, but they were significantly reduced in their ability to penetrate and produce symptoms in maize leaves, thus melanin is required for full virulence in this fungus (Rasmussen & Hanau, 1989).

Secondary metabolism (SM) products, including melanin, are required at different stages of pathogen development *in vitro* and *in planta* (Takano *et al.*, 1995, Money & Howard, 1996, Talbot *et al*., 1993, Baker *et al*., 2006). The role in pathogenicity of the *Ppt1* gene, which encodes a major regulator of SM key enzymes, Sfp-type 4′phosphopantetheinyl transferase (PPTase), was tested in *C. graminicola* (Horbach *et al*., 2009). PPTases are involved in post-translational modification of key enzymes involved in synthesis of secondary metabolites.

They activate conserved serine residues in the acyl carrier protein (ACP) domain of polyketide synthases (PKSs) and the peptidyl-carrier-protein (PCP) domain of non-ribosomal peptide synthases (NRPSs) (Lambalot *et al.*, 1996). Targeted deletion of *Ppt1* in *C. graminicola* produced mutants that had multiple defects in sporulation, produced non-melanized, non-functional appressoria, had high sensitivity to oxidative stress, and an inability to synthesize some secondary metabolites (Horbach *et al*., 2009). When inoculated into wounded maize leaves, *ppt1* mutants grew at rates comparable to the wild type, apparently developing both wide primary and narrow secondary hyphae, but they were unable to form acervuli or induce symptoms. The authors concluded that, although *Ppt1* was not required for growth *in planta*, synthesis of one or more SM-derived phytotoxins during colonization of maize is required to induce symptom development and allow sporulation (*C. graminicola* only produces acervuli on dead tissue).

Fungal Gene Expression Associated with Pathogenicity in *C. graminicola*

Tang and collaborators used laser capture microdissection to analyze the expression of *C. graminicola* genes during biotrophic colonization of maize stalks (Tang *et al.*, 2006) . Using microarrays, they compared expression of the fungal genes *in vitro* with fungal genes expressed in parenchyma cells containing biotrophic hyphae in stalks 2 dpi. Twenty-two percent of the 267 fungal genes induced specifically *in planta* were predicted to encode secreted proteins. The authors suggest that these proteins could be involved in the establishment of a compatible interaction with the host. The most highly expressed genes *in planta* were predicted to encode two secreted phytases (Wyss *et al.*, 1999). Phytases are acid phosphatases and are involved in conversion of organic forms of phosphorous, including phosphorylated compounds, to available inorganic phosphate (Abelson, 1999). Phytases could be induced as a result of an environment poor in phosphorous, and could enable the fungus to obtain phosphate from plant compounds for its growth (Mullaney *et al.*, 2000) (Mueller *et al.*, 2008). However, the precise role of phytases in the infection of maize by *C. graminicola* is not yet understood.

Krijger and collaborators used a method called yeast signal sequence trapping (YSST) to identify 103 genes encoding proteins secreted into culture media containing corn cell wall and leaf extracts (Krijger *et al*., 2008). These authors were able to demonstrate that most of these genes were also induced during growth *in planta*. The genes included a laccase that was constitutively induced at all stages of infection, two serine proteases and one peptidase induced during biotrophic and necrotrophic stages, and a hydrophobin and a glucanase induced during necrotrophy. Twenty-six secreted protein genes lacked homology to other sequenced genes, and they were induced at different stages of pathogen growth. Four genes predicted to encode cysteine-rich proteins were also induced *in planta*, two of them during biotrophy and two during necrotrophy. The roles of these proteins in establishment of the compatible interaction were not tested and remain unknown.

Another analysis described plant and fungal genes that were expressed during the first stages of colonization of maize leaves by *C. graminicola*. Thirteen fungal expressed sequence tags (ESTs) were identified, including genes associated with secondary metabolism, protein transport and cellular reprogramming, differentiation, and protein degradation. Genes associated with pathogen recognition, signal transduction, defense responses, protein turnover and carbon metabolism were expressed by maize during early infection (Sugui & Deising, 2002). Once again, the precise roles of these genes in the infection process are unknown.

A recent study used suppressive subtractive hybridization (SSH) to identify plant and fungal genes expressed during biotrophic and necrotrophic infection of maize leaves by *C. graminicola* (Vargas *et al.*, 2012). Among the 657 sequenced clones, 50 corresponded to *C. graminicola* genes. Ten of these encoded proteins predicted to be secreted that could be important for pathogenicity (Ellis *et al.*, 2009), including a hypothetical protein previously identified by Krijger and collaborators (Krijger *et al*., 2008). Two hundred and sixteen plant genes were differentially regulated during plant colonization, including genes associated with cell cycle, defense responses, and metabolism. Genes involved in signaling and

transport were significantly induced 48 hpi compared to 72 hpi, which the authors proposed is associated with reprogramming of host cells during the switch to necrotrophy. The study also evaluated the expression of several previously characterized plant defense associated genes including PR1, PR5, PR4, a chitinase, and a serine protease inhibitor (Muthukrishnan *et al.*, 2001, Wu *et al.*, 1994, Morris *et al.*, 1998, Torregrosa *et al.*, 2004). Expression of these genes was induced during biotrophic growth at 48 hpi, demonstrating that *C. graminicola* does not suppress defense responses during biotrophy like an obligate biotrophic pathogen (Doehlemann *et al*., 2008). These results were supported by observations indicating lignin deposition and accumulation of phytoalexins before the switch to necrotrophy.

These studies confirm that the biotrophic and necrotrophic phases of *C. graminicola* are highly complex and involve large shifts in gene expression. Some of the structural aspects of biotrophy resemble obligate biotrophs on a superficial level, and like obligate biotrophs, hemibiotrophic *Colletotrichum* fungi produce large numbers of secreted effectors during biotrophy that seem to be important for manipulating host metabolism and for suppressing some defense responses and host cell death. However, plant defenses seem to be induced during the biotrophic phase of growth, and it is still unknown how the pathogen neutralizes these defenses and how they compare in compatible and incompatible interactions.

Maize defense responses against *C. graminicola*

In a review published in 1999 by Bergstrom and Nicholson, it was noted that little is known about host factors involved in resistance or susceptibility of maize to *C. graminicola*. Although a few factors associated with resistance responses have been identified, their precise role in the disease interaction is unclear (Muimba-Kankolongo & Bergstrom, 2011, Balint-Kurti, 2009).

A recent study evaluated development of *C. graminicola* in stalks of resistant and susceptible maize inbreds and hybrids (Muimba-Kankolongo & Bergstrom, 2011).

Reduction of symptoms in resistant lines was associated with a delay in spore germination, appresoria formation, penetration, maceration of vascular tissues (72 to 96 hpi compared to 48 hpi in susceptible cultivars) and limitation of pathogen growth determined as fungal biomass detected in the inoculated stalks. The authors propose that mediation of resistance and susceptibility could be the result of the maize plant producing factors that limit (in resistant) or induce (in susceptible) fungal growth (Stoessl, 1983).

Defense-associated compounds that are known to be produced in maize upon fungal attack include zealexins, kauralexins, monorden and monocillins. Kauralexins, a class of diterpenoid phytoalexins, were induced by infection with *Rhizopus microsporus, Fusarium graminearum* and *C. graminicola*, as well as in response to external applications of jasmonic acid and ethylene (Schmelz *et al.*, 2011). In *in vitro* experiments, kauralexins affected feeding preferences of the European corn borer *Ostrinia nubilalis*, and growth *of C. graminicola* (Schmelz et al., 2011). Zealexins, a type of phytoalexin, were detected in maize stalks upon inoculation with the maize pathogens *F. graminearum, Ustilago maydis* and *Cochliobolus heterostrophus*, as well as non-maize pathogens including *Aspergillus flavus, Aspergillus sojae,* and *C. sublineola* (Huffaker *et al.*, 2011). Interestingly, among the seven assayed fungi, only *C. graminicola* failed to induce zealexin production in maize stalks. Zealexins exhibited antifungal activity *in vitro*, in some cases at lower concentrations than those found in infected maize tissue, but activity against *C. graminicola* was not tested.

Oxylipins, a class of polyunsaturated fatty acids, are involved in plant defense where they are believed to act as signaling molecules or as antimicrobial compounds (Kachroo & Kachroo, 2009). Most of them are synthesized via the lipooxygenase (LOX) pathway, which oxygenates polyunsaturated fatty acids. Analysis of the maize genome revealed the presence of twelve LOX genes, and expression of most of these is induced by pathogen attack (Kolomiets, 2004). However, few of these have been characterized. ZmLOX3 is induced after infection by *Aspergillus flavus* and *Fusarium verticilloides* (Wilson, 2001). This gene was found to be downregulated in maize lines resistant to aflatoxin

contamination, and the authors propose that products of this pathway could act as signaling molecules to induce aflatoxin production in *Aspergillus flavus* (Kolomiets, 2004). In fact, fungal oxylipins can act as hormone precursors to regulate sexual and asexual development (Champe & el-Zayat, 1989). Deletion of fungal oxylipin biosynthetic genes affected mycotoxin production in *A. nidulans* and pathogenicity of *A. flavus* on peanut seeds (Tsitsigiannis & Keller, 2006). Furthermore, it was recently proposed that plant-derived oxylipins could be used by pathogens to mediate their sporulation and mycotoxin biosynthesis (Christensen & Kolomiets, 2008). ZmLOX1 (Kim *et al.*, 2003) and ZmLOX10 (Nemchenko *et al.*, 2006) were induced after wounding and external application of jasmonic acid (JA), which is a type of oxylipin (Kachroo & Kachroo, 2009). ZmLOX10 was also induced upon external application of salicylic acid (SA), abscisic acid (ABA), and inoculation with *Cochliobolus carbonum*, suggesting that it plays a role in maize responses against necrotrophs (Nemchenko *et al*., 2006). Recently, maize mutants lacking the ZmLOX10 gene were shown to be more resistant to *A. flavus* and *C. graminicola,* and the authors propose the possibility that fungi developed the ability to utilize LOX pathway-derived compounds to promote pathogenicity (Christensen & Kolomiets, 2008).

Monorden and monocillins I, II and III were recently identified in maize stalks inoculated with *C. graminicola* (Wicklow *et al.*, 2009). Monorden, also known as radiciol, is a fungal secondary metabolite first identified in the Ascomycete *Monosporium* sp, isolated from soil samples in Africa in 1953 (Delmotte & Delmotte-Plaquee, 1953). Monocillins are intermediates in the radicicol biosynthetic pathway (Zhou *et al.*, 2010). *In vitro* analyses demonstrated their antifungal activity against maize stalk-rot and foliar pathogens (Wicklow *et al*., 2009). Monorden inhibits heat-shock protein 90 by competition with ATP for the binding site required for activation of Hsp90 (Roe *et al.*, 1999), suggesting that it can inhibit not only other fungi, but also plant Hsp90, interfering with mediation of defense responses. Wicklow and collaborators propose that *C. graminicola* secretes monorden and monocillins during early penetration and biotrophic stages of maize infection to suppress basal defense responses.

The availability of genome sequences for *Z. mays* and *C. graminicola*, and numerous fungal insertional mutants in our lab that are interrupted at different phases of hemibiotrophic development, make the economically important anthracnose disease interaction a useful model for exploration of the complexity of hemibiotrophy. Although many different approaches have been utilized to reveal aspects required for successful establishment of biotrophy and a transition to necrotrophy, the factors the regulate this complex lifestyle remain largely unknown. Development of a standardized assay to observe the cytology of the infection process at different stages of living wild type and mutant pathogen development in living host tissues would provide a useful tool to study this pathosystem in detail. In chapter 2 of this dissertation, I will describe such an assay, and the evidence it revealed for the induction of susceptibility in maize by *C. graminicola*.

Chapter 2

Evidence for a diffusible factor that induces susceptibility in the *Colletotrichum*/maize disease interaction

Introduction

The fungal genus Colletotrichum includes more than than 600 species that infect a wide variety of plant hosts (Farr & Rossmann, 2013).Within this group, *C. graminicola*, causal agent of anthracnose leaf blight (ALB) and anthracnose stalk rot (ASR) of maize is classified as one of the most economically important species, contributing to yield losses of up to 1 billion dollars in the United States alone in 2011(Bergstrom & Nicholson, 1999, Warren & Nicholson, 1975, Frey et al., 2011). *C. graminicola* is an intracellular hemibiotrophic pathogen. It begins the infection process as a biotroph, with primary invasive hyphae that are separated from the living host cytoplasm by a membrane. It then switches to necrotrophy, which is marked by the collapse of host cells, production of secondary invasive hyphae that are no longer enclosed by a membrane, and development of anthracnose symptoms (Venard & Vaillancourt, 2007a, Mims & Vaillancourt, 2002, Wharton *et al.*, 2001, Bergstrom & Nicholson, 1999).

The mechanisms involved in establishment of a successful *C. graminicola*-maize biotrophic interaction and further progression to necrotrophy are poorly understood (Krijger *et al.*, 2008, Horbach *et al*., 2009, Thon *et al*., 2002). Obligately biotrophic plant pathogens reprogram host cells, and suppress cell death and host defense responses in order to obtain the energy they require from living plant cells (Doehlemann *et al*., 2008, Eichmann *et al.*, 2004, Doehlemann *et al*., 2009). In contrast, many necrotrophs take advantage of plant defense responses to enhance their pathogenicity and to induce cell death by the secretion of phytotoxic secondary metabolites (SM) (Rolke *et al.*, 2004, Cessna *et al*., 2000, Amselem *et al*., 2011, Govrin & Levine, 2002).

The question arises as to whether the hemibiotrophic *Colletotrichum* fungi suppress cell death like obligate biotrophs do, or induce cell death like necrotrophs. The limited evidence in the literature suggests that they do both, first suppressing, and then later inducing cell death. For example, CgDN3, a small secreted protein that suppresses the hypersensitive response (HR), is required for establishment of *C. gloeosporioides* infection in *Stylosanthes guianensis* (Stephenson *et al*., 2000). Orthologs of CgDN3 were recently identified in *C. orbiculare* and *C. higginsianum*, and they suppressed cell death caused by exposure to fungal inducers of host cell necrosis (Yoshino *et al*., 2012, Wharton *et al.*, 2001) The genome of *C. graminicola* encodes several orthologs of these necrosis inducers, though it does not appear to contain an ortholog of CgDN3 (Wharton *et al*., 2001). Nonetheless, it seems reasonable to assume that *C. graminicola* behaves similarly to its *Colletotrichum* relatives, suppressing cell death during penetration and establishment of biotrophy, and then inducing (or at least not suppressing) cell death later, at the transition to necrotrophy. Both processes are likely to be regulated by secreted compounds (including proteins and/or secondary metabolites), the production of which is tightly regulated to provide the correct function at the appropriate time and place during the interaction.

In a previous study designed to discover fungal genes required for the establishment of a compatible *C. graminicola*-maize interaction, a restriction-enzyme mediated insertional (REMI) mutagenesis approach was utilized (Thon *et al*., 2000). This resulted in identification of a gene required for pathogenicity to maize stalks and leaves known as *Colletotrichum Pathogenicity Related 1* (*Cpr1*) (Thon *et al*., 2000). The predicted CPR1 protein is similar to the microsomal eukaryotic peptidase subunit Spc3p from *Saccharomyces cerevisiae* (E= 1e-22), one of the two essential components of the yeast signal peptidase complex (SPC) (Thon *et al*., 2002). The eukaryotic SPC is responsible for the processing of signal peptides as polypeptides cross the endoplasmic reticulum (ER) membrane (Fang *et al*., 1997, Meyer & Hartmann, 1997), the first step in protein transport and secretion (Zimmermann *et al.*, 2006). Thus, it is anticipated that

CPR1 has a role in protein transport and secretion in *C. graminicola*. The REMI insertion occurred in the 3'UTR of the *Cpr1* gene, and resulted in a leaky mutation in which expression was significantly reduced, but not completely eliminated in culture (Thon *et al*., 2002). The *cpr1* mutant was normal, other than a slight reduction in growth rate, when compared to the wild type (WT) *in vitro* (Thon *et al*., 2002). Moreover, there were apparently no significant differences between the mutant and WT strains up to 48 hours post inoculation (hpi) in maize leaves (Mims & Vaillancourt, 2002). However, by 72 hpi the WT had entered the necrotrophic phase of growth, characterized by the presence of thin, secondary hyphae, collapse of maize cells, and the appearance of the first small lesions. In contrast, hyphae of the *cpr1* mutant remained confined to a few cells, and there was no widespread tissue collapse or symptom development (Mims & Vaillancourt, 2002). Based on these observations, it was hypothesized that the *cpr1* mutant was altered in the secretion of compounds involved in biotrophic colonization and/or the switch to necrotrophy, but not required for initial penetration, or for growth *in vitro* (Thon *et al*., 2002, Mims & Vaillancourt, 2002). I propose two possibilities that could explain the behavior of the *cpr1* mutant. The first possibility is that the mutant fails to produce one or more substances that promote susceptibility during early infection. An alternative possibility is that the mutant produces inducers of defense and host cell death at an inappropriate time and place, that is, early during the infection process.

My first goal was to characterize and compare the phenotypes of the *cpr1* mutant and WT strain when inoculated on detached maize leaf sheaths. Initially developed by Sakamoto in 1950 (Sakamoto, 1950), similar detached leaf sheath assays have been extensively used to study plant-pathogen interactions. These assays provide a unique advantage because they facilitate detailed microscopic observations of living host and pathogen tissues, interacting in unfixed samples (Koga, 1994). Use of the optically clear leaf sheaths allowed unprecedented observation of details of the process of infection and colonization of rice by *Magnaporthe oryzae* (Koga, 2004, Kankanala *et al*., 2007, Takahashi *et al.*, 1999), and of sorghum by *C. sublineola* (Wharton & Julian, 1996, Wharton *et al*.,

2001). Use of leaf sheath assays has made possible the recent development of novel concepts in *M. oryzae* effector biology (Mosquera *et al*., 2009b, Khang *et al*., 2010). It has been reported that fungal penetration and colonization events are more synchronous in leaf sheaths than in leaf blades (Koga, 1994, Mosquera *et al.*, 2009b, Berruyer *et al.*, 2006). I used maize leaf sheath assays to test the hypothesis that *cpr1* is altered in the production of compounds that (a) suppress defense responses and promote compatibility (like a biotroph), or (b) induce host defense responses and cell death (like a necrotroph). To address this question, I conducted co-inoculation experiments. The observation that challenging a plant with a compatible pathogen can compromise resistance and lead to infection by a normally incompatible pathogen was first made by Tsuchiya and Hirata in 1973 (Tsuchiya & Hirata, 1973). This phenomenon, which has been observed with compatible and incompatible strains of powdery mildew and rust fungi, is known as induced susceptibility (Ouchi *et al.*, 1974b, Kunoh *et al.*, 1990, Lyngkjær & Carver, 1999a, Olesen *et al.*, 2003). Observations have been reported in other pathosystems in which inoculation with an incompatible strain has generated resistance against a normally pathogenic strain (Kunoh et al., 1990, Freeman & Rodriguez, 1993, Ouchi *et al.*, 1976a). This phenomenon is known as localized induced resistance.

I predicted that if the *cpr1* mutant is failing to secrete factors normally involved in suppression of maize defense responses and cell death during establishment of biotrophy, co-inoculation with the WT strain would allow the *cpr1* mutant to grow (induced susceptibility). On the other hand, if the *cpr1* mutant is inappropriately producing inducers of defense responses and cell death, co-inoculation with the mutant would prevent the WT *C. graminicola* from colonizing (localized induced resistance).

Materials and Methods

Fungal strains, fungal transformation, and spore suspensions

All fungal strains used in this study are listed in Table 2.1. *C. graminicola* strain M1.001 (a.k.a. M2) was the WT (Forgey, 1978). A strain of M1.001 expressing

modified red-fluorescent protein (mRFP) was obtained by using a polyethylene glycol-mediated transformation protocol (Thon *et al.*, 2000). M1.001 protoplasts were transformed with 3 μg of *EcoR*I-linearized pCA56, a plasmid containing the mRFP1 gene under the control of the TOXA promoter from *Pyrenophora tritici-repentis*, and the hygromycin B phosphotransferase gene from *Escherichia coli* as a selectable marker (Andrie *et al.*, 2005). Five different transformants were recovered and tested for pathogenicity. All behaved similarly *in planta*, and the strain with the strongest and most consistent fluorescence was chosen for subsequent analysis. The *cpr1* mutant strain was derived from M1.001 by REMI mutagenesis (Thon *et al*., 2000). The complemented *cpr1* mutant strain was produced by transformation of the *cpr1* mutant with a 3.6 kb DNA fragment containing the WT *Cpr1* gene (Thon *et al*., 2002). Fluorescent strains *cpr1*-ZsGreen and CgSl1-GFP1, the latter derived from CgSl1, a *C. sublineola* strain pathogenic to sorghum but nonpathogenic on maize, were previously described (Venard & Vaillancourt, 2007a). All strains were routinely grown on potato dextrose agar (PDA, Difco) and maintained at 23°C under continuous illumination. For plant inoculations, falcate spores were harvested and prepared as described (Venard & Vaillancourt, 2007a). Spore suspensions were adjusted to a final concentration of 5 x 10^5 spores/ml, unless stated otherwise. For experiments involving heat-killed spores, spore suspensions were boiled in 1.5 ml Eppendorf tubes for 5 min, then washed once by centrifugation, and resuspended in fresh sterile water. Heat-inactivated spores were produced by incubating the spore suspensions at 50°C for 10 min, and rinsed as described above (Bell & Presley, 1969).

Plant growth

The maize inbred Mo940 was used for this study. Mo940 is highly susceptible to ALB (Warren & Nicholson, 1975, Nicholson R. L, 1976) and was used previously to characterize the growth and pathogenicity of the M1001, *cpr1* mutant and *Cpr1*-C complemented strains in intact leaves (Thon *et al*., 2002). Plants were grown in the greenhouse in 3.8 x 21 cm Containers (Super SC-10 UV stabilized Stuewe & Sons, Inc. Oregon, USA), in a mixture of three parts Pro-Mix BX

(Premiere Horticulture, Ltd, Riviere du Loup, PQ, Canada) and two parts sterile topsoil. The plants were grown to the V4 stage under 14 hours of light. The seedlings were watered daily to saturation and fertilized two to three times per week with a solution of 150 ppm of Peters 20-10-20 (Scotts-Sierra Horticultural Product Co., Marysville, OH), beginning one week after seedling emergence.

Leaf sheath inoculations

Leaf sheaths from the second leaf of V3 maize seedlings were used. The plants were cut at the soil line and the tissue was processed and inoculated immediately, using the protocol described by Kankanala and collaborators (Kankanala *et al*., 2007), with the following modifications. Sheath pieces were cut into 5 cm segments, unfolded gently to expose the inner epidermal layer, and inoculated with 20 μl of a spore suspension. The inoculation drop was placed on the epidermis directly above the midrib, where it remained until observation. Inoculated sheaths were suspended horizontally in a Petri plate containing moistened filter paper (Whatman No.1) and incubated at 23°C with continuous illumination. For co-inoculation experiments using mixed spore suspensions, equivalent amounts of each individual suspension were combined prior to inoculation, and 20 μl of the mixture was inoculated as previously described. For co-inoculation experiments in which the spore drops were separated, 10-μl drops of each spore suspension were inoculated on the leaf sheath at a distance of approximately 250 mm apart. I determined that the average length of a maize epidermal cell was 309 ± 20 μm, by using the measure function in the AxioVision software (V4.8) to measure 11 epidermal cells on each of 15 leaf sheaths (165 cells). I multiplied this average by the number of cells between the inoculation drops on 20 sheaths at 24 hpi to determine the average distance between the drops. Individual inoculated leaf sheaths were prepared and observed under the microscope at various time points up to 72 hpi, as described below.

Paraquat and freeze injury experiments

The herbicide paraquat (1,1'dimethyl-4,4'-bipyridinium ion) was used to induce systemic cell death in maize leaf sheaths. Equivalent volumes of a paraquat

stock solution (10mM) and a spore suspension were either combined before inoculation, or co-inoculated as separate drops approximately 250 mm apart. For freeze-injury experiments, a metallic rod (3 mm diameter) dipped in liquid nitrogen was used to produce a localized injury in the center of the unfolded sheath, and a 10 μl drop of inoculum was immediately applied either to the same location, or to a location approximately 250 mm from the freeze-treated spot. Fungal colonization was assessed at 24 and 60 hpi.

Timed experiments

Ten-microliter drops of mutant spore suspensions were inoculated on maize leaf sheaths. At 0, 12, 24, or 36 hpi, a 10 μl drop of a WT spore suspension (or a drop of water as a control) was added at a distance of approximately 2.5 mm from the mutant inoculum drop. Control sheaths that were detached at time 0 were co-inoculated at 0, 12, 24, or 36 hours after detachment simultaneously with the mutant and WT (or water controls). All treatments were observed 60 h after the WT inoculum was added.

Spore concentration experiments

Three different inoculum concentrations (5×10^5, 1×10^5, and 5×10^4 spores/ml) of the WT and mutant strains were co-inoculated on maize leaf sheaths in all possible combinations. Fungal colonization was evaluated at 60 hpi.

Light microscopy and staining

Leaf sheaths were rinsed gently with deionized water in order to remove any superficial growth, and trimmed before observation, using the method described in (Kankanala *et al.*, 2007). Sheath pieces were mounted on slides under cover slips with the intact epidermal surface uppermost.

To detect the presence of H_2O_2, 3,3"-diaminobenzidine (DAB) staining was performed (Orozco-Cardenas & Ryan, 1999) with a few modifications. Non-trimmed leaf sheaths were stained for 8 hours in a DAB solution (1mg/ml), pH 3.8, at 25˚C under constant light. Sheaths where cleared in 96% boiling ethanol

for 5 minutes and then transferred to fresh 96% ethanol for 4 hours. Samples were mounted in 50% glycerol for observation.

Plant cell viability was evaluated by plasmolyzing with a solution of 0.75 M sucrose (Kankanala *et al*., 2007), or by using the viability dye neutral red. Leaf sheaths were stained for 1 hour in a neutral red solution (0.01%, 0.85M KNO_3), pH 7.5 (Stadelmann & Kinzel, 1972). Metabolically active cells plasmolyzed and accumulated neutral red inside the vacuole (Wharton & Julian, 1996). Fungal hyphae inside leaf sheaths were stained with lactophenol-trypan blue (Tong *et al*., 2009).

Epifluorescence and confocal laser scanning microscopy

Epifluorescence microscopy was conducted by using an Axioplan2 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) equipped with Chroma filter sets for GFP and DsRed (Chroma Technology Corp, Rockingham, Vt). Micrographs were obtained using an Axiocam MR monochromatic digital camera and the AxioVision software version 4.8. Software and filter parameters were as described by Tsai and collaborators (Tsai *et al.*, 2005).

Confocal laser scanning micrographs were acquired on a TCS SP2-AOBS microscope (Leica Microsystems, Bannockburn, Ill.). To visualize and image GFP, it was excited at 488nm, and dsRed was excited at 543nm, using helium/neon lasers. Emission conditions and filters are described in detail in (Goodin *et al.*, 2002).

Assessment of fungal growth and development *in planta*

Growth and development of fungal strains in maize leaf sheaths was routinely assessed at 20, 48 and 60 hpi, unless noted otherwise. More than 1,000 infected leaf sheaths were observed for this study. For statistical comparisons of the developmental timelines for the various strains *in planta,* 100 individual infection sites were evaluated on each of ten individual sheaths for each strain at 18, 24 and 48 hpi (a total of 1000 infection sites per time point per strain). To statistically compare the relative degree of colonization by the strains, the number of host cells colonized from each of 20 successful penetration sites was measured for 10

individual leaf sheaths (a total of 200 sites for each strain) at 48 hpi for the M1.001, WT-mRFP, and *Cpr1*-C strains, and at 60 hpi for the *cpr1* mutant and *cpr1*-Zsgreen strains. To quantify colonization by the *cpr1* mutant in the co-inoculation experiments, the number of host cells colonized from each of 20 successful penetration sites was determined on 10 individual leaf sheaths (a total of 200 sites) at 60 hpi. All experiments for statistical analyses were repeated at least twice.

Statistical Analysis

Differences among treatments were assessed using analysis of variance with SAS statistical package version 9.3 (SAS Institute Inc). Since many groups were compared, one-way analysis of variance was used, and if significant effects were detected, multiple comparisons of means were performed using Tukey and least significant difference (LSD) methods. Results were expressed as means with their corresponding standard deviations, and differences among or between means were considered to be significant if the probability, $p \leq 0.05$.

Results

The *cpr1* mutant strain germinated and penetrated maize epidermal cells as efficiently as the WT, but penetration was delayed

The percentage of spores that germinated to produce appressoria and primary infection hyphae over time in maize sheaths was measured for each strain. Development of the fluorescently-tagged strains did not differ statistically in this respect from the unlabeled parental strains, and the complemented *cpr1* strain containing an ectopic copy of the full-length *Cpr1* gene (Thon et al., 2002) was not statistically different from the WT strain (Figure 2.1). At 12 hpi, the *cpr1* mutant strain had a slight, but significant, delay in germination when compared with the WT (Figure 2.1). However, germination of the mutant was not reduced in comparison with the WT-mRFP or *Cpr1*-C strains (Figure 2.1). By 24 hpi, 41% of the WT appressoria had progressed to the production of invasive primary infection hyphae. At the same time point, 93% of the c*pr1* mutant spores had produced appressoria, but less than 5% of these had produced visible infection

hyphae (Figure 2.1). At 48 hpi, 36% of the *cpr1* mutant infection sites had progressed to the production of invasive primary hyphae, which is not statistically different from the rates of colonization achieved by the WT 24 h earlier (Figure 2.1). It could not be determined if the WT had initiated additional infections between 24 and 48 hpi, due to the extensive tissue colonization at that point from the original sites.

Inoculation of detached maize leaf sheaths with the CgSl1-GFP nonpathogen strain resulted in the production of appressoria by 24 hpi, but these appressoria only rarely (< 1%) produced primary infection hyphae within 48 hpi, and these rare hyphae never grew beyond the initially infected cell, even up to 96 hpi (not shown).

The mutant did not establish a normal biotrophic infection, or progress to necrotrophy in leaf sheaths

Inoculation of maize leaf sheaths with the WT, WT-mRFP, or *Cpr1*-C strains resulted in a visible water-soaked lesion at the inoculation site within 72 hpi (Figure 2.2A). In contrast, inoculation with the *cpr1* mutant, *cpr1*-Zsgreen, or CgSl1-GFP strains never resulted in the production of a visible lesion, even when the sheaths were retained for up to 96 hpi (Figure 2.2A).

Sucrose plasmolysis and the viability stain neutral red were used to determine the status of the host cells during the process of pathogen infection. Twenty hours after inoculation, apparently mature melanized appressoria of the WT and the *cpr1* mutant strains had formed on the inoculated tissue (Figure 2.2B). Host cells beneath and around these appressoria plasmolyzed and took up neutral red stain, indicating that they were alive. Forty-eight hours after inoculation, most WT infections had already entered and grown beyond one host cell via broad, branching hyphae, which narrowed when they passed across the apparently intact host cell walls (Figure 2.2B). Nearly half of the successful infections (45.7%) had colonized at least three host cells beyond the point of infection, and nearly 5% of the infections had already colonized five cells beyond the infection point (Table 2.2). The average number of cells beyond the infection point

colonized by the WT at 48 hpi was 3.8. The WT-mRFP and the *Cpr1*-C strains did not differ significantly from the WT in this respect. Thirty-six percent of the *cpr1* mutant appressoria had also entered host cells and produced primary infection hyphae by 48 hpi. However, even at 72 hpi, 96% of these mutant infections remained confined to a single host cell. The maximum number of cells beyond the infection site colonized by the mutant strains at 72 hpi was three, and less than 1% of the infection sites had progressed that far (Table 2.2). The average number of cells beyond the infection point colonized by the *cpr1* mutant strain at 72 hpi was only 0.6. The average for the *cpr1*-Zsgreen strain was not significantly different.

A combination of neutral red staining and plasmolysis confirmed that at least some of the host cells were alive at the time of invasion by either the WT or mutant strains (Figure 2.2B). Furthermore, most of the surrounding cells were also alive. Approximately 60 hours after inoculation, the centers of the WT colonies switched to necrotrophy, indicated by the appearance of thin, secondary hyphae, obvious tissue maceration and a lack of plasmolysis or staining of host cells (Figure 2.2B). The edges of the colony, however, remained biotrophic, indicated by the continued ability of newly invaded cells and cells beyond the colony borders to plasmolyze and take up the neutral red stain (Figure 2.2B). Leaf sheaths were routinely monitored up to 96 hpi, and on several occasions were retained for up to six days, but the *cpr1* mutant never produced symptoms on the leaf sheaths. The nonpathogen *C. sublineola* strain produced appressoria normally on maize sheaths, but only very rarely produced visible infection hyphae. Papillae could often be seen in the epidermal cells directly beneath the appressoria. The epidermal cells beneath and surrounding the appressoria appeared to be alive (See Figure 2.4N in this dissertation), while cells containing rare infection hyphae no longer plasmolyzed.

Pattern of ROS accumulation differs in mutant interactions

Plants respond to pathogen attack by accumulating ROS, especially H_2O_2 (Vargas et al., 2012). I used DAB staining to detect H_2O_2 production in sheaths

inoculated with WT and mutant strains. Leaf sheaths were inoculated as described and stained for observation at 12, 24, and 48 hpi. DAB precipitates could be observed by 12 hpi. The intensity of staining at this time was generally stronger in the WT than in the mutant inoculations (Fig 2.3 A,D). There was a noticeable increase in the amount of DAB staining by 24 hpi. Interestingly, a distinctive halo pattern was sometimes observed surrounding the penetration sites of the WT strain, but not the mutant strain, where a more diffuse accumulation of DAB-stained vesicles was typical (Figure 2.3 B,E). Forty-eight hours after inoculation, the halo pattern had disappeared, and very few precipitates could be detected in the WT-inoculated sheaths (Figure 2.3C). In contrast, an intense accumulation of DAB precipitates, especially in the cell walls of penetrated cells and within numerous vesicles beneath penetration sites, could be detected in the *cpr1*-mutant inoculated tissue (Figure 2.3F).

The mutant strain and the nonpathogen *C. sublineola* strain complete their life cycles on killed maize sheath tissue

To test the role of host cell viability and active host defenses, I inoculated freeze-killed and paraquat-treated leaf sheaths. Freezing was used to induce localized tissue damage, while paraquat, which is translocated, induced systemic damage. Both treatments resulted in the loss of ability of the affected host cells to plasmolyze (not shown). Both treatments allowed colonization by the mutant *cpr1*-ZsGreen strain, and the nonpathogen Cgsl1-GFP (not shown). The type and degree of colonization by these strains did not differ noticeably from the WT-mRFP strain. Colonization of damaged tissues was dramatically accelerated, with all three strains growing beyond the first invaded cell within just 24 hpi. By 60 hpi, all three strains had extensively colonized the freeze-killed and paraquat-treated tissues, and individual hyphae could no longer be easily distinguished. Freeze-killed tissue supported the formation of acervuli by all three strains, demonstrating that the *cpr1* mutant strain and the nonpathogenic *C. sublineola* strain are capable of completing their entire life cycles on maize sheath tissue, in the presumed absence of active host responses.

Co-inoculation of leaf sheaths with the WT strain induced susceptibility to the *cpr1* mutant

After observing the growth of the mutant and WT strains separately, I tested the effect of co-inoculating the strains on living maize leaf sheaths. So that they could be distinguished within the host tissue, the AFP tagged strains were used for co-inoculations and all following experiments, unless stated otherwise. Behavior of these strains on maize leaf sheaths resembled the non-tagged parental strains. Seventy-two hours after inoculation, the WT-mRFP strain had colonized more than 6 cells, while the cpr1-ZsGreen remained as appressoria or confined in the first invaded epidermal cell (Figure 2.4 A-C).

In experiments in which WT-mRFP and *cpr1*-ZsGreen spores were mixed in the same inoculation drop the latter was routinely observed growing beyond the first invaded cell, colonizing up to three cells by 60 hpi (not shown). After observing more than 100 co-inoculations, I concluded that the *cpr1*-ZsGreen strain only entered these additional cells when they were also colonized by the WT-mRFP, and no longer plasmolyzed. Co-inoculation with the mutant strain did not prevent the WT from colonizing maize normally (i.e. there was no evidence for localized induced resistance).

I next co-inoculated the mutant and WT strain on the same leaf sheath, but with the drops of inoculum separated. The drops were placed as close to one another as possible without having them drawn together by water tension (Figure 2.4 D). I determined that this distance was approximately 2.5 mm. I discovered that this co-inoculation “at a distance” induced susceptibility of the maize tissues to the *cpr1* mutant at 60 hpi. On average, about a third of the successful penetrations progressed from the initially infected cell to colonize two or more additional cells (Figure 2.4 E,F, Figure 2.5) In control sheaths in which *cpr1*-ZsGreen spore drops were paired with drops of water, growth was similar to that observed previously, in the absence of co-inoculation (Figure 2.5). Fewer than 5% of the mutant infections in these control inoculations grew beyond the first penetrated cell, even up to 72 hpi (Figure 2.5). Similar results were obtained when I used the untagged strains and the *Cpr1*-C strain in co-inoculations (not shown).

Plasmolysis (Figure 2.4 I-J) and vital staining (Figure 2.4 K-L) revealed that most of the host cells surrounding and between the WT and mutant colonies in the co-inoculations were alive (Figure 2.4 G-L). A localized freeze injury made at a distance of 2.5 mm from the inoculation drop did not induce susceptibility to the mutant (not shown).

Co-inoculations at a distance did not induce susceptibility to the nonpathogen

When spores from the nonpathogen and WT-mRFP were mixed in the same inoculation drop, CgSl1-GFP routinely penetrated and colonized the maize cells (not shown). However, as I had observed with the c*pr1*-Zsgreen strain previously, this only occurred when the cells had also been colonized by WT-mRFP and didn't plasmolyze. When spores from the nonpathogen and *cpr1-Zsgreen* were mixed, both strains germinated and form appressoria, but neither strain colonized the tissue to a greater extent than the controls (not shown).

To test interactions at a distance, I conducted triple-inoculation experiments, with spore suspensions of the nonpathogen, mutant, and WT strains inoculated at separate locations along the same leaf sheath (Figure 2.4 M-O). At 60 hpi, the growth of the nonpathogen was limited and indistinguishable from controls inoculated with drops of water on the sheaths (Figure 2.4 N). Rarely the nonpathogen produced a primary infection hypha, but in no case (N=50 sheaths) was it observed to grow beyond one cell (Figure 2.6). Growth of WT-mRFP in triple inoculations was normal, and induced susceptibility similar to that observed previously in the double inoculations was observed for the *cpr1*-Zsgreen strain (Figures 2.4 O and Figure 2.6). Co-inoculation of *cpr1*-ZsGreen and CgSl1-GFP1 at a distance, in the absence of the WT, did not induce susceptibility to either strain (not shown).

Induced susceptibility is dependent on distance, spore concentration, and timing of inoculation

The induced compatibility effect diminished as the distance between the inoculum drops was increased (Table 2.3). When the WT and mutant are initially separated by a distance of 2.5 mm, 33.4% of the infection sites grew beyond the

second colonized cell, compared to only 4% in the control. However, when the distance between the drops was doubled or tripled, the growth of the *cpr1* mutant was no longer different from the control.

The induced susceptibility effect depended on the initial spore concentration of both strains. A five-fold reduction in the WT inoculum, or a ten-fold reduction in either strain, significantly reduced the growth of the *cpr1* mutant in co-inoculations (Table 2.4).

Induced susceptibility was also affected by the amount of time that had elapsed between application of the mutant and WT inocula. The degree of colonization was significantly decreased when the mutant was alone for 12 h before the WT inoculum was added, and induced susceptibility was no longer observed when the time between inoculations was increased to 24 or 36 h (Figure 2.7). Control sheaths indicated that even by 96 hpi, the *cpr1* mutant inoculated alone only rarely (<5%) grew beyond the initially colonized cell.

Other mutant strains that are reduced in pathogenicity do not induce susceptibility to the *cpr1* mutant; the WT does not induce susceptibility to these other mutant strains

Several additional mutants of *C. graminicola* were tested for their effects in co-inoculations at a distance. A pyrimidine auxotrophic mutant (Rasmussen *et al.*, 1992), a melanin-deficient mutant (Rasmussen & Hanau, 1989) and several REMI mutants with unknown genetic defects, were tested (Thon *et al.*, 2000). All of these mutants were diminished in their ability to colonize leaf sheaths. By 60 hpi, spores of M1.201 (Pyr^-) failed to germinate on the surfaces of leaf sheaths, while REMI mutants 90-23 and 84-6 produced melanized appressoria but failed to penetrate the tissue. REMI mutant 80-37 only developed very short invasive hyphae, while 83-45 and 84-14 were able to produce primary infection hyphae of a normal size that, however, remained confined to the first invaded epidermal cell. The melanin-deficient mutant M1.502 produced non-melanized appressoria, but was eventually able to penetrate and colonize up to 2-3 epidermal cells by 60 hpi. The REMI mutant 9-4 was also delayed in penetration compared to the WT,

and colonized up to 2-3 cells by 60hpi. Both M1.502 and 9-4 mutants were visibly reduced in penetration efficiency and invasive growth rate compared with the WT.

