THE DEVELOPMENT OF COLLETOTRICHIUM GRAMINICOLA INSIDE MAIZE STALK TISSUES

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ABSTRACT OF DISSERTATION

Claire Marie-Pierre Venard

The Graduate School
University of Kentucky
2006
THE DEVELOPMENT OF *COLLETOTRICHUM GRAMINICOLA* INSIDE MAIZE STALK TISSUES

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ABSTRACT OF DISSERTATION

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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
Claire Marie-Pierre Venard
Lexington, Kentucky

Director: Dr. Lisa Vaillancourt, Associate Professor of Plant Pathology
Lexington, Kentucky

2006

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Colletotrichum graminicola is the causal agent of anthracnose stalk rot, and is one of the most common and aggressive pathogens of maize. The goal of my Ph.D. project was to contribute to a better understanding of the biology of the interaction between C. graminicola and its host. C. graminicola produces two types of asexual spores: one is produced on the surface of infected tissues and is thought to be involved in the spread of the disease in the field. The second type of spore, oval in shape, is produced inside the infected plant tissues, and was believed to be involved in the movement of the pathogen inside the plant tissues via the vascular system. I tested this hypothesis with both cytological and molecular approaches. I used strains of C. graminicola expressing green fluorescent proteins (GFP) to inoculate wounded plants, and followed the development of the pathogen over time. This study revealed that C. graminicola is not a vascular pathogen. C. graminicola primarily moved through the rind and vascular fibers. Oval spores were produced in colonized parenchyma cells and remained dormant, and did not appear to be involved in the movement of the pathogen, at least during the early stages of the disease development. I also studied pathogen ingress in the absence of a wound. I inoculated unwounded plants with the GFP expressing strains. C. graminicola efficiently colonized the epidermis and, given enough time, penetrated and colonized the deeper parenchyma tissues, after first moving through the fibers. To further test the role of sporulation in colonization of maize tissues, I used targeted mutagenesis to disrupt a major gene known to regulate sporulation and vegetative growth in several other fungi. The gene Cgg1, orthologue of the A. nidulans fadA, was disrupted using the split marker method. The Cgg1 mutants were less pathogenic than the wild-type to wounded plants. This was associated with an apparent increase in production of spores and primary infection hyphae. This suggests that Cgg1 signaling pathway plays a role in maximizing colonization of host tissues, and that this involves negative regulation of sporulation and primary hyphae production in planta.

KEYWORDS: Colletotrichum graminicola; anthracnose stalk rot; green fluorescent proteins; fibers; split marker method.
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THE DEVELOPMENT OF *COLLETOTRICHUM GRAMINICOLA*
INSIDE MAIZE STALK TISSUES

By

Claire Marie-Pierre Venard

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CHAPTER 1
INTRODUCTION TO MAIZE STALK ROT

Background

Maize (*Zea mays* L.) is economically the most important crop grown in the United States. The value of the U.S. maize crop in 2005 was $23 billion, ahead of soybeans ($17 billion) and wheat ($7.3 billion) (N.C.G.A. brochure, 2005). Maize has many important uses in addition to animal feed, including the production of oil, sweeteners, fibers, and even biodegradable plastics. The recent resurgence of interest in ethanol as an alternative fuel is likely to dramatically increase the demand for maize, and thus its value (I.P.M centers brochure, 2003). Numerous diseases impact maize yield and quality. Although it is impossible to precisely measure the losses caused by diseases, “best-guess” estimates range from 2 to 15% annually (White, 1999). Generally, maize diseases can be effectively managed through the use of host resistance and cultural practices, but new diseases frequently arise with little warning, and they can cause considerable damage. One example is the Southern Corn Leaf Blight (SCLB) epidemic that dramatically impacted maize production in the United States in the early 1970s (Ullstrup, 1972). Another is the emergence of gray leaf spot as a significant worldwide problem in the 1990s (Ward et al., 1999). High-yielding hybrid maize continues to be vulnerable to disease epidemics, due to its comparatively narrow genetic base, and also because we still understand so little about the biology of most maize diseases. Constant changes in agronomic practices, crop and pathogen genetics, and climate patterns promote the frequent emergence of new disease problems (White, 1999), requiring our continued vigilance to protect this valuable crop.

Stalk rots, caused by several different species of fungi, are among the most widespread and destructive diseases of maize (I.P.M brochure, 2003). Stalk rots usually do not cause major epidemics due to effective levels of quantitative resistance in most commercial hybrids (Kansas University Extension Service publication, 1999; University of Illinois Extension Service publication, 1995). However, stalk rot diseases are ubiquitous and chronic in maize fields worldwide, and they are estimated to reduce potential yields every year by at least 5% (White, 1999). That loss represents more than $1 billion in the U.S. alone, at 2005 prices. Stalk rots can cause yield losses by inducing premature plant death, which reduces grain fill, or by causing lodging, which makes harvesting difficult or impossible. Stalk breakage causes ears to touch the
soil, allowing ear rots to develop more easily (White, 1999). In some years, damaging epidemics of stalk rot occur. Widespread epidemics in the early 1970s caused significant damage to a crop that was still recovering from SCLB (Hooker and White, 1976; Perkins and Hooker, 1979). In 2000, stalk rot diseases were again particularly damaging throughout the United States (Malvick, 2001; Vincelli, 2000). That year, in Kentucky, stalk rot caused more than 90% lodging in some fields (P. Vincelli, personal communication).

Maize stalk rot epidemics are sporadic and relatively difficult to predict. The occurrence of stalk rot is associated with stresses during grain filling (White, 1999; Dodd, 1980). Conditions that reduce photosynthesis and the production of carbohydrates, including high plant population densities, foliar diseases, drought or soil saturation, reduced sunlight, cold weather, and nutritional imbalances, tend to promote the development of stalk rot (White, 1999; I.P.M. centers brochure, 2003). Insect damage is also associated with stalk rots because it creates wounds through which the pathogens can penetrate. The European corn borer (ECB) (*Ostrinia nubilalis*) is one of the most economically important insect pests of maize, causing an estimated loss of $1 billion annually (Gatch and Munkvold, 2002a). ECB larvae tunnel through the stalk tissues and cause physiological stress that predisposes the plants to stalk rot, and they can also serve as a vector for pathogens (Gatch and Munkvold, 2002 b). Genetically modified maize varieties carrying a *Bacillus thuringiensis* Bt toxin gene, which protects them from ECB damage, have a lower rate of infection by some, but not all species of stalk rot fungi (Munkvold and Hellmich, 1999; Gatch and Munkvold, 2002 a).

**The Challenge of Stalk Rot Management**

There are no viable chemical controls for management of stalk rots (White, 1999; I.P.M brochure, 2003). Reducing the stresses that predispose the plants, and using resistant or tolerant varieties, helps to minimize the impact of the disease. However, there is no maize variety that is resistant to all species of stalk rot fungi. Furthermore, even resistant maize plants become much more susceptible to stalk rots when they reach the grain-filling stage of development (Dodd, 1980). One hypothesis holds that carbohydrates are redirected to the ears during grain fill, to the detriment of the stalk and root tissues, which begin to senesce as a consequence and become more susceptible to opportunistic pathogens (Dodd, 1980; White, 1999). However, selecting for
post-anthesis stalk-rot resistance by increasing the persistence of carbohydrates in the stalk could be expected to negatively impact yield (White, 1999).

A major obstacle to developing new management strategies to control stalk rots is that we do not understand the basic biology of the disease. A diverse group of fungi cause stalk rot, including *Gibberella zeae*, *Fusarium graminearum*, *Fusarium verticillioides*, *Colletotrichum graminicola*, and *Stenocarpella maydis* (White, 1999; Agricultural Handbook, 1974). The mechanisms by which these pathogens infect and colonize stalk tissues are poorly understood. The goal of my Ph.D. research was to address this question for one of the most important stalk rot pathogens, *Colletotrichum graminicola* (Ces.) G. Wils (teleomorph *Glomerella graminicola* Politis). This pathogen, unlike other stalk rot fungi, is able to damage healthy and vigorous stalks, in addition to attacking senescing tissues (Dodd, 1980; Bergstrom and Nicholson, 1999). It also attacks other parts of the living maize plant including leaves, seedlings, and roots (Warren and Nicholson, 1975). It is one of the most common and aggressive of the stalk rot pathogens (White, 1999). On stalks, *C. graminicola* causes a disease called anthracnose stalk rot.

**The Impact of Anthracnose Stalk Rot**

Anthracnose stalk rot (ASR) caused by *C. graminicola* is a disease of worldwide importance on maize. ASR was considered as a minor disease in the United States until the early 1970s. It was first reported in the early 1940s in Illinois, associated with low phosphorus levels in the soil (Koehler, 1943). In the early 1960s, the disease was reportedly occurring more and more frequently in Arkansas (Dale, 1963). The first major epidemic occurred in 1972 in Indiana, where fields of sweet corn were completely devastated. More than 50% of the plants lodged, and stalks as well as ears and leaves were severely infected (Warren et al, 1973). ASR was first reported in North Carolina fields in 1968 (Leonard and Thompson, 1969), and in 1972 and 1973, severe epidemics of anthracnose occurred there. In 1975, *C. graminicola* was found in 78% of Illinois fields with rotting stalks and prematurely dying plants (Hooker and White, 1976). Field surveys conducted in 1980 and 1983 in Illinois revealed that *C. graminicola* was present in 73% and 80% of the maize fields, respectively, and was the predominant fungus in rotted stalks (Byrnes and Carroll, 1986). Today, *C. graminicola* is endemic in most areas worldwide where maize is grown, and continues to cause losses every year (Bergstrom and Nicholson, 1999).
*C. graminicola* is a very highly adapted and successful pathogen of maize. It survives well in the maize agro-ecosystem because it can infect living plants (Dodd, 1980) and it can also survive as a facultative saprophyte in maize residues (Naylor and Leonard, 1977). *C. graminicola* can infect any part of the plant at any time during the growing season (Warren and Nicholson, 1975, Bergstrom and Nicholson, 1999). However, anthracnose leaf blight (ALB) on seedlings and older plants, and ASR on mature plants at or near anthesis, are the most common diseases caused by *C. graminicola* (White, 1999; Bergstrom and Nicholson, 1999). A survey conducted in 16 maize-producing states in 1997 showed that anthracnose (including both ASR and ALB) was the third most damaging disease, after ear and stalk rots caused by two other common stalk rot fungi, *F. verticillioides* and *G. zeae* (I.P.M. brochure, 2003). *C. graminicola* also causes an increasingly common syndrome known as “top dieback” which resembles a vascular wilt since the upper leaves and stalk internodes die early during grain formation, resulting in poor grain quality and yield reduction, and frequently also in lodging of the prematurely killed upper parts of the plant (White, 1999; University of Illinois Extension Service publication, 1995).

Estimated losses due to maize anthracnose range from 0 to 40%, and depend on the susceptibility of the hybrid, environmental conditions, and timing of infection (White, 1999; Bergstrom and Nicholson, 1999). In highly susceptible inbreds such as C123 and Mo940, the lower stalk tissues quickly become severely rotted, and the plant will die before it reaches maturity, resulting in a total loss. Such highly susceptible inbred lines have now been largely removed from breeding programs. Today, most commercially available hybrids do not usually become susceptible to ASR until a point at or near anthesis, when the pith tissues start to senesce (White, 1999).

**Mechanisms of Resistance to Colletotrichum graminicola**

Maize genotypes vary widely in their susceptibility to ASR. The use of resistant hybrids is the best method for controlling maize anthracnose (White, 1999). Resistance to ALB and ASR appears to be inherited independently, and may be controlled by one or a few genes with major effects, or by several genes with additive effects (Bergstrom and Nicholson, 1999). Inbreds Mp305 and LB31 have been shown to have one or two dominant resistance loci (Badu-Apraku et al, 1987a, 1987b; Toman and White, 1993; White, 1999; Bergstrom and Nicholson, 1999).
Generally, very little is known about the mechanisms of resistance of maize to *C. graminicola*, or to any stalk-rot fungus. A breakthrough was made recently when a major gene for ASR resistance, *Rcg1*, was identified by a positional cloning approach (Abad et al., 2006).