None of these mutants were able to induce susceptibility to the *cpr1* mutant in co-inoculations at a distance, and the WT did not induce an increase in susceptibility to any of these mutants (not shown).

Heat-killed spores do not induce susceptibility

Previous studies have demonstrated that heat-killed or attenuated spores can still affect the outcome of subsequent pathogen inoculations (Bell & Presley, 1969). My results demonstrated that killed or inactivated WT spores did not induce susceptibility, and did not differ from the water controls when used in co-inoculation experiments with the *cpr1* mutant (not shown).

Discussion

Secreted compounds play important roles in fungal development and in the successful establishment of fungal interactions with plants (Condon *et al.*, 2013, Koeck *et al.*, 2011, Djamei *et al*., 2011). However, the mechanisms that regulate the production and secretion of these substances *in planta* are largely unknown.

In this study, I used detached maize sheath assays to study the behavior of the nonpathogenic *C. graminicola cpr1* mutant, which is predicted to be deficient in protein transport and secretion (Thon et al., 2002). The optically clear unfixed tissues, in which both the plant and pathogen were alive, allowed me to observe infection and colonization by both the mutant and the WT with unprecedented clarity (Mosquera *et al*., 2009b, Koga, 2004). Earlier studies using maize leaf blades and stalks (Mims & Vaillancourt, 2002, Thon *et al*., 2000, Thon *et al*., 2002, Venard & Vaillancourt, 2007a) indicated that the *cpr1* mutant resembled the WT during the first stages of infection and did not switch to necrotrophy; however, it had not previously been possible to observe the mutant in such detail.

When plant tissues are detached, they begin to senesce, and it is possible that assays using detached tissues will differ from those on the intact plant (Greenshields *et al.*, 2007, Audenaert *et al.*, 2002, Benito *et al.*, 1998). Nevertheless, detached leaf assays are frequently used for plant pathological studies (Fukuoka *et al.*, 2009, Benito *et al*., 1998, Audenaert *et al.*, 2002, Khang *et al*., 2010). *C. graminicola* infection and colonization of detached maize leaf sheaths closely resembled previous descriptions of the same isolate infecting intact leaf blades (Vargas *et al*., 2012). Appressoria were produced by 12 hpi, and melanized, mature appressoria were detected at 18-24 hpi. Thick, primary hyphae were observed by 48 hpi. These broad hyphae colonized up to five cells beyond the initial infection site, usually entering each new cell biotrophically, as evidenced by plasmolysis and vital staining assays. Colonized cells lost their ability to plasmolyze very quickly, usually before the hyphae entered the adjacent cell, indicating *C. graminicola* exists as a true biotroph only very briefly in these cells. The appearance of thin, secondary hyphae, marking the switch to necrotrophy, occurred at approximately 60 hpi, prior to obvious tissue collapse and lesion development, which occurred by 72 hpi. It has sometimes been stated that the switch to necrotrophy in *C. graminicola* occurs synchronously at approximately 72 hpi (Sugui & Deising, 2002, Horbach *et al*., 2009, Vargas *et al.*, 2012). However, my observations indicated that growth of *C. graminicola* resembles instead the colonization of sorghum by *C. sublineola* in which host cell death and cell wall degradation was localized to the center of the colony, while the colony margins continued to expand biotrophically (Wharton *et al*., 2001). Thus, biotrophy and necrotrophy co-exist in *C. graminicola* colonies *in planta*. The similarity in timing and events with descriptions of the disease on intact tissues in the literature gives me confidence that the sheath assay provides a relevant view of the disease process. Additionally, I note that the detached tissues continued to plasmolyze, and did not become susceptible to the *cpr1* mutant or to the nonpathogen *C. sublineola*, even when they had been detached for up to six days. This indicates that the cells remained alive for this period, and retained enough metabolic activity to express sufficient levels of resistance to

prevent growth by these strains. This differs from reports of detached leaf assays in Arabidopsis, where non-pathogens were able to infect the detached leaves (Greenshields *et al.*, 2007). Overall, it is my opinion that the advantages of the detached maize leaf sheath assay outweigh any disadvantages.

Germination and appressorial production were comparable in the mutant and WT strains. The mutant was slightly, but significantly, delayed in the formation of appressoria at 12 hpi when compared with the WT, but this small delay disappeared in comparisons with the fluorescently-labeled WT or complemented strains, both of which displayed normal pathogenicity. Major differences were seen in the timing of penetration and production of primary hyphae. This process was delayed by about 24 hours in the mutant compared with the WT. However, the efficiency of penetration did not appear to be negatively affected; approximately 40% of infection sites ultimately resulted in successful penetrations for both the WT and the mutant.

The delay in penetration by the mutant could result from an inability to secrete compounds required during the early stages of colonization. Komura and collaborators (Komura *et al.*, 1990) proposed that in powdery mildew, suppressors of host defenses were released at or before appressorial maturity. There is evidence that *Colletotrichum* appressoria also secrete effector proteins before penetration (Kleemann *et al.*, 2008, Kleemann *et al.*, 2012). At least seven *C. higginsianum* effector candidates (ChECs) are expressed in pre-penetration appressoria, and accumulate in the appressorial penetration pore during colonization of Arabidopsis leaves (Kleemann *et al.*, 2012). One of these effectors, *ChEC3*, was also induced in early primary invasive hyphae, and suppressed cell death induced by necrosis-inducing proteins in *N. benthamiana* (Yoshino *et al.*, 2012). I did not observe evidence of localized cell death or cytoplasmic disorganization upon inoculation with the *cpr1* mutant. However, little is known about HR and the reaction of maize plants to incompatible pathogens (Buckner et al., 1998).

Once the mutant and WT entered the host cells, another major difference between them became evident. The WT grew beyond the initially infected cell readily, progressing up to five cells beyond the infection point within 24 hours. In contrast, the mutant only rarely escaped from the initially infected cell (<5% of the time), and never caused symptoms on leaf sheaths kept for up to 6 days. Thus, it appears that the mutant may have a defect in the ability to induce accessibility of adjacent cells.

Various studies suggest that host cells can perceive fungal signals before penetration occurs (Kobayashi *et al.*, 1990), and that these signals trigger plant defense responses, including the accumulation of ROS and the activation of pathogenicity-related (PR) genes (Kunoh *et al*., 1990, Yamaoka *et al.*, 1994, Veneault-Fourrey *et al.*, 2005). Perception of defense elicitors is thought to cause the host cell to become inaccessible to a nonpathogenic fungus (Chappell & Hahlbrock, 1984, Cervone *et al.*, 1989, Bradley *et al.*, 1992, Ouchi et al., 1974b, Ouchi *et al*., 1976a). Inaccessibility can be blocked by suppressors produced by a compatible pathogen, and co-inoculation with a compatible pathogen can enable colonization by a normally incompatible one (Komura *et al.*, 1990, Yamaoka *et al.,* 1994, Lyngkjaer *et al.*, 2001). Most co-inoculation studies have been done with powdery mildew and rust fungi (Tsuchiya & Hirata, 1973, Ouchi *et al*., 1974b, Ouchi *et al.*, 1974a, Ouchi *et al*., 1976a, Ouchi *et al.*, 1976b, Yamaoka *et al*., 1994, Kunoh *et al.*, 1989, Kunoh *et al.*, 1990, Kunoh *et al.*, 1988, Kunoh *et al.*, 1985, Komura *et al.*, 1990, Kobayashi *et al.*, 1995, Kobayashi et al., 1990, Olesen *et al.*, 2003, Lyngkjaer *et al.*, 2001, Lyngkjær & Carver, 2001, Lyngkjaer & Carver, 1999, Carver *et al.*, 1999). Compatible interactions with the barley powdery mildew fungus *Blumeria graminis* f. sp *hordei* allowed infection by an otherwise incompatible pea powdery mildew, *Erisyphe pisi* (Kunoh *et al.,* 1985, Yamaoka *et al*., 1994, Kunoh *et al*., 1989, Kunoh *et al.*, 1990, Kunoh *et al*., 1988, Komura *et al*., 1990, Kobayashi *et al.*, 1995, Kobayashi *et al.*, 1990). Similar observations have been reported with cucumber (Ouchi *et al.*, 1976b), wheat, and oat powdery mildews (Olesen *et al.*, 2003), and with bean and cowpea rust fungi (Heath, 1983). This phenomenon has been named induced

susceptibility. The effect is generally localized, limited to the initially penetrated cell and up to three cells distant from the initial penetration site (Kunoh *et al*., 1988, LyngkjÆR & Carver, 1999b, Kunoh *et al.*, 1985, Kunoh *et al.,* 1989, Komura *et al*., 1990, Yamaoka *et al.*, 1994, Lyngkjaer & Carver, 1999, Lyngkjaer *et al.*, 2001). To our knowledge, only one study has reported a significant increase in susceptibility that extends as far as five cells, and none further (Heath, 1983). The substances responsible for induced susceptibility are unknown.

I designed co-inoculation experiments to test two possible explanations for the behavior of the *cpr1* mutant. The first hypothesis is that the WT *C. graminicola* secretes effectors to establish a compatible interaction with maize, and the mutant is unable to secrete these effectors. If this was true, I expected that co-inoculations of the mutant and WT would allow the mutant to grow. The second hypothesis is that the mutant inappropriately secretes elicitors of defense responses, which cause the neighboring cells to become inaccessible. If this was true, I expected that co-inoculations of the mutant and WT would prevent the growth of the WT. I labeled each strain with different AFPs so that I would be able to distinguish between them during the co-inoculation procedure.

I observed that when the WT and the *cpr1* mutant were co-inoculated in the same location, both strains were able to grow. This established that the mutant did not induce inaccessibility to the WT strain. I noticed that the *cpr1* mutant only entered cells that had already been colonized by the WT and that failed to plasmolyze. I had previously observed that the mutant was capable of growing in and completing its life cycle on dead maize tissue. The nonpathogen *C. sublineola* was also able to colonize dead maize sheath tissue, and it also grew when it was inoculated at the same site as the WT. Thus I could not eliminate the possibility that the mutant was growing in dead cells, rather than in cells that had been rendered accessible by WT effectors.

I next co-inoculated the WT and mutant strains at different locations on the maize sheaths, separated by 8.5 ± 1.5 sheath epidermal cells, a distance of

approximately 2.5 mm. I observed that these co-inoculations “at a distance” resulted in a significant increase in the growth of the *cpr1* mutant at 60 hpi. Vital staining and plasmolysis assays indicated that both strains usually invaded cells biotrophically, and also that cells between and surrounding the two colonies were alive. This argued against the possibility that the WT was producing a diffusible toxin that was killing the host cells in advance and allowing the mutant to grow. This was also supported by my observation that the nonpathogen did not grow in co-inoculations “at a distance”. I concluded that the WT was producing or eliciting the production of one or more diffusible substances that induced susceptibility of the host cells.

The induced susceptibility effect was observed consistently when the inoculum drops were separated by 8.5 ± 1.5 sheath epidermal cells. When I doubled or tripled the distance between the drops, the induced susceptibility effect disappeared. Thus, the inducing substance(s) has/have a limited ability for diffusion. Nevertheless, to my knowledge the effective distance observed here (8.5 cells) is the highest ever reported. Experiments in which I varied the inoculum concentrations suggested that the induced compatibility phenomenon was dosage-dependent. Reductions of either the WT or the *cpr1* mutant inoculums resulted in a decrease in the degree of induced susceptibility. These results were reinforced by my experiments with various other mutants of *C. graminicola* that all resulted in reductions in fungal biomass within the host tissue. None of these mutants were able to induce susceptibility. A suppressor of plant defense responses isolated from *Mycosphaerella pinodes* spore germination fluids acts in a dose-dependent manner (Oku *et al.*, 1977). Because reductions in *cpr1* inoculum also reduce the effect, it suggests that the mutant may be secreting reduced amounts of the effectors.

Most studies report that once a fungus is recognized by a plant cell, it is irreversibly reprogrammed towards accessibility or inaccessibility, and this state cannot by altered by successive inoculations *(Kunoh et al.,* 1988*,* Kunoh et al., 1989, Ouchi *et al*., 1976a, Ouchi *et al.*, 1976b). Application of the WT up to 12 hours after inoculation with the *cpr1* mutant still significantly increased the growth

of the mutant. However, longer intervals failed to induce susceptibility, suggesting that the host had become irreversibly programmed for inaccessibility at that time. This indicates that the mutant is producing elicitors of defense, although possibly at reduced levels. My DAB staining results, which show significant staining at sites inoculated with the mutant, support this idea. The staining results suggest that the mutant is slower to elicit responses than the WT, and this may explain why full inaccessibility is not induced until 24 hours after the mutant has been applied to the tissue. An alternative possibility is that the mutant was unable to survive in the appressorial state beyond 24 hours without penetrating. This seems less likely, since it is known that *Colletotrichum* appressoria can survive for extended periods (Binyamini & Schiffmann-Nadel, 1972, Zaitlin *et al.*, 2000, Muirhead & Deverall, 1981).

Suppression of defense responses by rusts and powdery mildews can induce local susceptibility to nonpathogenic species (Kunoh *et al*., 1985, Yamaoka *et al.*, 1994, Kunoh *et al.*, 1989, Kunoh *et al.,* 1990, Kunoh *et al.*, 1988, Komura *et al.*, 1990, Kobayashi *et al*., 1995, Kobayashi *et al.*, 1990). In my experiments, co-inoculation with *C. graminicola* “at a distance”’ did not induce susceptibility to the closely related nonpathogen *C. sublineola*. This suggests that the diffusible inducing substance(s) do/does not override the normal detection and defense response to nonpathogens in maize.

The nature of this diffusible inducer is unknown. It could be a plant signal, and not of fungal origin at all. It could also be a secreted fungal product. Numerous effector proteins (Perfect *et al*., 1998b, Bhadauria *et al.,* 2011, Bhadauria *et al.*, 2012, Yoshino *et al.*, 2012, Stephenson *et al.,* 2000, Kleemann *et al.*, 2012) and secondary metabolites (SM) (Rasmussen & Hanau, 1989, Takano et al., 1995, Horbach et al., 2009, O'Connell *et al*., 2012) are known to be produced during *Colletotrichum* disease interactions, including during the interaction between maize and *C. graminicola*. Laser capture microdissection (Tang *et al.*, 2006), yeast signal sequence trapping (Krijger *et al*., 2008) and suppressive subtractive hybridization (Vargas *et al.*, 2012), have been used to identify approximately 160 *C. graminicola* genes expressed during the establishment of biotrophy and the

switch to necrotrophy, many of which are predicted to encode secreted proteins Various effector proteins have been demonstrated in *M. oryzae* to move several cells beyond the infection site (Khang *et al.*, 2010). SM, many of which are secreted via membrane-bound transporters, have also been implicated in *C. graminicola* pathogenicity. Deletion of *Ppt1*, a major activator of polyketide synthases and non-ribosomal peptide synthetases in *C. graminicola,* led to the hypothesis that synthesis and secretion of one or more SM-derived compounds are required for pathogenicity (Horbach *et al*., 2009). The spore germination inhibitor mycosporin alanine (Leite & Nicholson, 1992), and the antifungal compounds monorden and monocillins I, II and III (Wicklow *et al*., 2009) identified in *C. graminicola*-infected stalks, are the only SM characterized from *C. graminicola*. Monorden inhibits heat-shock protein 90 (Hsp90) by competing with ATP for the binding site required for its activation (Roe *et al.*, 1999). Monorden could potentially inhibit other fungi, and also the maize Hsp90. Wicklow and his collaborators suggested that *C. graminicola* secretes monorden and monocillins during early penetration and biotrophic stages of maize infection to suppress basal defense responses.

My observations, described in this chapter, lead me to hypothesize that *C. graminicola* secretes (a) suppressor (s) of host defense responses that diffuses beyond the borders of the fungal colony, and predispose cells to fungal invasion. The *cpr1* mutant has a defect in one component of the signal peptidase, which is involved with protein transport through the ER. At least one previous study has directly implicated protein transport and secretion in pathogenicity. *Lhs1* is a molecular chaperone that mediates import and proper folding of proteins in the ER in *M. oryzae*. Strains with deletions of *lhs1* had severe defects in conidiation, ER protein translocation, and pathogenicity (Yi *et al.*, 2009). *M. oryzae lhs1* mutants were able to penetrate rice leaf sheaths but they remained confined to the first colonized cell, which is similar to the phenotype of the *cpr1* mutant in maize.

The ortholog of *Cpr1* in *A. niger* was dramatically up-regulated post-transcriptionally during chemically-induced ER stress, as was *LhsA*, the ortholog

of *Lhs1* (Guillemette *et al.*, 2007, Tyson & Stirling, 2000). It would not be surprising if *C. graminicola* (and *M. oryzae*) experience ER stress during appressorial and biotrophic development, when the requirement for secreted proteins increases rapidly. I propose a model in which the disruption of *cpr1* is associated with an inability of the mutant to adapt to ER stress, and results in a reduction in its ability to secrete diffusible substances that induce accessibility of host cells. Further characterization of this mutant could provide valuable information to help us understand the molecular mechanisms that the fungus utilizes to establish a compatible interaction with the plant. This could lead to the identification of targets that might be useful in the successful development of sustainable disease control strategies.

Table 2. 1. Fungal strains used in this study

Strain	Parental strain	Relevant phenotype	Relevant citation
M1.001	-	Pathogenic to maize	Forgey *et al*, 1978
WT-mRFP	M1.001	Transformed to exprpess RFP in planta. Pathogenicity normal	This study
*cpr1*mutant	M1.001	Nonpathogenic to maize. Obtained by REMI mutagenesis. Mutation in 3' UTR of the Spc3 otrhologous gene, encodes component of signal peptidase	Thon *et al*, 2000
cpr1-Zsgreen	*cpr1*mutant	*cpr1* mutant transformed to express ZsGreen fluorescent protein. Nonpathogenic to maize.	Venard and Vaillancourt, 2007
CgSl1-GFP1	CgSl1	Pathogenic to sorghum but nonpathogenic to maize	Venard and Vaillancourt, 2007
M1502	M5.002	Melanin-deficient, due to UV indced mutation in scytalone dehydrogenase gene	Vaillancourt and Hanau, 1990
M1201	M2.001	Pyrimidine biosynthetic mutant, due to a spontaneous mutation in orotate phosphoribosyl transferase gene. Nonpathogenic to maize. Spores don't germinate or adhere well on maize sheaths	Rasmussen *et al*, 1989
90-23	M1.001	Nonpathogenic to maize. Obtained by REMI mutagenesis. Specific mutation unknown.	Thon *et al*, 2000
9-4	M1.001	Reduced in pathogenicity to maize. Obtained by REMI mutagenesis. Specific mutation unknown	Thon *et al*, 2000, Thon *et al*, 2002
80-37	M1.001	Nonpathogenic to maize. Obtained by REMI mutagenesis. Specific mutation unknown.	Thon *et al*, 2000

Table 2.1. (continued)

Strain	Parental strain	Relevant phenotype	Relevant citation
83-45	M1.001	Reduced in pathogenicity to maize. Obtained by REMI mutagenesis. Specific mutation unknown	Thon *et al*, 2000
84-14	M1.001	Reduced in pathogenicity to maize. Obtained by REMI mutagenesis. Specific mutation unknown	Thon *et al*, 2000
84-6	M1.001	Nonpathogenic to maize. Obtained by REMI mutagenesis. Specific mutation unknown	Thon *et al*, 2000

Table 2. 2. Maximum number of cells colonized by the wild type (48 hpi) or *cpr1* mutant strain (72 hpi) on maize leaf sheaths.

	Maximum number of colonized cells (%)				
	1 cell	2 cells	3 cells	4 cells	5 cells
WT	3.8 ± 4.1	30.1 ± 7.1	45.7 ± 7.4	15.4 ± 5.4	4.7 ± 4.6
Cpr1	95.9 ± 3.7	3.5 ± 3.2	0.95 ± 2.0	0	0

Table 2. 3. Growth of the *cpr1* mutant in co-inoculations is affected by distance. Different treatments indicate numbers of cells and total distance separating both fungal colonies at the time of inoculation, and percentage of infection sites in which hyphae of the mutant colonized at least two cells. Treatments with different letters are different from each other with a significance of $p < 0.05$.

Treatment	No. cells	Distance	Infection sites beyond one cell	Class
D1	8.5 ± 1.5	2.6 ± 0.46 mm	33.5 ± 15	a
D2	13.3 ± 2	4.1 ± 0.62 mm	8.2 ± 14	b
D3	22.8 ± 4.9	7 ± 0.12 mm	9.9 ± 13	b
Control	N/A	N/A	4.6 ± 4.2	b

Table 2. 4. Growth of the *cpr1* mutant in co-inoculations is affected by inoculum concentration. Percentage of infection sites where hyphae of the mutant colonized at least two cells at different inoculum combinations. Treatments with asterisks (*) are different with a significance of p <0.05.

		WT inoculum		
	Spore concentration	5×10^5	1×10^5	5×10^4
***cpr1* mutant inoculum**	5×10^5	30.33 *	20.5*	7.33
	1×10^5	13.17	6.17	4.17
	5×10^4	14.5	7.71	3

Figure 2.1. Development of WT, *cpr1* mutant, *Cpr1*-C, and AFP-tagged versions of the strains on maize leaf sheaths. Percentage of ungerminated, adhered spores (white bar), appressoria (light gray bar), and invasive primary hyphae (dark gray bar) 12, 24 and 48 hours post inoculation (hpi). Error bars represent standard deviation.

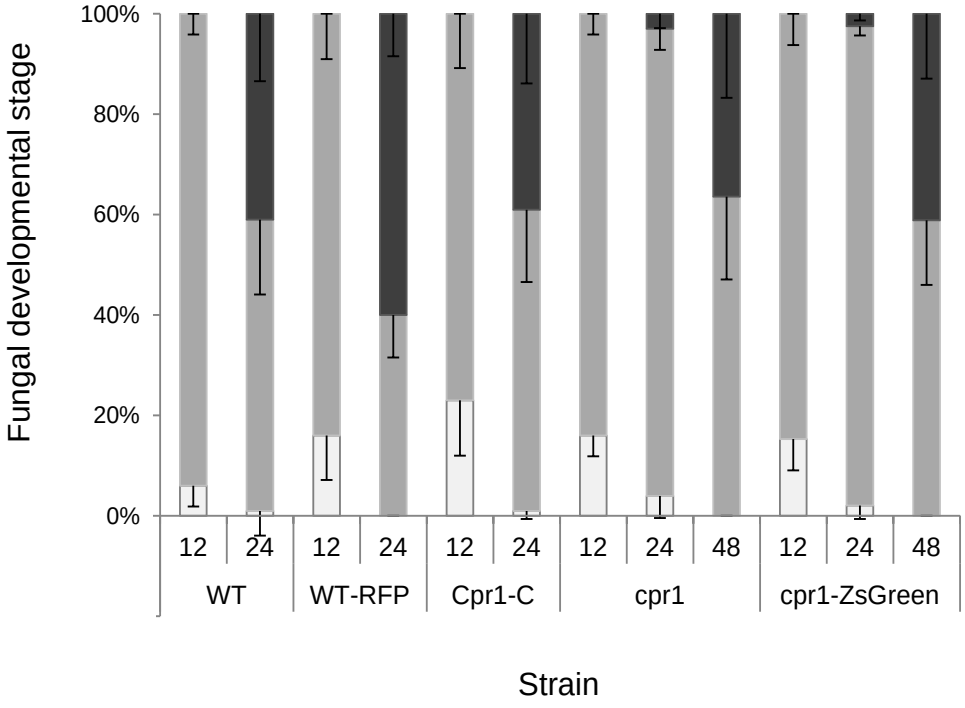


Figure 2.2. Phenotype of different strains on maize leaf sheaths. .**A**. Symptom development resulting from WT, *cpr1* mutant, *Cpr1*-C, nonpathogen CgSl1 and mock-inoculation on maize leaf sheaths 96 hpi. **B**. Phenotypes of the WT, and *cpr1* mutant on maize leaf sheaths 20, 48 and 60 hpi. Plasmolysis and neutral red uptake were used to determine host cell viability. Cells that are still not colonized (asterisk) or are just being invaded (arrows) usually remained alive. AP = appressoria, BH= biotrophic hyphae, NH= necrotrophic hyphae. **C**. Penetration of living cells at the edges of a necrotrophic colony.

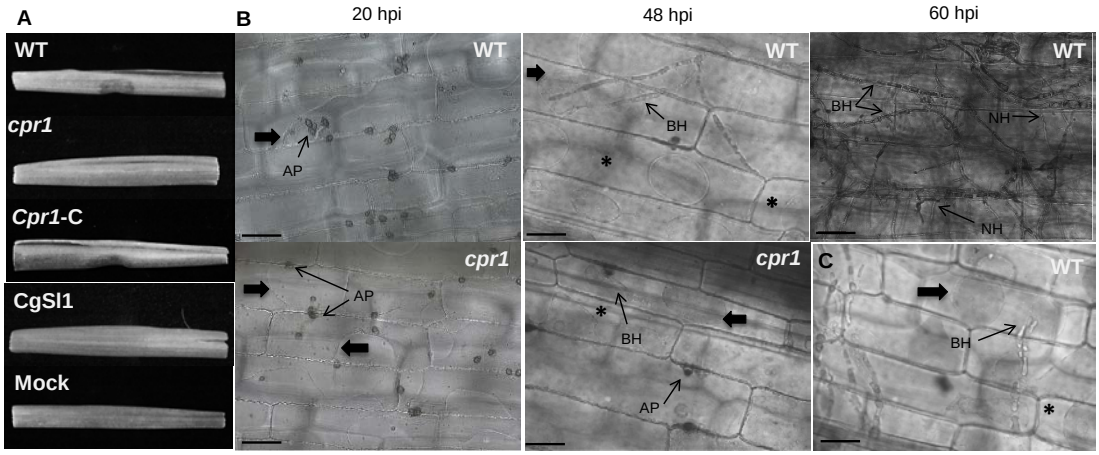


Figure 2.3. Pattern of ROS accumulation in maize leaf sheaths. **A-C** inoculated with WT or **E-G** inoculated with *cpr1* mutant, determined by DAB staining. AP= appressoria, BH= biotrophic hyphae. Scale bars equal to 20 µm.

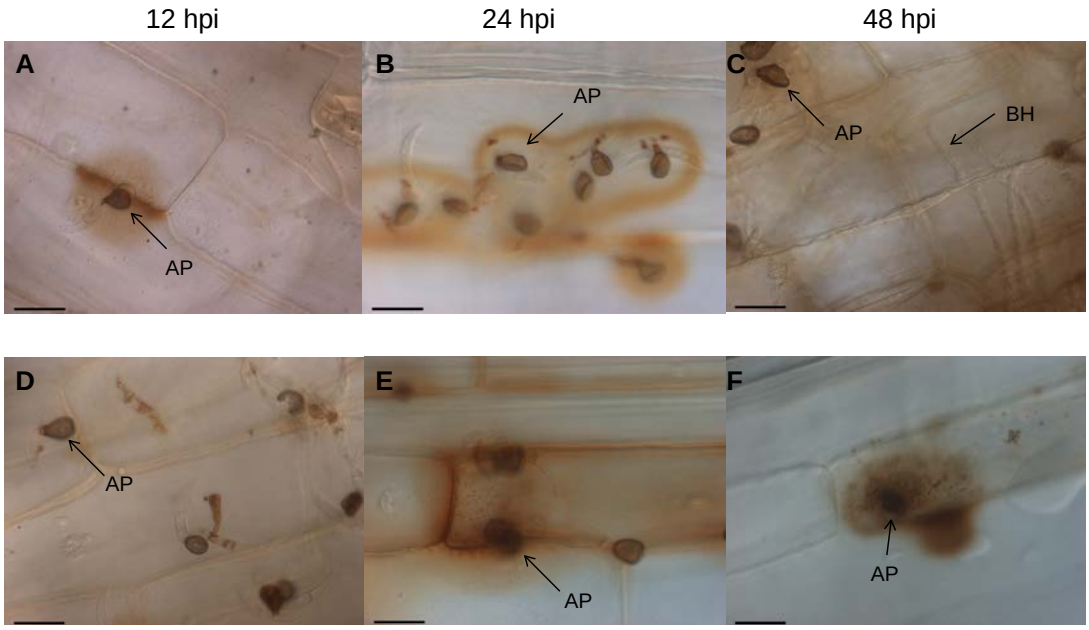


Figure 2.4. Leaf sheath co-inoculations. Schematic representation of **A**. leaf sheath inoculations and **D, G-H,** co-inoculations. **B**. Phenotype of WT-mRFP and **C**. *cpr1*-Zsgreen 72 hpi. **E.** *cpr1*-mutant colonizing more than one cell in co-inoculations with the WT. **F** Confocal image showing *cpr1* mutant crossing plant cell walls in co-inoculations. **I-J** Plant cell viability near WT and **K-L** *cpr1* fungal colonies in co-inoculation experiments 60 hpi. **M-O.** Triple inoculations with WT-mRFP, *cpr1*-Zsgreen and non-pathogen CgSl1-GFP. **N.** Non-pathogen failed to penetrate maize tissue. Cells beneath appressoria still plasmolyze. **O.** The *cpr1* mutant colonizing more than one cell. Cells that are not yet colonized (asterisk) or are just being invaded (arrows) still plasmolyze and uptake neutral red. Scale bars equal to 50 µm, except in F, where it is equal to 20 µm.

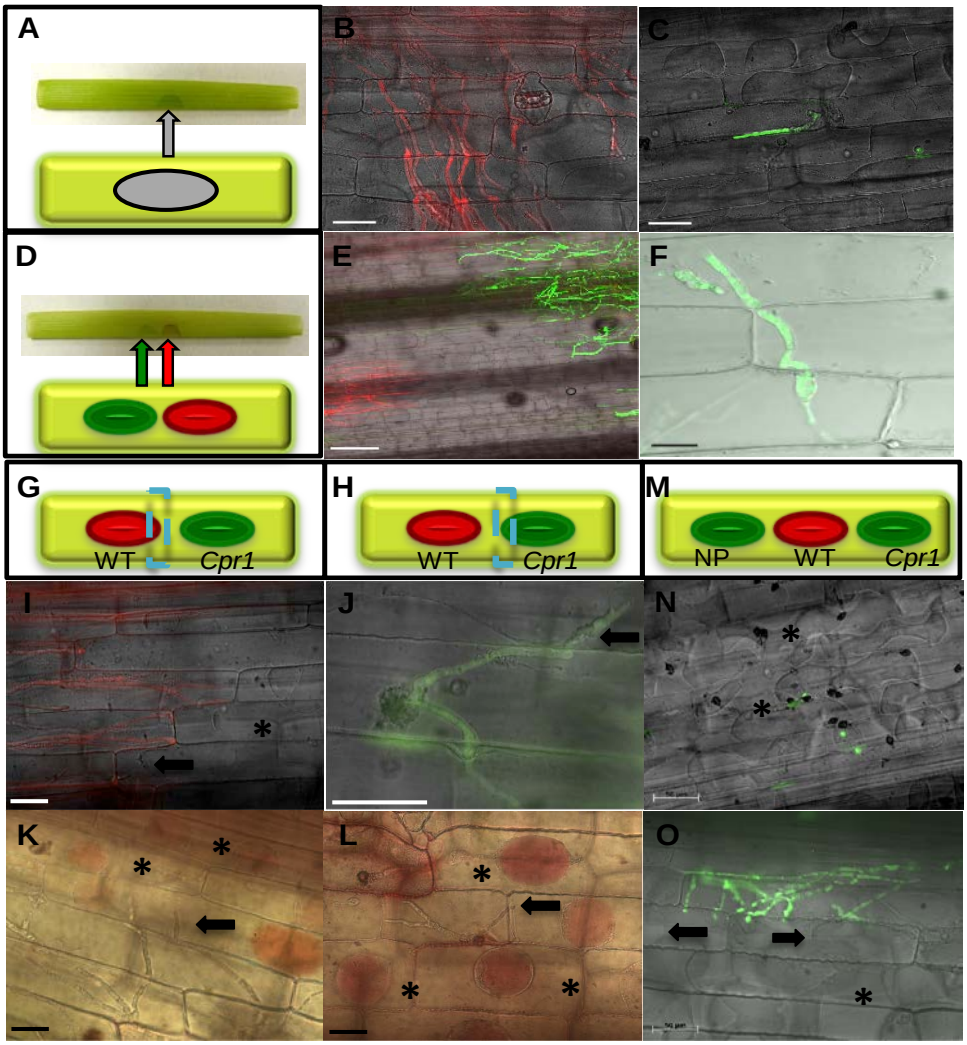


Figure 2.5. Percentage of penetration sites in which the *cpr1* mutant colonized two or more cells in co-inoculations. Co-inoculatsiation a distance from the WT (left) or water (right). Treatments with different letters are different gfrom each other with a significance of p (<0.05). Error bars represent standard deviations.

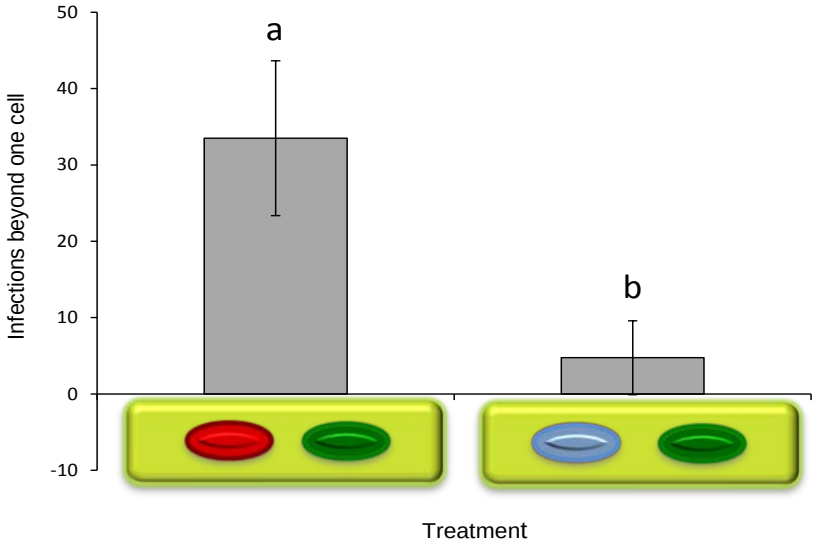


Figure 2.6. Average of the maximum number of colonized cells in triple inoculations. Cells colonized by *cpr1* mutant and the nonpathogen CgSl1. Treatments with different letters are different from each other (p <0.05). Error bars represent standard deviation.

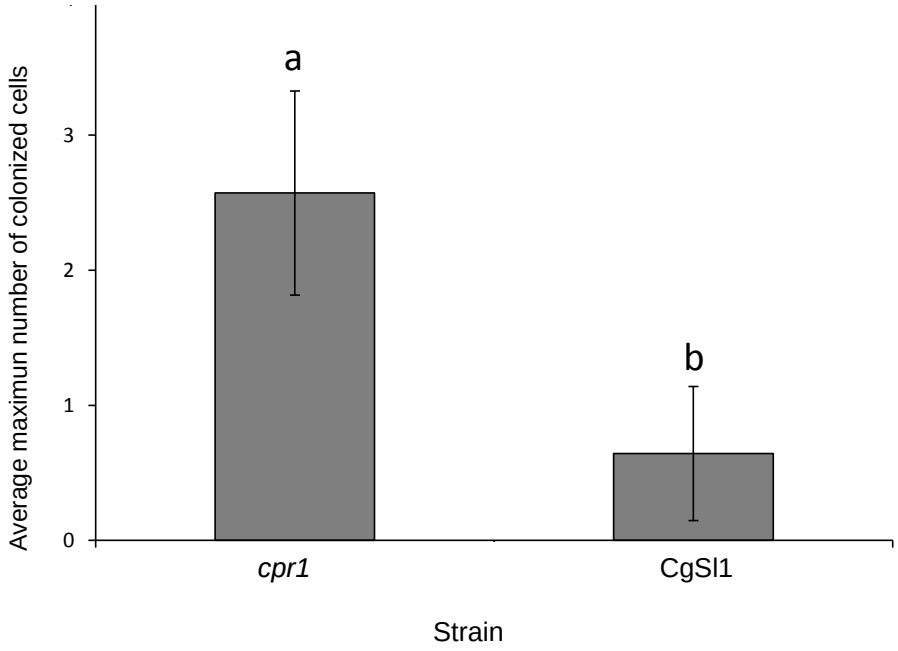
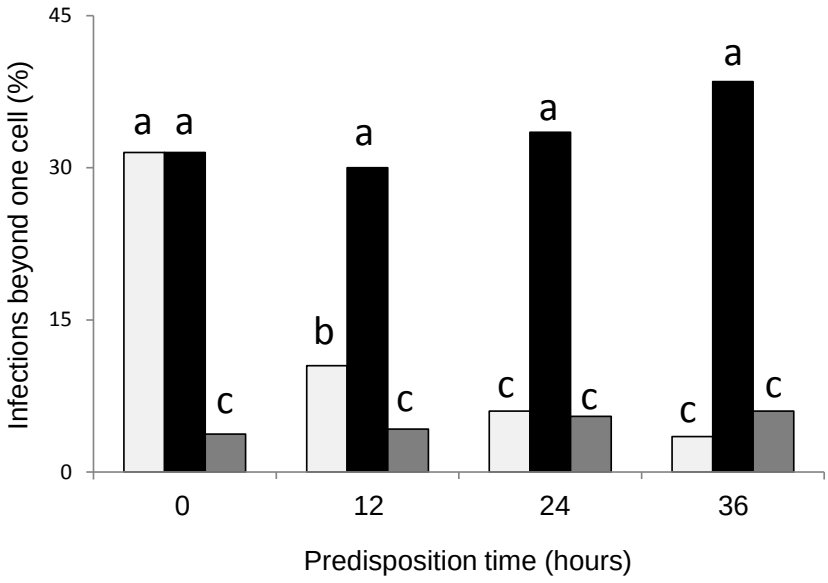


Figure 2.7. Growth of *cpr1* in co-inoculations is affected by predisposition time. Percentage of of infections in which hyphae of *cpr1* colonized at least two cells (white bars) is affected by the time it remains alone (predisposition time). WT inoculum was added 0, 12, 24 and 36 hours after mutant inoculum. In control sheaths, the mutant and WT strain (black bars), or *cpr1* mutant and water (dark gray bars) were added simultaneously, 12, 24 and 36 hours after sheaths were detached. All treatments were evaluated 60 hours after addition of the WT. Treatments with different letters are significantly different from each other (p <0.05).



Chapter 3

The Genomics of Secondary Metabolism in *Colletotrichum*

The findings described in chapter 2 of this dissertation led me to propose the hypothesis that the *cpr1* mutant is impaired in the secretion of one or more diffusible compounds required for establishment of compatibility and progression to necrotrophy *in planta*, but not for growth *in vitro.* Recent analyses of whole genome sequences from fungal plant pathogens with a variety of lifestyles has allowed identification of specific gene families that appear to be linked to these lifestyles (Kamper *et al.*, 2006, Spanu *et al.,* 2010, Amselem *et al.*, 2011, Bölker *et al.*, 2008). One striking observation was a dramatic increase in the number of genes associated with secondary metabolism in necrotrophs and hemibiotrophs versus biotrophs. Secondary metabolites (SM) often behave as toxins in necrotrophic disease interactions, inducing host cell death and necrosis (Markham & Hille, 2001). Examples include fumonisin B1,produced by different *Fusarium* species, (Chivasa *et al.*, 2005), cercosporin produced by *Cercospora nicotiniae* (Choquer *et al.*, 2005), and T-toxin, produced by *Cochliobolus heterostrophus* (Yang *et al.*, 1996, Baker et al., 2006) among others. To further explore the possibility that the *cpr1* mutant is defective in the production and/or secretion of one or more SM, I first identified and characterized the range of SM-associated genes in *C. graminicola.* In this chapter, I report the annotation of genes with the potential to encode key SM enzymes in the genomes of two *Collotrichum* species, *C. graminicola* and *C. higginsianum,* and I also identify *Colletotrichum*-specific and species-specific SM-associated genes and gene clusters. The results show that each *Colletotrichum* species has the potential to

unusually large and divergent spectra of SM, some of which may be previously unknown bioactive molecules. [1]

Introduction

The genome of the hemibiotrophic plant pathogen *C. graminicola* was recently sequenced by the Broad Institute, in collaboration with our laboratory, using Sanger and 454 pyrosequencing. The genome of a closely related *Colletotrichum* species, *C. higginsianum* was sequenced by the laboratory of Dr. Richard J. O'Connell at the Max Planck Institute, using 454 and Illumina technologies. Both genomes are available on the Broad *Colletotrichum* Comparative Genomics Websitehttp://www.broadinstitute.org/annotation/genome/colletotrichum_group/MultiHome.html. *C. higginsianum* infects dicotyledonous plants of the Brassicaceae family, including the model plant *Arabidopsis thaliana* (Huser *et al.*, 2009, O'Connell *et al.*, 2004). My approach was to conduct a comparative study of the putative SM genes of both species, and to identify genus-specific SM genes that might be associated with the hemibiotrophic lifestyle of *Colletotrichum*, as well as species-specific genes that could be important in the specific interaction of each fungal species with its respective host. A precedent is provided by the *ACE1* gene in *Magnaporthe oryzae*, which lacks orthologs in all other sequenced fungi (Collemare *et al.*, 2008a, Collemare & Lebrun, 2011). *ACE1* encodes a polyketide synthase that contributes to production of an avirulence factor required for avr-mediated resistance in rice cultivars that carry the Pi33 resistance gene (Collemare *et al.*, 2008b).