Interestingly, the gene appears to be a member of the large group of leucine-rich repeat (LRR) disease resistance genes that play roles in gene-for-gene interactions involving many different hosts and pathogens (reviewed by Martin et al., 2003). The *Rcg1* gene is highly significant because it is the first major gene for resistance to a *Colletotrichum* species that has ever been cloned.

The discovery that *Rcg1* is an LRR resistance gene was somewhat surprising because, notwithstanding one early suggestion to the contrary (Forgey et al., 1972), subsequent investigations have provided no evidence that physiological races of *C. graminicola* carrying different avirulence genes exist (Nicholson and Warren, 1976; White et al., 1987). Furthermore, resistance to *C. graminicola* is not generally associated with a recognizable HR reaction, and behaves as a quantitative trait. Even major-gene resistance like that in Mp305 can be defeated by host stress, high pathogen inoculum densities, or an environment that is particularly favorable for infection (Keller and Bergstrom, 1988; Bergstrom and Nicholson, 1999). For example, low light intensities decrease resistance to both ALB and ASR in a quantitative manner (Hammerschmidt and Nicholson, 1977a; Leonard and Thompson, 1976). The expression of resistance in leaves appears to involve the biosynthesis of phenolic compounds, which is light-dependent (Hammerschmidt and Nicholson, 1977b; Lyons and Nicholson, 1989; Lyons et al., 1993). Many phenolic compounds and their oxidation products are toxic to fungi. Evidence of a stimulation of phenylpropanoid synthesis during ALB is the formation of papillae in the wall of epidermal cells directly beneath fungal appressoria (Politis and Wheeler, 1973; Bergstrom and Nicholson, 1999; Mims and Vaillancourt, 2002). Expression of resistance also includes formation of stress lignin in cells surrounding lesions, which forms a barrier limiting the growth of the fungus, cell wall degradation, and lesion expansion (Muimba-Kankolongo and Bergstrom, 1990; 1992). In many maize cultivars, anthocyanin pigments are synthesized in a zone immediately surrounding the lesion (Hammerschmidt and Nicholson, 1977b). These pigments, which are also derived from the phenylpropanoid biosynthetic pathway, may act as scavengers of molecules that are toxic to the plants. All of these defense reactions occur in susceptible as well as resistant plants and the main difference appears to be in the speed with which they are induced.
(Bergstrom and Nicholson, 1999). Thus, the \textit{Rcg1} gene may act to recognize \textit{C. graminicola} and quickly activate these defense responses, before the pathogen has a chance to become established. The evidence suggests that the efficiency of the defense response pathway itself is significantly influenced by outside forces as well as by genetic limitations of particular host germplasm, and so it remains to be seen whether the \textit{Rcg1} gene will be equally effective in all genetic backgrounds and under a variety of conditions in the field. It also remains to be seen how quickly the pathogen will be able to adapt to widespread deployment of \textit{Rcg1} by production of a new race that lacks the putative avirulence gene product that \textit{Rcg1} recognizes.

\textbf{The Anthracnose Disease Cycle}

\textit{C. graminicola} overwinters as a saprophyte in infected maize residues on the soil surface (Naylon and Nicholson, 1977; Lipps, 1985; 1988). In spring, conidia are produced from acervuli that form on the debris. The horizontal dispersal of spores is limited to distances traversed by splashing and blowing raindrops. Anthracnose stalk rot epidemics develop rapidly under cloudy overcast conditions, but do not necessarily depend on prolonged periods of rain (Bergstrom and Nicholson, 1999). However, the disease is favored by extended periods of high humidity, a condition required for sporulation of \textit{C. graminicola} (Bergstrom and Nicholson, 1999; White, 1999).

Due to easier accessibility, the few studies of germination and infection of maize by \textit{C. graminicola} have been performed using leaves of highly susceptible seedlings (Politis and Wheeler, 1973, Mercure et al., 1994; 1995; Mims and Vaillancourt, 2002). Conidia of \textit{C. graminicola} adhere tightly to the leaf surface soon after contact, and usually require between 6 and 8 hours to germinate, as long as humidity levels are high (Mercure et al., 1994, 1995). When conidia germinate, they continue to secrete adhesive materials that bind the fungal germling to the plant surface. Germinating conidia often form a sessile appressorium, rather than a germ tube and an appressorium. The appressorium is induced by contact with a hard, hydrophobic surface (Chaky et al., 2000; Apoga and Hoch, 2004) An adhesive is also secreted to bind the appressorium to the plant surface. By 15 to 18 hours, the wall of the appressorium becomes fully melanized. The presence of melanin is believed to allow the development of high hydrostatic pressure within the appressorium, facilitating penetration inside the host cell (Bechinger et al., 1999). Infection is also facilitated by the production by the fungus of various lytic enzymes that
degrade the cuticle and cell wall (cutinase, cellulase, pectinase, and polygalacturonase) (Nicholson and Epstein, 1991; Nicholson, 1996). Optimal temperatures for spore germination and appressorium formation range from 15 to 35°C. Appressoria of *Colletotrichum* species remain viable for an extended period of time before penetration of the host tissues occurs (Prusky and Plumbey, 1992). Appressorial latency is prevalent on plant tissues inoculated during a period of rapid vegetative growth (Bergstrom and Nicholson, 1999).

Penetration into the host tissues and subsequent colonization are favored by a narrower temperature range than germination and appressorium formation, of between 25 and 30°C (Leonard and Thompson, 1976). Penetration occurs via an appressorial pore, a circular zone at the base of the appressorium that is not melanized (Politis and Wheeler, 1973; Mims and Vaillancourt, 2002). An infection hypha, also called a penetration peg, emerges through the pore and penetrates the host cell wall. A swollen primary infection hypha then develops inside the host cell. *C. graminicola*, like most *Colletotrichum* species, is a hemibiotroph, meaning that it infects its host initially as a biotroph and only later switches to necrotrophic growth (Perfect et al, 1999). During biotrophic development of the infection hypha, the host plasma membrane becomes invaginated and can be found in close association with the fungal cell wall. The host cell remains alive during this period, as evidenced by production of callose that surrounds the invading hyphae (Mims and Vaillancourt, 2002). The primary infection hyphae ramify and grow from cell to cell via narrow connections until, approximately 24 hours after initial invasion, the fungus switches to necrotrophic growth. Once this happens, the host cell walls become degraded and the pathogen is no longer surrounded by a host plasma membrane, but grows throughout the dead tissues as narrower, secondary hyphae (Mims and Vaillancourt, 2002). It is only during the necrotrophic phase that anthracnose symptoms appear. Symptoms of ALB typically consist of elongated oval lesions, parallel to the mid-vein, often with concentric zones of enlargement. Leaf blight can be severe in susceptible genotypes, even resulting in death of the leaf or the plant (White, 1999). However, plants at the seedling stage grow so rapidly that less susceptible genotypes will usually “outgrow” the disease.

Secondary inoculum is produced from lesions on the lower leaves. Conidia are disseminated vertically in the crop canopy by splashing rain, and repeated cycles of secondary inoculum production, dispersal and infection can occur throughout the season (Warren and Nicholson, 1975; Bergstrom and Nicholson, 1999). Leaves display a bimodal pattern of
susceptibility to ALB, with seedlings and senescing older leaves being more susceptible than leaves that are rapidly expanding (Leonard and Thompson, 1976; Bergstrom and Nicholson, 1999).

Infection of maize stalk tissues by *C. graminicola* has received much less attention than infection of the leaves. Pith infection certainly occurs via wounds that break the rind, and this appears to be the most frequent means of entry (Keller et al., 1986; Bergstrom and Nicholson, 1999). Wounds are most often the result of insect damage, particularly tunneling by ECB larvae. Conidia germinate at wound sites and develop long germ tubes (Bergstrom and Nicholson, 1999). Penetration of wounded tissues often occurs by hyphae directly, without the formation of an appressorium (Bergstrom and Nicholson, 1999). ASR also occurs commonly in the absence of injuries on the stem (Dodd, 1980; Gatch and Munkvold, 2002a, b), but the process of entry into unwounded stalks is much less understood. However, this question is likely to become increasingly important as the proportion of maize that contains *Bt* genes for resistance to insect feeding increases. This is because it was reported that, whereas the proportions of stalk rots caused by some other species of fungi decreased in Bt maize, the percentage caused by *C. graminicola* actually increased (Gatch and Munkvold 2002b). This suggests that *C. graminicola* is relatively efficient at penetrating and colonizing unwounded stalks. Conidia formed on leaves may be washed behind the sheath and it has been suggested that these are responsible for stalk infections. However, when conidial suspensions were deposited behind the sheaths of susceptible maize hybrids in the field, this resulted in infections less than 20% of the time, and most of those were associated with ECB damage (White and Humy, 1979). Thus, it appears that the intact maize rind is a very effective barrier to direct penetration by *C. graminicola*. It has also been suggested that the fungus first enters leaves and then moves systemically through the vascular tissues into the stalks. However, it is often possible to find severe ASR in the absence of significant levels of ALB (Carson and Hooker, 1981). Another suggestion is that the fungus enters roots and then enters the vascular system and becomes systemic. The ability of *C. graminicola* to infect roots has been established (Warren and Nicholson, 1975), but has not been further investigated.

Once *C. graminicola* has penetrated inside the maize stalk, a progressive rot of the pith tissues follows, rapidly expanding to encompass multiple internodes in a susceptible genotype. External symptoms of ASR include black discoloration of the rind tissues that may appear late in
the growing season. Internal symptoms include pith tissues that are blackened and easily crushed. Eventually the stalks may contain large cavities, with only the lignified bundle tissues remaining. At this point, the stems often become so weak that they collapse, and the plants lodge. Often, the external discoloration is not associated with pith rot. Conversely, rind tissues may appear green and healthy even when the pith tissues are rotten (White, 1999; Bergstrom and Nicholson, 1999). Systemic colonization at early developmental stages can result in a significant yield reduction (Bergstrom and Nicholson, 1999). *C. graminicola* is a particularly aggressive stalk rot pathogen that can rapidly destroy several internodes of a susceptible plant. In contrast, most of the other stalk rots will be confined to the basal internode. The means by which *C. graminicola* spreads so rapidly in stalks is not well understood, although several references demonstrate that discoloration associated with ASR proceeds initially along the bundles, with rot of the pith lagging slightly behind (Bergstrom and Nicholson, 1999; Tang et al., in press). *C. graminicola* can be cultured easily from isolated bundle tissues. This has led some to propose that *C. graminicola* is a vascular wilt, and spreads primarily via the vascular tissues.

### The Role of Sporulation in Pathogenicity of *C. graminicola*

Production and dissemination of spores is obviously a critical factor in the development of plant disease epidemics. The characteristic spores of *C. graminicola* are falcate to lunate in shape, and are produced from morphologically distinct conidiogenous cells in acervuli. Falcate conidia are produced in lesions on the plant surface, and are primarily responsible for dispersal of the fungus both among different parts of the same plant, and from plant to plant. Spores are carried primarily by splashing raindrops. Falcate conidia, whether produced in culture or in acervuli on the surface of infected tissues, are produced in an extracellular mucilaginous matrix (Nicholson, 1996). Upon drying, the matrix forms a film that surrounds the spores. The matrix’s antidessicant property allows the spores to survive through prolonged periods of drought. Because this mechanism allows the spores to survive as dry masses, it has been suggested that falcate spores might also be disseminated over long distances as particles by wind currents (Nicholson and Epstein, 1991).

Panaccione et al (1989) described a second, very distinct type of asexual conidium produced by *C. graminicola* during infection of maize leaves. This type is oval to elliptic in shape, multinucleate, and variable in size but usually smaller than falcate spores. Oval conidia
are produced from hyphae that lack distinct conidiogenous cells. Falcate conidia were reportedly produced only on the surface of solid media, whereas oval conidia were produced from submerged mycelium in either solid or liquid media (see Appendix A for some new observations I made regarding production of these spore types). Both types of propagule can be used to inoculate plants, and they induce similar symptoms on leaves, indicating that they are both pathogenic (Panaccione et al., 1989). The role of oval spores in the disease cycle, if any, is unclear. Because they are produced only inside tissues, and because they have reportedly been observed inside xylem vessels, it has been proposed that oval spores are involved in the movement of the pathogen systematically within the stalk, through the vascular system (Pannaccione et al., 1989; Bergstrom and Nicholson, 1999).