The *Colletotrichum* genome sequences were analyzed by a research team led by our laboratory and by Dr. O'Connell (O'Connell *et al.,* 2012). *C. graminicola* has a 57.4-Mb genome distributed across 13 chromosomes, including 3

[1] Some of the work reported in this chapter was included in O'Connell *et al*., 2012, Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analysis, Nature Genetics 44(9): 1060-1065

minichromosomes (>2kb), whereas *C. higginsianum* has a 49.3-Mb genome and 12 chromosomes, including 2 minichromosomes. Annotation of both genomes indicated the presence of 12,600 protein-coding genes in *C. graminicola,* and 16,172 protein-coding genes in *C. higginsianum.* The two predicted proteomes were analyzed by Dr. M. Thon by using Markov Clustering, which relies on similarity of the whole protein sequences to identify families of genes (Enright *et al.*, 2002).

Interestingly, the MCL analysis revealed that families of genes potentially associated with production of SM were significantly expanded in both *Colletotrichum* species when compared with other sequenced Ascomycetes (O'Connell *et al.*, 2012). Fungi make four major groups of SM: polyketides produced by polyketide synthases (PKS); peptides produced by nonribosomal peptide synthases (NRPS); alkaloids produced by dimethylallyl tryptophan synthases (DMATS); and terpenes produced by terpene synthases (TS) (Keller *et al.*, 2005). Recently, a fifth important class of fungal SM genes, PKS-NRPS hybrids with characteristics of both PKS and NRPS genes, was identified (Khaldi *et al.*, 2008). PKSs and NRPSs are the most abundant classes of secondary metabolite biosynthetic genes in fungi (Keller *et al*., 2005, Khaldi *et al.*, 2010). Products of SM genes are involved in multiple plant-pathogen interactions, and have a variety of functions including antibiosis, protection from stresses, and pathogenicity to animals and plants (Horbach *et al.*, 2009).

Colletotrichum species have been reported to produce a variety of SM, including flavones, peptides and terpenes, as well as DHN (1,8-dihydroxynaphthalene) melanin, which is essential for appressorium-mediated host penetration (Kubo *et al.*, 1991, Muimba-Kankolongo & Bergstrom, 2011, Singh *et al.*, 2010). Additional examples include the siderophore ferricrocin, isolated from *C. gloeosporiodes*, which has phytotoxic activity in grass cotyledons (Ohra, 1995), colletotrichins A, B and C from *C. nicotianae*, which produce symptoms resembling tobacco anthracnose when infiltrated in tobacco leaves (Goddard *et al.*, 1976, Kimura *et*

al., 1977, Kimura *et al.*, 1978, García-Pajón & Collado, 2003), and a tetrahydroxylated compound with antioxidant properties from *C. gloeosporioides* (Femenía-Ríos *et al.*, 2006) . Although SM have not been described in *C. higginsianum*, several have been characterized in *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 & Nicholson, 1992). It was recently reported that deletion of *Ppt1*, a gene encoding a cofactor essential for the enzymatic function of all PKS and NRPS, resulted in decreased pathogenicity in *C. graminicola,* providing support for the idea that SM play an important role in the regulation of pathogenicity to maize (Horbach et al., 2009).

Materials and Methods

The annotated genome sequences of *C. graminicola* and *C. higginsianum* were used to search for SM-associated genes. To improve the quality of the *C. higginsianum* genome assembly, it was re-annotated by Dr. Richard O'Connell and Dr. Michael Thon, using the Velvet assembler (Zerbino & Birney, 2008). This new version of the genome provided longer contigs and is referred to as the Velvet assembly. Candidate PKS and NRPS genes were initially identified by ortho-MCL analysis (performed by Dr. Thon) (Enright *et al*., 2002). This search relies on similarity of whole protein sequences to find families of genes. I conducted gene family searches using the Broad Institute's *Colletotrichum* database, BLAST searches against the NCBI databases, and InterproScan analysis, to further characterize and verify putative PKS, NRPS, PKS-NRPS hybrid, DMAT, and TS genes.

Annotation of secondary metabolism genes

Despite the relatively high degree of variability among SM genes, there are some conserved characteristics of each group that facilitated their identification and characterization.

Polyketyde Synthases – PKSs

PKS enzymes contain multiple domains, and the diversity of these domains defines the type and length of the polyketide that is synthesized (Keller *et al*., 2005). All known polyketides require the presence of 3 essential domains, namely the ketoacyl synthase (KS); acyltransferase (AT); and acyl carrier protein (ACP) domains (Khosla *et al.*, 1999). Some additional domains contribute to the reduction of the polyketide to various degrees, adding diversity to the final product. These additional domains are the ketoreductase (KS), dehydratase (DH), and enoylreductase (ER) domains (Gokhale *et al.*, 1999, Fox & Howlett, 2008). The highly conserved KS domain has been commonly used to infer ancestral relationships among PKS genes (Kroken *et al.*, 2003b). Figures 3.1A and 3.1B outline the procedure that I used for analysis of KS domains in *Colletotrichum* PKS genes.

Non-ribosomal Peptide Synthases - NRPSs

NRPSs are large modular enzymes that catalyze the condensation of amino acids to form a nonribosomal peptide (Caboche *et al.*, 2010). The diversity of products synthesized by these enzymes depends on the number of modules they contain (Caboche *et al*., 2010, Schwarzer & Marahiel, 2001). Each NPRS contains at least one module that includes an AMP-binding adenylation domain (A), a peptidyl carrier domain (T), and a condensation domain (C) (Brakhage & Schroeckh, 2011). The A domain recognizes and activates the residues, which are bound to the enzyme by the T domain, and finally the C domain condenses the peptide bond between the linked residues (Stachelhaus *et al.*, 1999, Lautru & Challis, 2004). A single thioesterase domain (TE) is located at the end of the enzyme. The TE domain is responsible for releasing the synthesized peptide from the complex (Cosmina *et al.*, 1993). Like PKSs, NRPSs can also have some additional domains that catalyze epimerization and N-methylations of the peptide (Konz & Marahiel, 1999). These additional domains account for the tremendous

complexity and diversity of known nonribosomal peptides (Weber & Marahiel, 2001). Figures 3.2A and 3.2B describe the process I used to analyze the NRPS genes of *C. higginsianum* and *C. graminicola*.

PKS-NRPS hybrids

Fungal PKS-NRPS hybrids were discovered a few years ago (Böhnert *et al.*, 2004, Kroken et al., 2003b, Schümann & Hertweck, 2007). They are typically composed of a fungal type I PKS fused to a single NPRS module that can be either complete or truncated (Collemare *et al*., 2008b). The NRPS portion is responsible for the formation of a peptide bond between a polyketide synthesized by the PKS portion, and a single amino acid (Sims & Schmidt, 2008). Usually, the resulting product is a tetramic acid (Song *et al.*, 2004, Bergmann *et al.*, 2007). Tetramic acids were initially identified in the early twentieth century, and they are normally associated with antimicrobial and antiviral activities (Royles, 1995). The criteria I used to classify PKS-NPRS hybrids were the presence of a complete KS domain, and at least one A, T or C domain. PKS-NRPS hybrids were re-annotated and confirmed by Dr. Marc Henri-Lebrun.

DMATs

DMATs synthesize a wide variety of SM using tryptophan as a starting point (Krupinski *et al.*, 1976). These products include alkaloids, compounds with powerful physiological activities (Tudzynski *et al.*, 1999, Wang *et al.*, 2004). The DMAT gene family encodes a group of enzymes that contain an aromatic prenyltransferase domain, responsible for the addition of prenyl groups during the first steps of alkaloid biosynthesis (Keller et al., 2005, Metzger *et al.*, 2009). I identified DMATs based on the presence of this conserved domain.

Terpene synthases - TSs

TSs are characterized by the presence of a prenyl synthase domain, which is responsible for the synthesis of terpenes from dimethylallyl diphosphates,

polymers of isopentyl units derived from the isoprenoid pathway (Tholl, 2006, Dairi, 2005). Terpenes have a wide variety of roles, including functions in defense, competition, and growth regulation (Greenhagen & Chappell, 2001). I identified TSs based on the presence of this conserved domain.

Phylogenetic analysis of secondary metabolite genes

Phylogenetic analyses of the *Colletotrichum* PKS, NRPS, TS, and DMATS predicted proteins were performed by using the software available on the phylogeny.fr website (http://www.phylogeny.fr/) (Dereeper *et al.*, 2008, Dereeper *et al.*, 2010). Amino acid sequences were aligned with Muscle (V3.7), and phylogenies were inferred by Maximum-likelihood using PhyML (V3.0). Trees were constructed using TreeDyn and statistical branch support was provided by a standard Likelihood Ratio Test, aLRT (Dereeper *et al.*, 2008). Trees were constructed from the AMP binding adenylation domains from NRPS genes, and the ketoacyl synthase N-terminal and C-terminal domains from PKS genes. PKS-NRPS, DMATS and TS were analyzed by aligning whole protein sequences. Due to the extreme length of the complete PKS-NRPS hybrid sequences, these proteins had to be analyzed by using different software. Alignments were made using Muscle (V3.8.3) (http://mobyle.pasteur.fr/) (Néron *et al.*, 2009) and Maximum-Likelihood phylogenies were calculated using PhyML 3.0 (http://www.atgc-montpellier.fr/) (Guindon *et al.*, 2010). The tree was built as described for other SM genes.

The relationships of the *C. graminicola* PKS and PKS-NRPS hybrids to 41 previously identified PKSs and PKS-NRPS hybrids from 10 different Ascomycetes were evaluated. The KS domains of genes involved in the synthesis of PM-toxin (*Didymella maydis*) (Yun *et al.*, 1998), alternapyrone (*Alternaria solani*) (Fujii *et al.*, 2005), melanin (*Colletotrichum lagenarium*) (Takano et al., 1995), lovastatin (*Aspergillus terreus*) (Hendrickson *et al.*, 1999, Kennedy *et al.*, 1999), zearalenone (*Giberella zeae*) (Kim *et al.*, 2005a, Kroken et al., 2003b), radicicol (*Chaetomium chiversii,* (Wang, 2008 #62) and *Pochonia*

chlamydosporia, (Reeves *et al.*, 2008)) fumonisin (*Giberella moniliformis*) (Proctor *et al.*, 2003), cercosporin (*Cercospora nicotiniae*) (Choquer et al., 2005), T-toxin (*Cochliobolus heterostrophus*) (Yang *et al.*, 1996, Baker *et al.,* 2006), ACE1 (*Magnaporthe oryzae*) (Böhnert et al., 2004), and four more PKS-NRPS hybrids from *M. oryzae* (SYN2, SYN6, SYN7 and SYN8) (Böhnert *et al.*, 2004), all characterized PKSs in *C. heterostrophus* (PKS3-PKS25) (Kroken *et al.*, 2003b), and 34 putative PKSs and 7 PKS-NRPS hybrids of *C. graminicola,* were included in the comparative analysis.

Predicted secondary metabolite clusters

The Secondary Metabolite Unknown Region Finder (SMURF) (Khaldi *et al*., 2010) has been used to predict clusters of genes associated with secondary metabolism in a wide range of fungi (von Döhren, 2009, Georgianna *et al.*, 2010). This web-based program was applied to both *Colletotrichum* annotated genome assemblies. SMURF was also applied to the most current annotated assemblies of several other sequenced fungi for comparisons. SMURF is available at http://www.jcvi.org/smurf.

Results

Manual curation indicated that initial MCL and Broad predictions of SM gene numbers were overestimated for both fungi, but especially for *C. higginsianum*

MCL analysis predicted 52 putative PKSs and 16 putative NRPSs in *C. graminicola,* and 120 putative PKSs and 48 putative NRPSs in *C. higginsianum*.

PKSs contain three essential conserved domains (KS, AT, ACP) and three optional conserved domains (KR, DH, ER). Manual checking of the initial MCL predictions revealed that four (8%) *C. graminicola* and 21 (18%) *C. higginsianum* predicted PKSs did not contain any of these conserved domains (Table 3.1). Additionally, 83 of the remaining predicted gene models in *C. higginsianum,* and 8 in *C. graminicola*, contained only one or two of the three conserved domains.

Probably due to the more fragmented nature of the *C. higginsianum* genome assembly (N50 contig = 6.15 Kb vs N50 = 228.96 Kb in *C. graminicola*), 43% of the 120 predicted *C. higginsianum* gene models were incomplete according to the BROAD institute annotation. In contrast, none of the predicted *C. graminicola* gene models were truncated (Table 3.1).

Phylogenetic analysis of the highly conserved KS domain from the PKS genes was performed using the 94 available complete KS N-terminal and C-terminal domains (O'Connell *et al*., 2012). This included 41 KS domains from *C. graminicola*, and 52 KS domains from *C. higginsianum*. Obviously truncated KS domains (<20 amino acids) were not included in the analysis (Figure 3.1).

A phylogenetic tree based on the alignments revealed apparent PKS gene family expansion, especially in *C. higginsianum.* For example, genes CH063_0051 and CH063_01345 appeared to be paralogs, as did GLRG_03511 and GLRG_05714 (Figure 3.3A). However, a more detailed examination revealed that many of these apparent clades of paralogous genes actually included fragments of PKS genes for which the initial predicted gene models were truncated or split. This was the case for CH063_14111 and CH063_06479, which could be aligned to one another and to a single predicted ORF in the Velvet assembly (Figure 3.3B). After identifying overlapping gene models and correcting them to the extent possible, I concluded that *C. graminicola* actually has 39 PKS genes, and *C. higginsianum* has 58 PKS genes. Sixteen of these genes appear to be orthologous in the two species (Figure 3.4).

Phylogenetic analysis was performed using the conserved AMP-binding domains from 7 and 26 putative NRPSs from *C. graminicola* and *C. higginsianum* respectively (Figure 3.5). A total of 56 AMP binding domains were included in the analysis. Initial MCL and Broad predictions and the phylogenetic analysis suggested gene expansion of this family, especially in *C. higginsianum*. For example, CH063_15443, CH063_10658, CH063_02723, CH063_10344 and CH063_02723 appeared to form a clade of paralogous genes that was unique to

C. higginsianum (Figure 3.5A). However, closer inspection revealed that, similar to the case with the PKS genes, multiple A domains from *C. higginsianum* predicted to belong to different ORFs, were actually fragments of larger NRPS genes. For example, A domains from CH063_15485, CH063_10172, CH063_01270, CH063_01271, CH063_10216, CH063_12138 and CH063_05450, appear to be orthologs of the seven A domains of GLRG_00469 (Figure 3.5B). Ultimately, I reannotated 23 *C. higginsianum* predicted gene models to form 9 NRPSs. Alignments to *C. graminicola* NRPS gene models, alignment to the Velvet assembly, and/or predictions of larger protein sequences by the FGNESH annotation program (http://linux1.softberry.com/berry.phtml), ultimately allowed consolidation of 32 of the 49 original gene models into 12 NRPSs for *C. higginsianum*, and 7 NRPSs for *C. graminicola*. Construction of a new tree using these reannotated gene models allowed me to conclude that only one A domain found in *C. graminicola* (GLRG_08225) apparently is not shared with C*. higginsianum,* while seventeen A domains in *C. higginsianum,* predicted to belong to 5 NRPSs, are not shared with *C. graminicola* (Figure 3.6).

Gene family searches using the Broad Institute *Colletotrichum* database, BLAST searches against the NCBI databases, and InterproScan analysis resulted in identification of 7 PKS-NRPS hybrids, 7 putative DMATs, and 14 TSs in *C. graminicola*. By using a parallel approach, 6 PKS-NRPS hybrids, 11 DMATs and 17 TSs were found in *C. higginsianum.*

Manual annotation of PKS-NRPS hybrids included evaluation for the presence of a KS domain and at least one NRPS A, T, or C domain. Phylogenetic analysis of whole protein sequences demonstrated that only two PKS-NRPS are found in both *Colletotrichum* species: GLRG_09715 is an ortholog of CH063_06174, and GLRG_11626 is an ortholog of CH063_05683. Four hybrids are found only in *C. higginsianum*, while 5 are present only in *C. graminicola* (Figure 3.7).

The initial automated prediction of DMATs suggested the presence of 7 DMATs in *C. graminicola* and 11 in *C. higginsianum.* BROAD gene models predicted a

truncated protein for CH063_11167 (missing the 5'end) and this gene was adjacent to CH063_15640 in the Velvet assembly. FGENESH predictions suggested a full-length DMAT encompassing both gene models. Alignment and phylogenetic analyses of whole protein sequences indicated that six DMAT proteins are shared between both species, one is found only in *C. graminicola*, and four are found only in *C. higginsianum* (Figure 3.8)

Searches for prenyl synthase domains predicted 14 TSs for *C. graminicola* and 18 for C. *higginsianum.* According to BROAD predictions, CH063_06400 and CH063_13385 were missing the 3' end and the 5' ends, respectively. FGENESH predicted a full-length TS encompassing both genes. Phylogenetic analysis of 31 TSs demonstrated that nine of them are shared between the two *Colletotrichum* species (Figure 3.9). Five are only found in *C. graminicola* while nine are only found in *C. higginsianum*.

A larger number of SM genes and SM clusters were predicted by SMURF for both *Colletotrichum* species than for other related sequenced fungi

In fungi, enzymes required for the synthesis, modification, regulation and transport of SM products are typically physically associated in co-expressed gene clusters (Hull *et al.*, 1989, Keller & Hohn, 1997, Tudzynski et al., 1999, Collemare *et al.*, 2008b, Khaldi et al., 2008, Bok *et al.*, 2006). I used a web-based software program (SMURF) to identify predicted SM gene clusters in both *Colletotrichum* species. SMURF was applied to the less fragmented Velvet assembly for *C. higginsianum*, after it failed to predict any gene clusters in the original assembly. The first step in SM cluster prediction by SMURF relies on the identification of SM key enzymes or "backbone genes" (PKSs, NRPSs, PKS-NRPS hybrids and DMATs) identified by the presence of the conserved domains described above (Khaldi *et al.*, 2010). SMURF identified 60 potential backbone genes in *C. graminicola* and 79 in *C. higginsianum*. For *C. graminicola*, 70% of SMURF-predicted backbone genes were included in potential SM gene clusters, compared with only 42% in *C. higginsianum* (Figure 3.10). SMURF predicted 42

SM clusters in *C. graminicola*, ranging from 2 to 24 genes (average 7) and 39 clusters from the Velvet assembly of *C. higginsianum*, ranging from 2 to 19 genes (average 8). SMURF identified a larger number of SM clusters in the two *Colletotrichum* species than in most other sequenced Ascomycete fungi (Figure 3.11). Assuming that clusters in which at least 25% of the genes were orthologous are shared, only 12 of the predicted clusters were found in both species, and in six of these less than half of the genes were syntenous.

Similarity of *C. graminicola* PKSs to some previously characterized fungal PKSs

Several SM gene clusters containing PKSs or PKS-NRPS hybrids have been described and characterized in other fungal species (Collemare *et al.*, 2008a, Baker *et al*., 2006, Proctor *et al.*, 2003, Hendrickson et al., 1999). These gene clusters are responsible for production of a range of compounds with a variety of biological functions. Phylogenetic analysis was performed to determine the relationships among the complete KS domains from 34 *C. graminicola* PKSs, the KS domains from the seven *C. graminicola* PKS-NRPS hybrids, and 41 additional KS domains from other fungal PKSs (Table 3.2).

Two major clades resulted from the phylogenetic analysis (Figure 3.12). The first major clade contained ten *C. graminicola* PKSs that all lack the reducing domains KR, DH and ER, and are thus classified as nonreducing PKSs (nrPKS). This clade contained nrPKSs responsible for melanin biosynthesis in *C. lagenarium* (C_lagenarium_PKS1) (Takano *et al.*, 1995) and *C. heterostrophus* (C_heterostrophus_PKS18) (Kroken *et al.*, 2003a).GLRG_04203 appeared to be orthologous with these two PKSs, and thus is likely to be responsible for melanin production in *C. graminicola*. GLRG_08620 is closely related to CTB1, the nrPKS responsible for the synthesis of cercosporin in *C. nicotiniae* (C_nicotiniane_CTB1) (Choquer *et al.*, 2005). P_chlamydosporia_RADS2, which is involved in radicicol biosynthesis (Reeves *et al.*, 2008), is orthologous to GLRG_11836. Interestingly, another *C. graminicola* nrPKS gene, GLRG_11778 also grouped in this sub-clade, together with RADS2, the nrPKSs involved in

radicicol biosynthesis in *C. chiversii* (C_chiversii RADS2) (Wang *et al.*, 2008) and G_zeae_PKS13, required for zearalenone biosynthesis in *G. zeae* (Kim *et al.*, 2005b). Another distinct sub-group within the reducing PKS clade contained 5 *C. graminicola* nrPKS, and three nrPKSs from *C. heterostrophus*, with only one pair (GLRG_09268 and C_heterostrophus_PKS21) that appears to be shared between the two species.

The second major clade is composed of three sub-clades that included most of *C. graminicola* reducing PKSs (rPKSs), and a fourth sub-clade that contained the seven *C. graminicola* PKS-NRPS hybrids together with the rPKS GLRG_08212. The first sub-clade included two *C. graminicola* genes (GLRG_11770 and GLRG_11840), together with the rPKSs required for synthesis of zearalenone (G_zea_PKS4) (Kim *et al*., 2005b) and radicicol (C_chiversii_RADS1 and P_chlamydosporia_RADS1) (Wang *et al*., 2008, Reeves *et al.*, 2008). Only two of the 13 *C. graminicola* rPKSs contained within the second sub-clade seemed to have characterized orthologs: GLRG_07171 and C_heterostrophus_PKS12; and GLRG_11435 and C_heterostrophus_PKS14.

The third sub-clade contains six *C. graminicola* rPKSs. GLRG_10317 is orthologous to C_heterostrophus_PKS6 and to A_solani_alt5. The latter is involved in the synthesis of alternapyranone in *A. solani* (Fujii *et al*., 2005). No orthologous *C. graminicola* rPKSs were identified for C_heterostrophus_PKS1 and PKS2, which are responsible for the synthesis of T-toxin (Yang *et al*., 1996, Baker *et a*l., 2006). Similarly, there were no *C. graminicola* orthologues of D_maydis_PKS1, required for the synthesis of PM-toxin in *Didymella maydis* (Yun *et al*., 1998). GLRG_09267 is orthologous to C. heterostrophus_PKS3, and both are related to A_terreus_lovF responsible for the synthesis of the diketide portion of lovastatin in *Aspergillus terreus* (Kennedy *et al.,* 1999). Four of the five remaining C. graminicola rPKSs in the third sub-clade also had apparent orthologs in *C. heterostrophus*.

All the *C. graminicola* PKS-NRPS hybrids grouped together in the fourth sub-

clade. GLRG_11507 and GLRG_09715 are orthologs of M_oryzae_SYN6 and M_oryzae SYN8, respectively, both of which have been identified as PKS-NRPS hybrids (Böhnert *et al*., 2004). Two more *C. graminicola* hybrids, GLRG_11890 and GLRG_11626 are orthologs of M_oryzae_SYN7, and also seem to be related to A_terreus_lovB, responsible for the cyclic nonaketide synthesis of lovastatin in *A. terreus* (Campbell & Vederas, 2010, Hendrickson *et al*., 1999). The two PKS-NRPS hybrids present in the ACE1 cluster in *M. oryzae* (M_oryzae_ACE1 and M_oryzae_SYN2) (Böhnert *et al*., 2004, Collemare *et al.*, 2008b), do not seem to have orthologs in *C. graminicola* or *C. heterostrophus*, although they do have orthologs in *C. higginsianum* (O'Connell *et al.*, 2012). Three more PKS-NRPS hybrids (GLRG_09842, GLRG_07434 and GLRG_01037) and one C. graminicola rPKS (GLRG_08212) were not related to any of the other KS domains analyzed here.

Two additional sub-clades included four CgrPKSs. GLRG_01860 is an ortholog of C_heterostrophus_PKS10; GLRG_10537 is an ortholog of C_heterostrophus_PKS25. Neither of these *C. heterostrophus* genes has been functionally characterized (Kroken et al., 2003b).

C. graminicola cluster 38 is similar to the *P. chlamydosporia* radicicol cluster

SMURF predictions indicated that three of the 42 SM clusters in *C. graminicola,* contain two PKSs: clusters 18, 35 and 38. Other known fungal SM clusters that include two PKSs are responsible for zearalenone, radicicol, T-toxin and lovastatin biosynthesis. My phylogenetic analysis indicated that the rPKS from cluster 38, GLRG_11840, is orthologous to the *P. chlamydosporia* and *C. chiversii* RADS1 genes, which are involved in radicicol production. The other PKS in cluster 38, nrPKS GLRG_11836, is orthologous to *P. chlamydosporia* RADS2 (Figure 3.12, clades A and B, sub-group 1). Comparisons of whole protein sequences suggest that four of the five genes in the SM gene cluster responsible for radicicol biosynthesis in *C. chiversii* exhibit a high degree of identity (62-72%) to genes in cluster 38 in *C. graminicola* (Table 3.3). The fifth

gene, radR, a transcription factor, seems to be missing from the *C. graminicola* cluster (Figure 3.13). The radicicol biosynthesis cluster in *P. chlamydosporia* also lacks the radR gene, and genes in the *P. chlamydosporia* cluster also exhibit a high level of identity (43-65%) and synteny to the genes in cluster 38.

C. higginsianum, seems to lack this cluster. Although all five genes in the cluster seem to have orthologs in *C. higginsianum*, with identity values between 32 and 50%, these genes are scattered throughout the genome, and three of them were predicted by SMURF to be parts of three different clusters.

C. graminicola cluster 18, orthologous to *C. higginsianum* cluster 10, is related to the cercosporin biosynthetic cluster of *C. nicotiniae*

C. graminicola cluster 18 and *C. higginsianum* cluster 10 are the most highly conserved clusters among the two *Colletotrichum* species. Both clusters are the largest predicted for each species, containing 24 and 19 genes, respectively. Fifteen of the 24 predicted genes in cluster 18 have orthologs in cluster 10 (GLRG_08610-GLRG_08624); other genes in the cluster have orthologs outside cluster 10. Only GLRG_08616 lacks an ortholog in *C. higginsianum* (Figure 3.14).

According to my phylogenetic analysis, a *C. nicotianae* nrPKS (*CTB1*) required for the synthesis of the non-selective phytotoxin cercosporin is orthologous to GLRG_08620, one of two PKSs in *C. graminicola* cluster 18 (Figure 3.12, clade A, sub-group 1). The cercosporin biosynthetic cluster consists of eight genes in *C. nicotiniae* (Chen *et al.*, 2007). GLRG_08620 exhibits a high level of identity (54%) with *CTB1*. Furthermore, CH063_02506, the othologous *C. higginsianum* nrPKS in cluster 10 (Table 3.4), is also very similar to *CTB1* (45% identity). Interestingly, four of the remaining seven genes in the *C. nicotianae* cercosporin cluster (*CTB3*, *CTB3*, *CTB5*, *CTB8*) also exhibit high levels of identity (47-55%) with genes in cluster 18. Levels of identity are a little lower with genes in *C. higginsianum* cluster 10 (ranging from 35-55%), but one additional cercosporin cluster gene, *CTB4*, has an ortholog in cluster 10 (51% identity).

Discussion

Genome wide inventory of SM-associated genes in *C. graminicola* and *C. higginsianum*

Automated predictions of SM-associated genes in *C. graminicola* and *C. higginsianum* indicated the presence of a surprisingly large number of these in both species, which could indicate that SM have an important role in *Colletotrichum* pathogenicity. The results of my more detailed manual analysis showed that, although the initial numbers were overestimated, both *Colletotrichum* species still have more SM genes than other closely related sequenced Ascomycetes (Spanu *et al*., 2010, Amselem *et al*., 2011, Collemare *et al.*, 2008a, Dean *et al*., 2005). It is important to note that the numbers might still be inaccurate, especially for *C. higginsianum.* The more fragmented genome (N50 contig = 6.15 Kb, vs N50 =228.96 Kb from *C. graminicola*), combined with the large size and multi-domain structure of PKSs, NRPSs and PKS-NRPS hybrids, resulted in less accurate predictions for *C. higginsianum* than for *C. graminicola.*

SM are usually low molecular weight molecules that are not essential for growth and survival of the producing organism in laboratory conditions, but become important for niche adaptation, and are associated with successful competition and toxic or inhibitory effects on other organisms (Shwab & Keller, 2008, Bölker *et al.*, 2008). Large numbers of SM-associated genes are usually found in necrotrophic plant pathogens (Amselem *et al.*, 2011), and SM are often implicated as phytotoxins with direct roles in pathogenicity (Daub, 1982, Gengenbach *et al.*, 1973, Matthews *et al.*, 1979, Scott-Craig *et al.*, 1992). In contrast, biotrophy seems to be associated with a loss of SM genes, as observed in *Blumeria graminis* (Spanu *et al*., 2010) and *Ustilago maydis* (Kamper *et al*., 2006, Bölker et al., 2008). Relatively little is known about the role of SM in hemibiotrophic plant pathogens (Böhnert *et al.*, 2004, Collemare *et al.*, 2008b). Until now, only a few SM produced by members the genus *Colletotrichum* have

been described, including the phytotoxins ferricrocin from *C. gloeosporioides* (Ohra, 1995), colletotrichins A, B and C from *C. nicotinae* (Goddard *et al.*, 1976, Kimura *et al.,* 1977, Kimura *et al.*, 1978, García-Pajón & Collado, 2003), and two antimicrobial methylflavonols from *C. dematium* (Abou-Zaid *et al.*, 1997). Another antimicrobial compound, colletotric acid from *C. gloeosporioides,* inhibited bacteria including *Bacillus subtilis* (Zou *et al.*, 2000). SM that have been characterized in *C. graminicola* include the spore inhibitor mycosporine alanine (Leite & Nicholson, 1992) and the antifungal compounds monorden and monicillins I, II, and III, which inhibited the growth of some seed-infecting and stalk-rot pathogens (Wicklow *et al.*, 2009). However, no specific *Colletotrichum* SM genes had been previously identified.

SMURF predictions are incomplete

The SM gene prediction software SMURF relies on common characteristics of SM synthesis in fungi in order to predict SM genes and gene clusters. Genes involved in the production of SM in fungi are commonly clustered and transcriptionally co-regulated (Hull *et al*., 1989, Proctor *et al*., 2003, Tudzynski *et al*., 1999). The complexity and diversity of SM is achieved by enzymes responsible for biosynthesis (backbones) and various combinations of modifying enzymes, responsible for further alterations to the initial product. Some of these modifying enzymes include oxidoreductases, P450 monooxygenases, methyl transferases, and esterases (Campbell & Vederas, 2010, Collemare *et al.*, 2008b, Fujii et al., 2005). Some clusters also include transcription factors and transporters, involved in regulation and translocation of the cluster product(s) (Shwab & Keller, 2008).

SMURF predicted 42 SM clusters in *C. graminicola* and 39 in *C. higginsianum.* However, some clusters were left out of these predictions. Melanin (1,8-dihydroxynaphthalene) is a SM required for appressorium-mediated host penetration in both *Colletotrichum* and *Magnaporthe* (Kubo & Furusawa, 1991). Based on similarities in gene content, order and orientation with the previously

characterized melanin biosynthetic clusters in *C. orbiculare* and *Magnaporthe oryzae*, our colleague Dr. Yasuyuki Kubo annotated the likely melanin clusters in *C. graminicola* and *C. higginsianum* (O'Connell *et al*., 2012). SMURF failed to predict either gene cluster. In all four fungi, five of the seven genes involved in melanin synthesis are physically associated in the melanin cluster. These five genes include two C6zinc binuclear transcription factors (*cmr1*, *cmr2*), one reductase (*t4hr1*), one PKS *(pks1)* and one laccase-like multicopper oxidase *(fet3*). The other two necessary genes, which include a scytalone dehydratase (*scd1*) and a trihydrooxynaplthalene reductase (*thr1*), are located outside the cluster (Kubo *et al.*, 1996, Perpetua *et al.*, 1996). SMURF failed to predict the melanin cluster in either *Colletotrichum* species. SMURF is trained to identify clusters based on similarity to previously identified clusters in *Aspergillus* (Khaldi et al., 2010). In *Aspergillus*, all seven genes responsible for biosynthesis of melanin are physically clustered (Tsai *et al.*, 1999). SMURF probably didn't recognize the *Colletotrichum* melanin clusters because of the two genes located outside the clusters.

Another cluster that SMURF missed was the carotenoid biosynthetic cluster, which was annotated in both *Colletotrichum* species by our colleague Dr. Robert Proctor, based on similarities to the *Fusarium fujikori* gene cluster (Linnemannstöns *et al.*, 2002). The carotenoid cluster is composed of four genes: a carotenoid dioxygenase, a phytoene synthase (a type of terpene synthase), a phytoene desaturase, and a rhodopsin. Carotenoids are terpenoid pigments synthesized by bacteria, fungi, and some insects (Moran & Jarvik, 2010). The carotenoid clusters in *C. graminicola* and *C. higginsianum* are highly conserved, but in *C. higginsianum* the phytoene synthase and phytoene desaturase appear to be physically separated from the carotenoid dioxygenase and the rhodopsin (although it is difficult to be certain of that, given the relatively poor quality of the *C. higginsianum* assembly). SMURF is not trained to recognize clusters that contain terpene synthase backbones.

Phylogenetic analyses of SM gene and gene cluster predictions provide relatively few insights into the classes of SM produced by the genus *Colletotrichum*

There are a number of well-characterized fungal SM biosynthetic clusters, some of which are responsible for the synthesis of phytotoxins with important roles in plant disease (Rebordinos *et al.*, 1996). Examples include T-toxin in *Cochliobolus heterostrophus* (Arntzen *et al.*, 1973, Watrud *et al.*, 1975, Turgeon & Baker, 2007), HC-toxin in *Cochliobolus carbonum* (Scheffer & Ullstrup, 1965) and cercosporin in *Cercospora* sp. (Daub, 1982). Other SM such as the siderophores NPS6 (Lee *et al.*, 2005, Oide *et al.*, 2006) and *sid1* (Tobiasen *et al.*, 2007, Greenshields *et al.*, 2007) from *C. heterostrophus* and *Fusarium graminearum*, respectively, are required for full virulence and resistance to oxidative stress. A phylogenetics approach was used to infer similarities between previously identified SM genes and gene clusters from ten different pathogenic Ascomycetes, and predicted SM genes and gene clusters in *Colletotrichum*.

The alternapyrone biosynthesis cluster in *Alternaria solani* is composed of five genes: three cytochrome P450 monooxygenases, one FAD-dependent oxygenase/oxidase, and one PKS (Fujii *et al.*, 2005). There were three *Colletotrichum* SM clusters containing three cytochrome P450 monooxygenases: clusters 23 and 27 from *C. graminicola,* and cluster 16 from *C. higginsianum. C. graminicola* clusters 23 and 27 include ten and 18 genes respectively, and only cluster 27 contains a PKS similar to that of the alternapyrone cluster. GLRG_10317, part of cluster 25, is an ortholog of A_solany_alt5, but none of the other genes in cluster 25 are shared with the atlernopyrone cluster. *C. higginsianum* cluster 16 contains 13 genes and it contains a DMAT rather than a PKS as a backbone gene. Thus, it does not appear that any of these clusters is related to the alternapyrone cluster, in spite of the presence of three cytochrome P450s.

A DMAT-containing SM cluster is responsible for synthesis of ergot alkaloids in *Claviceps purpurea*, which causes ergot disease of wheat and barley (Tudzynski

et al., 1999). The cluster contains a DMAT (*dmaW*), and four putative NRPSs (*lpsA*1, *lpsA*2*, lbsB, lpsC*) and one cytochrome P450 monooxygenase (*cloA*) (Tudzynski *et al.*, 2001, Schardl *et al.*, 2013). Four *C. graminicola* clusters (13, 14, 29, 33 and 39) and six *C. higginsianum* clusters (8, 13, 14, 29, 31 and 39) contain DMATs, however only two (cluster 33 of *C. graminicola* and cluster 8 in *C. higginsianum*) contain a second SM backbone enzyme, and in both cases this is a single NRPS. Thus, there is little evidence that any of the DMAT-containing clusters in *Colletotrichum* produce a product similar to lysergic acid.

Another SM-cluster that contains a DMAT, is the one responsible for the biosynthesis of siderodesmin, a non-host specific toxin involved in virulence of *Lepthosphaeria maculans*, the causal agent of blackleg disease(Gardiner *et al.*, 2004). This cluster contains 18 genes, including a DMAT (*sirD*), a PKS (*LmPKS*1) and an NRPS (*sirP*). GLRG_11574, a DMAT and GLRG_11575, a PKS from cluster 33 are 32 and 45% identical to *sirD* and *LmPKS*1, respectively. However, cluster 33 lacks an NRPS and is predicted to have only 10 genes, suggesting that this cluster is not related to the cluster from *L. maculans.*

The *C. graminicola* PKS GLRG_08620 is orthologous to C_nicotiniae_CTB1 and PKS13 of *C. higginsianum.* Cercosporin, a light-induced phytotoxin, is produced by *Cercospora* species that infect maize, coffee, and soybeans (Chen et al., 2007, Yamazaki *et al.*, 1975). Upon illumination, cercosporin induces the production of reactive oxygen species (ROS), which destabilize host membrane lipids and proteins, leading to membrane breakdown and cell death (Daub & Ehrenshaft, 2000). Of the eight genes that comprise the cercosporin biosynthetic cluster in *C. nicotiniae*, four have orthologs in cluster 18 of *C. graminicola* and five in cluster 10 of *C. higginsianum*. Both of these clusters are the largest ones predicted by SMURF, and they are highly conserved in the two *Colletotrichum* species. Additional modifying enzymes in both clusters, including five hypothetical proteins, two dehydrogenases, and an additional PKS in cluster 18 (GLRG_08632), suggest that the product of these clusters is different from

cercosporin. Low levels of amino acid identity in SM clusters (35-55%) might be an indication that although the cluster enzymes catalyze similar reactions, the final products are quite different.

There are four known fungal SMs that require the action of two PKSs for their synthesis: zearalenone (Kim *et al.*, 2005b); T-toxin (Baker *et al*., 2006); lovastatin (Hendrickson et al., 1999, Kennedy et al., 1999); and radicicol (Wang *et al.*, 2008, Reeves *et al.*, 2008). Four *C. graminicola* clusters (18, 21, 35 and 38) and 13 *C. higginsianum* clusters (4, 6, 7, 10, 15, 18, 23, 27, 33, 34, 35, 36 and 38) were predicted to contain two PKSs. However, a closer examination of the 13 *C. higginsianum* clusters revealed that only six of them really contained two PKSs (cluster 4, 6, 7,15, 23 and 36). Five of the clusters (10, 18, 27, 35, 38) actually contained two truncated fragments of a single larger PKS, and two more (clusters 33 and 34) contained fragments of a larger PKS-NRPS hybrid. Because of these ambiguities, further phylogenetic analysis for PKS and PKS-NRPS hybrid genes and gene clusters were performed using only *C. graminicola* predictions.

Our colleague Dr. Robert Proctor first noticed a *C. graminicola* predicted cluster, cluster 38, was similar to the previously characterized SM clusters responsible for the production of zearalenone, radicicol or monorden in other fungi. Phylogenetic analysis indicated that the non-reducing PKS GLRG_11836 in cluster 38 is orthologous to C_chlamydosporia_RADS2, and the second reducing PKS, GLRG_11840, is orthologous to C_chlamydosporia_RADS1 and C. chiversii_RADS1. The RADS cluster containing these PKSs is responsible for the biosynthesis of radicicol in these fungi (Wang *et al.*, 2008, Reeves *et al.*, 2008). The RADS cluster in *P. chlamydosporia* is identical in gene content, order, and orientation with *C. graminicola* cluster 38, with identity levels of 58-67%. Levels of identity with the genes in the RADS cluster in *C. chiversii* are higher (62-72%), but gene content and order are different. One gene (radR) is missing from cluster 38 and the other four genes are in a different order.

Radicicol, also known as monorden, is a SM with antifungal activity that was first isolated in 1953 from an *Ascomycete* fungus (*Monosporium* sp. nom. illegit.) found in soil samples (Delmotte & Delmotte-Plaquee, 1953). Interestingly, monorden and monocillins I, II and III, which are intermediates in the biosynthesis of monorden (Zhou *et al.*, 2010), were isolated from *C. graminicola*-inoculated maize stalks, and *in vitro* analyses demonstrated their antifungal activity against other maize stalk-rot and foliar pathogens (Wicklow et al., 2009). Monorden inhibits heat-shock protein 90, by competition with ATP for the binding site required for activation of Hsp90 (Roe *et al.*, 1999).This suggests it cannot only inhibit other fungi but also plant Hsp90, interfering with mediation of defense responses. Wicklow and collaborators suggested that during early penetration and biotrophic stages, *C. graminicola* could secrete these SM products in order to suppress basal defense responses. It seems very likely that cluster 38 is responsible for production of monorden and monicillins I, II, and III by *C. graminicola*.

There is a second cluster in *C. graminicola*, cluster 35 that also contains two PKS (GLRG_11770-11778). Phylogenetic analysis of their KS domains indicated these two PKSs are closely related to the two PKSs in cluster 38, and to the zearalenone and radicicol PKSs. GLRG_11778 is related to G_zeae_PKS13 and the RADS2 genes, and GLRG_11770 is related to G_zeae_PKS4 and the RADS1 genes. Phylogenetic analysis suggested that *C. higginsianum* lacks an ortholog of cluster 38, but that it has an ortholog of cluster 35. The two clusters (35 and 38) may be paralogous clusters, and one of the paralogs has been lost or gained since the divergence of *C. graminicola* and *C. higginsianum* from a common ancestor. Given the similarity of cluster 38 to the RADS cluster of the relatively distantly related *C. chlamydosporia*, it seems more likely that *C. higginsianum* has lost this cluster while *C. graminicola* has retained it.

Another SM that requires two PKSs for its synthesis is the T-toxin from *C. heterostrophus* (Baker *et al*., 2006). C_heterostrophus_PKS1 is ortholog of

D_maydis_PKS1 (60% identical), the gene responsible for the synthesis of PM-toxin in *D. maydis* (Yun *et al*., 1998). *C. heterostrophus* race T is the causal agent of southern maize leaf blight in maize carrying the Texas male sterile (Tms) cytoplasm (Miller & Koeppe, 1971). PM-toxin is structurally similar to T-toxin and also shows biological specificity towards maize with the Tms cytomplasm (Danko *et al.*, 1984, Mehrabi et al., 2011). Seven genes in two loci (Tox1A and Tox1B) are known to be required for the biosynthesis of T-toxin in *C. heterostrophus* race T: the PKSs ChPKS1 and ChPKS2, and the 3-hydroacyl CoA dehydrogenase ChLAM1 in Tox1A; the decarboxylase ChDEC1, and the dehydrogenases ChRED1, ChRED2 and ChRED3 in Tox1B (Turgeon & Baker, 2007). The *C. graminicola* PKS GLRG_03360 is related to these toxin-producing genes, but the *C. graminicola* gene is not predicted by SMURF to belong to a cluster. BLAST searches against the *C. graminicola* genome using the genes identified in ToxA and ToxB loci, indicated that none of the putative homologs in *C. graminicola* was predicted to be part of any SMURF predicted cluster.

Six putative homologs of these seven *C. heterostrophus* genes can be identified in the *C. graminicola* genome, with identities ranging from 41 to 51%, but none of these genes was predicted as part of a cluster, and none of them was located near GLRG_03360. C_heterostrophus_PKS2 was not closely related to any of the *C. graminicola* PKSs or PKS-NRPS hybrids, and none of the *C. graminicola* clusters with two PKSs contain orthologs of any of the genes in the T-toxin SM cluster. Thus, there is no evidence from my study that *C. graminicola* produces a SM similar to T-toxin.

Lovastatin is a SM synthesized by an *Aspergillus terreus* 18-gene cluster that also contains two PKSs, *lovF* and *lovB* (Kennedy *et al*., 1999, Hutchinson *et al.*, 2000, Auclair *et al.*, 2001). Lovastatin interferes with cholesterol biosynthesis and has important pharmaceutical applications (Tobert *et al.*, 1982, Tobert, 2003). *C. graminicola* does not have obvious orthologs of either *lovF* or *lovB*, but GLRG_09267 is related to *lovF* (40% identical) and the PKS-NRPS hybrids

GLRG_11890 and GLRG_11626 are related to *lovB* (50% identical). *ACE1*, a PKS-NRPS hybrid from *M. oryzae,* has been described as similar to LovB, with 37% identity (Böhnert *et al*., 2004). The *ACE1* cluster in *M. oryzae* is composed of 15 genes (Collemare *et al*., 2008b), and its product is an avirulence protein that confers Pi33-mediated resistance in rice (Berruyer *et al.*, 2003, Vergne *et al.*, 2007). Analysis of expression patterns of the *ACE1* PKS-NRPS hybrid indicated a strong induction of this gene during early stages of fungal penetration (Fudal *et al.*, 2007). *C. graminicola* does not have orthologs of *ACE1* or *SYN2*, another PKS-NRPS hybrid of *M. oryzae* found in the ACE1 cluster, but not required for avirulence in *Pi33* cultivars (Collemare *et al.*, 2008b). However, *C. higginsianum* has orthologs of both the *ACE1* (CH063_03067) and *SYN2* (CH063_03253/03254) clusters (O'Connell *et al.*, 2012). Three more characterized PKS-NRPS hybrids of *M. oryzae* (*SYN7, SYN6, SYN8*) were included in my phylogenetic analysis. *SYN8* and *SYN6* are also induced at early stages of rice penetration, and expression of the latter was also detected *in vitro* (Collemare *et al.*, 2008a). Deletion of *ACE1, SYN2*, or *SYN6* did not affect pathogenicity of *M. oryzae,* and only *ACE1* was required for resistance in *Pi33* cultivars*. C. graminicola* has two orthologs of *SYN7*: GLRG_11626 predicted as part of cluster 34 and GLRG_11890, predicted as part of cluster 39. GLRG_11507, part of cluster 31 and GLRG_09715, part of cluster 22, are orthologs of *SYN6* and *SYN8*, respectively.

Some *C. graminicola* NRPSs seemed to be related to other known fungal NRPS genes. A BLAST search identified GLRG_00469, an NRPS predicted as the backbone for cluster 2, as one of the first BLAST hits (58% identical) to *ABA1* from the yeast *Aureobasidin pullulans*. The reannotated *C. higginisanum* NRPS 2 was 62% identical to *ABA1*. *A. pullulans* is a saprophytic Ascomycete that produces aureobasidin, a SM with known antifungal activity against *Candida albicans, Cryptococcus neoformans* and *Aspergillus* sp., but with no toxicity to animals (Takesako *et al.*, 1991, Takesako *et al.*, 1993). The gene responsible for

the synthesis of this compound contains a single exon, and encodes a 1,200 kDa protein containing 9 ATC modules (Slightom *et al.*, 2009). GLRG_00469 is also predicted to have a single exon, and encode a 982 kDa protein, that contains only 7 ATC modules. Other BLAST hits to *ABA1* include *SIMA* from *Tolypocladium niveum* (Weber *et al.*, 1994), *NRPS3* from *C. heterostrophus* (Lee *et al.*, 2005) and uncharacterized NRPSs from *Trichoderma virens* and *M. oryzae.* In *T. niveum, SIMA* encodes a cyclosporine synthetase that is responsible for the synthesis of cyclosporine, a commercial drug used to suppress the immune system (Borel & Gunn, 1986). Targeted deletion of *NRPS3* in *C. heterostrophus* did not affect the phenotype of the fungus *in vivo* or *in planta* (Lee *et al.*, 2005). Although similar genes seem to be present in different Ascomycetes, the role of these in plant pathogenic fungi is still unknown.

Overall, few similarities were observed among *Colletotrichum* SM-associated genes and previously characterized genes. PKSs and PKS-NRPS hybrids from *C. graminicola* were analyzed for similarities to other Ascomycete genes, and I determined that fifteen (44%) of the 34 analyzed PKSs and four (57%) of the seven PKS-NRPS hybrids seem to be unique to *C. graminicola*. Three PKS-NRPS hybrids are shared with *M. oryzae*, whereas only two of them are shared with *C. higginsianum*. Twenty-nine percent of the PKS-NRPS hybrids, 45% of the PKSs, 64% of the TSs, 86% of the DMATS and 94% of the AMP binding domains from NRPSs in *C. graminicola* appear to be shared with *C. higginsianum.* Only five (15%) of the analyzed PKSs in *C. graminicola* were not shared with any other fungus included in this study.

SM products are often associated with phytotoxicity induced by necrotrophic plant pathogens. SM such as monorden and the *ACE1* gene product, have also been implicated in suppression or induction of resistance at early stages of fungal colonization. My goal in this chapter was to identify the putative SM-associated genes in *C. graminicola,* and to identify any SM with potential similarities to previously characterized SM that could be important in early stages

of penetration and establishment of a compatible interaction. Using this approach, I was able to identify a gene cluster that is probably responsible for production of monorden, a SM that has been proposed to suppress plant defense responses during early penetration events and during biotrophy (Reeves *et al.*, 2008, Wang *et al*., 2008, Wicklow *et al*., 2009). Particularly interesting was that the monorden cluster was not conserved in the closely related *C. higginsianum*, suggesting that it may be important specifically for *C. graminicola* on maize.

Other than this highly conserved cluster, very few firm conclusions could be drawn due to the extreme diversity of the very large array of SM genes and gene clusters in both *Colletotrichum* species. Thus, I next moved to use this information in an analysis of the expression of these genes during colonization of maize by *C. graminicola* to confirm the identities of co-regulated gene clusters, and to evaluate the potential role of SM at different stages of infection. Additionally I compared the transcriptome profiles of the WT and the *cpr1* mutant during sheath infection to provide insights into the potential relevance of SM during appressorial development and biotrophy in the WT versus the mutant. These experiments are described in the next chapter of my dissertation.

Table 3. 1. Number of required conserved domains identified in each of the PKSs predicted by MCL analysis for both *Colletotrichum* species. The more fragmented genome of *C. higginsianum* is evidenced by the number of incomplete predicted proteins in each of these categories. C = Predicts a complete gene, I = Predicts an incomplete gene. 0/3= No required conserved domains. 1/3= One of three conserved domains. 2/3= Two required conserved domains. 3/3= All required conserved domains.

	0/3		1/3		2/3		3/3		
	C	I	C	I	C	I	C	I	Total
C. graminicola	4	0	4	0	4	0	40	0	52
C. higginsianum	15	6	18	26	21	18	14	2	120

Table 3.2. Details and accession numbers of previously sequenced PKSs and PKS-NRPSs used in this study.

Gene name	Assigned gene name	Fungal species	Secondary metabolite	Accession number
PKS1	D_maydis_PKS1	*Didymella maydis*	PM-toxin	AY495642
ALT5	A_solani_ALT5	*Alternaria solani*	Alternapyrone	AB120221
PKS1	C_lagenarium_PKS1	*Colletotrichum lagenarium*	Melanin	BAA18956
LovB	A_terreus_lovB	*Aspergillus terreus*	Lovastatin	AAD39830
LovF	A_terreus_lovF	*Aspergillus terreus*	Lovastatin	AAD34559
PKS13	G_zeae_PKS13	*Gibberella zeae*	Zearalenone	ABB90282
PKS4	G_zeae_PKS4	*Gibberella zeae*	Zearalenone	ABB90283
RADS1	C_chiversii_RADS1	*Chaetomium chiversii*	Radicicol	EU980390
RADS2	C_chiversii_RADS2	*Chaetomium chiversii*	Radicicol	EU980390
RADS1	P_chlamydosporia_RADS1	*Pochonia chlamydosporia*	Radicicol	ACD39770
RADS2	P_chlamydosporia_RADS2	*Pochonia chlamydosporia*	Radicicol	ACD39770
FUM1	G_moniliformis_FUM1	*Gibberella moniliformis*	Fumonisin	AF155773
PKS1	C_heterostrophus_PKS1	*Cochliobolus heterostrophus*	T-toxin	U68040
PKS2	C_heterostrophus_PKS2	*Cochliobolus heterostrophus*	T-toxin	AY495643
CTB1	C_nicotiniane_CTB1	*Cercospora nicotiniae*	Cercosporin	AY649543
ACE1	M_oryzae_ACE1	*Magnaporthe oryzae*	ACE1	AJ704622
SYN2	M_oryzae_SYN2	*Magnaporthe oryzae*	N.A	AJ704623
SYN6	M_oryzae_SYN6	*Magnaporthe oryzae*	N.A	BN000505
SYN7	M_oryzae_SYN7	*Magnaporthe oryzae*	N.A	BN000506
SYN8	M_oryzae_SYN8	*Magnaporthe oryzae*	N.A	BN000507
PKS3-PKS25	C_heterostrophus_PKS3-PKS25	*Cochliobolus heterostrophus*	N.A	AY495643–AY495666

Table 3. 3. Comparisons between the radicicol (RADS) biosynthesis cluster from *C. chiversii, P. chlamydosporia* and SMURF cluster 38 from *C. graminicola*. Percent identity between each RADS cluster gene and the first two BLAST(p) hits in the *C. graminicola* database is indicated.

RADS cluster gene	*C. graminicola* closest hits	SMURF cluster	*P. chlamydosporia*	*C. chiversii*
			Identity (%)	
RadH	GLRG_11837	38	67	72
(Halogenase)	GLRG_08626	18	51	53
Rads2	GLRG_11836	38	58	62
(nrPKS)	GLRG_11778	35	37	38
RadE	GLRG_11838	38	60	65
(MFS trasnporter)	GLRG_06355	NC	43	44
Rads1	GLRG_11840	38	63	68
(Rpks)	GLRG_11770	35	44	45
RadP	GLRG_11839	38	65	70
(Cytochrome P450)	GLRG_06496	NC	39	38
RadR	GLRG_05281	NC	NC	25
(Transcription factor)	GLRG_10785	NC	NC	7

Table 3.4. Comparisons between the CTB1 cercosporin biosynthetic cluster and *Colletotrichum* clusters. Each gene of the *C. nicotiniae* cluster and first BLAST(p) hits in the *Colletotrichum* database.

CTB1 cluster gene	Predicted function	*Colletotrichum* closest hits	SMURF cluster	Identity (%)
CTB1	nrPKS	GLRG_08620	18	54
		CH063_02506	10	45
CTB2	*O*-methyltransferase	GLRG_08618	18	51
		CH063_07427	10	47
CTB3	*O*-methyltransferase	GLRG_08619	18	55
		CH063_11016	10	41
CTB4	MFS-transporter	GLRG_09290	NC	50
		CH063_00314	10	51
CTB5	FAD-dependent oxidoreductase	GLRG_11839	38	62
		CH063_02504	10	62
CTB6	NADPH-dependent oxidoreductase	GLRG_02545	NC	53
		CH063_07616	NC	55
CTB7	FAD-dependent oxidoreductase	GLRG_11528	NC	51
		CH063_00277	NC	51
CTB8	Transcription factor	GLRG_08617	18	47
		CH063_07428	10	35

Figure 3.1. Identification of KS domains. **A.** *C. higginsianum* and **B.** *C. graminicola* predicted PKSs using the NCBI conserved domain database (CDD). Proteins with no conserved domains, lacking the KS domain, or containing a truncated version, were not utilized for phylogenetic analysis.

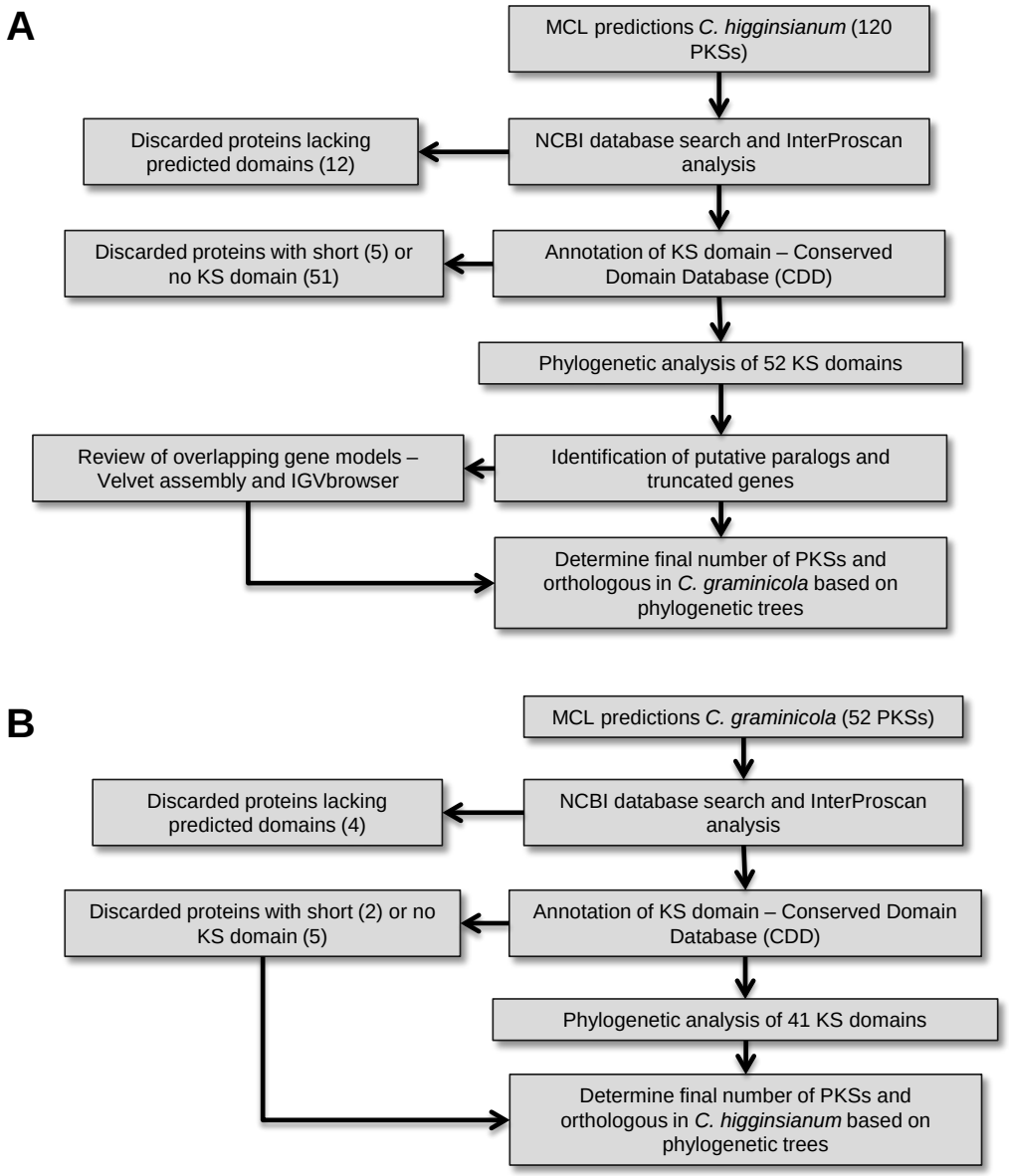


Figure 3.2. Identification of AMP-binding (A) domains. **A**. *C. higginsianum* and **B.** *C. graminiciola* predicted NRPSs using the NCBI conserved domain database (CDD). Proteins with no conserved domains, lacking the A domain or with truncated A domains were not included in the phylogenetic analysis.

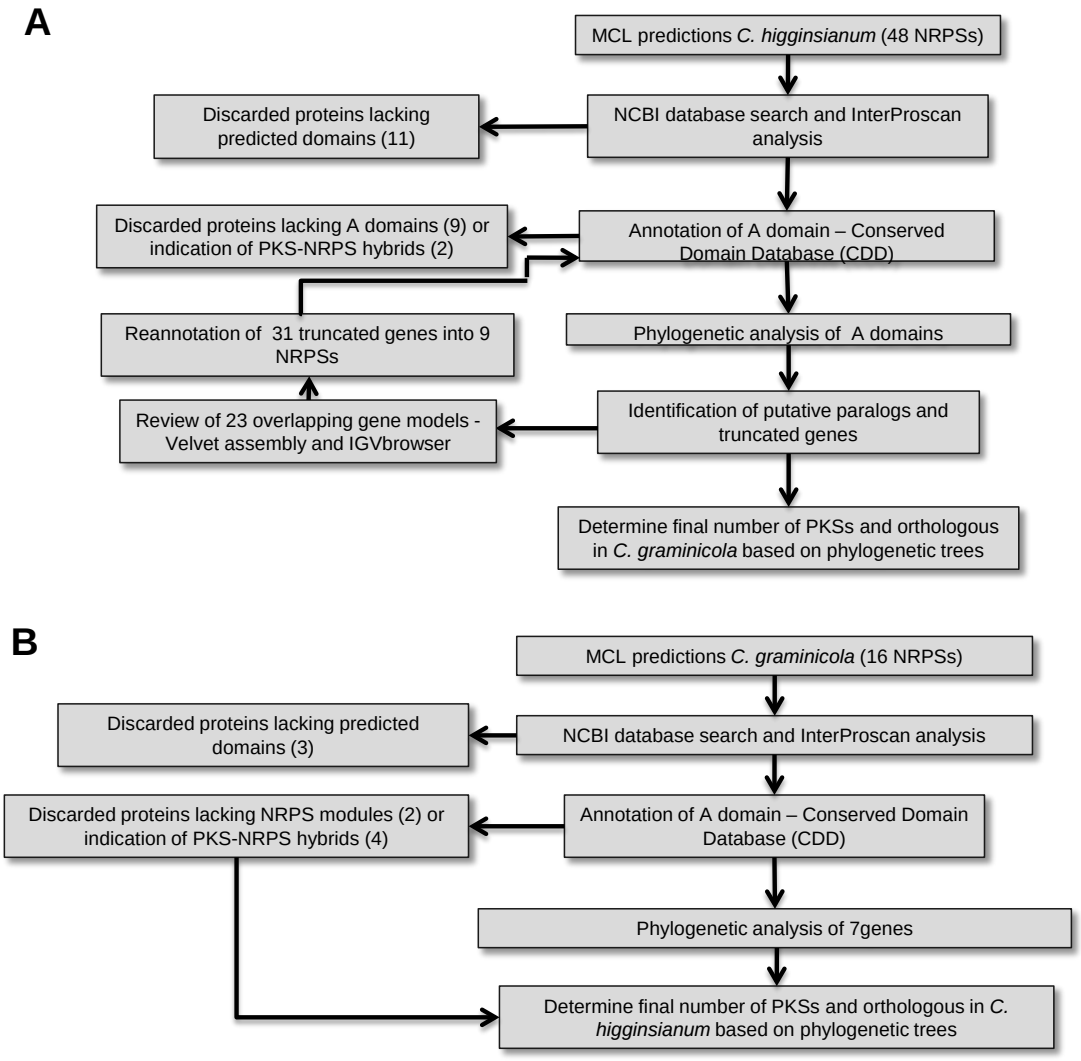


Figure 3.3. Details of phylogenetic tree alignments suggesting expansion of PKS families in *C. higginsianum*. **A.** Genes CH063_01990 and CH063_08736 (arrows)are paralogs, as indicated by alignment and visualization using IGVbrowser (http://www.broadinstitute.org/igv/). **B.** Genes CH063_06479 and CH063_14111 (arrows) are truncated fragments of a larger ORF.

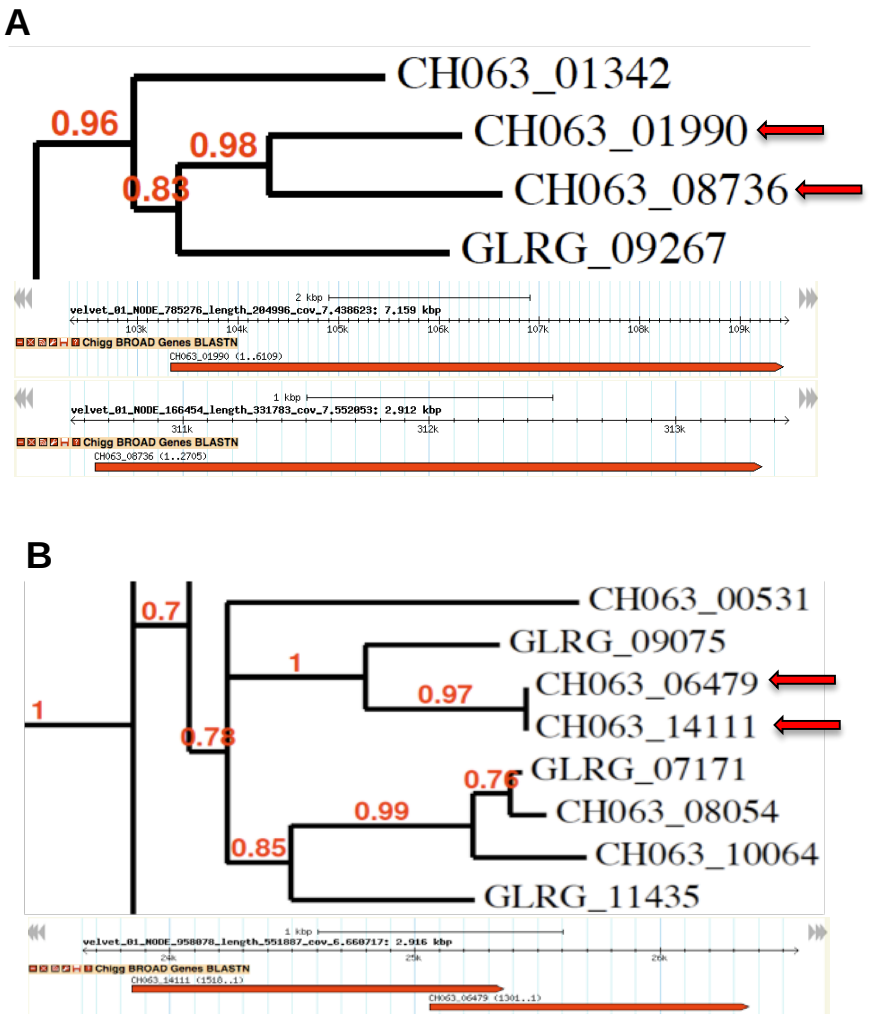


Figure 3.4. Phylogenetic analysis of PKSs from *C. graminicola* and *C. higginsianum,* based on alignment of the conserved KS domain. Sixteen putative orthologous PKSs were found between *C. higginsianum and C. graminicola* (blue boxes). Fragments of *C. higginsianum* PKSs were realigned and predicted to be part of a single ORF (orange boxes).

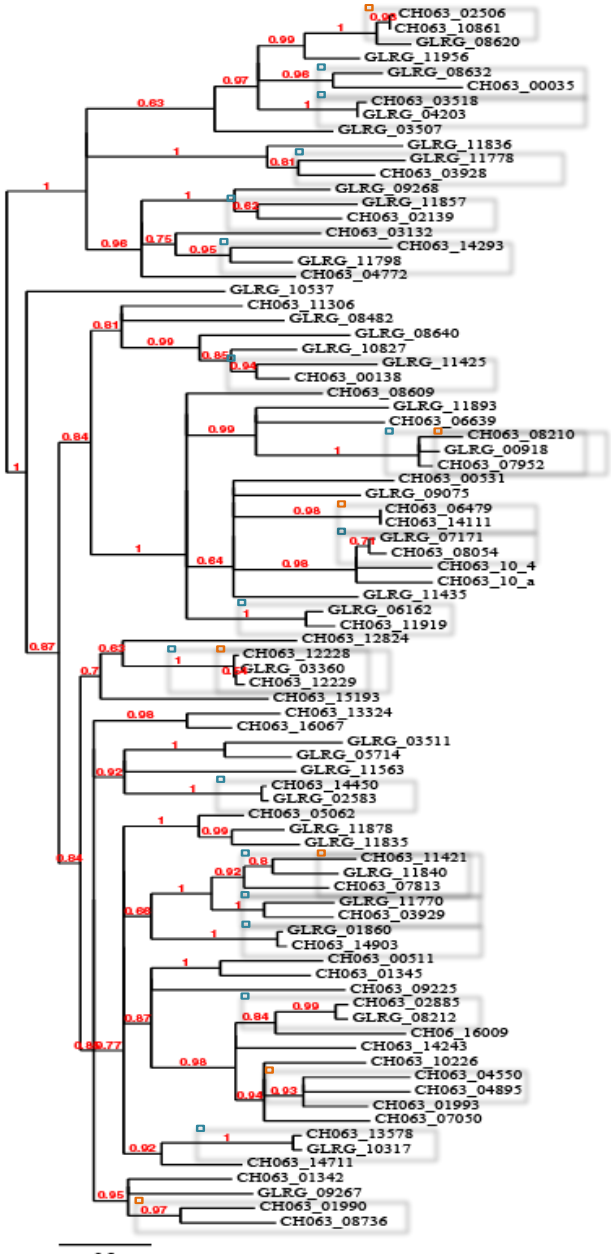


Figure 3.5. Details of phylogenetic tree alignments suggesting expansion of NRPS families in *C. higginsianum.* **A.** A clade of putative paralogous genes in C. higginsianum, as indicated by alignment and visualization using IGVbrowser (http://www.broadinstitute.org/igv/). **B**. Predicted AMP binding domains from five *C. higginsianum* NRPSs appear to be orthologs of the seven A domains of GLRG_00469 (GLRG_004691A to GLRG_004697A), but could be fragments of a larger gene.

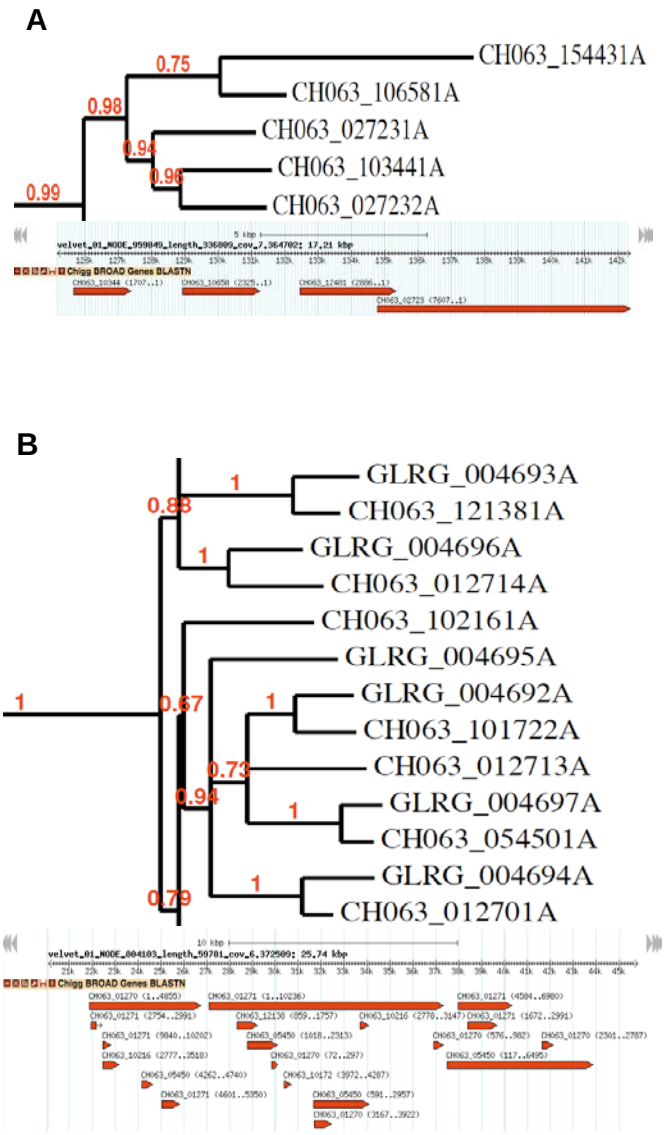


Figure 3.6. Phylogenetic tree based on alignment of AMP-binding domains from both *Colletotrichum* species. Putative orthologous AMP – binding domains are color-coded in boxes**.**

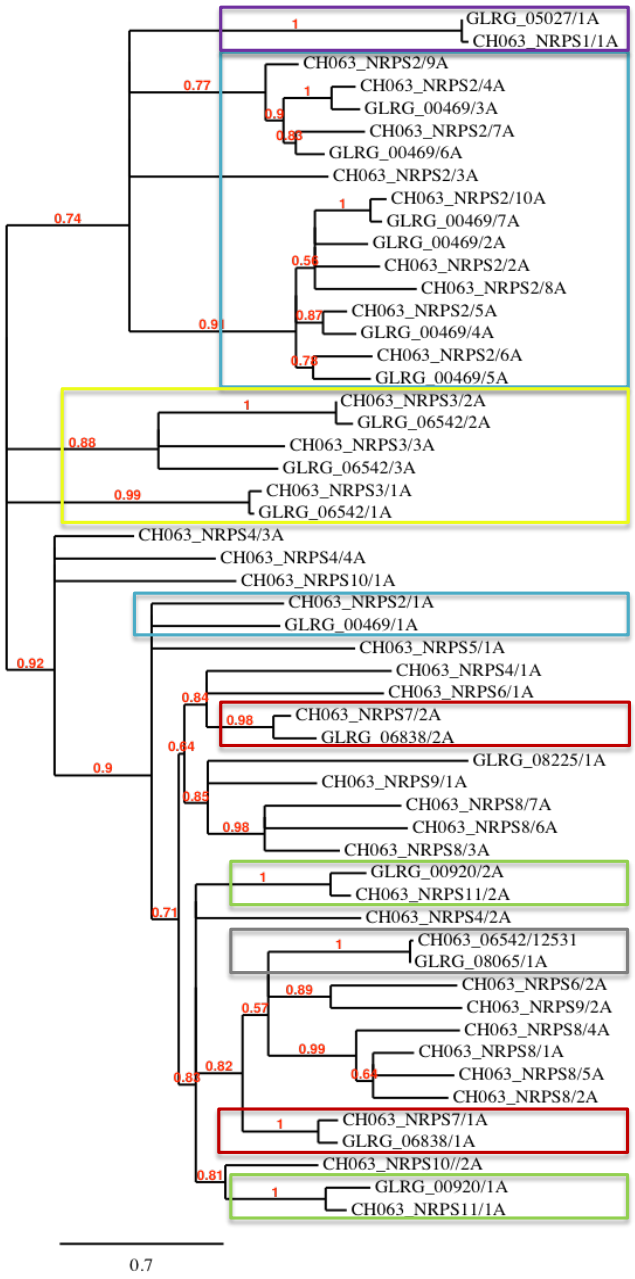


Figure 3.7. Phylogenetic tree based on alignment of PKS-NRPS hybrid protein sequences from both *Colletotrichum* species. Putative orthologs are color-coded in boxes.

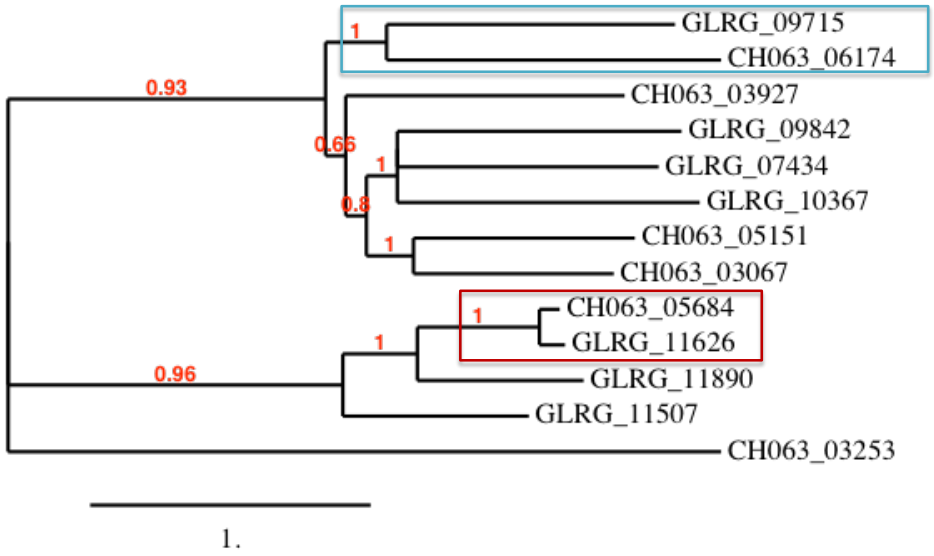


Figure 3.8. Phylogenetic tree based on alignment of DMAT protein sequences from both *Colletotrichum* species. Putative orthologs are color-coded in boxes.

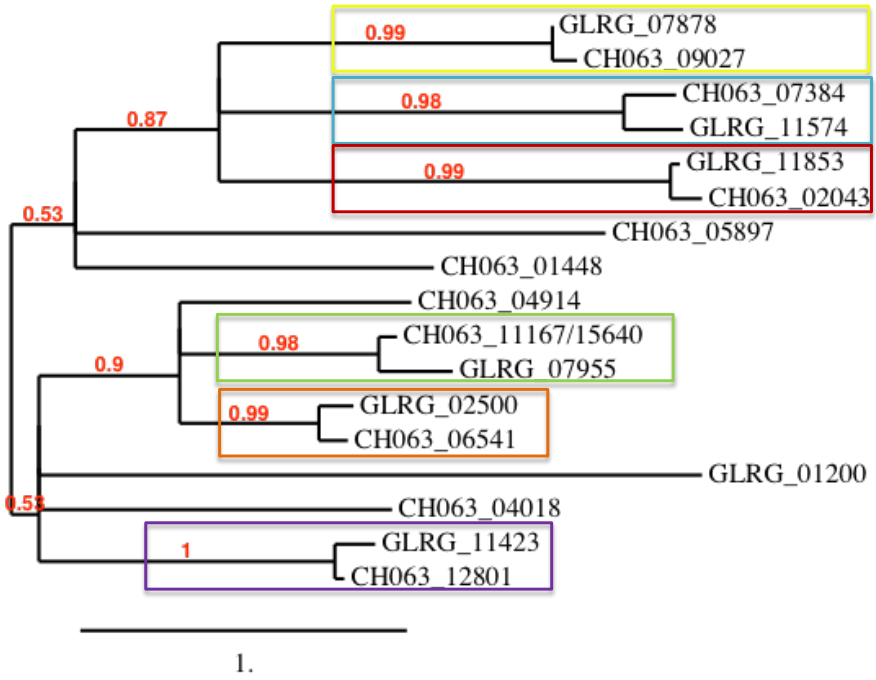


Figure 3.9. Phylogenetic tree based on alignment of terpene synthases protein sequences from both *Colletotrichum* species. Putative orthologs are color-coded in boxes.

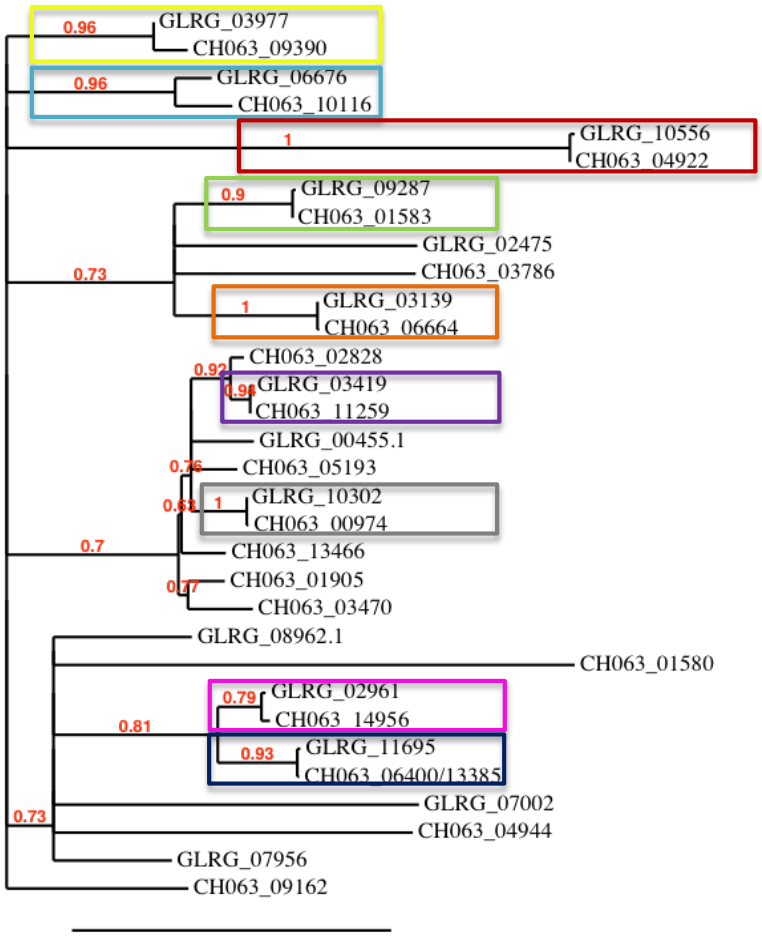


Figure 3.10. Number and classes of backbone genes predicted by SMURF. Clusters for *C. graminicola* (light gray) and *C. higginsianum* (dark gray).