Hypotheses and Objectives of This Work

As described above, several hypotheses have been presented in the literature, usually without very much supporting data, about how C. graminicola infects and colonizes maize stalks (White, 1999; Bergstrom and Nicholson, 1999). In the Vaillancourt lab, a method had been developed to produce stalk rot in the greenhouse (Thon et al., 2000; 2002). The goal of my Ph.D. research was to use this method together with fungal strains labeled with green fluorescent proteins (described in detail in appendix B) to observe for the first time the details of colonization of the maize stalk by C. graminicola, and so address some of these hypotheses.

There were some significant constraints on my experiments, which to some extent influenced my experimental design. Because I was working with transgenic fungi, I had to work in the greenhouse for containment purposes. This meant that I could only work with maize at or near anthesis, because we quickly discovered that trying to grow maize significantly into the post-anthesis senescence stage in the greenhouse resulted in major insect pest problems (J.D. Brown, personal communication). I chose the highly susceptible sweet corn variety Jubilee for my work because I wanted to be able to observe all stages in development of the disease over the relatively short time frame available for each experiment in the greenhouse. Jubilee sweet corn is also historically significant because it was this variety that was decimated by the first epidemics of ASR and ALB in Indiana in 1972 (Warren et al., 1973). The sweet corn plants are relatively small, and they generally thrive under greenhouse conditions, when compared with most varieties of field corn. The light intensity in the greenhouse is generally lower than in the field,
particularly in the winter, and I did see some quantitative variation in the relative susceptibility of the maize in the winter versus in the summer. I included in my work two nonpathogenic strains of *Colletotrichum* so that I could compare susceptible and resistant reactions in the same corn genotype. My microscopy data are by nature mostly descriptive, but to increase confidence I replicated most of my experiments many times, and quantified as many of my observations as possible.

I wanted to test the hypothesis that oval spore production plays a role in the spread of *C. graminicola* in maize stalks. One obvious approach would be to create a mutant that does not produce oval spores. However, I knew that numerous unsuccessful attempts had already been made by other researchers to create such a mutant (L. Vaillancourt, personal communication), and so I chose not to pursue that possibility further. I initially evaluated some pathogenicity mutants of *C. graminicola* that had been identified in a REMI screen (Thon et al., 2000). These mutants produced fewer oval spores in culture, but this did not seem to have a direct role in their lack of pathogenicity to maize stalks, and so that line of inquiry was abandoned (the nonetheless interesting results of the preliminary experiments can be viewed in Appendix A). I then decided to use a targeted mutagenesis approach to create a mutant that might over-produce oval spores *in planta*. My hypothesis was that I would observe more rapid spread of a mutant that produced more spores, if they had a role in systemic spread of stalk rot. I resolved to create a targeted mutation in the homologue of the *Aspergillus nidulans* FADA gene, which encodes a heterotrimeric G-protein regulating growth and development in that organism. I predicted that mutating the gene would result in production of more spores and less vegetative growth (Yu and Keller, 2005). FADA has pleomorphic effects on asexual and sexual development and on secondary metabolism in *A. nidulans*, and so I also hoped that mutating such a key developmental gene would reveal previously unsuspected connections among these various pathways and pathogenicity to maize.
CHAPTER 2
COLONIZATION OF FIBER CELLS BY COLLETOTRICHUM GRAMINICOLA IN WOUNDED MAIZE STALKS

Introduction

-Colletotrichum graminicola- is the causal agent of maize anthracnose, can infect any part of its host plant at any time during the growing season (Warren and Nicholson, 1975; Bergstrom and Nicholson, 1999). However, it occurs most frequently and causes the most damage as anthracnose leaf blight (ALB) on seedlings and older plants, and anthracnose stalk rot (ASR) on mature plants at or near anthesis (Warren and Nicholson, 1975; Leonard and Thompson, 1976; Keller and Bergstrom, 1988; Callaway et al, 1992; Bergstrom and Nicholson, 1999). Prior to the 1970s, localized epidemics of ALB and ASR had been reported from various countries around the world, but neither disease was considered to be a serious threat in the United States (Dale, 1963). This perception changed during the early-to-mid 1970s, however, when severe outbreaks of ALB and ASR occurred throughout the north central and eastern United States (Warren et al, 1973; Hooker and White, 1976). Removing highly susceptible germplasm from commercial hybrids has prevented further epidemics on the scale of those seen in the 1970s. Nonetheless, anthracnose is still very common in U. S. maize fields (Byrnes and Carroll, 1986; Anderson and White, 1987; Bergstrom and Nicholson, 1999). ASR in particular causes significant yield losses every year, and it ranks among the most important disease challenges for the maize breeding industry (Gatch and Munkvold, 2002a,b).

-C. graminicola- is one of the most aggressive of the stalk rot fungi affecting maize (Mduruma, 1984; Bergstrom and Nicholson, 1999; Gatch and Munkvold, 2002b). External ASR symptoms, when they exist, consist of a shiny black discoloration of the rind tissue. When the infected stalks are cut longitudinally, the interior pith appears dark and rotted (Bergstrom and Bergstrom, 1987; Muimba-Kankogo and Bergstrom, 1992; Anderson and White, 1994; Bergstrom and Nicholson, 1999; White, 1999). Severely rotted internodes are structurally weakened, and the plant may fall over or “lodge” as a result. Degeneration of the stalk tissues can also cause premature death of the upper parts of the plant, a symptom called top dieback.

Resistance to ALB and to ASR appears to be primarily polygenic (Carson and Hooker, 1980, 1981), although several genes with major effects have also been reported (Badu-Apraku et
al, 1978, 1986). Genes conferring resistance to ALB do not necessarily confer resistance to ASR, and vice versa (Bergstrom and Nicholson, 1999). This suggests that the mechanism of resistance to ALB may be different from that of resistance to ASR. In genotypes resistant to ASR, damage is usually limited to one or a few adjacent internodes, which may nonetheless become extensively rotted, especially post-anthesis (Anderson and White, 1994).

Very little is known about the process of infection and colonization by *C. graminicola* in maize stalks, although leaf infection has been somewhat better characterized (Politis and Wheeler, 1972; Mims and Vaillancourt, 2002). *C. graminicola* has been described as causing a vascular wilt in stalks because top dieback symptoms are reminiscent of a wilt disease, and because the fungus can be recovered by culturing isolated vascular bundles (Bergstrom and Nicholson, 1999). *C. graminicola* produces two types of asexual spore. Falcate spores are produced on the surfaces of host tissues in acervuli, and play the primary role in dissemination of the pathogen from plant to plant. Oval spores are produced only inside the infected plant tissues (Panaccione et al, 1989). The role of the oval spores in the fungal life cycle is unclear, but because they have been observed inside xylem vessels (Bergstrom and Nicholson, 1999), it has been suggested that lesion expansion in the stalk occurs as a result of systemic movement of these spores through the vascular tissues within and between internodes (Panaccione et al, 1989; Bergstrom and Nicholson, 1999). My objective in the work described in this chapter was to address these hypotheses by observing in detail the colonization of maize stalk tissues, and particularly of vascular tissues in the stalk, by *Colletotrichum graminicola*.

**Materials and Methods**

*Fungal cultures and production of spore suspensions:* *C. graminicola* strain M1.001, also known as CgM2 (Forgey et al, 1978), was obtained from the late Dr. Robert Hanau of Purdue University. Strain 6-2 is an insertional mutant derived from M1.001. This mutant causes no symptoms on maize leaves, and only very limited lesions on wounded maize stalks (Thon et al, 2000). The mutant was used here so I could compare a compatible with an incompatible interaction on a single susceptible maize genotype. *Colletotrichum sublineolum* isolate CgS11 was obtained from Dr. Ralph Nicholson of Purdue University. It is pathogenic on sorghum stalks and leaves, but non-pathogenic to maize in the field. It was used here as a control for maize stalk quality. If the plants become too environmentally stressed in the greenhouse,
M1.001, 6-2, CgS11, and their respective green-fluorescent transformants, were grown on oatmeal agar (Difco) plates for two weeks. Falcate spores were collected by adding 10 ml of sterilized water and rubbing the surface of the culture gently with a plastic mini pestle. The conidial suspension was collected, filtered though sterilized glass wool, and the conidia were washed 3 times in sterilized water. The concentration of conidia was adjusted to $5 \times 10^6$ spores per milliliter after the third wash.

**Fungal transformation:** Two different methods were used to produce transformants expressing green fluorescent proteins (the transformants resulting from these experiments are described in more detail in Appendix B of this dissertation). The first was a PEG-mediated protocol described previously (Thon et al, 2000). Protoplasts were transformed using 3-9 µg of linearized plasmids: pCT74 contains an SGFP gene under the control of the *Pyrenophora tritici-repentis* TOX-A promoter, and a hygromycin phosphotransferase gene as a selectable marker (Lorang et al, 2001). M1.001 was transformed with pCT74 linearized with EcoR1. Strain 6-2, which is already resistant to hygromycin (Thon et al, 2000), was co-transformed with pAN8-1 and pCT74, both linearized with EcoR1, and transformants were selected on phleomycin then screened for GFP expression. Plasmids pSM615 and pSM611 both contain the ZsGreen gene (Bourett et al, 2002). pSM615 also contains a hygromycin phosphotransferase gene and was used to transform M1.001. pSM611 contains a geneticin resistance gene and was used to transform strain 6-2. Both plasmids were linearized before use with BamH1. pSM615 and pSM611 were generously provided by J. Sweigard (Dupont Experimental Station, Wilmington, DE, U.S.A.)

I also used a protocol developed for *Agrobacterium*-mediated transformation of falcate spores (Flowers and Vaillancourt, 2005). Two different plasmids were used. The first, pJF1, contains the same SGFP and hygromycin resistance genes and promoter elements as pCT74 (Flowers and Vaillancourt, 2005). The second plasmid, pBin-GFP-hph, also contains GFP and hygromycin resistance genes, under the control of other promoters (O’Connell et al, 2004). All transformants were single-spored and stored on silica at –80°C.

**Plant growth and inoculation:** The sweet corn hybrid Jubilee was chosen for this study because it matures quickly; it does not exceed a manageable size for the greenhouse; it is highly significant rotting of stalks inoculated with *C. sublineolum* can result (L. Vaillancourt, unpublished results).
susceptible to ASR and ALB; and it has a historical association with the first epidemics of anthracnose in the United States (Warren et al, 1973). Jubilee sweet corn seed was a generous gift of Syngenta, and was obtained from Rogers Seed Company (Rogers Seed Co., PO Box 4727, Boise, ID 8371). Plants were grown in the greenhouse in 10-inch pots in a mixture of 3 parts Pro Mix BX (Premiere Horticulture Ltd., Riviere du Loup, PQ, Canada) / 2 parts sterilized topsoil. Three seeds were planted in each pot. After germination, the seedlings were watered daily to saturation with a solution of 4.75g of Miracle-Gro 18-18-21 formulation for tomatoes (Stern’s Miracle-Gro Products Inc., Port Washington, NY, USA) per liter. Two plants per pot were ultimately retained until they reached a late vegetative stage (V11-V12). Just prior to anthesis, the sheath tissue was stripped from the second internode above the soil line. The plants were placed on their sides, and a small wound was made in the center of the stripped internode with a sterile dissecting needle (2 mm in depth). A 10 µl drop of conidial suspension, or sterile water as a control, was applied to the wound. The wound was covered with a detached microfuge tube cap and sealed with parafilm to create a moist chamber. The plants were left overnight. The tube caps were then removed, and the plants were placed upright. The inoculated internodes from two plants were harvested each day starting at 6 days after inoculation (dpi). For each experiment, a minimum of 20 plants was inoculated with each fungal strain or with water. Each experiment was repeated at least three times.

Statistical analysis: Nine different strains of C. graminicola were used in this study, including the three non-fluorescent strains (M1.001, 6-2, and CgSl1), and two independent SGFP or ZsGreen transformants of each. The lengths of the primary lesions produced by each strain were measured after splitting inoculated stalks longitudinally. Because measuring required the destruction of the stalks, the expansion of individual lesions could not be analyzed over time. Instead, each data point represents one separate stalk, collected beginning at 6 dpi up to 15 dpi. Lesion length data were analyzed using the General Linear Model (GLM) procedure that is part of the SAS statistical analysis software package (SAS Institute Inc., 1997). The Tukey mean separations test was applied to the data, with a significance level of P < 0.05. The effects on average lesion length of different strains (also including the water control) and of incubation time were analyzed.

Fluorescent microscopy: Fungal development in stalk tissues was monitored in a few cases by using a Zeiss laser scanning microscope model 410 (Carl Zeiss Inc., Thornwood NY),
or an Olympus FV1000 (Olympus America Inc., Melville, NY). A Leica TCS NT confocal microscope (Leica Microsystems Inc., Exton PA) was used for a majority of the observations. Infected tissue sections were cut by hand with a razor blade and observed without further treatment, or after staining with acridine orange according to the protocol of Guenther and Trail (2005) to visualize the fungal nuclei. Green fluorescent proteins (GFP and ZsGreen) were excited at 488 nm. Plant cell walls and chloroplasts autofluoresce at this excitation wavelength, and this property was used to visualize the plant tissues. However, at lower magnifications, the autofluorescence of the plant cell walls was usually not intense enough to be captured in the micrographs.

Resin embedding and sectioning: I used a tissue fixation and embedding procedure originally developed by Spurr (1969). Small pieces of tissue, 2-3 mm square, were dissected from inoculated and uninoculated stalk internodes. The fragments were stored prior to embedding in 2.5% gluteraldehyde in 100 mM KH$_2$PO$_4$ buffer, pH 6.8, at 4ºC. Resin-embedded specimens were cut into 1-2 µm thick sections with a Leica Reichert Ultracut microtome (Leica Microsystems, Inc., USA) and tungsten carbide knives (Delaware Diamond Knives, Wilmington, DE). The sections were stained with toluidine blue, air-dried and mounted, then observed with a light microscope (Zeiss AxioSkop Microscope; Carl Zeiss Jena GmbH, Zeiss Gruppe, 07740 Jena Germany).

Results

The following descriptions represent the consensus of thousands of observations of hundreds of individual inoculated plants over the course of more than two years (Tables 2.2, 2.3). There were two recognizable phases that occurred during the infection and colonization of wounded maize stalks by *C. graminicola*. A primary lesion formed at the wound site, which elongated by colonization of, and expansion through, the fibers associated with the vascular bundles and rind. Primary lesion development was followed by production of discontinuous secondary lesions at a distance from the wound site.

Primary lesion development: The development of lesions was monitored in living plants inoculated with fluorescent transgenic strains derived from the wild type *C. graminicola* isolate (M1.001), the *C. graminicola* mutant (6-2), and the *C. sublineolum* control isolate (CgSl1). The timing of the earliest stages of establishment of the fungi in maize stalks varied among replicate
experiments. However, by nine days after inoculation there were consistent and obvious quantitative differences between M1.001 and its fluorescent derivatives and the other strains (Figure 2.1A,B). Statistical analysis revealed that transformation with fluorescent proteins had no significant effect on the virulence of CgSl1 or 6-2 to maize stalks (measured as lesion length at 13 dpi, Table 2.1), but caused a slight decrease in the virulence of M1.001 (Table 1.1). M1.001 and the fluorescent strains derived from it were all significantly more virulent than the CgSl1 or 6-2 strains (Table 1.1). All of the treatments produced lesions that were significantly larger than those resulting from the water control.

The development of the lesion from a wound site inoculated with M1.001 or its fluorescent derivatives was primarily longitudinal (Figure 2.1B). The fungus established within the wounded tissues and progressively colonized and destroyed the pith tissues (Figure 2.2E, 3G). This decay eventually resulted in the formation of an elongated primary lesion inside the stalk (Figure 2.1C). The rind and the epidermal cell layer became desiccated, but remained intact above the disintegrated tissues at the center of the lesion (not shown). A broad darkly discolored zone surrounded the lesion (Figure 2.1C). Fluorescent mycelium was visible in this zone, but never beyond it (Figure 2.2A-D). Further observations of stained, embedded tissue sections confirmed that fungal hyphae were confined to the discolored areas (not shown). Falcate spores, together with setae, were produced on the surfaces of intact cells at the margins of the central lesion cavity, and also on aerial mycelium that traversed the spaces among the remaining vascular bundles within the cavity (Figure 2.1D, Table 2.2). Oval spores were produced inside some of the parenchyma cells within the discolored lesion border (Figure 2.2L, Table 2.2). In some cases, a few of the oval spores had germinated at the site of development (not shown). Fungal hyphae developed densely within the fiber cells beneath the rind (Figure 2.2H-J). This colonization was associated with a dark discoloration of the epidermis, and accumulation of thick-walled fungal material (stromata) in the epidermal cells (Figure 2.2M). Acervuli with setae and falcate spores were produced from these stromata if they were incubated in high humidity (not shown). Hyphae could sometimes be observed growing intercellularly in the primary lesions (Figure 2.2K).

The development of the mutant *C. graminicola* strains and the *C. sublineolum* strains was qualitatively similar to that of M1.001. A primary lesion developed (Figure 2.1E,F), in which falcate spores were produced. Oval spores were also produced in the discolored cell layers.
surrounding the lesions (Table 2.2). However, the size of the lesions, the frequency of colonization of the tissues surrounding the wounds, and the frequency of spore production, were all substantially reduced in plants inoculated with the 6-2 or CgSl1 strains in comparison with plants inoculated with M1.001 strains (Tables 2.1, 2.2).

Observations of sections of embedded tissue revealed that parenchyma cell walls were thicker in samples that had been wounded and treated with water than in samples from unwounded controls (Figure 2.3A-F). Cell walls did not appear to be thicker than normal in regions surrounding primary lesions formed by M1.001 (Figure 2.3G, I). In samples wounded and inoculated with CgSl1 or 6-2, the walls of cells surrounding the lesions did appear thickened, but cells containing fungal mycelium within the lesions appeared thinner-walled. In all inoculated tissues, numerous apparent occlusions were observed in the intercellular spaces (Figure 2.3I-K). These occlusions were not observed in wounded tissues treated with water alone, or in unwounded tissues. Papillae similar to those observed in leaf cells undergoing C. graminicola infection (Mims and Vaillancourt, 2002) were not found in stalk samples.

Two types of hyphae were observed colonizing the parenchyma cells at the margins of primary lesions (Figure 2.2N-P). Staining with acridine orange revealed that one hyphal type was multinucleate, with most of the cells (89%) containing two nuclei (Figure 2.2N,O). These hyphae were relatively thick, averaging 3.7 (± 0.8) mm in diameter. The second type was uniformly monokaryotic and noticeably thinner, averaging only 1.9 (± 0.6) mm in diameter (Figure 2.2O,P). Aerial hyphae traversing the spaces within the disintegrated centers of the lesions were all of the thinner, mononucleate type: the thicker, multinucleate hyphae were not observed outside of host cells. Thicker hyphae were frequently observed in fibers (Figure 2.2H, 2.4E,F), but nuclear condition could not be discerned in these cases: perhaps acridine orange failed to penetrate these highly lignified tissues.

Longitudinal development of the pathogen in the maize stalk: By 6 dpi, the C. graminicola M1.001 strains had heavily colonized the lignified fibers beneath the rind and surrounding the vascular tissues within the primary lesion (Figures 2.2, 2.4). The fungus progressed longitudinally in the stalk mainly by growing through these cells. Formation of secondary lesions was frequently observed at some distance from the primary lesion (Figure 2.1H,I, Table 2.3). The presence of these secondary lesions was sometimes, but not always, associated with dark rind discoloration. Secondary lesions were only formed along the same side
of the stalk as the original wound (Figure 2.1I), and were associated with bundles that were linked to the primary lesion at the wound site. Two types of secondary lesion could be recognized: the first was relatively small and light brown (Figure 2.1H), and contained no fluorescent hyphae. The second type was larger and darker in color (Figure 2.1I), and contained fluorescent fungal material (Table 2.3). Hyphae were observed apparently emerging from colonized fibers within the vascular bundles or beneath the rind and giving rise to this second type of secondary lesion (Figure 2.4A-F). At the point of emergence, the hyphae were narrow and seemed to be passing through very small openings, possibly pits, in the walls of the fiber cells (Figure 2.4F). The primary lesion at the wound site and the secondary lesions eventually merged, and in this way the entire internode could become rotted within only two weeks after inoculation.

Fungal hyphae were sometimes seen within xylem elements and other vascular tissues (Figure 2.4B-D). However, only vessels directly damaged by the wound or by the subsequent degradation in the primary lesion were ever colonized. Bundle fiber cells were always colonized before xylem or phloem, and xylem or phloem colonization only occurred in areas that were already significantly degraded, whereas fiber colonization occurred ahead of significant damage to the tissues. Oval spores were observed only once inside the xylem vessels, just below a nodal plate, where at least one of them had germinated (not shown).

When the fungus reached the node, mycelium proliferated in the parenchyma cells just below it, and this was accompanied by a dark discoloration of the plant tissues (Table 2.3) and formation of secondary lesions (Figure 2.1I, K-L). On several occasions, the fungus progressed beyond the node into the internode above (Figure 2.1J-L, Table 2.3). Movement into the upper internode appeared to result after a breakdown of tissues at the nodal plate, causing a breach that allowed the fungus to pass through via the disintegrated cells (Figure 2.1K-L). Secondary lesions were formed in the upper internode (Figure 2.1K), and fluorescent fungal hyphae were observed in some of these lesions (Table 2.3). Very rarely, the fungus also spread downward through the lower node and formed secondary lesions in the internode below (Table 2.3).

In contrast to M1.001, spread of the mutant C. graminicola and the C. sublineolum strains through the maize stalk was extremely limited. These strains colonized fibers under the rind and along bundles within the primary lesion, but to a much more limited extent than M1.001 (not shown). Vascular bundles containing fibers colonized by the mutant or C. sublineolum strains
often exhibited a very dark discoloration (Figure 1E-G, Table 3). Discontinuous secondary lesions were never observed with either strain, but occasionally new disease foci formed that were contiguous with the original lesion (Figure 1G, Table 3).

Discussion

The work described in this chapter represents the first detailed cytological study of the colonization of living maize stalks by *C. graminicola*. Experiments were performed in the greenhouse due to the necessity for containment of the transgenic fungal strains. The general course of symptom development I observed was similar to descriptions of disease progress on highly susceptible maize genotypes in the field (Anderson and White, 1984; White, 1999). It also matches descriptions of disease progress in the susceptible dent corn inbred B73 in the greenhouse (Tang et al., in press). However, it is important to point out that, because of the constraints of my experimental design, I looked only at infection of green maize stalks inoculated prior to anthesis. In the field, ASR is also often a problem on older, senescing stalks, and the process of colonization in senescing stalks could differ from my observations reported here. Furthermore, Jubilee is a highly susceptible variety of sweet corn, and for these experiments it was grown under relatively low light intensity conditions that are likely to increase its susceptibility to ASR. Thus, it will be important to confirm these observations with other maize genotypes and under other conditions to determine how generally they apply.

For these experiments, a small wound was created to allow fungal ingress. There is an established relationship between wounding caused by insects such as the European corn borer and the incidence of ASR and other stalk rot diseases (Bergstrom et al., 1983). Although the pathogen can directly infect and colonize unwounded stalks (White and Humy, 1976; and Chapter 3 of this dissertation), the results of my experiments with intact stalks were generally less consistent and predictable, and so I included wounding as part of the inoculation protocol for the work in this chapter.

*C. graminicola* is a hemibiotroph in leaves (Politis and Wheeler, 1973; Mims and Vaillancourt, 2002). In wounded stalks, I noted the presence of thick, multinucleate hyphae apparently giving rise to thinner, mononucleate hyphae within parenchyma cells at the edges of the primary lesions. This hyphal dimorphism is reminiscent of *C. graminicola* in leaves (Mims and Vaillancourt, 2002), and of hemibiotrophy in other *Colletotrichum* species (O’Connell et al.,
2000). However, morphology alone does not prove that the thicker hyphae are biotrophic: thus, the question of whether *C. graminicola* is hemibiotrophic in stalks awaits further investigation. It is worth noting that Tang et al., (in press) noted that cells containing *C. graminicola* hyphae within the secondary lesions, which they referred to as “break out zones”, were capable of plasmolysis, which supports the idea that they are initially colonized biotrophically.