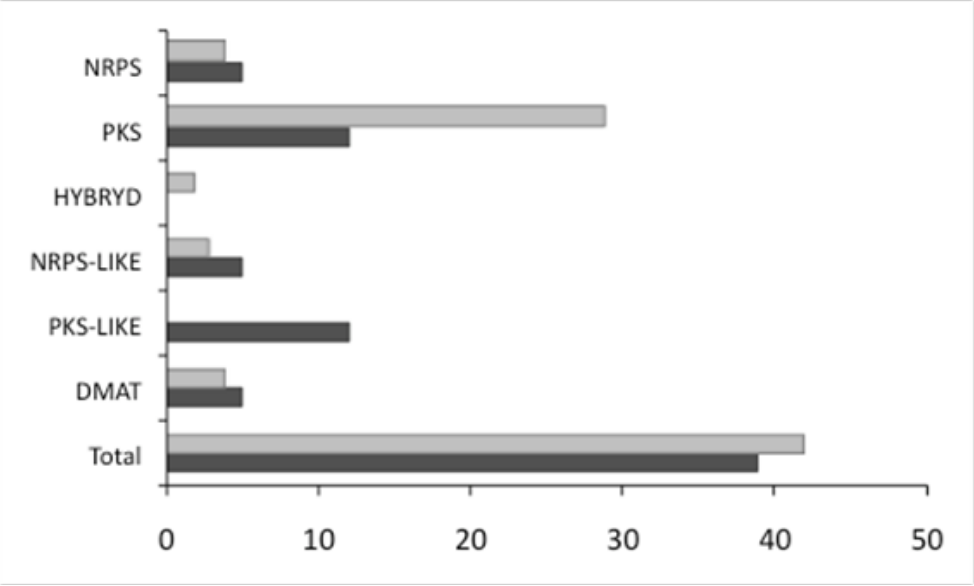


Figure 3.11. Total number of clusters predicted by SMURF in *Colletotrichum* and 8 more Ascomycetes

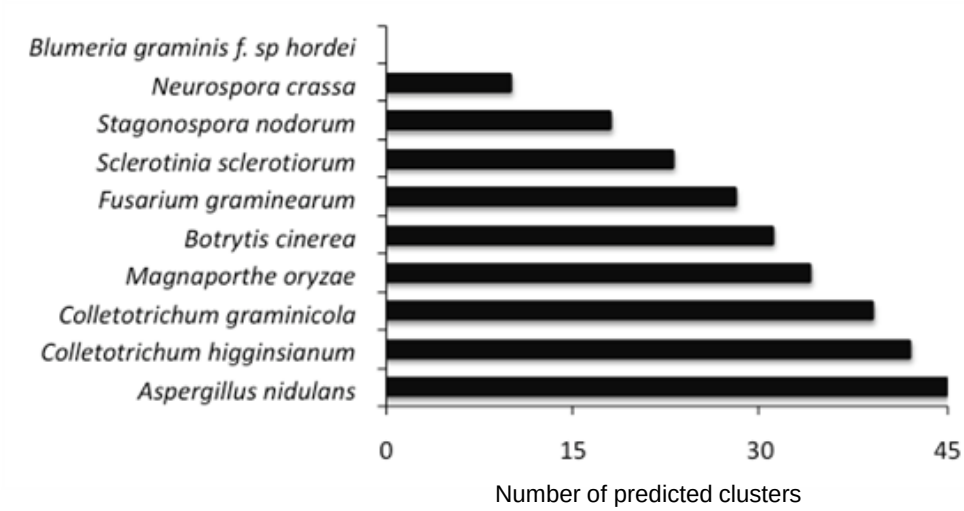


Figure 3.12. Phylogenetic analysis of amino acid sequences of KS domains from PKSs and PKS-NRPS hybrids from *C. graminicola* and 10 other Ascomycetes.

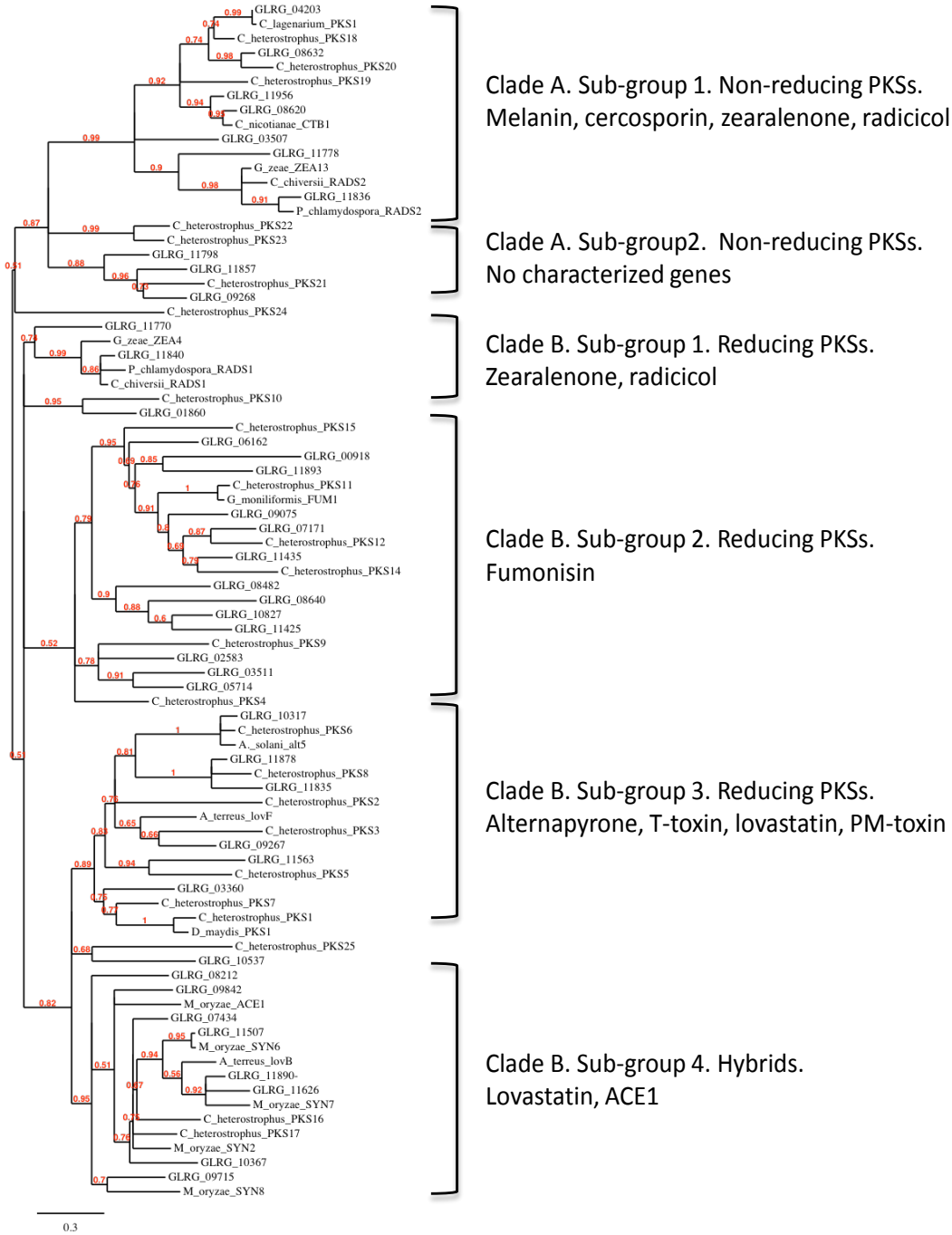


Figure 3.13. Comparisons between the radicicol (RADS) biosynthesis cluster from *C. chiversii, P. chlamydosporia* and SMURF cluster 38 from *C. graminicola*. Microsynteny is indicated by gray bars.

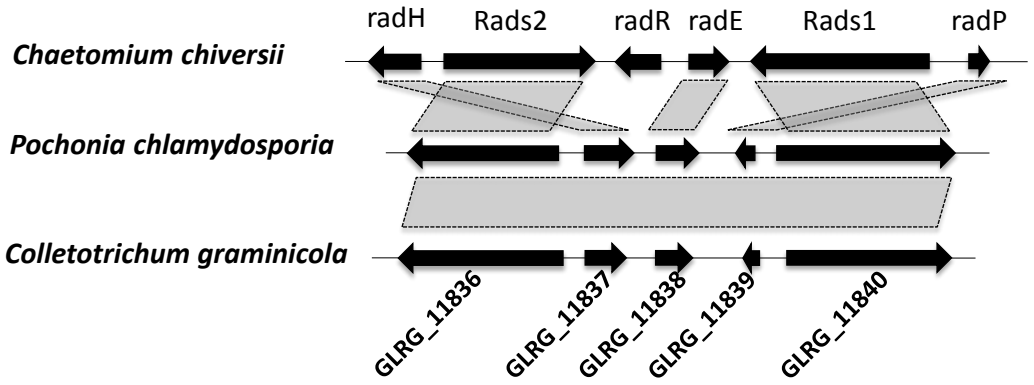
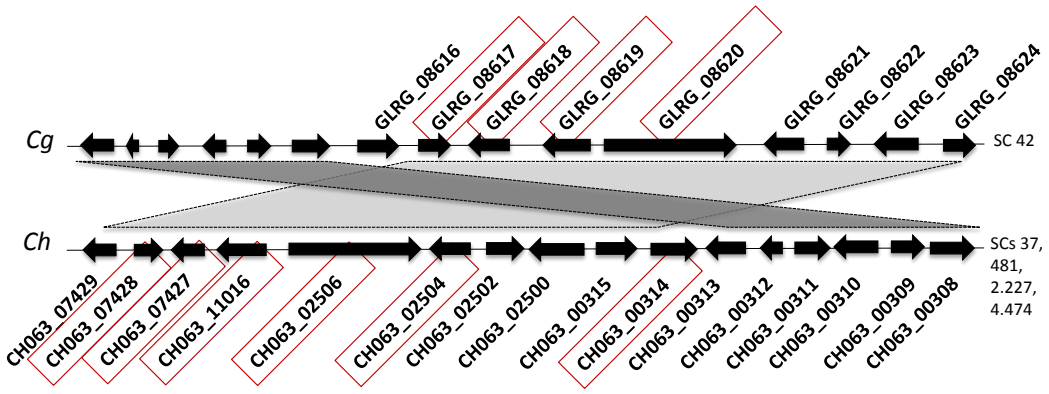


Figure 3.14. Structure of *C. graminicola* cluster 18 and *C. higginsianum* cluster 10. Initial SMURF predictions were modified and only shared portion of the clusters is shown. All 15 genes are in the same scaffold in *C. graminicola* whereas *C. higginsianum* cluster was reconstructed from 4 different scaffolds. Putative orthologs of genes of the cercosporin biosynthetic cluster are shown in red boxes. Microsynteny is indicated by gray bars.



Chapter 4

Expression of potential pathogenicity determinants during compatible and incompatible interactions between *C. graminicola* and maize

It has generally been assumed that hemibiotrophic pathogens behave initially like biotrophs, and then switch later to necrotrophic development (Koeck *et al*., 2011, Horbach *et al.*, 2011). My findings in Chapter 3 indicated that the genome of *C. graminicola* encodes a large number of genes associated with secondary metabolism (SM), a feature that is typical of necrotrophic plant pathogens that secrete phytotoxic compounds to activate host defensive apoptotic pathways and kill plant cells in advance (Markham & Hille, 2001, Amselem *et al*., 2011). Unlike necrotrophs though, *C. graminicola* does not kill host cells in advance, and each new cell is typically invaded while it is still alive. This behavior is more like a biotroph. Biotrophs suppress host defense responses by producing a range of secreted effectors that manipulate host defense pathways (Spanu *et al*., 2010, Doehlemann *et al*., 2009, Djamei *et al*., 2011). However, recent studies have indicated that *C. graminicola* induces maize defense responses even during early stages of colonization, which is more like a necrotroph (Vargas *et al.*, 2012). Thus, the theory that *C. graminicola* behaves first as a biotroph, and then as a necrotroph, seems to oversimplify the true nature of this interaction.

To better understand biotrophic versus necrotrophic development in *C. graminicola*, we conducted a transcriptome analysis of the pathogen during pre-penetration, biotrophic colonization, and necrotrophic colonization stages of development in maize sheaths[2]. In this chapter, I have characterized the genes that were differentially expressed during these different phases, and I also used the data to validate co-regulation of predicted SM clusters, and to clarify potential

[2] Some of the data included in this chapter were published in O'Connell *et al.,* 2012, Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analysis. Nature Genetics 44 (9): 1060-1065

roles of secondary metabolism, especially during the early stages of penetration of *C. graminicola.* I also compared the transcriptome of the pathogenic (WT) strain with that of a non-pathogenic mutant strain that is impaired in establishment of biotrophy and the switch to necrotrophy.

Introduction

Colletotrichum graminicola, the causal agent of anthracnose leaf blight and anthracnose stalk rot of maize, is a hemibiotroph. It grows initially as a biotroph, producing thick, primary hyphae that invade living host cells, and are separated from the host cytoplasm by a membrane. Later, it switches to necrotrophic growth, producing thin, secondary hyphae that are no longer surrounded by a membrane (Mims & Vaillancourt, 2002, Venard & Vaillancourt, 2007a). Symptoms are produced only during the necrotrophic phase (Bergstrom & Nicholson, 1999, Münch *et al.*, 2008). Biotrophic plant pathogens are known to reprogram host metabolism and suppress host defense responses in order to obtain nutrients from living plant cells (Kamper *et al.*, 2006, Doehlemann *et al*., 2008, Spanu *et al.*, 2010). There is evidence that, during early infection stages, *C graminicola* avoids activation of plant defense by replacing the PAMP chitin with chitosan in the appressorial wall and in walls of primary hyphae. This may avoid recognition by plant chitinases (El Gueddari *et al.*, 2002b). However, basal plant defense responses are activated during early stages of infection of maize leaves, when the fungus appears to be growing biotrophically (Vargas *et al*., 2012), suggesting that *C. graminicola* does not suppress host defense responses at this stage like a biotroph. Furthermore, I found that the *C. graminicola* genome encodes a very large number of enzymes associated with secondary metabolism (SM) (O'Connell *et al.*, 2012), a feature normally associated with necrotrophic pathogens (Amselem *et al.*, 2011, Bölker *et al.*, 2008). These observations suggest that the interaction of maize with the hemibiotrophic *C. graminicola* pathogen does not precisely match the characteristics of either biotrophic or necrotrophic interactions.

I predicted 42 SM-clusters in the *C. graminicola* genome, based on gene content and sequence similarities to other identified SM-biosynthetic clusters (O'Connell *et al*., 2012, Khaldi et al., 2010), which suggests that this fungus has the potential to produce a wide variety of SM. However, only three SM have been identified in *C. graminicola* to date: melanin, required for appressorial maturation (Rasmussen & Hanau, 1989); the spore germination inhibitor mycosporin alanine (Leite & Nicholson, 1992); and the antifungal compounds monorden and monocillin (Wicklow *et al.*, 2009). Several approaches have been used to identify *C. graminicola* genes that are expressed during growth *in planta,* including laser capture microdissection (Tang *et al*., 2006), yeast signal sequence trapping (Krijger *et al.*, 2008) and suppressive subtractive hybridization (Vargas *et al.*, 2012). Approximately 170 fungal genes have been identified as a result of these studies, a very small number considering that the genome of *C. graminicola* is predicted to encode 12,006 proteins (O'Connell *et al.*, 2012). Mutants of *C. graminicola* lacking *Ppt1*, a major activator of polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) (Lambalot *et al.*, 1996) had multiple developmental defects and were non-pathogenic to maize leaves, suggesting that the synthesis and secretion of one or more SM-derived compounds is required for pathogenicity and for multiple stages of *C. graminicola* development (Horbach et al., 2009). However, few SM-key enzymes were identified in these expression studies, with the exception of a PKS involved in melanin biosynthesis (Sugui & Deising, 2002). Previous approaches utilized to characterize fungal genes expressed *in planta* have been limited in part because fungal transcripts are underrepresented in infected samples.

Next generation sequencing (NGS) protocols can generate millions of reads, providing a much more comprehensive catalogue of fungal gene expression in infected plant tissues (Kawahara *et al.*, 2012). We generated Illumina RNA-seq transcriptome data representing three stages of fungal infection of detached maize leaf sheaths: pre-penetration melanized appressoria; late biotrophic

development (when the fungus had colonized 3-4 cells beyond the infection site); and necrotrophy (when the fungus had begun to produce secondary hyphae). A preliminary analysis of the WT transcriptome data was published in *(O'Connell et al.,* 2012). The data are available from the NCBI Gene Expression Omnibus under accession number GSE34632. Here I have compared these data with a parallel set of RNA-seq data that was generated for the nonpathogenic *cpr1* mutant fungus during two stages of infection: pre-penetration melanized appressoria; and biotrophic development, in which approximately 95% of the successful penetration sites consisted of primary hyphae that were limited to the initially infected cell.

I had several goals for the work described in this chapter. First, I wanted to characterize in detail the fungal genes that were differentially regulated across all stages of development in order to compare these with genes that are reportedly expressed by biotrophs versus necrotrophs *in planta*. This type of in-depth analysis had not been done previously. I was particularly interested in genes that encoded putative effectors (typically associated with biotrophy) and SM-associated genes (typically associated with necrotrophy). I anticipated that this would help to explain the nature of these shifts in *C. graminicola*. Second, I wanted to evaluate and validate the co-expression of SM-associated gene clusters in *C. graminicola*. In fungi, SM-associated genes are not only clustered, but also co-regulated (Chen *et al*., 2007, Proctor *et al*., 2003, Khaldi *et al*., 2008). Finally, I expected that comparisons of the mutant and WT transcriptomes would give additional clues about the nature of the mutation, and also might reveal genes that are expressed early in biotrophy, in the first infected cell, versus later, when the fungus has colonized several cells, most of which contain intercalary hyphae and have already died. Since the mutant appeared to be defective specifically in the ability to move from the first cell into the adjacent cells, I hoped to identify candidate genes involved in this process.

Materials and Methods

Plants and fungal strains

The highly susceptible maize inbred Mo940 was used for this study. Plants were grown to the V3 stage in the greenhouse, with a 14-hour day length, in 3.8 x 21 cm plastic Conetainers (Super SC-10 UV stabilized Stuewe & Sons, Inc. Oregon, USA), containing a mixture of three parts Pro-Mix BX (Premiere Horticulture, Ltd, Riviere du Loup, PQ, Canada) to two parts of sterile topsoil. Plants were watered daily to saturation and fertilized two to three times per week with a solution of 150 ppm of Peters 20-10-20 (Scotts-Sierra Horticultural Products Co., Marysville, OH), beginning one week after germination.

C. graminicola strain M1.001 was the WT strain (O'Connell *et al.,* 2012). A mutant derived from M1.001 by restriction-enzyme mediated insertional mutagenesis (REMI) is nonpathogenic to maize stalks and leaves due to an insertion in the *Cpr1* gene, which is predicted to encode one component of the microsomal signal peptidase (Thon *et al*., 2000, Thon *et al.*, 2002, Mims & Vaillancourt, 2002). A complemented strain (*Cpr1*-C), generated by transformation of the mutant with a 3.6 kb fragment of genomic DNA containing the WT *Cpr1* gene, is fully restored in pathogenicity and comparable to the WT strain both *in vitro* and *in planta* (Thon *et al*., 2002). All fungal strains were routinely cultured on potato dextrose agar (PDA, Difco) at 23ºC under continuous light.

Transcriptome profiling

Sample preparation and RNA extraction

C. graminicola falcate spores were harvested and inoculated on maize leaf sheaths as described in chapter 2 of this dissertation. Two 20-µl inoculum drops were applied to each leaf sheath, approximately 1 cm from either end, and the inoculated sheaths were incubated in a moist chamber at 23°C under continuous light. Sheaths with mature pre-penetration appressoria (AP, approximately 20

hpi); intracellular biotrophic hyphae, before symptoms or secondary hyphae were visible (BT, approximately 36 hpi); or necrotrophic hyphae, in which initial browning of the tissue and secondary hyphae were visible (NT, approximately 60 hpi), were collected. The *cpr1* mutant does not progress to the production of necrotrophic hyphae, and so only the AP and BT stages were collected for that strain. Each infected leaf sheath was inspected under the microscope to confirm the staging and to determine the extent of colonization. For the BT and NT samples, each examined sheath was gently cleaned with a moistened sterile cotton swab to remove unattached spores and superficial mycelia. The process of trimming, cleaning, and examination did not take more than two minutes per sheath. Approximately six trimmed tissue pieces were pooled into a single microfuge tube, flash-frozen in liquid nitrogen, and maintained at -80°C until RNA extraction.

Total RNA was extracted from the tissue pieces by crushing the frozen tissue followed by grinding in TRIzol (1 ml per 100 mg sample) (Invitrogen). To increase RNA yield, samples were incubated for 7 hours in isopropanol followed by 2 hours in 100% ethanol, both incubated at -20 ºC. To obtain high quality RNA, samples were purified and treated with DNAse using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's protocol (Metz *et al.*, 2006), and resuspended in 50µl of RNase free water. Extracts from approximately 30 leaf sheath pieces were pooled for each experimental repetition. RNA integrity number (RIN) and quantity were measured with an Agilent 2100 Bioanalyzer before sequencing.

RNA sequencing

Three-hundred µg of total RNA from three replicates of each treatment was submitted for sequencing to the Texas AgriLife Genomics and Bioinformatics Service Center (Texas A&M System). Fifteen *C. graminicola* libraries (3 WT-developmental stages, 2 MT developmental stages, 3 biological replicates of each) were prepared by using the Illumina TruSeqTM RNA Sample Preparation

Kit and the manufacturer's instructions. Data were generated from ten lanes of Illumina GAII sequencing, in two separate runs, with barcoding to multiplex biological replicates. Read lengths were 76 bp (including 7 bp for the barcode adaptor). For the first run, eight lanes of a flowcell were used and lane five was spiked with 1% PhiX as a control. For the second run, in which additional data for the WT AP was obtained, two lanes were used. Data were processed using the Illumina software CASAVA-1.7.0 for base calling and de-multiplexing, and the final results were stored as individual files for each sample in FASTQ format. These data were subjected to a statistical reanalysis for this dissertation by Noushin Ghaffari, Scott Schwartz, and Charlie Johnson, of the Texas AgriLife Genomics and Bioinformatics Service Center.

Alignment to reference genome and modeling

Fungal reads were mapped to the reference *C. graminicola* genome with TopHat (Trapnell *et al.*, 2009) by Stefan Amyotte. The counts per annotated gene per sample were obtained with the “coverageBed” function from the “BEDtools” suite and custom R scripts (O'Connell *et al.*, 2012). Using the total number of reads that mapped to each annotated *C. graminicola* gene (for each sample), we examined differential expression across multiple comparisons using R (Team, 2012). The comparisons we investigated are shown in Table 4.1, column 2.

We used a “Mixed Effects” Generalized Linear Model (GLM) to account for biological replication, timepoint and genotype effects. The goal of the analysis was to identify the genes that showed the strongest experimental responses between time points and genotypes. The mixed-effect GLM allowed us to leverage data from all time points simultaneously, in a unified analysis framework. The GLM improves the statistical power and inference capabilities, and can protect against multiple testing as compared to the pairwise testing paradigm through a single “no effects” test, of the type used in (O'Connell *et al.*, 2012). On the other hand, pairwise comparisons can be derived from the GLM structure where warranted.

We used the GLM implemented in the R package, edgeR (version 3.0.8) (Robinson *et al.*, 2010). The methods implemented in edgeR assume that the underlying distribution for the discrete count measures of the next generation sequencing (NGS) is negative binomial (NB). In conjunction with an internal, model-based normalization method, edgeR estimates mean and variance of the NB distribution for each gene (Dillies *et al.*, 2012).

Experimental analysis

Using the GLM we examined main effects and interactions across fungal genotype, developmental phase, and time. The Genotype variable captures the WT versus MT effect. The Time variable represents the effect of time points 20, 48, 60 hai: AP, BT, and NT. Therefore, our “Design_Variable”, which covers all the interactions between genotypes and time points, can be represented as the following equation: Design_Variable = Genotype*Time [http://www.clcbio.com]. The Design_Variable also included a coefficient for the MT-NT interaction, which does not exist in our dataset, so this column was manually removed. The final model includes four coefficients and their combinations. Each coefficient, or a combination of them, can produce a list of statistically significant genes for the comparisons that we were interested in. Using this model, we first tested the hypothesis that a given gene was changing at all among any of the groups: we filtered out the genes that were not significantly different among any of the groups, represented by the above-mentioned four coefficients of the model. We started with 12,006 genes, and this initial filtering reduced the set to 2,778 genes.

Additional Filtering

The default filtering method of the edgeR package is called “cpm”, which stands for Counts Per Million. This method takes a matrix of read counts and calculates the cpm for each sample. The user specifies a threshold for a minimum number of samples that should have a certain number of counts per million. This cpm method does not take the existence of sample replicates into account, and sometimes passes through samples in which one or more of the replicates have

unacceptably low read counts (i.e., background noise). Samples with very low read counts cannot be subjected to a valid statistical analysis for significance.

We developed a new filtering method that filters out genes based on the number of reads mapped to them individually, while considering the samples and their replicates. In this method, there were two levels of examination for each gene. In the first level, genes were retained if the total number of reads for all the replicates of at least one sample group was greater than 20. In the second level of filtering, the genes that didn't pass the first step were retested. In this round, genes that did not have at least three sample groups with a minimum of 15 total reads in each were filtered out. This mechanism ensured that samples with very few reads did not pass filtering. Applying our filtering method to the fungal dataset changed the number of differentially expressed genes from 2778 to 2442. The minimal number of reads for each step was determined empirically, to provide the best compromise between keeping reasonable genes and eliminating genes with too few reads for meaningful statistical analysis. When we examined the set of the filtered genes, we found that most of the excluded genes had very few, e.g. less than ten reads for all replicates of a sample, in most cases in more than one group.

Normalization and statistical testing

For the next step, we used the "DGEList" function of edgeR to create an object for each group. The DGEList object takes the table of counts and its grouping information as its minimum information. In our case, we had five groups: WT-AP, WT-BT, WT-NT, MT-AP and MT-BT.

The DGEList object contains count data that are filtered and ready for normalization. The default normalization method of the edgeR package is Trimmed Mean of M values (TMM). The TMM method uses a sample as the reference, and it assumes that most genes are not differentially expressed. As the result of the normalization, scaling factors are calculated for each gene (Robinson & Oshlack, 2010). The edgeR package provides a function to

calculate the scaling factors called “calcNormFactors”. With this function, normalization factors that account for different library sizes will be added to the DGEList object. Dividing the counts by the normalization factors normalizes the read counts (Dillies *et al*., 2012).

The edgeR package assumes that the underlying distribution for the RNASeq data is a negative binomial distribution. The dispersion for each gene needs to be estimated from the data. The package uses quantile-adjusted conditional maximum likelihood (qCML), and it is implemented within two functions, “estimateGLMCommonDisp” and “estimateGLMTagwiseDisp”. We decided to use “estimateGLMTagwiseDisp” because it estimates the dispersion for each gene individually, and the estimated value is more reliable for gene-by-gene comparisons.

Finally, the statistical testing was done using the “glmFit” and “glmLRT” functions. These functions use the gene-by-gene dispersions that were estimated in the previous step, the count data, and the normalization factors, and also the comparison at hand. The comparison is specified through the coefficient of the GLM model, and the glmFit function fits the negative binomial GLM for each gene. The results can be classified as the group of differentially expressed genes (up or down regulated) using the “decideTestsDGE” function. This function contains a multiple testing procedure and therefore, provides options to correct for the multiple testing effect. The default is false discovery rate (FDR) (Benjamini & Hochberg, 1995). In order to generate a list of significant genes, each gene needs to be tested multiple times against different competing cases. This will increase the possibility of randomly selecting genes. This is the multiple testing phenomenon. FDR is one way to correct for this issue. The FDR method adjusts the threshold so that the expected number of false positives will be acceptable with respect to the *p*-value and the total desired significant number of genes. It is one of the most popular and powerful statistical methods for p-value adjustment.

Heatmaps

Heatmaps were built by using Genesis (Release 1.7.6) (Sturn *et al.*, 2002), and represent log2-fold changes of a transcript in each of three fungal developmental stages, relative to the average expression across all stages.

Quantitative RT-PCR

RNA extraction from inoculated leaf sheaths

Leaf sheaths were inoculated with WT and MT strains, incubated, trimmed, and flash-frozen for RNA extraction as described above. Additional treatments were also tested in the (q)RT-PCR experiments. Tissue inoculated with the *Cpr1*-C strain, and sheaths that were mock-inoculated with water, were also collected, trimmed, and frozen for RNA extraction as described, at 20, 36 and 60 hpi.

RNA extraction from in vitro appressoria

Appressoria of the WT, MT, and *Cpr1*-C strains were produced *in vitro* on polystyrene Petri dishes as described by Kleeman and collaborators (Kleemann *et al*., 2008), with some modifications. *C. graminicola* spores were collected and washed as previously described in chapter 2 of this dissertation, and 40 ml of a spore suspension at a concentration of 2 x 10^4 spores/ml was added to each Petri dish. Twenty hours later, each plate was inspected under the microscope to verify the presence of mature melanized appressoria. Appressoria were broken and scraped from the bottoms of ten Petri plates, using a sterile culture spreader, into a total of five ml of TRIzol. Appressoria collected from 40 Petri plates were combined into each replicate. RNA purification, DNase treatment, and testing for RNA integrity and concentration were performed as described above.

cDNA synthesis and cycling reactions

The SuperScript II reverse transcriptase kit (Invitrogen) was used to synthesize the first strand of cDNA from 1 µg of DNase and RNase-treated total RNA in a volume of 20 µl. Primers were designed to amplify 100-200 bp fragments, using PrimerQuest (Integrated DNA Technologies) software. The reaction mix for real-

time PCR contained 0.4 mM of each primer, 10µl of SYBR green PCR Master Mix (AppliedBiosystems), 5 µl of a 1:5 dilution of the cDNA product, and DEPC water to a final volume of 20 µl. Cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. The reactions were carried out in fast 96-well reaction plates on the ABI 7900HT fast RT-PCR system (Applied Biosystems). Fungal transcript levels were normalized by using the fungal actin gene as an internal standard, and relative expression was calculated using the Pfaffl method (Pfaffl, 2001). Maize genes were normalized against the maize actin gene (Kankanala et al., 2007), and expression was calculated relative to mock-inoculated plants.

Validation of RNA sequencing data

To validate the RNAseq data, I used qRT-PCR to evaluate the expression levels of 34 *C. graminicola* transcripts encoding various secondary and primary metabolic proteins and house-keeping proteins.

Functional annotation and gene ontology

Nucleotide sequences similar to differentially expressed genes ($P<0.05$), were identified by BLASTx searches of the non-redundant database (E value 1E-3)of the Blast2go suite (Conesa & Götz, 2008). Functional characterization and gene ontology (GO) categories for cellular functions, cellular components, and biological processes, were also assigned using this platform. The GOSSIP function was utilized to determine GO term enrichment in different comparisons (Blüthgen *et al.*, 2004). Manual annotation of specific genes was performed using BLAST searches against the NCBI databases and InterproScan analysis. Cellular localization of significantly expressed transcripts was predicted by using WoLF PSort (http://wolfpsort.org/) (Horton *et al.*, 2007). Comparisons to *C. higginsianum* were performed by using the online Broad Institute *Colletotrichum* database(www.broadinstitute.org/annotation/genome/colletotrichum_group/Multihome.html).

Results

Nearly all of the predicted *C. graminicola* genes are transcribed during host colonization by the WT

A major advantage of the detached sheath assay for the transcriptome analysis was the ability to evaluate each infection site individually to confirm the stage of development. As I had observed previously (see chapter 2 for more details) there was no noticeable difference between the WT and MT strains in the timing or efficiency of appressorial production on maize sheaths at 24 hpi (Figures 4.1A and D). Thirty-six hours after inoculation, the WT had colonized 3-4 cells beyond the initial point of infection (Fig 4.1B), while the MT remained confined to the first invaded epidermal cell (Fig 4.1E). Sixty hours after inoculation, I observed thin secondary hyphae in the centers of the WT colonies, characteristic of the necrotrophic phase (Figure 4.1C). However, the MT still remained in the first invaded cell.

Ninety percent of the predicted *C. graminicola* genes (10,810/12,006) were transcribed *in planta* during at least one phase of development by the WT (O'Connell *et al*., 2012). After applying the statistical model described in the Materials and Methods section, above, a total of 2,355 genes (20%) were identified as statistically differentially expressed. Of these, I selected 1,883 genes that had a log2 fold change ≥ 2. My primary interest was in the differentially expressed genes that were associated with the transitions from pre-penetration appressoria to biotrophy, and from biotrophy to necrotrophy. There were 672 genes that were differentially expressed in appressoria versus biotrophy (WTAP-WTBT), and 821 in biotrophy versus necrotrophy (WTBT-WTNT) (Table 4.1). One hundred and fifty three genes were differentially expressed in both comparisons. The expression levels of most of these increased progressively from the appressorial to the biotrophic to the necrotrophic phases (Figure 4.2). A few genes had the opposite pattern, progressively decreasing in expression from the appressorial to the biotrophic to the necrotrophic phases (Figure 4.2). Among

the genes that were differentially regulated in only one comparison, 367 were significantly higher in appressoria than biotrophy, and 486 were significantly higher during necrotrophy than biotrophy. Only nine genes were identified that were significantly more highly expressed during biotrophy than in either the appressorial or the necrotrophic phases (Figure 4.2). These included two MFS transporters, three oxidoreductases, one gene involved in cobalamin (vitamin B12) biosynthesis, and three hypothetical proteins lacking any conserved domains. Two of these have orthologs in other *Colletotrichum* fungi, but the third gene had no homology to other reported fungal sequences.

For the MT, only two phases of development occurred in leaf sheaths (appressorial and biotrophy). Somewhat surprisingly, there were no significant differences in gene expression between the WT and MT appressoria, and only one gene was statistically differentially expressed in the MT fungus during the transition from appressoria to biotrophy (MTAP-MTBT). I also compared gene expression between WT biotrophy and MT biotrophy (WTBT-MTBT). A total of 267 genes were differentially expressed in this comparison.

Predicted secreted and plasma membrane-bound proteins are over-represented among differentially expressed genes during growth *in planta*

The *C. graminicola* genome is predicted to encode 1,650 secreted proteins, accounting for 13.7% of the total predicted genes. Another 14% are predicted to encode proteins that localize to the plasma membrane (Figure 4.3A). Analysis of the predicted localization of proteins encoded by fungal transcripts that are differentially expressed *in planta* revealed that secreted proteins are over-represented. In the WT, 36% and 33% of the transcripts that are differentially expressed in WTAP-WTBT, and in WTBT-WTNP, respectively, are predicted to encode secreted proteins (Figure 4.3B). Similarly, plasma-membrane associated proteins accounted for 23% and 19%, respectively, of these differentially expressed fungal transcripts. Among the 267 genes that are differentially expressed in the comparison of WTBT-MTBT, the degree of over-representation

was even higher, with 41% predicted to encode secreted proteins, and 26% predicted to encode plasma membrane proteins.

Gene ontology analysis of differentially expressed genes

I performed a functional annotation of differentially expressed transcripts by using Blast2GO (Conesa & Götz, 2008). Gene ontology (GO) terms were applied to 40%-67% of the differentially expressed transcripts (Table 4.2) in three GO categories: biological process (P); molecular function (F); and cellular component (C). Given that the number of transcripts in each of the comparisons varied, the cutoff in the annotation analysis also varied in each set, for both biological process and molecular function. Only GO categories that included at least 8% of the annotated sequences were analyzed.

Genes that were differentially expressed in the appressoria to biotrophy (WTAP-WTBT) comparison were associated with 22 GO terms that were significantly (P<0.05) overrepresented in appressoria-expressed genes (WTAP-WTBT_down), relative to biotrophy-expressed genes (WTAP-WTBT_up). The molecular function category included genes associated with oxidoreductase activity, oxidation-reduction processes, monooxygenase and transferase activity, and binding to iron ion, heme, proteins, vitamins, amino acids, carboxylic acid, and amines (Figure 4.4). For the biological process category, there were genes associated with electron transport and metabolism of amines, organic acids, carboxylic acids, acyl-carrier protein, and cellular amino acids. For the cellular component category, genes encoding intracellular and cytoplasmic proteins were enriched.

The transition to biotrophy was characterized by an overrepresentation of terms associated with hydrolase activity (Figure 4.5). Also, primary metabolism and transmembrane transport genes were enriched. Overall, biotrophy-expressed genes included more terms related to carbohydrate and protein metabolism and catabolism. In comparison with the appressoria-expressed genes, the biotrophy-expressed genes were enriched for the extracellular region.

Relative to necrotrophy, biotrophy-expressed transcripts in the WT (WTBT-WTNT_down) were enriched in 20 different GO terms, including oxidoreductase, cellular catabolism, peptide and phosphate hydrolytic activities, and vitamin metabolism (Figure 4.6). Two categories potentially associated with secondary metabolism, dimethylallyltransferase activity and alkaloid metabolism, were also overrepresented in this comparison.

The necrotrophic stage in the WT was accompanied by a significant enrichment in terms related to hydrolase and glycosidase activities and cellulose binding (Figure 4.7). Consistent with this, genes encoding proteins associated with the extracellular region were overrepresented among necrotrophy-expressed transcripts.

In the comparison of the mutant appressoria to mutant biotrophic phase, only a single gene was significantly differentially expressed. This gene was not annotated by Blast2GO, but appears to encode a small, secreted hypothetical protein that is unique to *C. graminicola* according to NCBI Blast searches. The gene was more highly expressed during MT biotrophy.

Most of the genes that were not annotated by Blast2GO are predicted to encode secreted proteins

A total of 676 of the differently expressed genes (38%) were not assigned GO terms by Blast2GO (Table 4.2). Among these non-annotated genes, 278 (41%) were predicted to encode secreted proteins (Figure 4.8A-B). Many fungal effector proteins are small (<300 aa), secreted, cysteine rich (>3% cys) hypothetical proteins, induced *in planta* and functioning to facilitate pathogen colonization (Kamper et al., 2006, O'Connell *et al.*, 2012, De Wit *et al.*, 2009, Rooney *et al.*, 2005). Forty-two of the non-annotated genes (6%) are predicted to encode small, cysteine rich, secreted putative effector proteins (Table 4.3). The smallest group of these putative fungal effectors (five) was found among the set of genes more highly expressed in necrotrophy versus biotrophy (WTBT-WTNT_up) (Figure 4.8B, Table 4.3). The largest group (18) was found among genes that were more

highly expressed in biotrophy versus necrotrophy (WTBT-WTNT_down) (Figure 4.8B, Table 4.3). Comparisons of the WT and MT during biotrophy revealed that five putative effectors were more highly expressed in the WT (WTBT-MTBT_down), while two were more highly expressed in the mutant (WTBT-MTBT_up) (Figure 4.8C).

Twenty of the 676 non-annotated genes had previously been recognized in *C. graminicola* as predicted secreted proteins, including ten that had been identified as putative effectors (Krijger *et al*., 2008, Vargas et al., 2012) (Table 4.3). Four were similar to effectors identified in *C. truncatum* on lentil (Bhadauria *et al*., 2011) and ten were similar to effectors previously reported from *C. higginsianum* on Arabidopsis (Kleemann *et al*., 2012) (Table 4.3).

Most genes differentially expressed between WT biotrophy and MT biotrophy are also differentially expressed between WT appressoria and WT biotrophy

Blast2GO analysis was applied to the group of genes that were differentially expressed in WT biotrophy relative to the biotrophy in the MT (WTBT-MTBT). Genes with a higher level of expression in the MT (WTBT-MTBT_up) relative to the WT were enriched in 3 GO terms: nucleotide binding, acyl carrier protein biosynthesis, and vitamin binding (Figure 4.9A). No significant enrichment in GO terms was identified among genes with higher levels of expression in WT biotrophy relative to MT biotrophy (WTBT-MTBT_down).

I found that 224 (89%) of the 267 genes that were differentially expressed in the WTBT-MTBT comparison were also differentially expressed in the WTAP-WTBT comparison (Figure 4.9B). All of the genes that were significantly more highly expressed in biotrophy versus appressoria in the WT (WTAP-WTBT_up), were also significantly higher in the WT biotrophic phase compared with MT biotrophy (WTBT-MTBT_down) (Figure 4.10A). Similarly, all of the genes that had lower expression in biotrophy versus appressoria in the WT, also had lower expression in the WT biotrophic phase relative to MT biotrophy (WTBT-MTBT_up) (Figure 4.10A).

Analysis of predicted cellular localization for the 43 genes that were unique to the WTBT-MTBT comparison did not suggest any obvious differences compared to the 224 genes that were shared (Figure 4.10B). No significant enrichment in GO terms was found for these 43 genes, compared to genes that are differentially expressed in the transition to biotrophy in the WT (WTAP-WTBT).

Patterns of expression of predicted SM clusters

Forty-two putative SM clusters were predicted by the SMURF program, based on identification of key SM enzymes and similarities of neighboring genes to other identified fungal clusters (see chapter 3 of this dissertation for details).

Only four SMURF predicted clusters were obviously co-regulated, which I defined as having most or all of the genes in the cluster significantly differentially expressed, and with the same pattern of expression. These clusters will be discussed individually below.