The mutant strain of *C. graminicola* was included in this study so that I could compare a compatible and an incompatible interaction in a single maize genotype. The mutation in strain 6-2 affects a gene that encodes a component of the microsomal signal peptidase, and thus is likely to affect protein transport and secretion (Thon et al., 2002). It was rather surprising that the *C. graminicola* mutant and *C. sublineolum* strains were both able to complete their life cycles on wounded maize stalks, since neither of these strains sporulates on living maize leaves (Mims and Vaillancourt, 2002). However, although the lesions were qualitatively similar, lesion expansion was significantly reduced for both strains in comparison with the wild type *C. graminicola* strains. Thus, resistance to both of these strains in wounded stalks is quantitative rather than qualitative.

The lesions formed by all of the strains were surrounded by broad zones of darkly discolored parenchyma cells. This discoloration may have been the result of an active defense mechanism in the plant cells, since it was not observed in inoculated detached stalk pieces (see Appendix A of this dissertation). Another possible sign of an active host defense response was thickening of the walls of cells surrounding the wound site. Wall thickening due to deposition of lignin is a general wound and stress response in monocots (Whetten and Sederoff, 1995). Lignin production occurs via the phenylpropanoid pathway, which is also responsible for production of other defense metabolites that appear to play a role in the anthracnose disease (Lyons et al., 1990; Whetten and Sederoff, 1995; Hipskind et al., 1996; Bergstrom and Nicholson, 1999). The precise biochemical nature of the cell wall thickening I observed in this study requires further study. It is possible that wound-healing, a phenomenon in which wounded stalks become increasingly resistant to infection by *C. graminicola* with time, may be associated with this response (Muimba-Kankolongo and Bergstrom, 1990, 1992; Bergstrom and Nicholson, 1999).

ASR and top dieback in the field are frequently associated with vascular discoloration (Muimba-Kankolongo and Bergstrom, 1992; Bergstrom and Nicholson, 1999). I observed relatively little discoloration in my experiments, except for darkening of bundles immediately
adjacent to lesions caused by the avirulent or non-pathogenic strains. Light brown discolorations observed along vascular bundles at a distance from primary lesions caused by M1.001 did not appear to contain fungal material, suggesting the presence of a transported signal or toxin. *C. acutatum* is known to produce compounds that are toxic to several plant species including *Cacao* sp and *Polyathia* sp (Jayashinge and Fernando, 2001). Because bundle discoloration appeared to precede fungal spread through the fibers and emergence into the adjoining parenchyma cells, it is possible that it is a prerequisite for the formation of secondary lesions.

It has been reported that *C. graminicola* can be recovered from isolated vascular bundles (Bergstrom and Nicholson, 1999). Because oval spores have been observed inside xylem, it was hypothesized that *C. graminicola* moves systemically primarily as oval spores through the xylem vessels. It was also suggested that the oval spores would be trapped at nodes, and form new colonies at those sites that could spread the disease into the next internode (Panaccione et al, 1989; Bergstrom and Nicholson, 1999). Although *C. graminicola* certainly used bundle tissues for rapid spread through the internode, I found that it spread primarily as hyphae within the mostly non-living fiber cells. New disease foci (secondary lesions or “break out zones”) were formed when the fungus emerged from the fibers in both the bundles and the rind at intervals and began to attack adjacent cells. The ability to establish in the fiber cells, and to move from fiber to fiber both along the vascular bundle and beneath the rind, were negatively affected in the mutant 6-2 and in the *C. sublineolum* strains.

Fungal progression was usually stopped at the node. The vascular organization in the nodes is extremely complex. Vascular bundles are discontinuous at this point, a feature that limits the impact of damage to the vascular system in one internode, and blocks the movement of large pathogens, including fungal spores, in the sap (Shane et al, 2000). Occasionally, the fungus was able to enter the upper internode beyond the node. This resulted after significant degradation of parenchyma cells at the nodal plate, causing a breach in the node through which the fungus could penetrate. It seems likely that such breaches would be common, given enough time, and this might be the major way in which the fungus progresses between internodes.

My study has not clarified the role of the oval spores in the disease cycle of *C. graminicola*. Certainly my observations do not prove that oval spores are not involved in systemic movement, but the primary way that I observed *C. graminicola* moving through the stalk is as hyphae in the fibers. Oval spores were only observed once within the xylem elements
of the stalks in my experiments. In that case it appeared that the spores had collected below the nodal plate, and at least one had germinated and seemed to be in the process of initiating a new disease focus. This was the only evidence in favor of the hypothesis that oval spores do play some role in spreading the pathogen within the stalks. However, because they appear to be produced primarily in parenchyma cells, it is likely that the stalks would have to be thoroughly macerated before the spores would be released, and then there is the question of how they could be carried passively upward once the flow within the xylem had been broken in the degraded vessels. Falcate conidia were also produced abundantly in primary lesions. Production of falcate spores is dependent on light and high humidity (Panaccione et al, 1989). Presumably, the rind covering the incubation chamber maintains the necessary humidity, and also allows enough light penetration to induce spore production. Because both types of spores are produced in these wound sites, it is possible that they could be picked up on or in the bodies of insects feeding at these sites and vectored to new wounds on other plants. It is known that spores of this pathogen retain viability after passing through the gut of the European corn borer (Bergstrom and Nicholson, 1999).

This study revealed that *C. graminicola* is an extremely efficient colonizer of fiber cells within the maize plant beneath the rind and in association with vascular bundles. Using these cells, it moves rapidly through the internode from an initial inoculation point. I suggest that fiber colonization could be a way for the pathogen to “move behind enemy lines”. The plant appears to set up a defensive perimeter, visible as discolored cells with thickened cell walls, surrounding the primary lesion. However, the mostly nonliving fibers may be left relatively “unguarded”, so that if the pathogen can move through the defensive line by this route, it can break out in a new area and begin the process of degradation again. As a potentially important pathogenicity determinant, fiber colonization by *C. graminicola* deserves further study.
Table 2.1: Statistical analysis of lesion lengths caused by inoculation of wounded maize stalks by *Colletotrichum*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No of samples</th>
<th>Mean lesion length (cm)</th>
<th>Standard deviation</th>
<th>LS</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1.001</td>
<td>9</td>
<td>4.52</td>
<td>1.61</td>
<td>4.08  a</td>
</tr>
<tr>
<td>M1.001 GFP</td>
<td>10</td>
<td>4.15</td>
<td>2.49</td>
<td>3.59  b</td>
</tr>
<tr>
<td>M1.001 ZsGreen</td>
<td>12</td>
<td>4.14</td>
<td>2.15</td>
<td>3.68  b</td>
</tr>
<tr>
<td>6-2</td>
<td>7</td>
<td>1.40</td>
<td>0.40</td>
<td>1.10  c</td>
</tr>
<tr>
<td>6-2 GFP</td>
<td>7</td>
<td>1.47</td>
<td>0.73</td>
<td>1.20  c</td>
</tr>
<tr>
<td>6-2 ZsGreen</td>
<td>7</td>
<td>0.95</td>
<td>0.28</td>
<td>0.84  c, d</td>
</tr>
<tr>
<td>CgSl1</td>
<td>9</td>
<td>0.89</td>
<td>0.31</td>
<td>1.01  c, d</td>
</tr>
<tr>
<td>CgSl1 GFP1</td>
<td>7</td>
<td>0.87</td>
<td>0.32</td>
<td>0.64  d</td>
</tr>
<tr>
<td>CgSl1 GFP2</td>
<td>7</td>
<td>1.04</td>
<td>0.31</td>
<td>0.69  d</td>
</tr>
<tr>
<td>Water</td>
<td>9</td>
<td>0.25</td>
<td>0.16</td>
<td>0.23  e</td>
</tr>
</tbody>
</table>

Lesion lengths were measured at 13 days after inoculation.

LS means and classes were obtained using the GLM procedure that is part of the SAS statistical analysis software package. P=0.001. Means with different letters were significantly different from one another.
Table 2.2: Local lesion development for *Colletotrichum* strains in wounded inoculated maize stalks

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mycelium in necrotic parenchyma cells (primary lesion)</th>
<th>Oval spores produced</th>
<th>Falcate spores produced&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1.001</td>
<td>150/150 (100%)</td>
<td>50/150 (33%)</td>
<td>174/246 (71%)</td>
</tr>
<tr>
<td>6-2</td>
<td>71/88 (81%)</td>
<td>6/88 (7%)</td>
<td>38/179 (21%)</td>
</tr>
<tr>
<td>CgS11</td>
<td>47/79 (60%)</td>
<td>2/79 (3%)</td>
<td>57/193 (30%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The strains name refers to the strain together with its fluorescent derivatives. Because the virulence in each group was similar (see Table 1), I grouped them for presentation in Table 2. Furthermore, since there was no relationship between observation of the phenomenon and the days after inoculation, I pooled the observations for all days in these data.

<sup>b</sup> The total numbers of observations in this column are larger because falcate spores could be observed in stalks inoculated with the non-fluorescent strains as well as fluorescent strains (while oval spores and mycelium could only be observed for the fluorescent strains).

The data are presented as number of stalks in which the phenomenon was observed / total number of stalks observed (percentage).

The first column shows that 6-2 and CgS11 sometimes failed to establish in the necrotic tissues surrounding the lesion. In these cases, they did not produce spores. Data in the columns “Oval spores” and “Falcate spores” include the stalks in which the fungus failed to establish.
Table 2.3: Longitudinal colonization of wounded maize stalks by *Colletotrichum* strains.

<table>
<thead>
<tr>
<th>Strains&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Secondary Lesions Present:&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fluorescent Mycelium In the Secondary Lesions:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculated Internode</td>
<td>Internode Above</td>
</tr>
<tr>
<td>M1.001</td>
<td>143/235 (25%)</td>
<td>20/235 (9%)</td>
</tr>
<tr>
<td>6-2</td>
<td>13/173 (8%)</td>
<td>/</td>
</tr>
<tr>
<td>CgSl1</td>
<td>6/193 (3%)</td>
<td>/</td>
</tr>
</tbody>
</table>

The data are presented as the number of stalks in which the phenomenon was observed / total number of stalks observed (percentage).

**a.** The strain name refers to the original strain together with its fluorescent derivatives. I grouped the strains for presentation in this table because the degree of virulence within groups was very similar (see Table 2.1). Because there was no significant relationship between the number of days after inoculation and occurrence of these phenomena, I pooled the observations across all days.

**b.** For the pathogenic M1.001 strains, secondary lesions were discontinuous with the primary lesion at the wound site. For the avirulent mutant and non-pathogenic CgSl1 strains, secondary lesions were always contiguous with the original primary lesion: separate secondary lesions were never formed by these strains.