The *C. graminicola* PKS cluster 18 was predicted by SMURF to contain 24 genes (O'Connell *et al.*, 2012). Fourteen of the genes were orthologous to genes in cluster 10 of *C. higginsianum* [15]. These genes in the *C. higginsianum* cluster 10 were highly expressed, and co-regulated, during appressorial development in that fungus [15]. However, the orthologous genes in cluster 18 were expressed poorly, or not at all, in maize sheaths during all three phases of development [15]. I tested three of these genes (GLRG_08617, GLRG_08620, and GLRG_08621) by qRT-PCR, and I confirmed that expression was not detectable during any stage (not shown). The other ten genes that were predicted to belong to cluster 18 had orthologs in *C. higginsianum*, but these were scattered throughout the genome. Interestingly, 90% of these genes were expressed at a significantly higher level in appressoria relative to biotrophy (WTAP-WTBT_down) (Figure 4.11A). Only one gene, a putative transcription factor, was not differentially expressed at any stage of fungal colonization analyzed (Figure 4.11A).

All of the genes predicted to belong to PKS cluster 35 were significantly more highly expressed in pre-penetration appressoria compared with biotrophy (WTAP-WTBT_down) (Figure 4.11B). Eleven genes were predicted as part of the PKS-NRPS hybrid cluster 22. Nine of these were significantly induced during necrotrophy (WTBT-WTNT-up) (Figure 4.11C). Four of the five genes predicted as part of cluster 38 were also significantly induced in necrotrophy (WTBT-WTNT_up) (Figure 4.11D). Only GLRG_11836, a putatative PKS, was not differentially expressed at any stage of fungal colonization.

Only one gene from cluster 22 (GLRLG_09715, a PKS-NRPS hybrid) and one from cluster 35 (GLRG_11776, a putative salicylate hydroxylase) were significantly different in WT versus MT biotrophy. Five additional SM-associated genes were differentially expressed between the WT and mutant biotrophic stages: these included GLRG_08063 (cluster 15); GLRG_08212 (cluster 16); GLRG_09073 (cluster 20); GLRG_09837 (cluster 23); and GLRG_11503 (cluster 31).

RNA sequencing validation

Thirty-four genes were selected to validate the RNA-sequencing results. Log2 transcript fold-changes (AP vs BT, BT vs NT, AP vs NT) measured by both RNAseq and qRT-PCR were plotted to measure the correlation between gene expression profiles (Figure 4.12). A linear regression value of R^2=0.84, and a slope of y=0.75, indicated that the data were consistent [15].

Quantitative reverse transcriptase PCR analysis of selected genes

The expression of selected genes was also evaluated by (q)RT-PCR. RNA isolated from mature appressoria induced on artificial surfaces (IV-AP) and leaf sheaths inoculated with the *Cpr1*-C strain were also included. The actin gene was utilized for normalization of CT values. This gene has been identified to be a good normalization factor in different RT-PCR experiments in other fungi, and in leaf sheath experiments (Kankanala et al., 2007, O'Connell et al., 2012, Balmer

et al., 2013). Each experiment was repeated twice. Table 4.4 contains the average relative level of expression, standard deviation, and fold change of the averaged value for each gene in each comparison.

Expression of *Cpr1* was increased in all three strains in appressoria *in planta* relative to appressoria on artificial surfaces (IV-AP) (Figure 4.12). During biotrophy, expression was reduced in all three strains, and then it increased slightly during necrotrophy in the WT and Cpr1-C strains. Expression of *Cpr1* in MT IV-AP was reduced 8-fold, compared with the WT and *Cpr1*-C strains, but surprisingly, levels of expression *in planta* for all three strains during AP and BT were similar.

Expression of SM-associated genes in mutant versus WT

Two genes from cluster 18, two from cluster 22, and two from cluster 35, were confirmed to be differentially expressed between WTAP and WTBT (Figure 4.14). Five of the six genes were more highly expressed in appressoria *in planta*, relative to IV-AP, in all three strains. The exception was GLRG_08628, one of the cluster 18 genes. I was able to confirm that both genes in cluster 18 and both genes in cluster 35 are expressed at lower levels during biotrophy relative to appressoria (Figure 4.14). However, expression of genes in cluster 22, indicated in the RNAseq analysis to have higher expression in necrotrophy relative to biotrophy, was in fact higher during necrotrophy according to the (q)RT-PCR data (Figure 4.14). Expression of these SM-associated genes in the MT was comparable to the WT in all cases. However, I observed a few differences between the *Cpr1*-C strain and the WT. For example, expression of the cluster 35 genes in the *Cpr1*-C strain during biotrophy relative to necrotrophy was much lower, compared with the WT and mutant.

Expression of effector proteins in mutant versus WT

Expression of six putative effector proteins was evaluated by (q)RT-PCR. GLRG_06284 and GLRG_00201, homologs of two biotrophy-associated

secreted (BAS2 and BAS3) protein effectors in *M. oryzae* (Mosquera *et al.*, 2009b), and GLRG_07767 and GLRG_01192, homologs of two *C. higginsianum* effector candidates (Kleemann et al., 2012), were chosen for analysis. In addition, the single gene that was differentially regulated in the MT during biotrophy relative to appressoria (GLRG_03688), and one of the most highly expressed putative fungal effector genes (GLRG_07776), were also tested.

Expression of most of the putative effectors was higher in appressoria *in planta* versus in IV-AP in all three strains. The exceptions were GLRG_01192 and BAS2 (Figure 4.15) Expression levels of the BAS2 and BAS3 orthologs were 2-3 fold higher in appressoria than during biotrophy. Additionally, expression of BAS3 was 3-4 fold higher during biotrophy relative to necrotrophy. GLRG_07767, a predicted LysM containing protein, was progressively reduced in expression during biotrophy and necrotrophy. GLRG_07776, one of the most highly expressed fungal genes, was induced *in planta*. Expression of this gene was slightly reduced in biotrophy relative to appressoria, and expression in necrotrophy was reduced 2.2-fold compared with biotrophy.

Expression patterns for most of the effector genes did not seem to differ much in the MT compared with the WT and Cpr1-C complemented strains. An exception was GLRG_01192, a predicted HR-inducing protein, which was 2-3-fold more highly expressed during biotrophy versus appressoria in the WT and *Cpr1*-C complemented strain, but appeared unchanged in the mutant. Expression of the putative small secreted effector GLRG_03688 was highly induced in the WT and *Cpr1*-C complemented strain, by 4.2 and 4.4-fold, respectively. In the MT, expression of this gene is reduced 3.3-fold in comparison with the WT. Comparison of GLRG_03688 with other sequenced *Colletotrichum* genomes, indicated that this gene is present only in the closely related sorghum pathogen *C. sublineola.*

Maize leaf sheaths respond differently to WT and *cpr1* mutant infections

Basal host defense responses to the MT and WT strains at different stages of infection were studied by evaluating expression of resistance genes by (q)RT-PCR. The genes were chosen for the study based on reports in the literature of their response to *C. graminicola* or to biotrophic pathogens (van der Linde *et al.*, 2011, Vargas et al., 2012, Balmer et al., 2013, Doehlemann et al., 2008). Levels of expression for genes *in planta* were normalized against RNA extracted from mock-inoculated leaf sheaths.

Expression of the salicylic acid (SA)-associated genes PR1, PR3, and PR5; the jasmonic acid (JA)-induced Bowman-birk trypsin inhibitor (*Bti*); and a cell death inhibitor (*Bi-1)* (van der Linde et al., 2011) in WT, MT and Cpr1-C strain interactions, relative to mock-inoculated plants, is shown in Figure 4.16. Although in some cases there were significant differences in the values, overall patterns of relative expression of maize defense genes in response to the WT and the Cpr-1 strains were similar. All the defense genes were highly expressed in response to appressoria, prior to penetration. Expression of PR3, Bti, and Bi1 was reduced during BT vs AP, while PR1 expression was increased, and PR5 was unchanged. During NT, expression of PR3 increased relative to BT, while PR1 expression decreased, and expression of the rest of the genes was unchanged.

Expression of maize defense genes in response to the MT versus the WT differed in several cases. In appressoria, expression of PR3, *Bti*, and *Bi-1* was at least twice as high in plants that were responding to WT appressoria relative to the MT. Expression of PR3 was reduced during BT in the WT to approximately half of the expression level in the MT strain during BT.

Discussion

”Waves” of fungal genes are differentially expressed across sequential stages of maize infection and colonization

C. graminicola is a hemibiotroph, and various studies, including this dissertation, have described the process by which it infects and colonizes maize tissues (Politis & Wheeler, 1973, Mims & Vaillancourt, 2002, Venard & Vaillancourt, 2007b). There are three recognizable phases of pathogenic development: appressoria that form on the surface prior to penetration; biotrophy, characterized by thick primary hyphae, surrounded by a membrane, that initially colonize living host cells; and necrotrophy, identified by production of narrow necrotrophic hyphae and concurrent degradation of host cell walls and development of symptoms. In this study, I identified and characterized 1,340 genes that are differentially expressed in “waves” across these three phases of development, including 193 genes that are expressed primarily or only in pre-penetration appressoria, and 608 that are expressed primarily or only during necrotrophy. Only nine genes were identified as biotrophic specific. I attribute the relatively low number of statistically significant biotrophy-specific genes to the fact that in *C. graminicola*, biotrophy consists of a mixture of cell types, including numerous pre-penetration appressoria, biotrophic primary hyphae entering living host cells, and intercalary primary hyphae that are occupying cells that are already dead or dying. Furthermore, necrotrophy is also a mixed culture, with necrotrophic hyphae produced in the center of the colony, but persistence of biotrophy at the colony edges. This lack of synchronicity would be expected to mute potential differences in gene expression across cell types. In *C. higginsianum*, which infects Arabidopsis, the biotrophic phase is limited to a single epidermal cell, and then there is a complete switch to necrotrophy (O'Connell *et al.*, 2012). Production of primary hyphae from appressoria is also more synchronous and efficient in Arabidopsis, which does not seem to have germination self-inhibitors like *C. gramincola* does. Many more genes were statistically differentially

expressed in biotrophy in *C. higginsianum*, probably because of this relative synchronicity(O'Connell et al., 2012).

Functional interpretation of the patterns of fungal gene expression during developmental transitions *in planta*

Pre-penetration appressoria were highly active in expression of a unique set of genes, including some genes that would be expected to play roles in signaling or manipulating the host. Thus, functional annotation and significant enrichment analysis indicated that SM-associated and detoxification proteins seem to be important during appressorial stages. GO terms related to monooxygenase activity, acyl carrier protein biosynthesis, phosphatentheine binding and amino acid binding, mostly described cytochrome P450s, PKSs and NRPSs. Iron ion binding and heme binding categories also included cytochrome P450s and peroxidases. Both of these classes of genes are associated with oxidation of potential toxic compounds (Meunier *et al.*, 2004), and defense against plant ROS. Deletion of a secreted peroxidase leads to an increase in H_2O_2 in appressoria of *M oryzae* infecting rice leaf sheaths, and delayed host colonization, but it did not prevent symptom development (Tanabe *et al.*, 2011). Other GO terms enriched in appressoria-induced genes were amino acid biosynthetic processes and cellular amine metabolism. Genes in this category included an aminotransferase (GLRG_09800), ProDH (GLRG_07259) and P5CDH (GLRG_06830), all genes involved in proline degradation (Deuschle *et al.*, 2004). In plants, proline accumulation is linked to the synthesis of phenolic compounds, callose deposition and ROS accumulation (Shetty, 2004, Deuschle *et al*., 2004).

During appressorial development, *C. graminicola* seems to encounter oxidative stress, possibly originating from the host, and an active detoxification system could be required for successful colonization. Recent studies have demonstrated that putative effectors can be detected in the appressorial pore, even before penetration (Kleemann *et al.*, 2012), and the authors suggest that active

secretion of fungal effectors could be important to suppress host defense and reprogram host metabolism to predispose host cells for fungal invasion.

The transition from appressoria to biotrophy appears to involve increased transcription of genes associated with activation of secreted proteases, indicated by enrichment in GO terms associated with peptidase activity, protein metabolism, proteolysis, exopeptidase and carboxypeptidase activities. Fungal proteases have been associated with various roles in pathogenicity of plants (ten Have *et al.*, 2004, Murphy & Walton, 1996, Van den Ackerveken *et al.*, 1993). Some proteases from plant pathogenic fungi can degrade host chitinases and induce host cell death (Movahedi & Heale, 1990, Valueva & Mosolov, 2004). Many of the genes within these categories are predicted subtilisin-like proteases, a class of serine proteases. Subtilisins are pathogenicity determinants in the entomopathogenic fungus *Metarhizium anisopliae*, where the serine protease Pr1 is induced during host-cuticle penetration (St. Leger *et al.*, 1992). Subtilisins have been implicated in penetration and colonization by plant pathogenic fungi, since they have the potential to degrade cell wall proteins and plant defense proteins (Olivieri *et al.*, 2002).In *M. oryzae*, targeted deletion of the subtilisin *Spm1* severely compromised pathogenicity in rice plants (Oh *et al.*, 2008)

Carboxypeptidases were also significantly induced in the transition to biotrophy. Carboxypeptidases cleave the C-terminus peptide bonds of polypetides (Folk *et al.*, 1960), but their role in fungal plant pathogens is not clear. Mutant rice plants that transiently expressed a carboxypeptidase inhibitor from potato (PCI), were highly resistant to infection by the blast fungus *M. oryzae* and the root pathogen *F. verticillioides* (Quilis *et al.*, 2007). The authors proposed that fungal carboxypeptidases could be involved in cell wall biosynthesis, by processing and maturation of chitin synthase (Machida & Saito, 1993), degradation of plant cell wall proteins, and amino acid uptake.

One third of the biotrophy-specific genes in *C. graminicola* were predicted to encode putative effectors. Other genes with expression elevated during biotrophy

included some associated with aspartic peptidase activity. Aspartic proteases can have lytic activity against peptide bonds of multiple proteins, and could be involved in host cell wall degradation, or inactivation of host proteases (Movahedi & Heale, 1990, Plummer *et al.*, 2004). GO terms associated with vitamin B6 biosynthesis (vitamin biosynthetic process, pyridoxal phosphate biosynthetic process and vitamin B6 biosynthetic process), were also enriched in biotrophy. Vitamin B6, also known as pyridoxine, is a cofactor in multiple enzymatic reactions, especially associated with amino acid metabolism (Percudani & Peracchi, 2003). Pyridoxine has also recently been associated with antioxidant activities and resistance to oxidative stress in plant and fungi (Bilski *et al.*, 2000, Titiz *et al.*, 2006).

I observed that ROS seems to accumulate even before appressorial penetration, but is no longer detected in maize leaf sheaths at 48hpi, when biotrophic growth is established. This suggests that fungal proteases and active detoxification mechanisms could be important to overcome defense mechanisms and establish a successful biotrophic interaction.

Finally, the switch to necrotrophy was associated with an enrichment in GO terms related to glycosyl hydrolase activities, most likely involved in degradation of host cell walls. Cell wall degrading enzymes are utilized for host penetration and colonization, and also to obtain nutrients from plant polymers (Walton, 1994). This observation is similar to what has been described for necrotrophic plant pathogens, which normally secrete large quantities of cell wall degrading enzymes during host colonization (Amselem *et al*., 2011, Daub, 1982).

Genes encoding putative secreted effectors are usually expressed at higher levels during early stages of development *in planta*

Bioinformatic predictions of cellular localization of proteins encoded by the 1,383 differentially expressed genes suggested that one third of these proteins are secreted. These results are similar to a previous study that investigated genes induced in *C. graminicola*-inoculated maize stalks (Tang *et al*., 2006). Those

authors reported that 22% of the *in planta* induced fungal genes were predicted to encode extracellular proteins, versus only 13% of the transcripts induced *in vitro*. All three stages of development were associated with expression of numerous genes predicted to encode secreted and membrane-bound proteins. Each stage was characterized by production of a distinctive subset of these genes, presumably with distinct functions in the host-pathogen interaction.

During host colonization, biotrophic plant pathogens secrete small effector proteins that are involved in suppression of the host defense response and reprogramming of host metabolism (Doehlemann *et al*., 2009, Catanzariti *et al.*, 2006, Kamper *et al.*, 2006). These effector proteins are typically cysteine-rich, and poorly conserved among different species or genera of fungi. Among the non-annotated genes, I identified 37 predicted to encode small, cysteine rich, secreted hypothetical proteins, the majority of which were expressed more highly in appressoria and during biotrophy relative to necrotrophy. Two of these, GLRG_03688 and GLRG_07776, were also evaluated by (q)RT-PCR, which confirmed this pattern of early expression. GLRG_07776 was one of the most highly expressed fungal genes *in planta*.

I also identified orthologs of several known fungal effector protein genes, including BAS2 (GLRG_06284) and BAS3 (GLRG_00201) from *M. oryzae* (Mosquera et al., 2009b). Expression of both of these genes was elevated in appressoria and biotrophy of *C. graminicola*. In *M. oryzae*, BAS2 and BAS3 were two of the most highly expressed genes during biotrophic colonization of rice plants (Mosquera *et al*., 2009b). However, deletion of either gene did not affect pathogenicity in rice leaf sheaths.

Another conserved non-annotated gene, GLRG_01192, is an ortholog of CgEC91, a hypersensitive-response inducing protein effector, induced during the switch to necrotrophy in *C. higginsianum*. Expression of this gene was 6-fold increased in biotrophy relative to appressoria in the RNAseq data, although my (q)RT-PCR results suggested it was only 1.8-fold higher. GLRG_07767 is an

ortholog of ChEC90, a LysM containing protein similar to CIH1 from *C. lindemuthianum*, involved in fungal cell wall protection against plant chitinases (Perfect *et al.,* 1998b). GLRG_07767 was 2-5 fold more highly expressed in biotrophy and appressoria relative to necrotrophy.

Analysis of the expression of these six putative effectors in appressoria produced *in planta* vs. *in vitro* indicated that all of them were plant-induced, another typical characteristic of fungal effectors. In both *C. higginsianum* and *C. obiculare,* appressoria and primary hyphae were the primary sites for expression of effectors (Gan *et al.*, 2013). These organs were proposed to function primarily as secretory organs for the production of these proteins for modification of the plant environment and induction of compatibility (Gan *et al*., 2013). My data suggest a similar pattern in *C. graminicola*.

Predicted SM clusters are expressed at both early and late stages of host colonization

In *C. higginsianum*, expression of numerous SM-associated genes occurred primarily during the appressorial and biotrophic phases of development. It was suggested that these SM acted as effectors that promoted susceptibility, rather than as toxins that killed host tissues (O'Connell *et al.,* 2012). I verified co-regulation of four potential SM clusters in *C. graminicola*. Clusters 18 and 35 were significantly induced in appressoria, while clusters 22 and 38 were induced during necrotrophy. Induction of SM during early stages of development has been described in other intracellular hemibiotrophs including *C. higginsianum* (O'Connell *et al*., 2012); *C. obiculare* (Gan *et al*., 2013); and *M. oryzae* (Fudal *et al.*, 2007, Collemare *et al.*, 2008a). This behavior is not typical of biotrophs, which produce relatively few SM. Expression of SM genes during necrotrophy is more expected, given that SM are usually associated with necrotrophic plant pathogens (Brosch *et al.*, 1995, Amselem *et al.*, 2011).

Only four of the 42 clusters predicted by SMURF were clearly co-regulated. However, this may be due to low to moderate levels of expression for many of

the genes, which made it impossible to distinguish them statistically across the different phases of development. The highly conserved melanin cluster was not predicted by SMURF. Only two genes from the cluster (GLRG_04203, a PKS and GLRG_04204, a multicopper oxidase) had significantly higher levels of expression in the appressorial stage relative to biotrophy and necrotrophy, as I would have expected. This suggests that low levels of fungal biomass, and high levels of variation, especially during the early stages of infection, may affect my ability to statistically identify genes that are differentially expressed.

Cluster 18 contains 24 genes and is orthologous to cluster 10 in *C. higginsianum* (O'Connell *et al.*, 2012). Cluster 10 was highly induced *in planta*, during appressoria and biotrophic stages in Arabidopsis (O'Connell *et al.,* 2012). According to the RNAseq data, the 15 genes that are highly conserved between cluster 18 and cluster 10 were not expressed above background levels at any stage of development in *C. graminicola*. However, expression of the 10 genes predicted in cluster 18 by SMURF that are not shared with the *C. higginsianum* cluster, was increased significantly in appressoria relative to biotrophy. The single exception was GLRG_08631, a putative fungal transcription factor (TF). TFs are sometimes regulated post-transcriptionally: thus, a lack of co-expression doesn't necessarily mean that this TF doesn't play a role in the cluster. Interestingly, *C. higginsianum* does not have this part of cluster 18, suggesting that the 24 genes predicted as part of cluster 18 could actually represent two separate clusters in *C. graminicola*. The backbone in this second cluster is the nrPKS GLRG_08632. My phylogenetic analysis determined that this gene is an ortholog of C_heterostrophus_PKS20, an uncharacterized gene, and is related to the PKS required for melanin biosynthesis. An extensive phylogenetic analysis (Kroken *et al*., 2003b), determined that C_heterostrophus_PKS20 was related to PKS14 and PKS19 in the necrotrophic pathogen *B. cinerea*. None of these genes has been functionally characterized.

Phylogenetic analysis of the two PKSs in cluster 35 indicated that GLRG_11778 is related to G_zeae_PKS13 and the RADS2 genes, and GLRG_11770 is related to G_zeae_PKS4 and the RADS1 genes. G_zeae_PKS13 and G_zeae_PKS4 are required for the synthesis of zearalenone (ZEA) in *Fusarium graminearum* (Kim *et al.*, 2005b). Two additional genes, ZEB1, an isoamyl alcohol oxygenase and ZEB2, a fungal transcription factor, are part of the ZEA biosynthesis cluster. BLAST searches of the *C. graminicola* genome indicated that genes in cluster 35 did not contain putative homologs of ZEB1 or ZEB2. No similarities to any other known SM biosynthetic cluster were found for this group of genes.

Four PKS-NRPS hybrids (ACE1, SYN2, SYN6 and SYN8) were found to be highly induced during early penetration stages in rice (Böhnert *et al.*, 2004, Collemare *et al*., 2008a). My phylogenetic analysis indicated that the backbone of cluster 22, GLRG_09715, is related to SYN8. In a phylogenetic study, comparisons among some previously characterized PKS-NRPS hybrids indicated that SYN8 did not seem to have orthologs in other fungal species (Collemare *et al*., 2008a). Other identified SM products that require the action of a PKS-NRPS hybrid include compactin in *Penicillium citrinum* (Abe *et al.*, 2002), equisetin in *F. heterosporum*(Sims *et al.*, 2005) and fusarin C in *F. moniliforme* (Song *et al.*, 2004). BLAST searches of the *C. graminicola* genome, indicated that cluster 22 did not contain orthologs of any of the genes in these biosynthetic clusters.

Based on sequence analysis of the RADS cluster in *P. chlamydosporia*, in chapter 3, I described the possibility that cluster 38 is involved in the synthesis of monorden and monocillins in *C. graminicola*. Although the nrPKS GLRG_11836 was not significantly induced during necrotrophy, results from phylogeny and synteny analyses suggest that this gene is also part of the biosynthetic cluster (Reeves *et al.*, 2008). Monorden and monicillins I, II and III had been identified in *C. graminicola*-infected stalks, and it was proposed that this inhibitor of HSP90, could be involved in suppression of basal defense responses during early infection and biotrophic colonization of maize (Wicklow *et al.*, 2009). However,

my expression analysis suggested that genes in cluster 38 are most highly expressed during necrotrophy. Radicicol has been involved in generation of reactive oxygen species (ROS) and induction of programmed cell death in animal systems (Kim *et al.*, 2012, Compton *et al.*, 2006). It is possible that monorden plays a role in induction of host cell death during the switch to necrotrophy in maize. The role of monorden in late stages of maize colonization needs further study.

Transcriptome profiling reveals similarities and differences to both biotrophic and necrotrophic fungal plant pathogens

Genes that are expressed during early stages of infection in *C. gramicola* (appresoria and biotrophy), have some similarities to genes expressed in plant-biotrophic interactions. Analysis of gene expression of the biotroph *Uromyces fabae* colonizing bean leaves allowed the identification of various *in planta* induced genes (PIGs) (Hahn & Mendgen, 1997). These included genes involved in amino acid metabolism, electron transport, transmembrane transport and detoxification (Jakupović *et al*., 2006). Detoxification genes included cytochrome P450s and peroxidases, also overrepresented in the appressorial stage of *C. graminicola.* A gene involved in H_2O_2 detoxification is also required for virulence of the biotrophic pathogen *Ustilago maydis* (Molina & Kahmann, 2007). Amino acid metabolism and electron transport are also among the overrepresented categories in *C. graminicola* appressorial stage. Two of the most highly expressed genes in the haustoria of bean rust are predicted to be involved in vitamin B1 (thiamine) biosynthesis. Thiamine biosynthesis genes were also highly expressed in wheat leaves inoculated with the rust pathogen *Puccinia triticina* (Thara *et al.*, 2003). Vitamin metabolism also appears to be important during the biotrophic phase of *C. graminicola* in maize. As vitamins B6 and B2, vitamin B1 is a cofactor of different enzymes, many of them involved in carbon metabolism (Sohn *et al.*, 2000). It is suggested that induction of thiamine biosynthetic genes in rust fungi is associated with synthesis of metabolites to

support fungal growth *in planta* (Sohn *et al*., 2000, Thara *et al*., 2003). Genes associated with transmembrane transport were also overrepresented in biotrophy relative to appressoria.

Interestingly, biotrophy-expressed genes in *C. graminicola* also share similarities with transcripts expressed during plant colonization by necrotrophic pathogens. Zhuang and collaborators recently reported a transcriptome analysis of lesions colonized by the necrotrophic pathogen *Sclerotinia sclerotiorum* on stems of pea plants (Zhuang *et al.*, 2012). Predicted functions of fungal genes expressed during colonization included host cuticle and cell wall degradation, fungal cell wall biosynthesis, transport, regulation of transcription, and acid proteolysis. Membrane transporters and aspartic acid proteases were also significantly more highly expressed during biotrophy in *C. graminicola*. Aspartic proteases (*Apr*) have been identified in *B. cinerea* inoculated carrots, cabbage and grapes (Movahedi & Heale, 1990). Application of the purified enzyme from *B. cinerea* induced cell death in carrot cell cultures, and inhibition of the enzymatic activity significantly reduced virulence. The authors suggest that *Apr* is crucial for induction of cell dead during tissue colonization by *B. cinerea*. In contrast, in *C. gloeosporioides* isolated from apple (*Malus domestica*), the aspartic protease Cgsap was expressed during appressorial formation, but targeted deletion had no effect on symptom development compared to the wild type (Plummer *et al.*, 2004). As mentioned previously, aspartic proteases can have lytic activity on peptide bonds from multiple proteins, and could be involved in host cell wall degradation, or inactivation of host proteases.

An RNA-sequencing study of *Sclerotinia homeocarpa*, the causal agent of dollar spot disease in creeping bentgrass, indicated that 22% of the transcripts induced *in planta* 96 hpi encoded glycosyl hydrolases (Orshinsky *et al.*, 2012). Secreted proteases and transporters were also overrepresented in infected tissue compared to mycelium grown *in vitro*. In our study, necrotrophy-expressed genes were enriched in glycosyl hydrolase activities, and this expression was

associated with visible host cell wall degradation. The behavior of *C. graminicola* during this phase resembles the descriptions of necrotrophs. However, my cytological studies described in chapter 2 also show that the borders of the fungal colony remain biotrophic, a behavior that seems to be shared by *C. graminicola* and the closely related *C. sublineola*, but not by *Colletotrichum* species infecting dicots. I assume that genes are differentially expressed in the hyphae in the colony centers versus at the colony edges, so that these two stages can coexist. Further, my observations in chapter 2 suggest that genes are expressed, most likely in these leading hyphae, that encode substances that diffuse out ahead of the colony and promote susceptibility of the surrounding living cells. In some way, the fungus must "disguise" the extreme damage that it is causing in the necrotrophic center from the cells beyond the colony border, so that they do not become sensitized, resulting in induced inaccessibility.

Plant defense responses also suggest a combination of both lifestyles

Expression of some plant defense-associated genes was induced even before pathogen penetration. PR1, PR3 and PR5 were also reportedly induced during early infection stages of *C. graminicola* on intact leaf blades (Vargas *et al*., 2012). PR1 was detected by 12 hpi, and expression of this gene continued to increase until 72 hpi. Another study indicated that PR1 was induced by 24 hpi, increasing up to 800-fold by 48 hpi, and then decreasing 500-fold by 96 hpi (Balmer et al., 2013). Expression of PR genes is associated with induction of the salicylic-acid (SA) defense pathway. Balmer and collaborators determined that in maize, salicylic acid accumulated at 36 and 96 hpi, while jasmonic acid (JA) was detected 96 hpi with *C. graminicola* (Balmer et al., 2013). The Bowman-birk trypsin inhibitor (*Bti*) is a marker for induction of jasmonic acid (JA) (Rakwal *et al.*, 2001). Expression of *Bti* was significantly higher in response to the appressorial stage in the WT compared with the MT. Expression of *Bti* was also significantly induced in maize leaves 24 hpi with the biotrophic *U. maydis*, however silencing of this gene did not affect pathogen colonization (van der Linde *et al.*, 2011). SA-

dependent pathways are typically deployed against biotrophic plant pathogens, while JA-mediated pathways are usually associated with plant responses against necrotrophic pathogens (Govrin & Levine, 2000). Arabidopsis plants that are impaired in SA-accumulation are more susceptible to biotrophic pathogens, while defects in JA-signalling result in increased susceptibility to necrotrophic pathogens (Thomma *et al.*, 1998). The role of SA and JA signaling defense pathways is less clear for hemibiotrophic plant pathogens, but my data suggests that both are active, possibly in different parts of the infection.

Bax-inhibitors (*Bi*), can suppress programmed cell death (PCD) in plants (Hückelhoven, 2004). Expression of the Bax-inhibitor *Bi1* was significantly induced in response to appressoria of the WT versus the MT. The MT was still able to penetrate maize epidermal cells, suggesting that expression of this gene is not crucial for initial penetration: however, it is interesting to speculate that it plays some role in delaying cell death to allow establishment of the primary hypha. In barley, expression of the Bax-inhibitor *Bi1* was induced during infection by the obligate biotrophic pathogen *Blumeria graminis* f.sp. *hordei* (Eichmann *et al.*, 2004). Similary, silencing of *Bi1* significantly reduced infection while overexpression induced susceptibility to biotrophic pathogens (Eichmann *et al*., 2004, Doehlemann *et al.*, 2008). Another Bax-inhibitor was expressed in maize leaf blades 48 hpi with *C. graminicola* (Vargas *et al.*, 2012). This suggests that cell death inhibitors in addition to the one tested in my study could be important during biotrophic colonization by *C. graminicola.*

<u>The *cpr1* mutant fails very early in biotrophy</u>

The *cpr1* mutant has a defect in one component of the signal peptidase complex, and it is expected, as a result, to be deficient in protein transport and secretion. This defect could have a feedback suppression effect on the expression of genes encoding secreted proteins, via activation of a secretion stress response (Pakula *et al.*, 2003, Martínez & Chrispeels, 2003, Schröder & Kaufman, 2005). Indeed, transcripts that were expressed at a significantly lower level in MT versus WT

biotrophy, were enriched for secreted or membrane-localized protein-coding genes (more than 65%).

Secreted and membrane protein transcripts are enriched in appressoria and biotrophic hyphae of both the MT and WT strains, suggesting that even at this early phase of development, the pathogen is already communicating with the host and preparing it for invasion. I had speculated that the MT might differ from the WT in the expression of secreted protein effectors or SM-associated genes, which could explain the induced susceptibility effect induced by the WT (see chapter 2). However, there were relatively few differences in the expression of these gene classes between the MT and WT. No significant differences were detected in expression of the early-induced SM clusters in the mutant, or in any SM-associated gene. Only seven putative effector genes were differentially regulated: five were more highly expressed during WT biotrophy, while two were more abundant during MT biotrophy. The majority of effectors had similar patterns of expression. It is possible that one of the seven differentially expressed effectors has a highly significant role in establishment of compatibility, and this will be tested in future with functional analysis of these genes.

There were no genes statistically differentially expressed between WT and MT appressorial stages. This observation is consistent with descriptions of the process of WT and MT infection in leaf blades (Thon *et al.*, 2002, Mims & Vaillancourt, 2002) and in my observations leaf sheaths in chapter 2. In both cases, no significant differences were observed in timing or efficiency of appressorial formation by the WT versus MT at 24 hpi. The percentage of the total reads in both samples that were fungal was around 2% [17], so differences in biomass are unlikely to explain the lack of differentially regulated genes. Instead, it appears that transcriptional development of the mutant and WT are similar pre-penetration. This may be related to the surprising finding that expression of the *Cpr1* gene was similar in both mutant and WT in appressoria *in planta*. In contrast, transcript levels were 8-fold lower in the mutant versus WT

appressoria *in vitro*. This suggests that plant signals may regulate turnover of *Cpr1* transcripts *in planta*. Since levels of transcript are similar, I propose that differences in phenotype in the *cpr1* mutant may relate to post-transcriptional aspects of *Cpr1* regulation and function. Some possibilities could include alternate splicing and protein modification.

Observations from Chapter 2, indicated that the MT displayed a 24 hour delay in formation of primary hyphae relative to the WT, but by 48 hpi, 36% of the appressoria had colonized the first epidermal cell. However, only one gene was significantly differentially expressed at this stage relative to appressoria. Interestingly, the gene is predicted to encode a putative small cysteine-rich effector. GLRG_03688 was 6.1-fold more highly expressed in biotrophy versus appressoria in the MT. This gene was also expressed at higher levels during biotrophy in the WT. Expression in WT biotrophy was 3.3-fold higher than in the MT. BLAST searches against other sequenced *Colletotrichum* species indicated that GLRG_03688 has an ortholog in *C. sublineola*, the closely related causal agent of sorghum anthracnose, but lacks orthologs in *C. higginsianum* and *C. gloeosporioides,* both pathogens of dicots.

The identification of only one gene may relate to the relative fungal biomass produced in these stages. Only about a third of appressoria had produced primary hyphae, and these were small and confined only to a single cell. Additionally, their viability status is unknown so it is possible that many of them were undergoing cell death, which would reduce transcript representation. The biotrophic sample from the MT had much less biotrophic tissue in proportion to appressoria than the biotrophic sample from the WT did. It is possible that the majority of sequences in MT-BT were from the majority of pre-penetration appressoria in the MT sample, and this may have muted any statistical differences. Relaxing the statistical stringency may reveal additional genes that are potentially expressed in biotrophic hyphae of the MT.

GO terms identified in genes with lower expression in the WT biotrophy relative to the MT, overlapped with the terms identified in genes with lower expression in WT biotrophy relative to appressoria. Together, these results suggest that the differences in gene expression observed in the comparisons between WT and MT biotrophic stages, are likely to be associated with the mutant stopping development very early in the colonization process, and failing to establish an effective biotrophic relationship with the plant. I had anticipated that comparative analysis of the mutant and WT transcriptomes would reveal more about the precise nature of the mutation. Although it didn't pinpoint the defect, it did reveal with much more precision the point at which the mutant is affected. It appears to be very early during the initial establishment of the biotrophic phase. Although the mutant has apparently normal development up until that point, it is possible that the mutant fails to secrete proteins even earlier, at the appressorial phase, that are necessary to fully prepare the epidermal cell for invasion and establishment of biotrophy. Some evidence in support of this was the observation that host tissues exposed to mutant appressoria generally accumulated less defense gene transcripts. Plant defense genes are activated before penetration occurs in response to fungal elicitors, and differences in expression of PR3, *Bt1* and *Bi-1* could be result either of the WT suppressing defense responses, or of the mutant failing to elicit a strong response.

I conclude that the *C. graminicola* hemibiotrophic infection appears to encompass aspects both of biotrophy and of necrotrophy, simultaneously. I speculate that biotrophy in this fungus is a very highly localized phenomenon, which involves only the cells at the leading edge of the developing colony. Likewise, necrotrophy is highly localized and isolated to the colony centers. It will be important in future to investigate localized expression of genes involved in SM and genes encoding putative fungal effectors, in order to understand the behaviors of the different cell types during infection. Sheath infections provide an ideal bioassay for these studies, allowing visualization of labeled proteins in a

living host interacting with the living pathogen. Similar studies of *M. oryzae* on rice sheaths have led to recent breakthroughs in our understanding of effector biology in that pathosystem. The mutant appears to have a deficiency in establishment of biotrophic infection in the first cell, but I discovered that this can be rescued by activity of a nearby WT colony, suggesting that the MT is failing to secrete diffusible substances that are necessary to prepare the cell for its occupation. The identity of these substances does not appear to be revealed by the expression data, and it is possible that post-transcriptional events are of primary importance in this phenomenon. Thus, the mutant may be failing to translate, process, or secrete proteins that are being transcribed. The difference in the way the plant perceives the MT and WT appressoria, even though transcriptionally they appear identical, suggests there may be differences in the types and quantities of proteins that are being produced by these strains *in planta*. Clearly, much more work is necessary before we can understand the basis for the mutant behavior and for the induced susceptibility phenomenon, but the data generated in this study will provide an excellent foundation for those future investigations.

Table 4.1. Genes with significantly different expression between different fungal stage comparisons. Higher and lower expression refers in each case to the first term in the comparison. WT=wild type. MT= Mutant. AP=Appressoria. BT=Biotrophic. NT=Necrotrophic.

Comparison	Fungal stages compared	Lower expression	Higher expression	Differentially expressed genes
1	WTAP-WTBT	193	479	672
2	WTBT-WTNT	213	608	821
3	MTAP-MTBT	0	1	1
4	WTAP-MTAP	0	0	0
5	WTBT-MTBT	233	34	267

Table 4.2. Number of genes in each comparison that were annotated using Blast2GO. WT=wild type. MT= Mutant. AP=Appressoria. BT=Biotrophic. NT=Necrotrophic.

	Total genes		Percentage	
Stage transition	Annotated	Not annotated	Annotated	Not annotated
WTAP-WTBT_down	119	74	61.7	38.3
WTAP-WTBT_up	328	151	68.5	31.5
WTBT-WTNT_down	100	113	46.9	53.1
WTBT-WTNT_up	365	243	60	40
WTBT-MTBT_down	151	82	64.8	35.2
WTBT-MTBT_up	21	13	61.8	38.2

Table 4. 3. Predicted secreted hypothetical proteins that were not annotated, and previously described as putative secreted effectors in *Colletotrichum* fungi. [1] Krijger et al, 2008. [2] Bhadauria et al, 2011 [3] Kleeman et al, 2012 [3]. (E value 1e-4). WT=wild type. MT= Mutant. AP=Appressoria. BT=Biotrophic. NT=Necrotrophic.

Comparison	This study	*C. graminicola* [1]	*C. truncatum* [2]	*C. higginsianum* [3]
WTAP-WTBT_up	5	4	2	1
WTAP-WTBT_down	6	0	0	2
WTBT-WTNT_up	6	1	1	0
WTBT-WTNT_down	18	2	1	5
WTBT-MTBT_up	2	1	0	1
WTBT-MTBT_down	5	3	1	1

Table 4.4. Expression of selected genes, secondary metabolites and putative effector proteins by quantitative RT-PRC. Values indicate averages of relative expression from two biological replicates, and the calculated standard deviation. Fold change (FC) values of the averaged value are indicated as Log2. WT=wild type. MT= Mutant. C= Complemented strain. IV-AP= *In vitro* appressoria.AP=Appressoria. BT=Biotrophic. NT=Necrotrophic.

		WT-AP/IVAP		C-IVAP/AP		MT-IVAP/AP		WT-BT/AP		C-BT/AP		MT-BT/AP		WT-NT/BT		C-NT/BT	
Gene ID	**Function**	**RE**	**FC**	**RE**	**FC**	**RE**	**FC**	**RE**	**FC**	**RE**	**FC**	**RE**	**FC**	**RE**	**FC**	**RE**	**FC**
GLRG_04964	CPR1	1.35±0.71	0.43	1.59±0.12	0.66	12.06±8.83	3.59	0.88±0.44	-0.18	0.54±0.06	-0.87	0.65±0.58	-0.62	0.94±0.2	-0.08	0.95±0.39	-0.07
GLRG_08626	Cluster 18	3.08±0.85	1.620	6.26±3.08	2.640	2.77±0.47	1.470	0.28±0.08	-1.820	0.22±0.08	-2.160	0.22±0.07	-2.21	1.45±0.39	0.530	1.69±0.58	0.760
GLRG_08628	Cluster 18	1.74±0.17	0.800	0.84±0.09	-0.240	0.81±0.07	-0.360	0.41±0.01	-1.280	0.3±0.04	-1.740	0.21±0.02	-2.11	1.77±0.15	0.820	1.31±0.12	0.390
GLRG_09710	Cluster 22	10.73±5.87	3.420	10.47±0.47	3.380	19.47±9.97	4.280	2.55±2.61	1.350	2.13±0.33	1.080	1.77±0.65	0.82	180.2±69.5	7.490	73.8±7.28	6.200
GLRG_09715	Cluster 22	1.46±0.49	0.540	0.80±0.59	-0.310	1.86±0.09	0.890	1.14±0.11	0.190	2.12±0.78	1.080	2.14±0.2	1.09	25.83±15.4	4.690	19.1±14.33	4.250
GLRG_11770	Cluster 35	3.29±0.55	1.710	4.55±0.03	2.180	6.06±1.22	2.590	0.21±0.11	-2.240	0.03±0	-5.040	0.35±0.1	-1.49	0.78±0.57	-0.350	0.7±0.41	-0.530
GLRG_11778	Cluster 35	3.52±1.46	1.810	19.37±6.38	4.270	10.85±2.19	3.430	0.16±0.08	-2.610	0.09±0.08	-3.390	0.53±0.15	-0.92	0.79±0.64	-0.320	1.14±1	0.190
GLRG_06284	Effector- BAS2	3.78±1.1	1.920	1.89±1.15	0.910	133.3±19.7	7.050	0.31±0.11	-1.660	0.18±0.02	-2.460	0.52±0.16	-0.95	0.18±0.02	-2.490	0.49±0.16	-1.040
GLRG_00201	Effector - BAS3	7.38±1.15	2.880	5.63±1.08	2.490	16.02±3.5	4.000	0.35±0.02	-1.480	0.35±0.01	-1.500	0.25±0	-2	0.09±0	-3.350	0.11±0.03	-3.140
GLRG_01192	Effector- ChEC91	0.92±0.5	-0.110	1.51±0.44	0.590	0.18±0.17	-2.420	3.53±1.89	1.820	8.25±0.54	3.040	0.99±0.26	0	0.63±0.02	-0.670	0.7±0.29	-0.540
GLRG_07767	Effector- ChEC90	3.28±2.48	1.710	5.37±2.64	2.520	10.77±6.72	3.420	0.28±0.11	-1.790	0.026±0.01	-5.260	0.81±0.53	-0.3	0.24±0.1	-2.070	0.41±0.21	-1.280
GLRG_03688	Effector	12.62±5.92	3.650	2.2±1.11	1.130	2.23±0.69	1.150	19.50±7.29	4.280	20.96±9.68	4.390	6.71±1.61	2.74	0.51±0.2	-0.960	0.41±0.52	-1.270
GLRG_07776	Effector	10.45±0.19	3.380	1.43±0.22	0.510	55.3±30.36	5.780	0.64±0.06	-0.640	0.30±0.05	-1.710	1.28±0.28	0.35	0.17±0.04	-2.560	0.85±0.27	-0.230

Figure 4.1. Phenotype of WT and cpr1 mutanat strains in leaf sheaths. WT **(A-C)** and MT **(D-E)** **o**n maize leaf sheaths. Appressoria **(A and D)** samples were collected 18-24 hpi. Biotrophic samples **(B and E)** were collected 36-48 hpi. Necrotrophic samples (C) were collected 60-65 hpi. AP= Appressoria. BH=Biotrophic hyphae. NH= Necrotrophic hyphae. Bars equal to 50 µm.

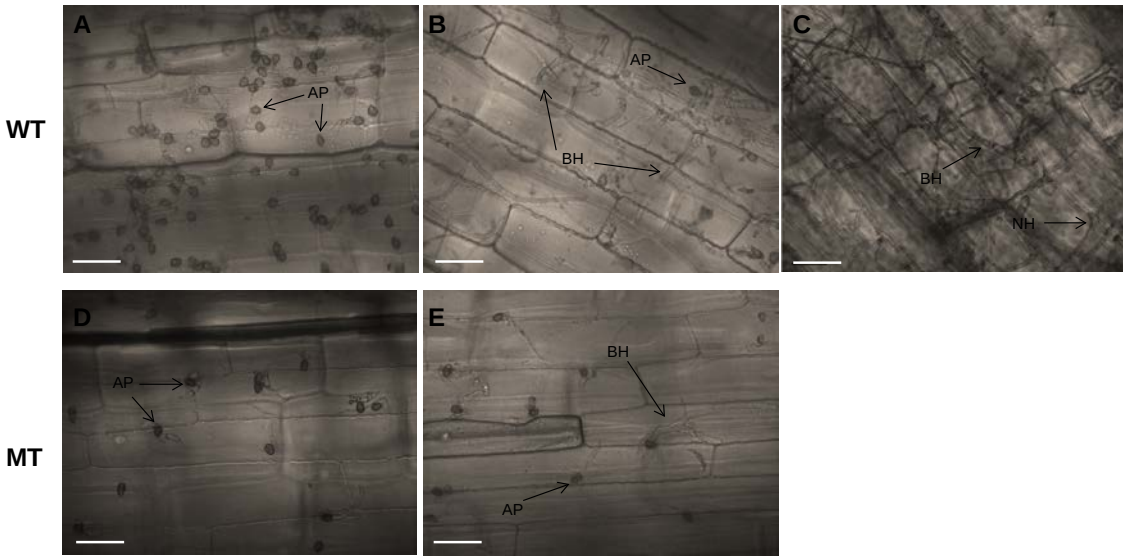


Figure 4.2. Patterns of relative expression of the 153 genes that were differentially expressed in both WT comparisons (WTAP-WTBT and WTBT-WTNT). WT=wild type. AP=Appressoria. BT=Biotrophic. NT=Necrotrophic.

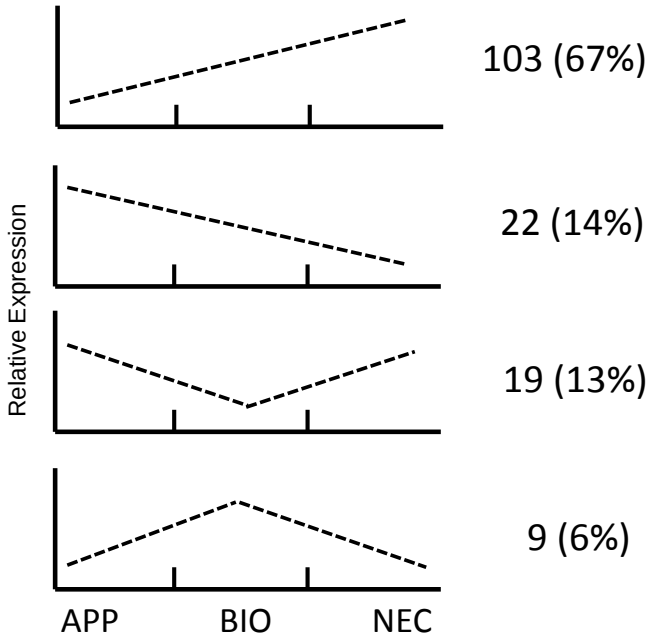


Figure 4. 3. Predictions of cellular localization. **A**. The entire proteome of *C. graminicola* and **B.** Predicted proteins from genes differentially regulated at some stage of fungal colonization. WTAP-WTBT= transition from appressoria to biotrophy. WTBT-WTNT= transition to necrotrophy. WTBT-MT-BT= Comparison between WT biotrophy and MT biotrophy.

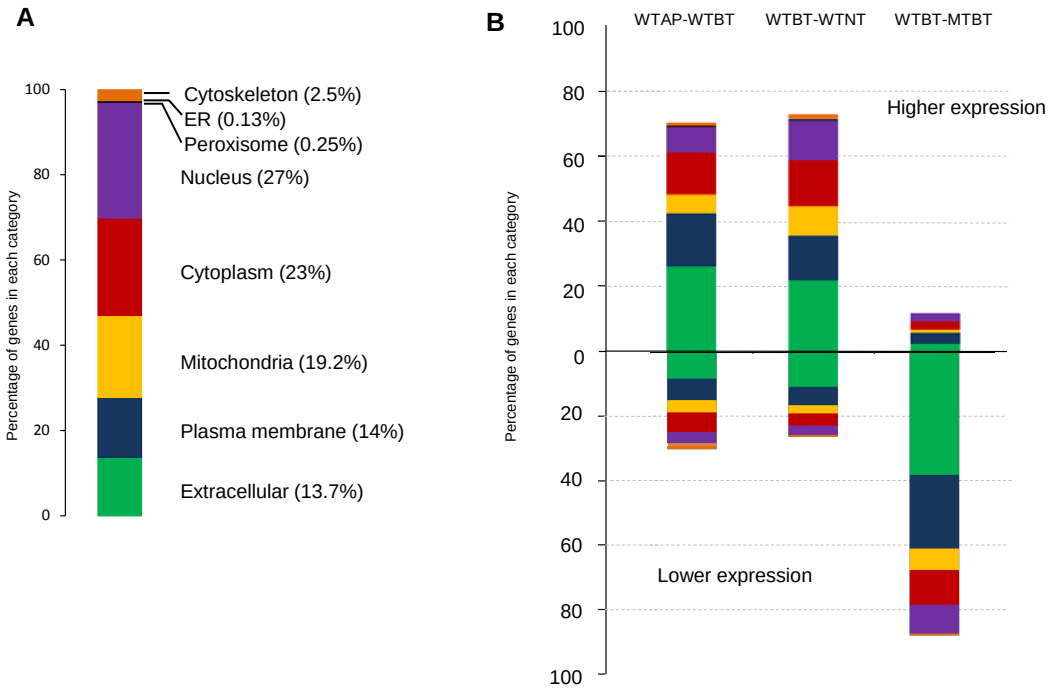


Figure 4.4. GO terms significantly overrepresented in appressoria-expressed genes. Comparisons represent appressoria-expressed genes (WTAP-WTBT_down, black bars) relative to biotrophy-expressed genes (WTAP-WTBT_up, white bars).

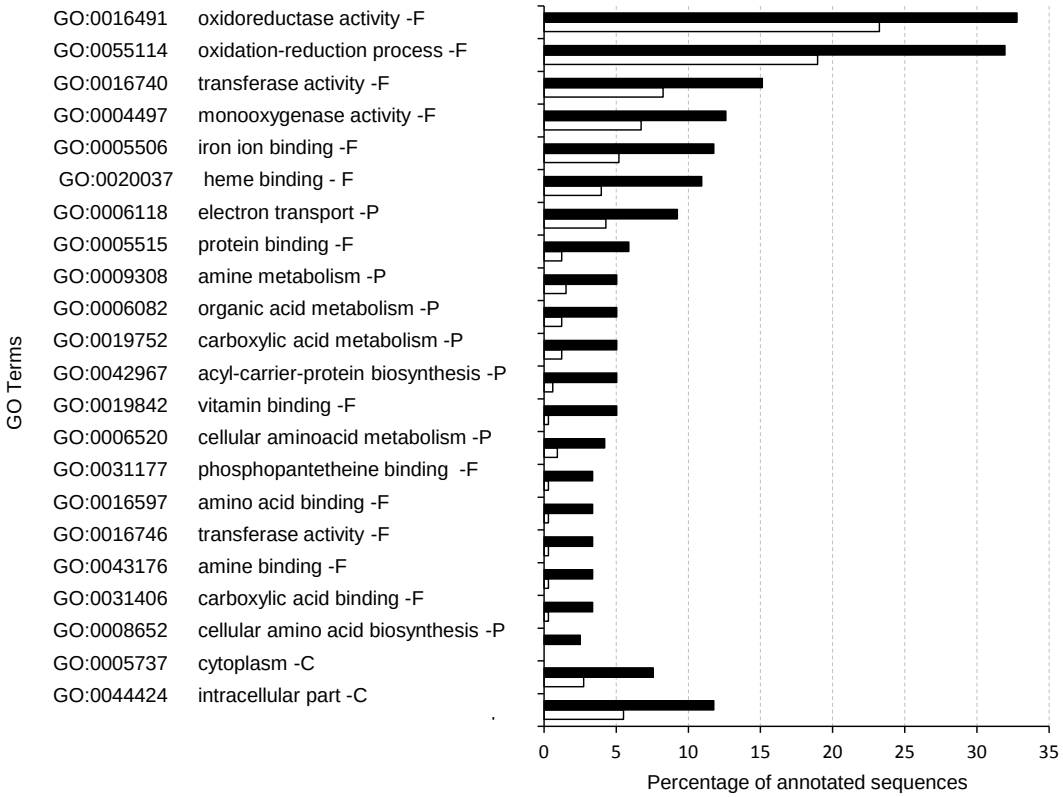


Figure 4.5. GO terms significantly overrepresented in biotrophy-expressed genes. Comparisons represent biotrophy-expressed genes(WTAP-WTBT_up, black bars) relative to appressoria-expressed genes (WTAP-WTBT_down, white bars).

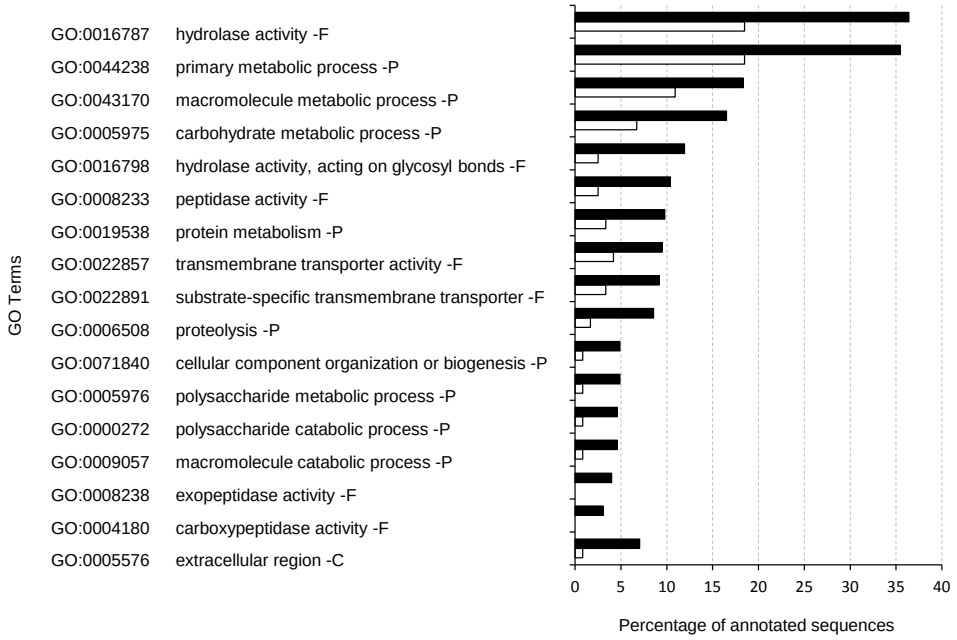


Figure 4.6. GO terms significantly overrepresented in biotrophy-expressed genes. Comparisons represent biotrophy-expressed genes (WTBT-WTNT_down, black bars) relative to necrotrophy-expressed genes (WTBT-WTNT_up, white bars).

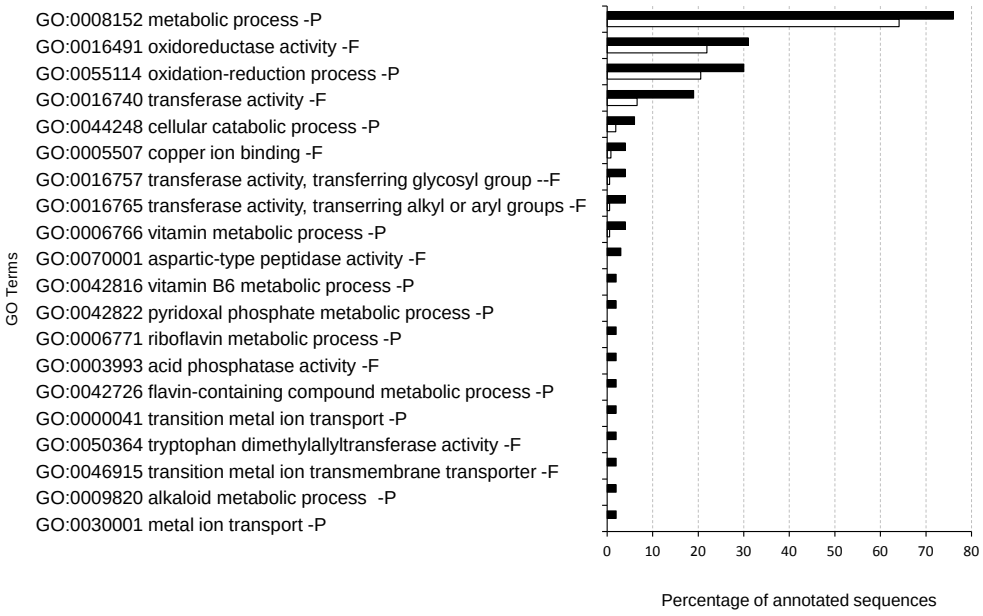


Figure 4. 7. GO terms significantly overrepresented in necrotrophy-expressed genes. Comparisons represent nectotrophy-expressed genes (WTBT-WTNT_up black bars) relative to biotrophy-expressed genes (WTBT-WTNT_down, white bars).

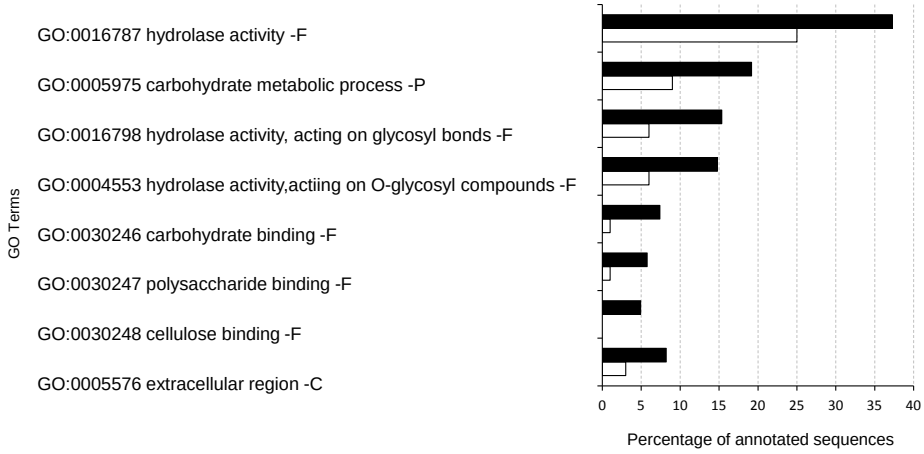


Figure 4.8. Characterization of genes that were not annotated by Blast2GO. Light gray: secreted hypothetical proteins. Blue: small, secreted, cysteine rich proteins. Dark gray: Other secreted proteins Black: Non-secreted proteins with other predictions. WT=wild type. MT= Mutant. AP=Appressoria. BT=Biotrophic. NT=Necrotrophic.

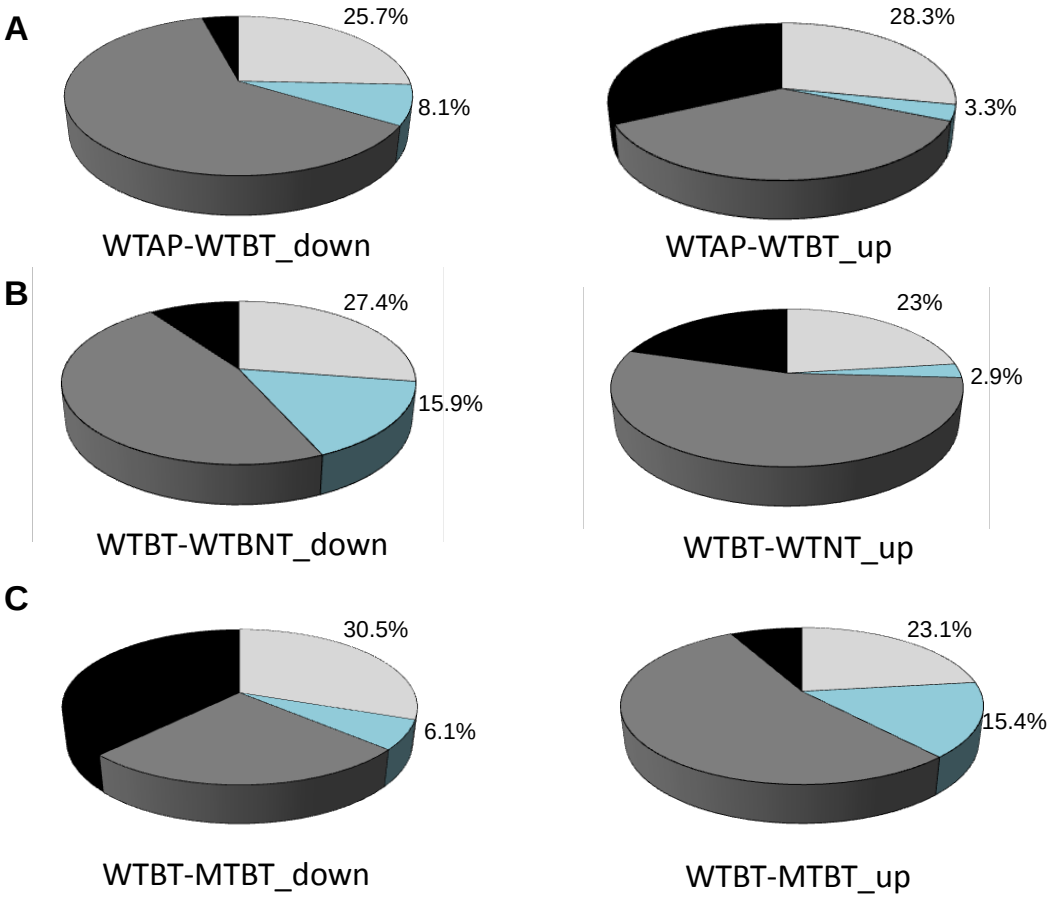


Figure 4.9. Genes differentially expressed between WT and MT. **A**. GO terms significantly overrepresented in genes with higher expression in the MT (WTBT-MTBT_up) relative to genes with lower expression in the MT (WTBT-MTBT_down) **B.** Number of genes differentially expressed in the transition to biotrophy in the WT (white) and between biotrophic stages in WT and MT (Buckner *et al.*). Intersection represents number of genes that were common in both comparisons.

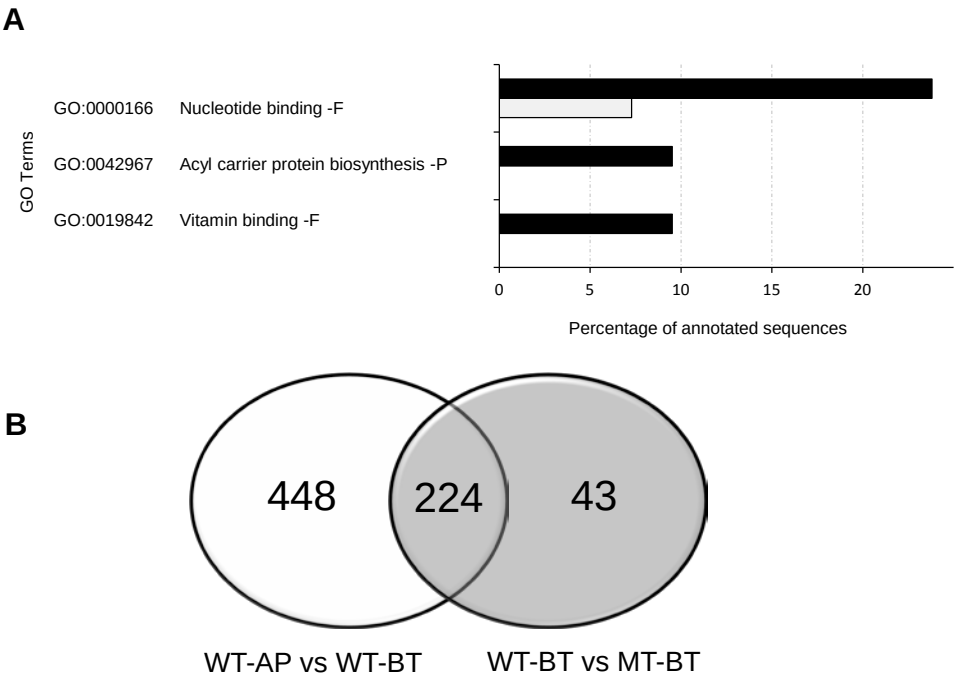


Figure 4.10. Characterization of genes differentially expressed between WT and MT. **A.** Patterns of expression of genes differentially expressed in the transition to biotrophy in the WT (graphs) and in the comparisons between biotrophic stages in the WT and MT (black triangles). **B**. Predicted cellular localization of WTBT-MTBT genes also present in WTAP-WTBT (outer ring) compared to genes only found in the WTBT-MTBT comparisons (inner ring).

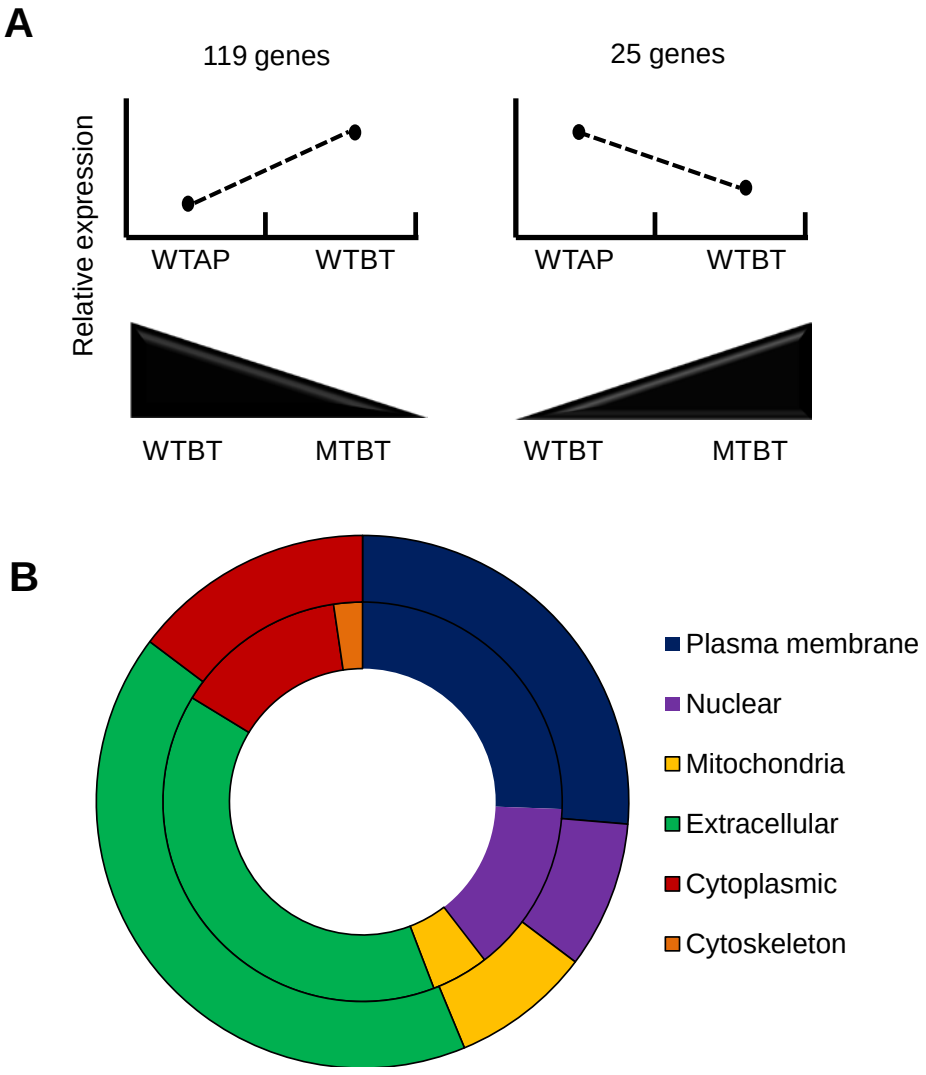


Figure 4.11. Heatmaps of gene expression of SMURF clusters **A.** cluster 18. **B.** cluster 35. C. Cluster 22. D. Cluster 38. Transcript representation is shown as fold changes in each repetition (Log2) relative to the average number of normalized reads for each gene across all stages. * Expression significantly higher in AP (AP-BT_down) + Expression significantly higher in NT (BT-NT_up).

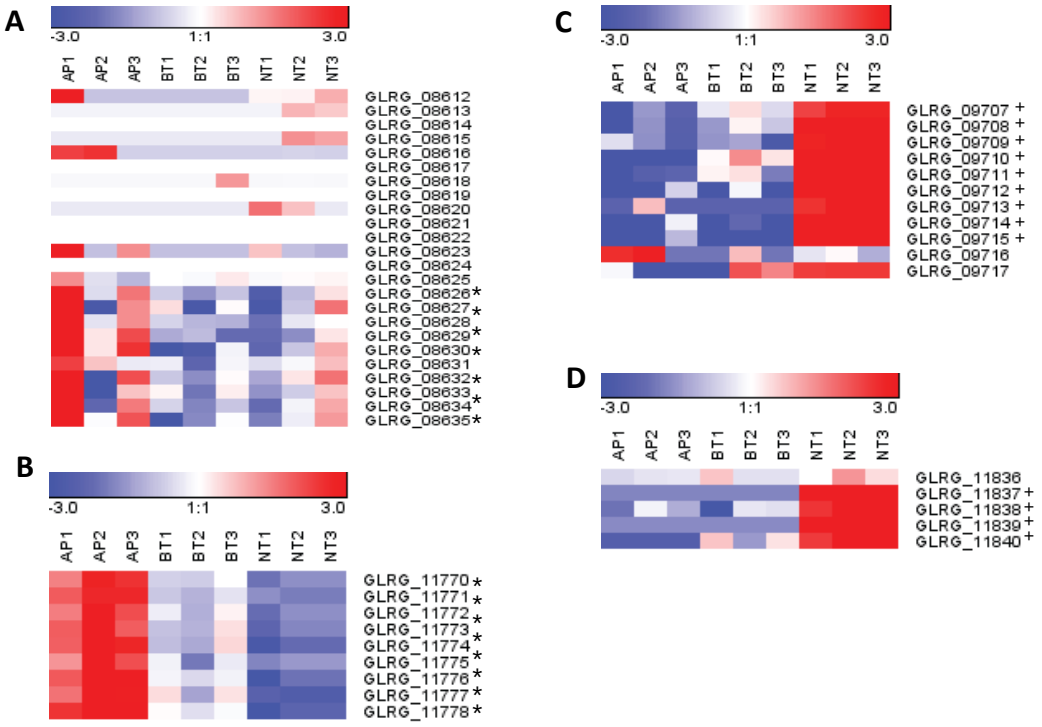


Figure 4.12. Correlation analysis of fold changes determined by RNA sequencing and RT-PCR. Log2 fold changes as determined by RT-PCR are plotted in the x-axis and determined by RNAseq are plotted in the y-axis.

Figure 4.13. Relative expression of *Cpr1* during different stages of fungal infection. WT (black bars), *Cpr1*-C (white bars) and *cpr1* mutant (dark gray bars), measured by quantitative RT-PCR. Expression values are shown as fold changes relative to expression in other fungal stages. IVAP= *in vitro* appressoria. AP= appressoria . BT= biotrophic stage NT= necrotrophic stage.

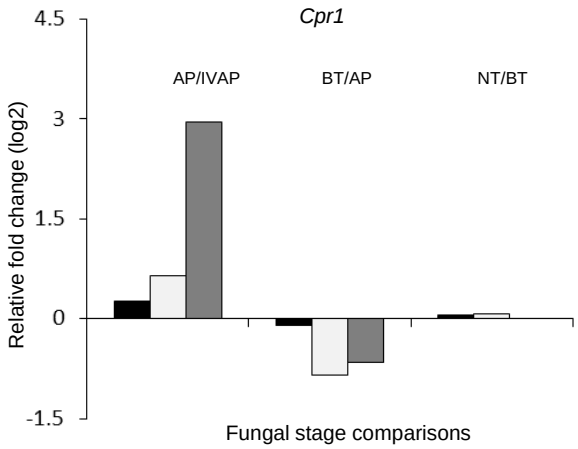


Figure 4. 14. Quantitative RT-PCR of selected SM fungal genes. Genes from clusters 18, 22 and 35 in WT (black bars), *Cpr1*-C (white bars) and *cpr1* mutant (dark gray bars). Expression values are shown as fold changes relative to expression in other fungal stages. IVAP= *in vitro* appressoria. AP= appressoria . BT= biotrophic stage NT= necrotrophic stage.

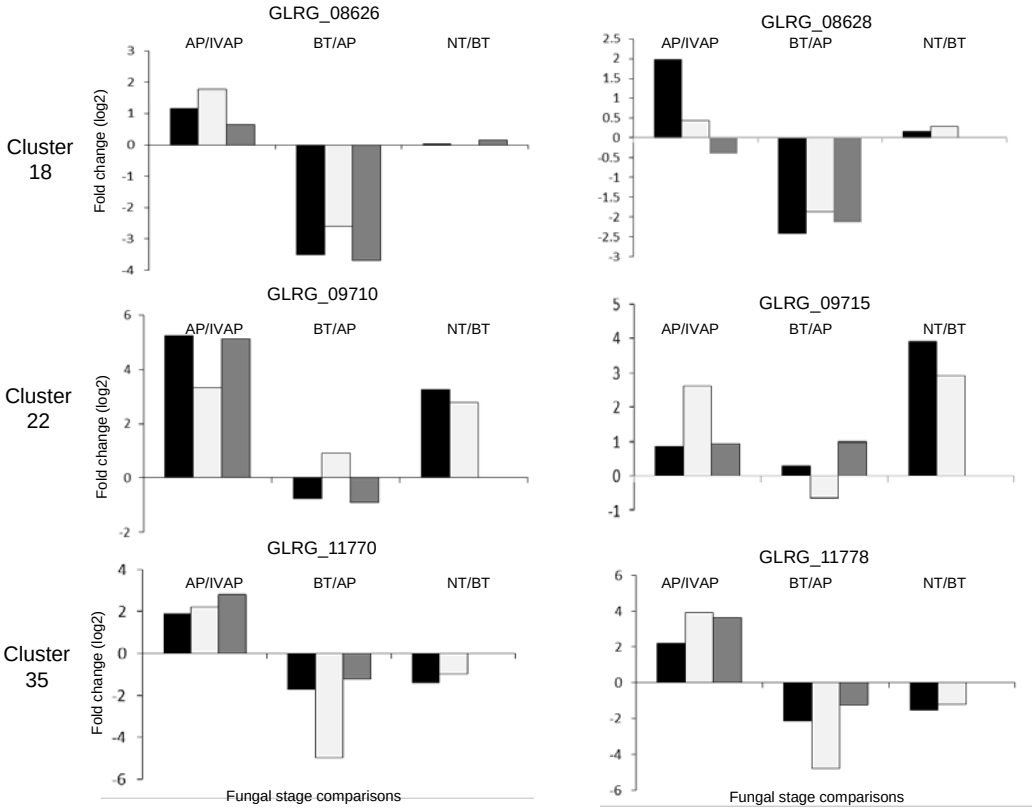


Figure 4.15.Quantitative RT-PCR of putative secreted effector protein genes. BAS2 and BAS3= biotrophy-associated proteins 2 and 3. Expression in different stages of fungal infection in the WT (black bars), *Cpr1*-C (white bars) and *cpr1* mutant (dark gray bars). Expression values are shown as fold changes relative to expression in other fungal stages. IVAP= *in vitro* appressoria. AP= appressoria . BT= biotrophic stage NT= necrotrophic stage.

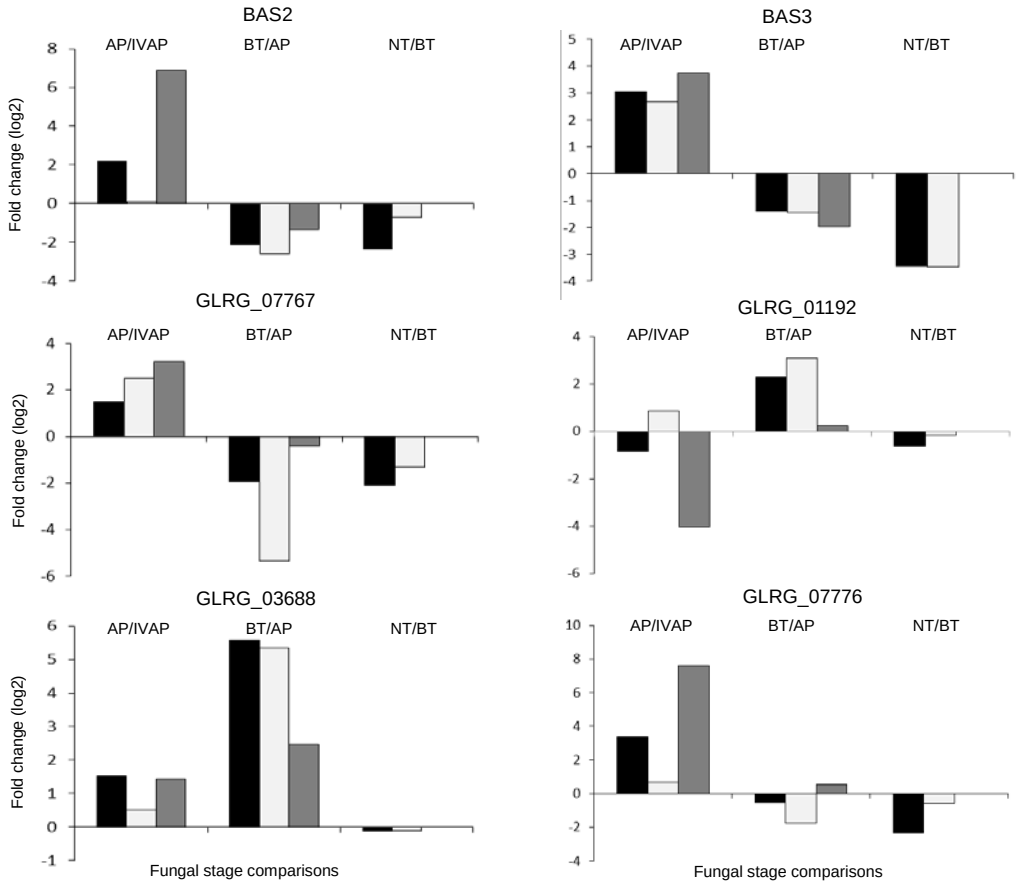
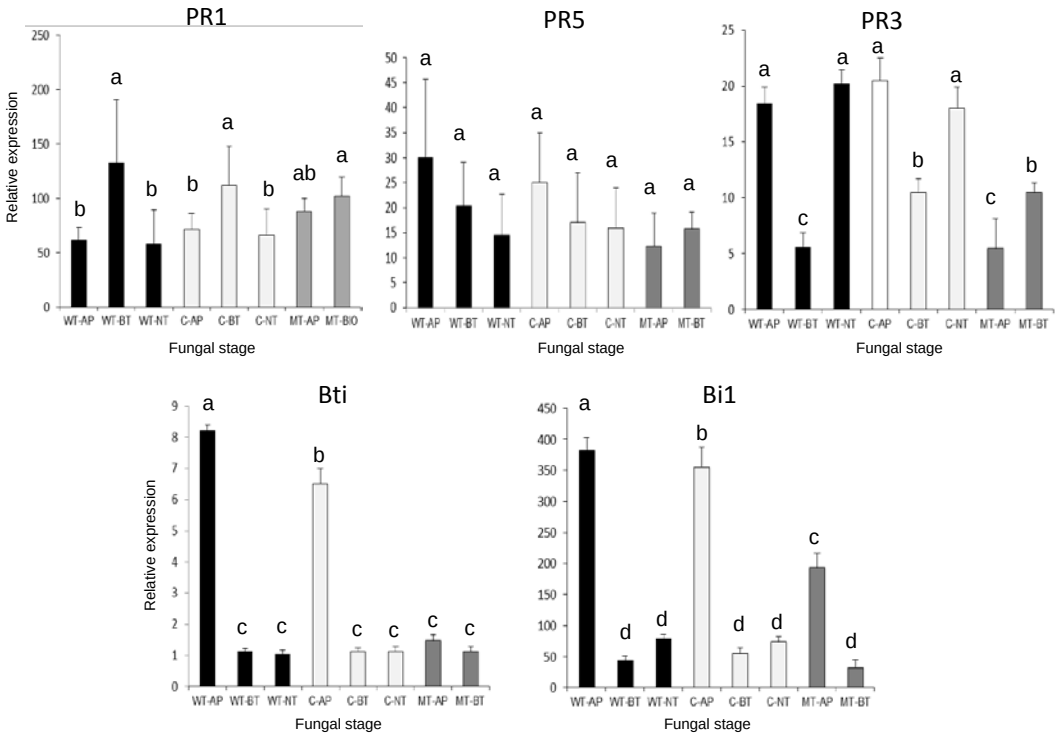


Figure 4.16. Maize defense responses are activated early into the infection process. Bars represent expression of each gene, relative to mock-inoculated plants. PR: pathogenicity-related. Bti: Bowman-birk trypsin inhibitor (JA marker). Bi1: Bax inhibitor (cell death inhibitor).



Chapter 5

Concluding Remarks

The genus *Colletotrichum* includes hundreds of species that cause anthracnose and reduce yields in a variety of hosts, including fruits, legumes and cereals. In spite of their importance as pathogens, relatively little is understood about the mechanisms that are involved in establishing the hemibiotrophic lifestyle that characterizes this genus.