**c.** Not all secondary lesions could be observed for the presence of fluorescent mycelium because some stalks were inoculated with non-fluorescent strains.
**Figure 2.1:** Symptoms caused by inoculation of maize stalks with pathogenic, avirulent, and non-pathogenic strains of *Colletotrichum*. **A:** Typical external symptoms, 13 days after inoculation. Left to right: pathogenic *C. graminicola* strain (M1.001), avirulent mutant strain (6-2), non-pathogenic *C. sublineolum* strain (CgS11), and water control. The arrow indicates an area containing abundant stromata in the epidermal cells. **B:** The same stalks as in panel A, split in half with the longitudinal cut centered on the wound site and both halves shown. **A** and **B**: scale bars = 5 cm. **C:** Closer view of a primary lesion formed by a pathogenic *C. graminicola* strain. The lesion developed beneath the rind and was surrounded by a layer of dark parenchyma cells (arrow). The picture was taken 13 days after inoculation with the GFP transformant of M1.001. **D:** detailed view of acervuli (arrows) formed inside the primary lesion. Acervuli were produced on the surfaces of the discolored cells at the lesion margins, as shown here, as well as on aerial hyphae that traversed the cavity left after parenchyma cells in the center of the lesion had disintegrated (not shown). This picture was taken 11 days after inoculation with M1.001. **E** and **F:** lesions formed by the avirulent mutant and the non-pathogenic *C. sublineolum* strains, respectively. Discolored bundles (arrows) that extended beyond the edges were observed accompanying 61% of the lesions formed by mutant 6-2, and 38% of the lesions formed by CgS11. Similar discolored bundles were never seen associated with lesions produced by the pathogenic M1.001 strains. These pictures were taken 11 days after inoculation with the ZsGreen and GFP transformants of the mutant strain (E) and the non-pathogenic strain (F). Scale bar = 0.5 cm. **G:** The avirulent mutant and the non-pathogenic strains never produced discontinuous secondary lesions. Sometimes, however, secondary infection foci were formed that were contiguous with the original lesion (the wound site is marked with an asterisk). This picture was taken 15 days after inoculation with the avirulent mutant. **H:** Formation of secondary lesions (arrow) in an internode that was inoculated with the ZsGreen transformant of M1.001 (the original wound site is indicated with an asterisk). These lesions were very small and lightly pigmented. This picture was taken 15 days after inoculation. **I:** This sample was observed 15 days after inoculation with the ZsGreen transformant of M1.001 (the original wound site is indicated with an asterisk). The secondary lesions were larger and darker and contained green fluorescent fungal hyphae (not shown). Scale bar = 5 cm. **J:** This sample was observed 15 days after inoculation with M1.001. The fungus had crossed the node, and produced secondary lesions in the internode above. Significant damage
was visible at the node (arrow), which had apparently provided an opening for the pathogen to cross the node plate. Scale bar = 5 cm. **K:** Closer view of a “nodal breach”. The pathogen had apparently progressed to the node and produced a cavity containing disintegrated cells just beneath the node plate. The cavity is surrounded by a layer of darkly pigmented cells. Note the presence of secondary lesions (black arrow) in the internode above. This picture was taken 12 days after inoculation with M1.001. Scale bar = 1 cm. **L:** Closer view (detail of the region contained in the black box in panel K) of the cavity and the damage apparently caused by the fungus at the node plate. Aerial hyphae are visible in the cavity (arrow).
**Figure 2.2:** *Colletotrichum* colonizing living maize stalk tissues. **A to D:** longitudinal median sections through primary lesions resulting from infection by the ZsGreen transformant of M1.001 (**A** and **B**) and ZsGreen transformants of the avirulent mutant strain 6-2 (**C** and **D**). Fluorescent fungal mycelium did not extend beyond the edges of the lesions. Both samples were observed 8 days after inoculation. The pictures were taken with a dissecting microscope equipped with a mercury lamp. Scale bars = 0.5 cm. **E to G:** transverse sections through the margins of primary lesions resulting from inoculation with a pathogenic *C. graminicola* strain, an avirulent mutant strain, and a non-pathogenic *C. sublineolum* strain, respectively. **E:** this picture was taken 10 days after inoculation with the ZsGreen transformant of M1.001. Fluorescent green fungal mycelium can be seen colonizing the discolored tissues at the lesion margin. Note that the lignified bundle tissues persist within the otherwise disintegrated portions of the lesion. The arrows indicate acervuli forming on the surface of the cells at the lesion margin. Scale bar = 100 µm. **F:** This picture was taken 6 days after inoculation with the ZsGreen transformant of the avirulent mutant strain. The asterisk indicates the site of the original wound. Fungal hyphae have colonized the parenchyma cells that have been damaged directly by the wound. Scale bar = 100 µm. **G:** This picture was taken 13 days after inoculation with the ZsGreen transformant of the non-pathogenic *C. sublineolum* strain. Fungal mycelium has colonized host parenchyma cells only to a very limited extend. However, note the presence of an acervulus with setae (arrow) on the surface of the colonized tissues, at the site of the original wound. Scale bar = 100 µm. **H to J:** colonization of fibers and bundle tissues by pathogenic *C. graminicola* strains. **H:** Section from the edge of a lesion, in which a hyphae can be seen colonizing a bundle fiber. Note the short branches with swollen tips emerging from the main hypha. There are no hyphae visible in the xylem (asterix). This picture was taken 14 days after inoculation with the GFP transformant of M1.001. Scale bar = 100 µm. **I:** Section from the edge of a lesion, in which a hyphae can be seen colonizing a bundle fiber. This picture was taken 11 days after inoculation with the ZsGreen transformant of M1.001. No fungal hyphae are visible in the xylem (asterisk). Scale bar = 100 µm. **J:** Hyphae colonizing fibers in the rind. This view is of uncut tissue, through the epidermal cells into the fibers below. The picture was taken 11 days after inoculation with the ZsGreen transformant of M1.001, at the apex of the external lesion. Scale bar = 100 µm. **K:** Growth of hyphae between cells. This picture was taken 8 days after inoculation with the GFP 1 transformant of M1.001, in the darkly pigmented parenchyma layer.
Scale bar = 100 µm. L: Production of oval spores by the pathogenic strain in parenchyma cells. This picture was taken 8 days after inoculation with the ZsGreen transformant of M1.001. Note that the oval spores usually did not germinate \textit{in situ}. Scale bar = 100 µm. M: Stromata produced by the pathogenic strain in colonized epidermal cells. Stromata were produced only in the darkly discolored area of the rind. Stromata appeared as a very dense accumulation of fungal hyphae that eventually became melanized. This picture was taken 13 days after inoculation with the ZsGreen transformant of M1.001. Scale bar = 100 µm. The samples in panels N-P were stained with acridine orange. N: Thick, multinucleate hypha colonizing parenchyma cells at the margin of a primary lesion. The black arrows indicate septa, and white arrows indicate nuclei. This picture was taken using epifluorescence microscopy 8 days after inoculation with M1.001. Scale bar = 10 µm. O: An intact parenchyma cell at the edge of a primary lesion colonized by both types of hyphae. The white arrows indicate multiple nuclei per cell in the thicker hyphae, and the orange arrows indicate single nuclei in the thinner hyphae. This picture was taken with a confocal microscope 7 days after inoculation with M1.001. Scale bar = 10 µm. P: A thin, monokaryotic hypha located outside the host cells, within the disintegrated center of a primary lesion. The black arrows indicate septa, the white arrows show the nuclei. The picture was taken using epifluorescence microscopy 8 days after inoculation with M1.001. Scale bar = 10 µm
**Figure 2.3:** Thick sections of embedded maize stalk tissues. A to L: scale bar = 110µm. A: Transverse section demonstrating the normal organization and appearance of unwounded, uninoculated maize stalk tissue, including the rind, vascular bundles, and pith (parenchyma cells). B: Transverse section of maize stalk tissues 7 days after wounding with a dissecting needle. C: A closer view of a typical vascular bundle in the unwounded maize stalk (detail from panel A, upper rectangle). The thick-walled cells surrounding the bundles are the fibers (F), which form the bundle sheath. The four large cells are xylem vessels (Xy). Adjacent to the xylem is the phloem (P), composed of companion cells, the sieve plate, and sieve tubes. Fibers (F) are also present in the rind just below the epidermis (arrow). D: A closer view of a vascular bundle in the wounded maize stalk (detail from panel B, upper rectangle). All the cells appear to have much thicker walls than in the unwounded control (compare with panel C). PP: primary phloem. E: A closer view of healthy parenchyma cells (detail from panel A, bottom rectangle). Note the relatively thin walls, and the clear intercellular spaces (IS). F: A closer view of the thick-walled parenchyma cells near the wound (detail from panel B, lower rectangle). Compare to panel E. G-I: stalks wounded and inoculated with the pathogenic *C. graminicola* strain, the avirulent mutant strain, and the non-pathogenic *C. sublineolum* strain respectively. G: Transverse section from the lower edge of a primary lesion. The white arrow indicates an acervulus. Note the degree of degradation of the colonized parenchyma cells (compare to panel A). There are numerous hyphae visible in the cells (black arrows). The intercellular spaces between the parenchyma cells are often filled with dark occlusions, magnified in panel J (panel J, black arrows). Cell walls do not appear to be very thick (compare with panels B, C, and F). The sample was collected 9 days after inoculation with the GFP transformant of M1.001. H: Transverse section from the lower edge of a primary lesion resulting from infection by the avirulent mutant, 9 days after inoculation. Walls of the parenchyma cells surrounding the primary lesion (magnified in panel K) are quite thick. Intercellular spaces were filled with dense occlusions (panel K, black arrow). Hyphae were only visible in a few cells nearest to the wound site (panel H, black arrows). I: Transverse section from the lower edge of a primary lesion resulting from inoculation with the non-pathogenic *C. sublineolum* strain, magnified in panel L. This sample was collected 7 days after inoculation with CgSl1.
**Figure 2.4:** Colonization of bundle fibers by *C. graminicola*.

Scale bars for figures A-D = 100 µm. **A and B:** Transverse sections through the edges of the discolored cell layers surrounding primary lesions resulting from inoculation with the pathogenic *C. graminicola* strains. **A:** Hyphae are visible inside several fibers surrounding this bundle. Hyphae appear to have emerged from the fibers to colonize the surrounding parenchyma cells (white arrows). Note that the xylem has not been colonized. **B:** A view of a colonized bundle at the edge of the primary lesion. Numerous hyphae are visible in the fibers (white arrows), and some hyphae are also visible in tracheids near the center of the bundle (orange arrows). The large xylem vessels do not appear to be colonized. L = lacunas. Pictures **A** and **B** were both taken 8 days after inoculation with the ZsGreen transformant of M1.001.

**C and D:** apparent early stages in the formation of a secondary lesion. The pictures are of two adjoining sections from the same discolored bundle just beyond the edge of a primary lesion. The section in panel **C** was closer to the primary lesion than the section in panel **D.** **C:** this part of the bundle is quite heavily colonized. Proliferation into the adjacent parenchyma tissues has apparently just begun. Note that the large xylem vessels contain some hyphae (white arrows). **D:** In this section, the fibers are densely colonized but not the large xylem vessels. Thus, progression of the fungus upward in the stalk appears to be primarily via the fibers, from which hyphae eventually emerge to colonize the adjacent parenchyma and xylem and other vascular tissues.

**E:** Hypha apparently in the process of “breaking out” of a fiber (below) into adjacent parenchyma cells (arrow). This sample was observed 9 days after inoculation with the GFP strain of M1.001. Scale bar = 10 µm. **F:** The hyphae emerge from the fibers via narrow extensions (arrows) that may be passing though pits. This sample was observed 9 days after inoculation with the GFP strain of M1.001. Scale bar = 10 µm.
CHAPTER 3

PENETRATION AND COLONIZATION OF INTACT MAIZE TISSUES BY COLLETOTRICHUM GRAMINICOLA

Introduction

Stalk rot is one of the most economically important diseases affecting maize worldwide, and anthracnose (ASR), caused by Colletotrichum graminicola, is among the most common and severe of the stalk rot diseases. There is an established relationship between wounding caused by insects such as the European corn borer and the incidence of ASR and other maize stalk rot diseases (Bergstrom et al, 1983; Keller and Bergstrom, 1986; Gatch and Munkvold, 2003a,b). Controlling insect damage by the use of transgenic maize expressing the Bacillus thuringiensis Bt toxin reduces the incidence of some stalk rots (Bergstrom et al 1997; Munkvold et al., 1997; Gatch and Munkvold 2003a,b). However, ASR often occurs in the absence of insect wounding, and the proportion of stalk rots that are caused by C. graminicola actually increases in Bt maize (Dodd, 1980; Gatch and Munkvold, 2003a). We do not understand much about the process by which C. graminicola gets into unwounded stalks. C. graminicola also causes leaf blight (ALB) and seedling blight of maize, and it has been suggested that the pathogen enters stalks via the leaves or roots (Bergstrom and Nicholson, 1999). It has also been proposed that it gets in by direct penetration through the rind (consisting of the stalk epidermis and subtending fiber cells) (White and Humy, 1976). However, when attempts were made to produce infections in the field by placing inoculum behind leaf sheaths in contact with the rind, stalk infections occurred in less than 20% of the inoculated plants, and these were usually associated with corn borer injuries (White and Humy, 1979). Thus, the rind appears to be an effective barrier to direct penetration by C. graminicola. Understanding how C. graminicola enters stalks in the absence of wounds will become increasingly important as production of Bt maize continues to expand, and direct penetration presumably becomes the predominant means of infection by this pathogen. After observing that C. graminicola enters and grows rapidly through fibers in the rind of wounded maize stalks, and had the ability to emerge from them at a distance into adjacent parenchyma (Chapter 2 of this dissertation), I used a similar cytological approach to study the ability of the fungus to penetrate uninjured stalk epidermal cells, and to move from there through the rind fibers into the parenchyma cells below. I discovered that, although C. graminicola has a
significant capacity for penetration of intact rind tissues, this mode of entry is neither as rapid nor as efficient as wound-associated infection.