In my dissertation research, I utilized a combination of cytological and genomic approaches to describe the biotrophic and necrotrophic phases of *C. graminicola* growing in maize with a level of detail that had never been achieved previously. Thus, my findings from chapter 2 clearly established that *C. graminicola* initially invades host cells biotrophically, as indicated by plasmolysis and vital staining. I was able to show that a previously characterized nonpathogenic mutant germinated and penetrated normally, and that it was disrupted specifically in its ability to establish a biotrophic infection. My work with this nonpathogenic mutant of *C. graminicola* also showed, for the first time, that *C. graminicola* produces diffusible factors that modify host metabolism in order to predispose plant cells for fungal invasion. Even more importantly, my work resulted in a bioassay that can be used in the future to characterize these substances.

My cytological observations allowed me to determine that hemibiotrophy in *C. graminicola* differs from that in most other *Colletotrichum* species, and from previous descriptions of *C. graminicola* in the literature. Thus, infection of new cells, even at 60 hpi when the centers of the fungal colonies had become necrotrophic, still occurred biotrophically. Biotrophic stages in *C. higginsianum* and *C. destructivum* are limited to the first invaded cell, and in C. *lindemuthianum* biotrophy persists during the invasion of several subsequent cells. In these cases, the necrotrophic switch is complete, unlike *C. graminicola*, and after the

switch newly invaded cells are killed in advance. I found that hemibiotrophy in *C. graminicola* was more similar to the behavior of its close relative *C. sublineola*, which had been described in the literature previously.

Part of my dissertation research contributed to the recently published genome sequencing project for *C. graminicola* and *C. higginsianum,* which provided the first insights into the potential classes of proteins that are synthesized by these pathogens, and that could be important for their growth *in planta*. A large number of potential secreted fungal effectors, similar to those associated with biotrophic plant pathogens, and an expansion of secondary metabolites, usually associated with necrotrophic pathogens, suggested that these hemibiotrophic pathogens express aspects of both lifestyles. My analysis of our transcriptome data representing three major stages of infection also supported this conclusion, and further indicated that the pathogen first behaves more like a biotroph, and then later switches to behavior that is more like a necrotroph. Thus, gene expression during appressorial and biotrophic stages resembled previous descriptions of strict biotrophs, with prominent expression of functions related to detoxification, nutrient uptake and nutrient transport. Similarly, gene expression during biotrophic and necrotrophic stages shared similarities with necrotrophic pathogens, including the preferential expression of transcripts involved in transport, proteolysis, and cell wall degradation. Study of early infection stages *in planta*, in particular, has proven quite challenging, and to my knowledge, mine was one of the first studies that attempted to characterize and compare gene expression *in planta* during all three stages of development.

My work also established that the early, biotrophic stages of colonization by *C. graminicola* differ in some important respects from true biotrophy. For example, secondary metabolism appeared to be important during pre-penetration stages in *Colletotrichum*. This was an unexpected result, since secondary metabolism products are usually associated with phytotoxic activity in necrotrophic pathogens. The role of secondary metabolism in the early stages of maize

infection remains to be characterized. Additional evidence that Colletotrichum hemibiotrophy differs from biotrophy was my demonstration of expression of host defense genes, even prior to fungal penetration, that have been previously associated with both the SA response to biotrophs and the JA response to necrotrophs.

It was disappointing that my gene expression analysis did not reveal the specific nature of the defect in the nonpathogenic mutant, although it did help to pinpoint the precise stage at which the defect occurs. Although the leaf sheath assay allowed me to maximize the amount of fungal biomass in these samples, some limitations (i.e. very low fungal biomass) still exist. Thus, I think that differences between the two strains at the early infection stages might exist, but that the small proportion of fungal transcripts caused a large amount of variation resulting in low statistical significance. Furthermore, although transcript levels of some crucial pathogenicity factors appear to be normal in the *cpr1* mutant, some of them could be failing to be transported to the right place in the right time (i.e. secreted). This hypothesis could be tested by transforming candidate genes to express fluorescent proteins, and comparing expression and localization in the mutant versus the wild type. Post-transcriptional differences between the wild type and mutant are also likely to occur. Alternative splicing is a possibility that cannot be ignored. Differences could also be occurring at the level of protein modification. The likelihood of post-transcriptional differences is supported by evidence I generated showing that expression of plant genes is different in response to appressorial stages of the wild type versus the mutant.

My work indicates that hemibiotrophic development in *C. graminicola* is highly localized, with biotrophy at the borders of the expanding colony, and necrotrophy in the center. The nature of the intercalary primary hyphae just behind the advancing tips, occupying host cells that no longer plasmolyze but are not yet colonized by necrotrophic hyphae, remains mysterious. I speculate that gene expression in these fungal cells may be in a transitional state between biotrophy-

associated gene expression (i.e. suppressing host defense responses and modifying host metabolism) and necrotrophy associated expression (i.e. inducing cell death). To address some of these questions, specific fungal and host cell types should be isolated, perhaps using laser capture microdissection microscopy. I tried, but was not successful in standardizing this technique for utilization on maize leaf sheaths. Better protocols are needed.

The development and standardization of the leaf sheath assay in the *C. graminicola*-maize pathosystem, in my opinion, will be the most enduring contribution of my dissertation work for the future. This assay was crucial for my detailed characterization of the infection processes of the wild type and mutant strains. Additionally, it facilitated my analysis of transcript expression during growth *in planta*, allowing the observation and classification of each individual sample, as well as increasing the relative amount of fungal biomass for the analysis. Furthermore, co-inoculation experiments using the mutant strain will provide a useful tool for testing and characterization of effector candidates. With the genome and the assay, our future progress on understanding the nature of the maize-*Colletotrichum* interaction, and our ability to identify novel targets for therapies manage this disease, seems assured.

Appendix 1

C. graminicola modulates environmental pH, but apparently not by the production of ammonium

In *C. gloeosporioides* infecting avocado, and *C. coccodes* infecting tomato, tissue alkalinization and the induction and function of certain pathogenicity factors seem to be related. An increase in environmental pH in lesions produced on ripe fruit by these fungi occurs via secretion of high concentrations of ammonia by the fungus. Some of the ammonium is thought to be produced via the reduction of nitrate, because nitrate reductase (*nit*) mutants cannot accumulate ammonium and are significantly reduced in virulence (Alkan *et al*., 2008, Kramer-Haimovich *et al*., 2006). Increased pH due to ammonium production induces expression of fungal pathogenicity genes, including lytic enzymes that have high pH optima (Kramer-Haimovich *et al.*, 2006, Yakoby *et al.*, 2000, Drori *et al.*, 2003). Deletion of nitrate reductase, the nitrogen regulator area and the ammonium transporter. Targeted deletion of AREA, a major nitrogen regulator, and AMET, an ammonium transporter, affects ammonium accumulation and virulence, suggesting that ammonium secretion is pathogenicity factor in *Colletotrichum* fungi infecting avocado and tomato fruits (Alkan *et al*., 2008, Shnaiderman *et al.*, 2013).

In our recent article comparing the genomes and transcriptomes of *C. graminicola* and *C. higginsianum*, it was reported that both species cause local increases in pH and secrete ammonia during necrotrophy, but that *C. graminicola* does so to a lesser extent than *C. higginsianum* (O'Connell *et al.,* 2012*)*. Several fungal genes involved in nitrate reduction and ammonium export were reportedly significantly induced during tissue alkalinization by *C. gloeosporioides* (Miyara *et al.*, 2012) .I used the transcriptome data to compare the expression of *C. graminicola* and *C. higginsianum* orthologues of these genes *in planta* (Figure A1.1). The patterns of expression for these genes were similar for the two species, with some exceptions. First, the gene encoding nitrate reductase

(GLRG_02724/CH063_08184) was significantly more highly expressed during appresorial and biotrophic stages in *C. higginsianum*, while in *C. graminicola* the levels of expression were similar, and moderate, across all stages of development. Second, an ammonium importer (GLRG_03954/CH063_12269) was significantly more highly expressed in *C. higginsianum* during appressorial formation and biotrophy, whereas in *C. graminicola* this gene was siginificantly more highly expressed during necrotrophy. GDH2, involved in synthesis of ammonium, had higher expression in biotrophy, while in *C. graminicola* this gene was highly expressed in appressoria. A glutamate importer (GLRG_04076/CH063_12275) had significantly higher expression levels during necrotrophy in both species.

C. gloeosporioides nit mutants are impaired in ammonia secretion and tissue alkalinization, involved in activation of some pectate lyases (Kramer-Haimovich *et al*., 2006, Yakoby *et al.*, 2001). Induction of nitrate reductase during biotrophy of *C. higginsianum* could suggest that nitrate metabolism also plays a role in tissue alkalinization and activation of some lytic enzymes. Pectate lyases also appear to be more important during colonization of Arabidopsis leaves by *C.higginsianum* than of maize by *C. graminicola*, and it is proposed that it could be related to the larger proportion of pectin in Arabidopsis tissue compared to maize (O'Connell *et al*., 2012).

The ammonium importer MEP is induced during ammonium uptake and colonization of avocado fruits (Shnaiderman *et a*l., 2013). Induction of this gene during necrotrophy could be associated with nutrient uptake from the host. Additionally, in *C. gloeosporioides*, secretion of ammonia is associated with induction of glutamate dehydrogenase and glutamine synthase (Miyara *et al.*, 2010). None of these genes was significantly induced at any stage of infection in *C. graminicola*, suggesting that pH increase and ammonium secretion differs from the process described for *C. gloeosporioides*.

I tested the hypothesis that *C. graminicola* modulates environmental pH *in vitro* by the production of ammonium. *C. graminicola* strain M1.001 was grown in different media, under different pH conditions and with different sources of nitrogen. Changes in pH and ammonium production in each treatment were measured. Results were compared with a *C. gloeosporioides* strain AVO06, collected from avocado in Israel, provided by Stanley Freeman (The Volcani Center, Bet Dagan, Israel).

C. graminicola and _C. gloeosporioides_ both increase the pH of solid media during growth in the presence of yeast extract up to pH=8.0

Low ambient pH is reported to induce alkalinization and ammonium production by *C. gloeosporioides* and *C. coccodes* (Alkan *et al*., 2008, Prusky *et al.*, 2001). I inoculated *C. graminicola* and *C. gloeosporioides* on solid complete media containing 1% yeast extract (complete medium, 3% agar)(Miyara *et al.*, 2008), and with a starting pH of 4.5, 6.0, or 8.0. The plates were cultured for 4 days, and the pH was measured at 1.5, 3.0 and 4.0 cm from the inoculation point using a pH indicator paper. The results obtained showed that, when the initial pH values were 4.5 and 6.0, both strains increased the pH of the medium out to several millimeters ahead of the leading edge of the colony (Figure A1.2). However, no change in pH was detected in yeast extract medium at an initial pH of 8.0 (Figure A1.2). In all cases, non-inoculated controls remained at the starting pH values, except for pH 8.0, where at day 4 after inoculation, the pH had decreased slightly to 7.5.

Nitrogen starvation induces an increase in pH and ammonium production by _C. gloeosporioides_, but not by _C. graminicola_

Nitrogen starvation is reported to induce the production of ammonium by *C. gloeosporioides* (Drori *et al*., 2003, Kramer-Haimovich *et al*., 2006).I tested the effect of nitrogen starvation by two different approaches.

In the first experiment, *C. graminicola* and *C. gloeosporioides* were grown in rich medium containing 1% yeast extract, starved for nitrogen for 15 hours (induction treatment) and then transferred to fresh 1% yeast extract medium with an initial pH of 4.0. Details of 1% yeast extract medium are published elsewhere (Miyara *et al.*, 2010).

Changes in pH and ammonium accumulation were compared with non-inoculated controls, and with treatments that had not been starved for nitrogen.

A continuous increase in pH was detected in media inoculated with either strain, whether nitrogen-starved or not (Figures A1.3A and A1.3B). The increase in pH was correlated with accumulation of ammonium ion in both cases. Nitrogen starvation did not appear to significantly affect ammonium production by either strain.

In a second experiment both strains were pre-grown in MS medium, starved for 15 hours, and then transferred to SM medium at an initial pH of 4.0. MS medium is a nitrogen-rich medium and contains sucrose as source of carbon, while SM medium contains KNO_3 as the sole source of nitrogen, and glucose as a carbon source. Details of MS medium, starvation (intermediate medium) and SM medium are published elsewhere (Kramer-Haimovich *et al*., 2006). The pH and ammonium concentration were measured for 5 days.

C. gloeosporiodes and C. graminicola both increased the pH of the media, whether nitrogen-starved or not (Figures A1.3C, A1.3D). Five days after *C. gloeosporioides* was transferred to the SM medium, the pH had increased to 6.42 under induced conditions, and to 6.25 under non-induced conditions (Figure A1.3C). After five days, pH in SM medium inoculated with C. graminicola increased from 4.0 to 5.15 in treatments that were starved for nitrogen and from 4.0 to 4.91 in the non-starved treatments. *C. graminicola* had a significantly lower growth rate in this medium than *C. gloeosporioides* did, which might account for the slower changes in the pH (Figure A1.4).

Ammonium ion was detected in SM medium inoculated with *C. gloeosporioides*, but only in the nitrogen starvation-induced treatments (Figure A1.3C). The accumulation of ammonium was first detected after 96 hours of incubation and reached 30 mg/L by 5 dpi. No ammonium ion was detected in any of these flasks inoculated with *C. graminicola* (Figure A1.3D).

Conclusions

As previously reported, *C. gloeosporioides* seems to modulate environmental pH in vitro by secretion of ammonium, and ammonium production seems to be triggered by nitrogen starvation.

Ammonium was produced by *C. gloeosporioides* in both yeast extract and SM medium (the latter only under starvation conditions). However, ammonium was only produced by *C. graminicola* in yeast extract. Production of ammonium by both fungal species in yeast extract was not affected by nitrogen starvation. These observations suggest that the ammonium produced in the yeast extract may have resulted from deamination of amino acids, and not production via the nitrate reduction pathway. Increase in pH due to production of ammonia from oxidative deamination aminoacids has been reported in other fungi growing at low pH in media containing yeast extract (St Leger *et al.*, 1999). This possibility has also been considered in *C. gloeosporioides* (Prusky *et al*., 2001).

C. graminicola was able to increase environmental pH *in vitro*, but to a lesser extent than *C. gloeosporioides.* This ability was not affected by nitrogen starvation in either fungus in my experiments. This does not agree with previously published studies that suggest that nitrogen limiting conditions induce a more rapid and higher alkalinization by *C. gloeosporioides* (Drori *et al*., 2003, Kramer-Haimovich *et al*., 2006) . Overall, I did not find significant evidence to conclude that nitrogen starvation played a role in the increase of pH, as has been described for *C. gloeosporioides.* The change in pH also was not consistently associated with production of ammonium. *C. graminicola* was able to increase the pH of SM medium, but apparently not by the production of ammonium. I do

not know what *C. graminicola* might have been producing instead of ammonium to change the pH of the SM medium.

The detection of pH and ammonium *in vivo*, reported in the paper by O'Connell collaborators (O'Connell *et al.*, 2012), relied on measurements with a fluorescent dye in whole inoculated tissues. I failed to obtain consistent results using this dye. Gene expression data do not support the hypothesis that the nitrate utilization pathway is involved in alkalinization during necrotrophy by *C. graminicola*. My results suggest that nitrogen metabolism and pH modulation by *C. graminicola* in maize may occur by different mechanisms than in some other *Colletotrichum* fungi.

Table A1.1. Gene expression of nitrogen metabolism associated genes. Summary of expression showing *C. higginsianum* and *C. graminicola* orthologs of *C. gloeosporioides* genes associated with ammonium synthesis and secretion (Nit, GDH2, AMET), ammonium uptake (MEP, GLT), and ammonium regulation (GLRG_04139). Significant higher expression (red) or lower expression (green) are shown as log2 fold changes.

Predicted function	Gene ID	AP/BT	BT/NT	AP/NT
Nitrate reductase (Nit)	CH063_08184	0.90	2.53	3.43
	GLRG_02724	1.48	-0.50	0.98
Glutamate dehydrogenase (GDH2)	CH063_04868	-2.14	0.23	-1.92
	GLRG_00422	1.57	-0.81	0.76
Ammonium exporter (Spanu *et al.*)	CH063_14932	-1.04	0.51	-0.52
	GLRG_06038	1.87	-0.86	1.01
Glutamate importer (MEP)	CH063_12269	0.80	3.81	4.62
	GLRG_03954	0.60	-2.52	-1.92
Glutamate importer (GLT)	CH063_12275	-0.09	-3.72	-3.81
	GLRG_04076	-2.92	-3.45	-6.37
Glutamine synthase (GS1)	CH063_04573	-0.28	-0.45	-0.74
	GLRG_04139	0.09	0.30	0.38

Figure A1.1. Growth of *C. graminicola* in 1% yeast extract medium affected pH. **A.** pH increased from 4.0 to 8 (green) below the media where the fungus had grown, and to pH 6.0 (yellow) milimiters ahead of the fungal colony to pH 6.0 (yellow). **B.** pH changes induced by *C. gloeosporioides* and *C. graminicola* in media with initial of pH 8.0 (dark gray line), pH 6.0 (black line) and pH 4.5 (light gray bar). Edge of the colony is shown with a dotted line. Error bars represent standard deviations.

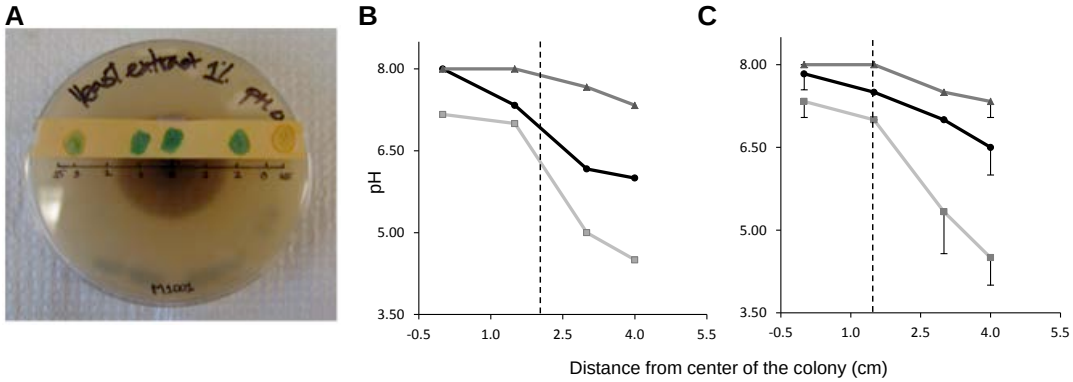


Figure A1.2. pH changes and ammonium accumulation. Induced by **A** and **C.** *C. gloeosporioides* and **B** and **D**. *C. graminicola* in 1% yeast extract **(A-B)** or SM medium **(C-D)** after nitrogen starvation treatment (black bar), without nitrogen starvation treatment (white bar) or uninoculated media (gray bar). Lines represent the accumulation of ammonium after nitrogen starvation treatment (black line), without nitrogen starvation treatment (white line) and uninoculated media (gray line). Error bars represent standard deviations.

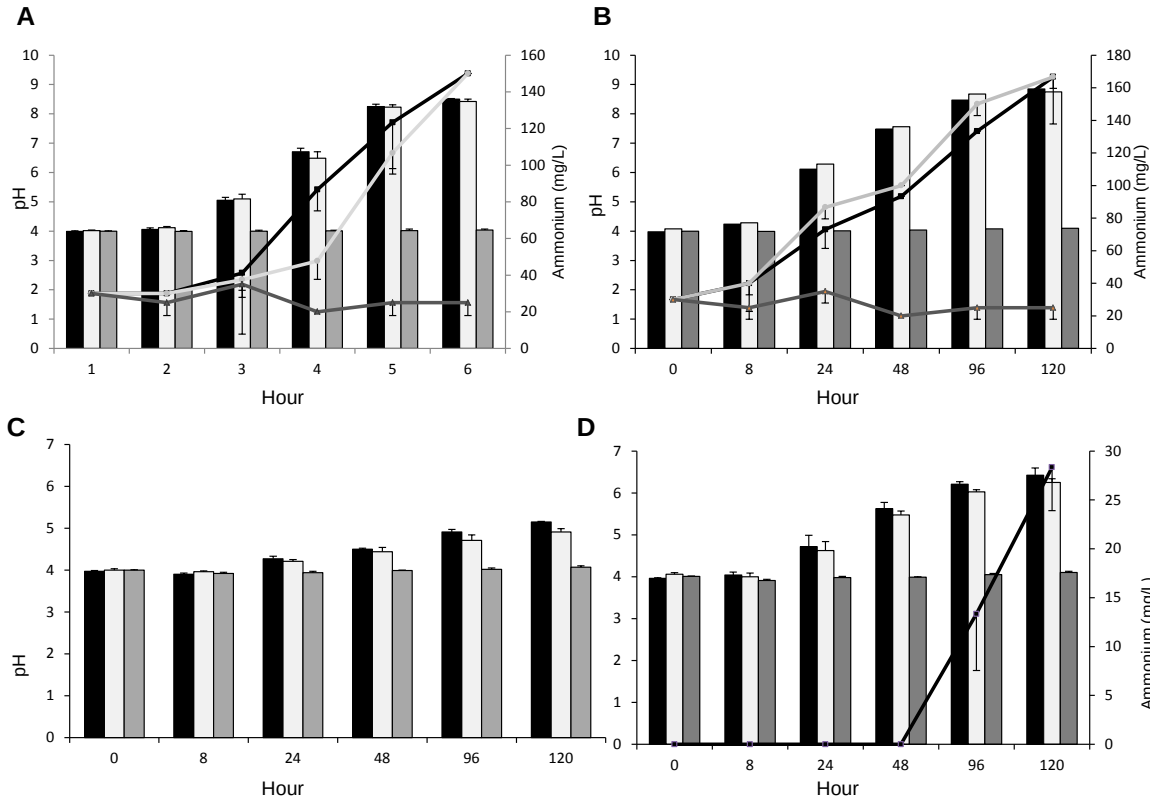
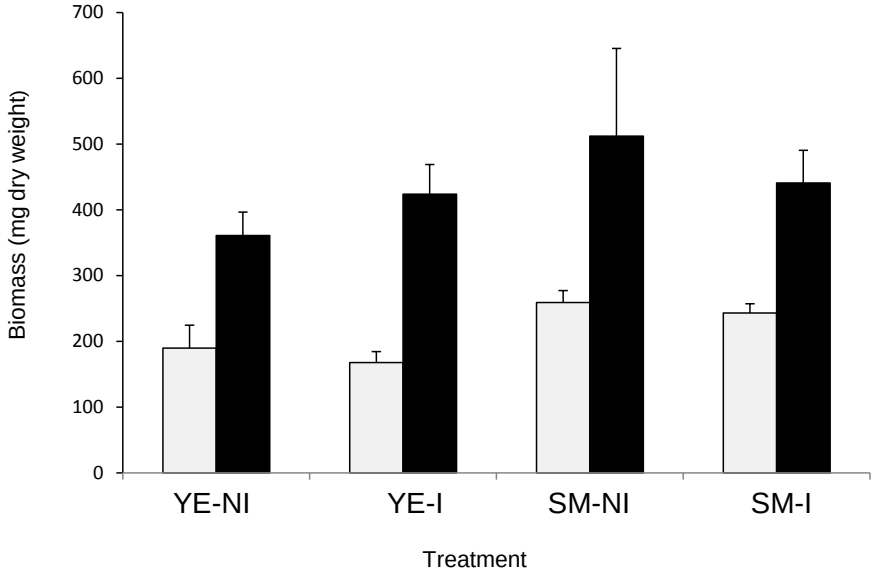


Figure A1.3. Biomass production by *C. graminicola* and *C. gloeosporioides*, under different nitrogen conditions. *C. graminicola* (white bars) and *C. gloeosporioides* (black bars) produced on yeast extract (YE) or secondary medium (SM), five days after nitrogen starvation (induction-I) or without nitrogen starvation (non-induction-NI).



Appendix 2

A potential role for fungal nitrate metabolism in pathogenicity of *C. graminicola*

It is known that nitrogen has a significant effect on *C. graminicola* pathogenicity. When levels of host nitrogen are low, plants become predisposed to infection by *C. graminicola* (Inguagiato *et al.*, 2008). It is known that expression of fungal pathogenicity genes is often induced by nitrogen starvation (Talbot *et al*., 1993, Stephenson *et al*., 2000, Pérez-García *et al.*, 2001, Donofrio *et al.*, 2006). Nitrogen regulation has been implicated directly in pathogenicity of *C. lindemuthianum*: a mutation in the AreA master regulator, which functions to switch on genes that are under nitrogen catabolite repression, resulted in a nonpathogenic strain that could not colonize or switch to necrotrophy(Pellier *et al*., 2003) .Genes that are under the regulation of AreA include genes involved in nitrate utilization. The nitrate reductase gene, the first gene in the nitrate utilization pathway, is expressed at moderate levels during all stages of *C. graminicola* development on maize sheaths, including necrotrophy (Figure A1.1).

To better understand the role of the nitrate utilization pathway in pathogenicity, I tested the ability of several independently generated nitrate reductase mutants to infect and colonize leaf sheaths from different resistant and susceptible maize lines. The mutant strains are described and characterized in (Vaillancourt & Hanau, 1994)

Previous studies have reported that *nit* mutations have varying effects on pathogenicity. Thus, *nit* mutants of *C. coccodes* secrete less ammonium and have lower infection rates, when inoculated in tomato fruits (Alkan *et al.*, 2008). *C. gloeosporoides nit* mutants are also unable to accumulate ammonium, secrete pectate lyase PelB, and were significantly less virulent in avocado fruits (Kramer-Haimovich *et al.*, 2006). In contrast, *nit* mutants of *C. graminicola* were reported

to have normal levels of pathogenicity on leaf blades of a highly susceptible inbred maize line (Vaillancourt & Hanau, 1994).

For my experiments, I used three independently generated *nit* mutant strains, and their progenitor strains as controls (Table A2.1). I included two versions of the M1.001 strain: M1.001 was the strain from which two of the nit mutants were produced, and was stored on silica in a -80 freezer at the same time as the *nit* mutants, in 1991. It was the best control for these mutants, therefore. During the ten-year period that these strains were left in the freezer before revival, M1.001BH was in daily use in the laboratory of Dr. R. Hanau at Purdue (thus M1.001BH). Another graduate student had noted that there were minor differences between these two versions of the M1.001 strain, perhaps due to accumulation of mutations during culture of the M1.001BH strain. Leaf sheaths from the susceptible maize varieties Jubilee and Mo940, and from the resistant varieties Mp305 and H99, were harvested and inoculated as described in chapter 2 of this dissertation.

The *nit* mutants were more aggressive on leaf sheaths of the resistant variety H99

All the strains were able to germinate, penetrate, and colonize maize leaf sheaths of all four maize varieties. I determined the number of cells colonized by each strain 48 hours post inoculation (hpi). All strains colonized the susceptible varieties to a significantly greater extent than the resistant ones (Figure A2.1) All strains seemed to have similar colonization rates in three of the maize varieties tested (Figure A2.1). However, all three *nit* mutants appear to have a higher rate of colonization in the resistant variety H99, when compared to their progenitor strains (Figure A2.1D), routinely colonizing up to 6 or 7 cells when the progenitors had typically colonized only up to 4 or 5 cells.

The *nit* mutants induce susceptibility to *cpr1*-ZsGreen

The *nit* mutants M1.401 and M1.402 were able to induce susceptibility to *cpr1*-ZsGreen in M0940 and H99 leaf sheaths when co-inoculated "at a distance" (not shown). The level of susceptibility did not appear to differ from that induced by the WT progenitor M1.001.

The *nit* mutants are also more aggressive to intact leaf blades than their progenitor strains.

To determine if the difference in aggressiveness to leaf sheaths of maize variety H99 also occurred in whole plants, I performed quantitative whorl inoculations in the maize varieties Mo940 and H99, using four different spore concentrations. Eight days after inoculation, the plants were evaluated for the presence or absence of anthracnose symptoms. Three plants per treatment were used in each experiment, and the experiment was repeated twice.

There were significant differences in disease incidence at different spore concentrations ($P < 0.05$) between the *nit* mutants and their respective progenitor strains in the H99 plants (Figure A2.2A), but not in the Mo940 plants (Figure A2.2B).

Discussion

Three independently generated *nit* mutants were tested, including two different loci (nitrate reductase enzyme and molybdenum cofactor) that affect nitrate reductase activity. All three produced similar results, strongly suggesting that the increased aggressiveness I observed in the leaf sheath and whorl assays are due to the defect they share in the nitrate utilization pathway. In the future it will be important to create a targeted disruption of the nitrate reductase gene, and compare the knockout (KO) with ectopic and complemented strains to confirm these results.

My observations confirmed previous reports that the *nit* mutants had normal pathogenicity to the susceptible maize variety Mo940. They were also normal on

the highly susceptible sweet corn hybrid Jubilee and on the resistant inbred Mp305. This is added evidence that the nitrate utilization pathway does not play an important role in alkalinization of tissue as an aid to pathogenicity in *C. graminicola* (see Appendix 1 of this dissertation).

In sheath assays I saw significant levels of colonization of the resistant varieties Mp305 and H99, even though leaf blades of these varieties exhibit very high levels of resistance to the pathogen. This indicates that leaf sheaths are more susceptible than leaf blades, perhaps due to their status as sink rather than source tissues, which are known to express defense genes to a lesser degree (Coleman, 1986, Fischer *et al.*, 1999). Nevertheless, the degree of colonization of the resistant lines was significantly reduced in comparison with the susceptible lines, demonstrating that sheaths react to the disease in way that is correlated with their field reactions. Furthermore, sheaths may provide a suitable model for stalk tissue, which is also a sink. ASR is a much more economically important problem than ALB. This hypothesis needs to be tested by comparing the interactions of the pathogen with stalk and sheath tissues in more detail.

It is not clear why the *nit* mutants were more aggressive specifically to H99. H99 is a maize inbred line that is highly susceptible to anthracnose stalk rot (ASR), but highly resistant to anthracnose leaf blight (ALB) (Warren & Shepherd, 1976, Sukno *et al.*, 2008).The other resistant line, Mp305, is highly resistant to both phases of the disease, due to the activity of a single resistance locus that contains two LRR-type R genes (Broglie *et al.*, 2011, Frey *et al.*, 2011)The genetic basis for foliar resistance in H99 is unknown, but its expression is associated with a rapid and extensive production of ROS and induction of HR. DAB staining on sheaths inoculated with the WT and the *cpr1* mutant strains, indicated a strong accumulation of H_2O_2 48 hpi, compared to the observations made on the susceptible cultivar Mo940 described in chapter 2. By 24 hpi, H_2O_2 was detected on sheaths inoculated with both strains (Figure A2.3A,D). Forty-eight hours post inoculation, a wide distributed, strong accumulation of H_2O_2 was

indicated by dark brown precipitates. DAB precipitates were localized, and were only observed in cells in the proximity of the fungus. Interestingly, despite of the strong DAB precipitates, the WT was able to colonize H99 leaf sheaths, while the mutant did not (Figure 2.3C, F). Accumulation of DAB precipitates was previously described in *C. graminicola* infecting maize leaf blades (Vargas *et al.*, 2012). Vargas and collaborators, suggest that plant-derived vesicles were responsible for accumulation of H_2O_2 around fungal hyphae which increased during the switch to necrotrophy. The mechanisms that *C. graminicola* uses to overcome ROS accumulation and colonize the plant tissue remain unknown.

Nitrate-reductase deficient fungal mutants tend to accumulate nitrate. (Schinko *et al.*, 2013). In animal systems, accumulation of nitrate increases activity of nitric oxide synthase (NOS), which can generate superoxides and induce oxidative stress (Schinko *et al.*, 2010). Therefore, it is possible that *nit* mutants are under a state of constitutive oxidative stress, and this may "prime" them so that they can adapt to the oxidative stress that occurs during host defense more efficiently. This hypothesis should be tested in the future by evaluating the expression of NOS and also the generation of ROS and resistance to oxidative stress of these mutants and targeted KO strains.

Table A2.1. Details of *nit* mutant strains used to inoculate sheaths from anthracnose susceptible and resistant maize cultivars

Parental strain	*nit*$^-$ strain	Mutation
M9.001	M9.401	Nitrate reductase
M1.001	M1.401	Nitrate reductase
M1.001	M1.402	Molybdenum cofactor

Figure A2.1. Average number of maximum colonized cells (%) by the different *nit* mutants and thei parental strains in leaf sheaths of four the maize varieties. **A.** Jubilee, **B.** Mo940, **C.** Mp305 and **D.** H99. Arrows indicate *nit* mutants. ■ Appressoria, ■ 1 cell, ■ 2 cells, ■ 3 cells, ■ 4 cells, ■ 5 cells, ■ 6 cells, ■ 7 cells. Arrows indicate *nit* mutants**.**

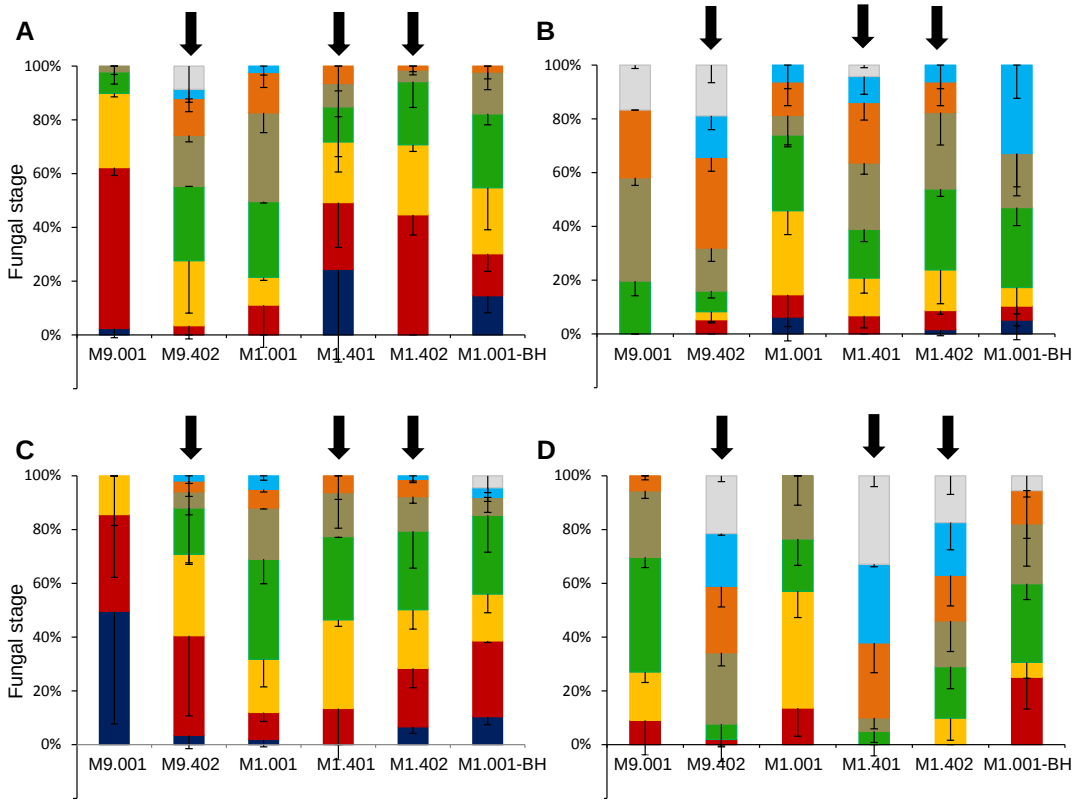


Figure A2.2. Incidence of anthracnose symptoms caused by the nit mutants and parental strains on the maize varieties **A.** H99 and **B.** Mo940, inoculated with different spore concentrations. 1 x 10^6 spores/ml (light gray bars), 5 x 10^5 spores/ml (black bars), 1 x 10^5 spores/ml (white bars) and 5 x 10^4 spores/ml (dark gray bars). Asterisks represent strains with significant differences (*P* <0.05) in pairwise comparisons.

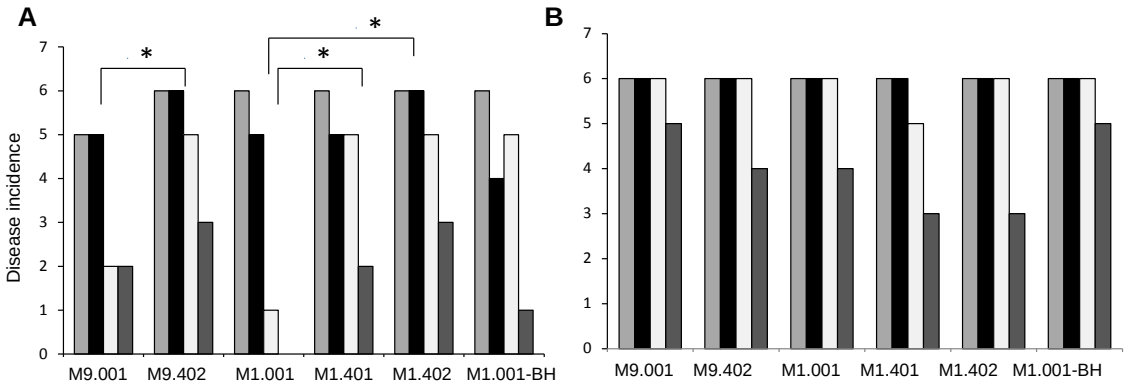
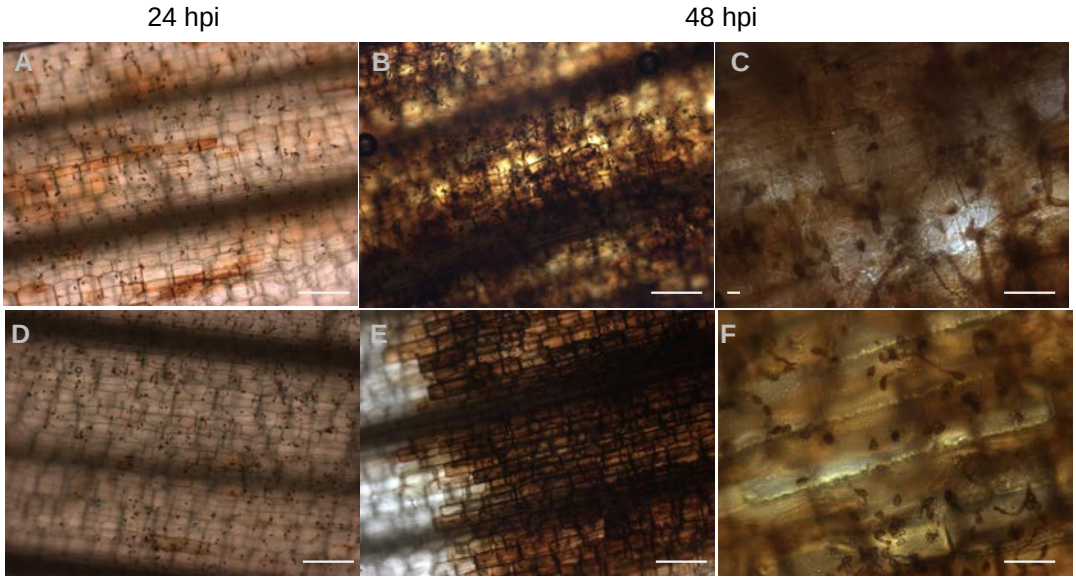


Figure A2.3. Patterns of ROS accumulation on H99 leaf sheaths. Leaf sheaths inoculated with the WT **(A-C)** and *cpr1* strains **(D-F).** Scale bars equal to 200µm in A-**E**, and 50µm in **C** and **F**.



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EDUCATION
B.S. Industrial Microbiology. 2006. Pontificia Universidad Javeriana. .Colombia

PROFESSIONAL EXPERIENCE
August 2007-Present. Graduate Research Assistant, University of Kentucky.

January 2006 -June 2007. Research Assistant. Colombian Corporation for Agricultural Research, CORPOICA. Colombia.

January 2005 – January 2006. Internship. Colombian Corporation for Agricultural Research, COPOICA.ColombiaJuly 2003 – December 2004. Student Worker. Colombian Corporation for Agricultural Research, COPOICA. Colombia.January 2003 – June 2003. Teaching Assistant. Pontificia Universidad Javeriana. Colombia.June 2001 – December 2001. Internship. Colombian Corporation for Agricultural Research, COPOICA. Colombia

RESEARCH PUBLICATIONS

O'Connell, J. O., Thon, M. R., Hacqard, S., Ver Lore van Theemaat, E., Amyotte, S. G., Kleemann, J., Torres, M. F., (57 more authors), Vaillancourt, L. J. 2012. Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. Nature Genetics44, 1060–1065

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PUBLISHED CONFERENCE PROCEEDINGS

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PROFESSIONAL SERVICE

Student representative on the Department of Plant Pathology Academic Program Committee, Jan. 2012-present.

PROFESSIONAL ACTIVITIES

- Member of the Mycological Society of America (2010)
- Member of the American Phytopathological Society (APS) since 2012
- Member of APPS (Association of Plant Pathology Scholars) at UK since 2010

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