*C. sublineolum*, which is closely related to *C. graminicola*, causes stalk rot and leaf blight on sorghum, but does not appear to infect maize in the field (Vaillancourt and Hanau, 1992). As described in Chapter 2, we routinely use *C. sublineolum* as a control for stalk quality in our inoculation experiments. I was very surprised to discover in the course of my investigation that colonization of healthy, unwounded maize stalk epidermal cells by *C. sublineolum* was almost as efficient as by *C. graminicola*, and *C. sublineolum* also had some ability to penetrate the rind into deeper tissues. This was such an interesting result that I decided to pursue further by comparing the ability of this *C. sublineolum* strain versus *C. graminicola* to infect unwounded maize leaves (either seedling or mature) and seedling roots. *C. graminicola* efficiently infected and colonized all of these tissues, but *C. sublineolum* did not successfully colonize any of them. This suggests that non-host resistance of maize to *C. sublineolum* is conditional, and perhaps tissue specific.

**Materials and Methods**

*Fungal cultures and production of spore suspensions:* Green-fluorescent protein (GFP) expressing transformants of two *Colletotrichum* species were used for this study. A GFP transformant of *C. graminicola* strain M1.001 was produced using a PEG-mediated protocol described previously (Thon et al, 2000). Protoplasts were transformed with 3-9 µg of plasmid pCT74, which contains an SGFP gene under the control of the *Pyrenophora tritici-repentis* TOX-A promoter, and a hygromycin phosphotransferase gene as a selectable marker (Lorang et al, 2001). The plasmid was linearized with *EcoR*1 before use in transformation. To produce a transformant of *C. sublineolum* strain CgS11, a protocol developed for *Agrobacterium*-mediated transformation of falcate spores was used (Flowers and Vaillancourt, 2005). Plasmid pBin-GFP-hph contains GFP and hygromycin resistance genes under the control of the *A. nidulans* trpC promoter (O’Connell et al, 2004). Transformants were single-spored and stored on silica at −80°C. These same transformants were also used for the work that was described in Chapter 2 of this dissertation.

Fungal strains were grown on 1/2 strength oatmeal agar (Difco) at 23°C for two weeks. Falcate spores were collected by adding 10 ml of sterile water, and rubbing the surface of the
culture gently with a plastic mini pestle. The conidial suspension was collected, filtered though sterile glass wool, and the conidia were washed 3 times in sterile water. The concentration of conidia was adjusted to 5 x 10^6 spores per milliliter after the third wash. For leaf and root inoculations, 0.01% Tween-20 was added. 10µl of spore suspension was used to inoculate the plants. Controls were inoculated only with water (or water with 0.01% Tween, for the leaf and root experiments).

**Plant growth and inoculation:** The sweet corn hybrid Jubilee was chosen for this study because it is highly susceptible to ASR and ALB; it matures quickly; it does not exceed a manageable mature size for the greenhouse; and it has a historical association with the first epidemics of anthracnose in the United States (Warren et al., 1973). Jubilee sweet corn seed was a generous gift of Syngenta, and was obtained from Rogers Seed Company (Rogers Seed Co., PO Box 4727, Boise, ID 8371).

For stalk inoculations, plants were grown in the greenhouse in 10-inch pots in a mixture of 3 parts Pro Mix BX (Premiere Horticulture Ltd., Riviere du Loup, PQ, Canada) / 2 parts sterilized topsoil. Three seeds were planted in each pot. After germination, the seedlings were watered daily to saturation with a solution of 4.75 g of Miracle-Gro 18-18-21 formulation for tomatoes (Stern’s Miracle-Gro Products Inc., Port Washington, NY, USA) per liter. Two plants per pot were ultimately retained until they reached a late vegetative stage (V11-V12). The sheath tissue was stripped from the second and third internodes above the soil line just prior to anthesis. The plants were placed on their sides, and a drop of spore suspension, or water as a control, was placed in the center of each of the stripped internodes. For wounded control treatments, a small puncture wound 2mm deep was made through the rind with a dissecting needle, and drop of spore suspension was placed on the wound. All drops were covered with detached microfuge tube caps and sealed with parafilm to create a moist chamber, and the plants were left overnight. The tube caps were then removed, and the plants were placed upright. Individual inoculated internodes were harvested for observation each day starting at 1 day after inoculation (dpi), and continuing for up to 9 dpi.

For leaf inoculations, 10 µl drops of spore suspension were placed into individual wells of microhumidity chambers (Bergstrom and Nicholson, 1983). Inoculations were done late in the afternoon. The chambers were clamped onto the leaves, and the leaves remained attached to the plants. The chamber wells were covered with adhesive tape, which remained in place.
overnight. Chambers were removed from the leaves the next morning (14-18 hours later), and individual infection sites were excised with a scalpel and examined between 2 and 14 dpi. Inoculated mature leaves were located at the 2nd internode position of plants just prior to anthesis, grown as described above. Inoculated seedling leaves were the first, fully expanded true leaves of Jubilee seedlings at the V2 leaf stage in conetainers (one plant per conetainer) containing a mixture of 3 parts Pro Mix BX / 2 parts sterilized topsoil.

For seedling root inoculations, Jubilee seeds were surface-sterilized by soaking in 10% bleach solution for 10 min., followed by three rinses in sterile milli-Q water. The seeds were rolled into several layers of water-saturated germination paper (Anchor Paper Co., St. Paul, MN), forming a loose tube. The tube was placed upright in a beaker of sterile tap water, so that the seeds were not in direct contact with the water but were kept moist by the wicking action of the paper, and kept in the dark at 25ºC. Water was replenished as needed to maintain constant saturation of the germination paper. After 2 days, primary radicles had emerged from the seeds, and by 5 days they were between 1 – 1.5 inches long. These primary roots were inoculated with 10 µl drops of spore suspension just behind the root elongation zone. The roots were left exposed for about an hour to let the spores attach, and then the inoculated roots were wrapped again in the saturated germination paper and kept moist in the dark until observation. Individual seedlings were removed from the paper, and infected roots were observed under the microscope at 1, 2, and 3 dpi.

**Microscopy:** The development of fungi in aerial plant tissues was monitored by using a Leica TCS NT confocal microscope (Leica Microsystems Inc., Exton PA). Infected leaf pieces were observed without sectioning or staining. Infected stalk tissue sections were cut by hand with a razor blade and observed without further treatment. In both cases GFP was excited at 488nm. Plant cell walls and chloroplasts autofluoresce at this excitation wavelength, and this property was used to visualize the plant tissues. However, at lower magnifications, the autofluorescence of the plant cell walls was usually not intense enough to be captured in the micrographs. Root tissues were monitored under a Zeiss Axioscop compound microscope equipped with epifluorescence. Longitudinal sections of infected roots were produced by hand with a razor blade. Transverse sections of infected roots were obtained by embedding roots in 5% agarose in PBS buffer, and sectioning with a vibratome (using a protocol adapted from Koltai and Bird, 2000). Inoculated roots were cut into 2-3 mm long pieces, and immediately
fixed in fresh FAA solution at 4°C for at least 24 h. The fixed samples were rinsed in PBS buffer and embedded. The agarose was kept at 45°C, just above its solidification temperature, to avoid damaging the samples. When they were completely solidified, the blocks of agarose were divided so that each section contained an individual root sample. These smaller blocks were glued for sectioning on a Vibratome, according to the instructions provided by the manufacturer (Vibratome® 1000 Plus Sectioning System, The Vibratome Company, St Louis, MO). Sections of 50-100 µm were cut in a distilled water bath. The agarose usually detached from the root tissues at this point, and the released sections were mounted for viewing in a drop of distilled water under a cover glass.

Statistical analysis of stalk infections: Lesion lengths were measured as the maximum length of visible discoloration on the surfaces of uncut, inoculated stalks. Colonization of epidermal cells, and of tissues below the epidermal cells, was recorded as a percentage, consisting of the number of plants in which these events were seen as a proportion of the total number of plants that were observed. Each experiment included two or three replications of each treatment, and each experiment was repeated at least twice. Data were analyzed using the Mixed procedure that is part of the SAS statistical analysis software package (SAS Institute Inc., 1997). The effects of different strains, different internodes, and the time elapsed post inoculation, on lesion length, and on the degree of colonization of epidermal cells and deeper tissues, were analyzed.

Results

For this study, I used two GFP-transformants that I also used for the work that is described in Chapter 2 of this dissertation. In that study, the *C. graminicola* GFP-transformant was slightly less pathogenic than the wild-type strain in wounded stalks, and the *C. sublineolum* GFP transformant strain was equivalent to its wild-type progenitor (**Table 2.1**). A total of 88 individual infection sites on unwounded stalk tissues that I observed, only 29 of them (76%)
actually became infected, even though the fungus germinated and produced abundant appressoria on all of them within 24 hours (Table 3.1). In contrast, all 25 of the control stalks that I inoculated after wounding were colonized by the fungus within 2 days (not shown).

There was no statistically significant relationship between the time that had elapsed since inoculation and the infection of epidermal cells (Table 3.1). There was surprisingly little difference between *C. graminicola* and *C. sublineolum* in the ability to colonize epidermal cells of unwounded stalks in the long run, with *C. graminicola* colonizing 76% of the inoculation sites, and *C. sublineolum* 62% of sites, within 9 days. The *C. sublineolum* strain produced lesions on unwounded stalk tissues that were similar in size to those caused by *C. graminicola* early in the experiment (P = 0.7669 at 1-2dpi; P = 0.0771 at 3-5dpi). However, the *C. graminicola* lesions expanded more quickly, so that by the end of the experiment they were significantly larger than those caused by *C. sublineolum* (P < 0.0001, 6-9dpi). Nevertheless, there was an upward trend for both strains over time, suggesting that in both cases lesions were still expanding at the end of the experiment (P < 0.0001) (Figure 3.1 A and B).

Colonization of the epidermal cells of unwounded stalks always preceded invasion of rind fibers and deeper tissues, but deeper colonization did not always occur when epidermal infection was observed, at least within the time course of my study. The percentage of deeper colonization did increase for both strains over time (P = 0.0023). Inoculation with *C. graminicola* resulted in colonization of deeper tissues 60% of the time at 7-9 days after inoculation. Deeper colonization was significantly less frequent for *C. sublineolum* (P = 0.0062), occurring in only 15% of inoculation sites at 7-9 dpi, with no deeper colonization appearing earlier than 7 dpi, whereas for *C. graminicola* colonization of deeper tissues occurred in as little as 2 dpi.

Infection of unwounded maize stalks by *C. graminicola* and *C. sublineolum*: Infections of control wounded maize stalks by both strains proceeded in the same way as already described in Chapter 2 of this dissertation. The process by which epidermal cells of unwounded stalks were infected was very similar to published descriptions of infection of leaf epidermal cells (Bergstrom and Nicholson, 1999; Mims and Vaillancourt, 2002; Politis and Wheeler, 1973), and also to my own observations of this process (see below). Appressoria formed on stalk epidermal cell surfaces within 24 hours after inoculation, and the first infection hyphae were visible within 48 hours (Figure 3.1 C). Appressoria were formed by both strains, with no obvious difference.
between them in the efficiency of this process. Within two days, a network of hyphae was visible for both strains within the epidermal cells in a percentage of the stalks (Figure 3.1 D, Figure 3.2 A). The hyphae passed from cell to cell via extremely narrow connections (Figure 3.1 F). Discoloration of the epidermal tissues occurred within a few days (Figure 3.1 A and B), and then formation of stromata by both strains was observed (Figure 3.1 E, Figure 3.2 C). These stromata gave rise to acervuli and conidia if they were incubated in a moist environment (not shown).

In as little as 2 days after inoculation with \( C. graminicola \), invasion of the underlying fibers and parenchyma could be observed (Figure 3.1 G-I, Figure 3.2 B). Movement from cell to cell again occurred via narrow connections through host cell walls that otherwise appeared to remain intact (e.g. see Figure 2.4 E,F). Once the parenchyma was invaded, signs of rotting finally appeared, including destruction of cell walls and formation of a cavity within the pith. \( C. sublineolum \) only rarely entered the tissues beneath the epidermis, but when it did so it appeared to use a mechanism similar to that of \( C. graminicola \).

**Infection of leaves by \( C. graminicola \):** The ability of \( C. sublineolum \) to colonize unwounded stalk epidermal tissues so efficiently was surprising, and I decided to investigate further by comparing this strain with \( C. graminicola \) regarding its ability to colonize other types of unwounded maize tissues. A total of 38 seedling leaves were inoculated and examined, and the following descriptions represent the consensus of my observations. It has already been reported that \( C. sublineolum \) does not cause anthracnose on maize leaves (Thon et al., 2002). I confirmed this, observing that \( C. sublineolum \) formed abundant appressoria on all of the inoculated leaves, but only three successful penetrations into the tissues resulted (out of many hundreds of appressoria on each leaf), and only one primary infection hypha (Figure 3.3 A). \( C. graminicola \), in contrast, entered all of the seedling leaves within 48 hours after inoculation, and had proliferated and begun to cause significant tissue collapse 24 hours later. The fungus was observed to progress from cell to cell in the mesophyll in a manner similar to that described for epidermal colonization, i.e. through very narrow connections (Figure 3.3 B and C). At 3 dpi, typical anthracnose lesions were observed on these leaves, consisting of a necrotic lesion surrounded by a yellow halo. In the necrotic center of the lesion, \( C. graminicola \) produced stromata (Figure 3.3 D). Colonization of the mesophyll cells continued to expand at the edges of the lesion, and hyphae were often observed in the bundle fibers (Figure 3.3 E).
To test whether it was the relative age of the tissue that was important, I also inoculated 30 leaves that were of the same physiological age as the stalk internodes I had been inoculating. *C. sublineolum* failed to germinate on these leaves, whereas *C. graminicola* germinated and developed appressoria within 24 h. In contrast to the rapid development in the young leaf tissues, *C. graminicola* penetrated the epidermal cells of these older leaves only after 7 dpi. Once established inside the leaf tissues, *C. graminicola* developed in a manner similar to that described above for the seedling leaf tissues. By 8 dpi, necrotic lesions surrounded by a yellow halo were observed on some of the leaves. In the necrotic centers of the lesions, the fungus produced stromata ([Figure 3.3 G](#)). The fungus could frequently be seen inside the fibers surrounding the vascular bundles ([Figure 3.3 H, I](#)). At the margins of the lesions, the fungus continued to expand and colonize the mesophyll cells ([Figure 3.3 F](#)).

*Infection of maize roots by C. graminicola:* It has been suggested that *C. graminicola* might enter stalks via roots, and it is known that the pathogen can cause decay of the roots of highly susceptible seedlings (Warren and Nicholson, 1975), but the process of root infection has not been closely investigated. I examined a total of 71 infection sites on seedling roots, and the following descriptions represent the consensus of my observations. At 24 h after inoculation, I found that conidia of both *Colletotrichum* species germinated on roots, and formed melanized appressoria. I observed this in 82.3% of the samples for *C. graminicola*, and 60% of the samples for *C. sublineolum*. The appressoria in both cases appeared to be identical to those formed on aerial plant parts. As early as 24 hpi, *C. graminicola* had invaded the root epidermal cells (in 13.6% of the samples) and cortical cells (in 31.7% of the samples). I had to end the experiment at 3 dpi because the seedlings only stayed healthy in the germination papers for that long. I never saw the fungus move into the stele where it might be possible to initiate systemic movement into the above-ground portions of the plant. In contrast to the stalks, but like the leaves, *C. sublineolum* did not colonize root tissues: development was arrested at the point of appressorium formation, and structures that may have been papillae were usually observed associated with the arrested appressoria. In a total of 30 samples, I never observed production of primary infection hyphae by *C. sublineolum* in root tissues.
Discussion

My goal in this study was to use cytological methods to describe the nature of infection of intact stalk tissues by *C. graminicola*. My results demonstrated that the pathogen is capable of infecting intact stalk tissues, although it entered wounded sites much more quickly and efficiently. The high degree of variation in the timing of initial infection of individual inoculated stalks suggests that there are uncontrolled genetic and/or environmental variables that have a significant influence on fungal penetration. Once penetration occurs, the outcome seems to be more assured in that the fungus progresses through the epidermal cells at what appears to be a fairly constant rate, as the lesions enlarge with time. Lesion expansion was somewhat slower for *C. sublineolum* than *C. graminicola*, suggesting that quantitative resistance may be operating to slow the growth of this organism. However, it should be mentioned that *C. sublineolum* also grows more slowly than *C. graminicola* in culture (Vaillancourt and Hanau, 1992). The fact that not all inoculations of unwounded stalks resulted in infections shows that, even in this very susceptible corn genotype, the intact rind has some capacity to protect the inner stalk tissues. White and Humy (1976) reported that only about 20% of the Jubilee sweet corn plants they inoculated behind leaf sheaths in the field developed stalk rot after four weeks, and most of those were associated with corn borer damage. They reported a higher degree of darkening of the stalk and adjacent leaf epidermis, which could correlate with the epidermal infection and sclerotial formation that I observed, although it is impossible to be sure because unfortunately they did not describe this darkening further. I observed a much higher rate of infection of unwounded inner tissues at 7-9 dpi than White and Humy (1976) did in more than twice the amount of time (60% versus 20% of the inoculated plants), and it’s possible that this was either because my plants were more environmentally stressed, and therefore less able to resist pathogen ingress, or because there are moderating influences of other organisms living behind intact leaf sheaths in the field that weren’t duplicated in my study where the rinds were exposed and the leaf sheaths removed. Thus, even though I can show that there is significant potential for direct invasion of stalks by *C. graminicola*, this does not necessarily mean that direct invasion occurs commonly in the field.

It has been suggested that stalks may become infected via leaves with ALB (Bergstrom and Nicholson, 1999). Fibers associated with vascular bundles in leaves were readily colonized by *C. graminicola*. Given that there is a direct connection between leaf bundles and those in the
sheaths, it seems quite possible that the pathogen could be introduced into the sheaths from the leaves, and perhaps from there into the stalk epidermal cells, which are typically in contact with sheath tissues. However, ASR often occurs in the absence of significant ALB (Carson and Hooker, 1981) and so this is probably not the only way that the pathogen enters.

Entry into the stalk via the roots has also been suggested (Bergstrom and Nicholson, 1999), and although it is known that *C. graminicola* can infect roots, there appears to have been no prior investigation of this possibility. I found that conidia of both *Colletotrichum* species germinated on roots, and formed melanized appressoria that appeared identical to those formed on aerial plant parts. This is different from a report of root infection by *Magnaporthe grisea*, which also produces melanized appressoria on leaves, but which form non-melanized penetration structures on roots (Sesma and Osbourne, 2004). *C. graminicola* was able to invade the root epidermal and cortical cells and grow within them, but within the time constraints of the experiment I never saw the fungus move into the stele where it might be possible for it to initiate systemic movement, as was described for *M. grisea* (Sesma and Osbourne, 2004). It is possible that if I could have waited longer I might have seen this, since I could observe hyphae very close to the stele. I did plant one batch of 12 inoculated seedlings in soil, and grew the plants to post-anthesis in the greenhouse, but no sign was ever observed of stalk rot development, and GFP-expressing fungus could not be recovered from samples of stalk and leaf tissues of the mature plants. However, the plants did become severely infested with spider mites and thrips as they senesced, and so that experiment was not repeated. Thus, my results are inconclusive on the question of whether roots are a viable entry point for stalk rot.

The ability of *C. sublineolum* to infect stalk epidermal cells so efficiently was surprising. This fungus is not normally found as a pathogen of maize, even in areas where maize and sorghum are commonly grown together (Lebeau, 1950; Williams and Willis, 1963; Jamil and Nicholson, 1987). These observations suggest that non-host resistance to *C. sublineolum* by maize is conditional, and perhaps also tissue specific. One possibility I have also considered is that the lower light conditions under which the stalk epidermal cells are produced may decrease basal resistance. Low light is known to increase susceptibility of leaves to *C. graminicola* (Hammerschmid and Nicholson, 1977a). Since the stalk epidermis develops beneath the sheath, it is not exposed to as much light as the leaves and so it may not have the same capacity to mount an effective defense. This is something that needs further investigation.
It is interesting that *C. sublineolum* did not actually germinate on older leaves, even though the same batch of inoculum applied to unwounded stalks germinated and formed appressoria efficiently. Spores of *C. graminicola* must sense a hydrophobic substrate in order for germination to be induced (Chaky et al., 2001), and so it is possible the older leaves are not sufficiently hydrophobic to induce germination of this species; leaves do become less hydrophobic as they age and as the cuticle erodes. Additionally, it is known that older leaves express a greater degree of resistance to *C. graminicola* than younger leaves, and so it is possible that the lack of germination was directly due to expression of resistance by the leaves, though it would be surprising to see it expressed even pre-germination, since typically resistance to *Colletotrichum* occurs at the stage of initial penetration.

Conditional non-host resistance is a very interesting finding, which is, however, consistent with previous observations from our lab that suggested that highly stressed maize stalks were prone to significant rotting by *C. sublineolum* (Vaillancourt, unpublished) and that *C. sublineolum* can complete its life cycle in wounded stalk tissues (Chapter 2 of this dissertation). Conditional non-host resistance may explain the controversy in earlier papers where some sources suggested that maize was susceptible to isolates of *Colletotrichum* from sorghum, whereas others found strict host specificity (Chowdery, 1936; Wheeler et al, 1974; Williams and Willis, 1963; Bergstrom and Nicholson, 1999; Munkvold and Hellmich, 1999). Although there is no evidence that cross-infection occurs routinely in the field, my work shows that the potential exists and this may pose a risk, particularly in areas where both crops are commonly grown, and where conditions for maize may be sub-optimal so that the maize may be stressed and less able to mount a defensive response. Evidence has been increasing that host resistance and non-host resistance are actually very closely related processes in many cases (reviewed by Thordal-Christensen, 2003). There may be less difference than we thought between host-specific resistance of maize to *C. graminicola*, and non-host resistance to *C. sublineolum*: *C. sublineolum* clearly has the potential to cause disease in maize, and maize clearly has the potential to be susceptible. Why then, is this interaction usually incompatible? Understanding the answer to this question could help us to develop improved methods for control of the anthracnose stalk rot disease in the field.
Table 3.1: Statistical analysis of lesions produced by *C. graminicola* and *C. sublineolium* on unwounded maize stalks.

<table>
<thead>
<tr>
<th></th>
<th>Number of samples</th>
<th>Average Lesion Length (cm)</th>
<th>LS Mean</th>
<th>% Epidermal Colonization</th>
<th>% Deeper Colonization</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Colletotrichum graminicola</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (1-2 dpi)</td>
<td>12</td>
<td>0.03</td>
<td>0.03</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>Mid (3-5 dpi)</td>
<td>21</td>
<td>0.4</td>
<td>0.59</td>
<td>90</td>
<td>19</td>
</tr>
<tr>
<td>Late (6-9 dpi)</td>
<td>5</td>
<td>1.42</td>
<td>1.42</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Total number of samples</td>
<td>38</td>
<td></td>
<td></td>
<td>76</td>
<td></td>
</tr>
<tr>
<td><em>Colletotrichum sublineolium</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (1-2 dpi)</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mid (3-5 dpi)</td>
<td>21</td>
<td>0.2</td>
<td>0.36</td>
<td>71</td>
<td>0</td>
</tr>
<tr>
<td>Late (6-9 dpi)</td>
<td>20</td>
<td>0.56</td>
<td>0.59</td>
<td>80</td>
<td>15</td>
</tr>
<tr>
<td>Total number of samples</td>
<td>50</td>
<td></td>
<td></td>
<td>62</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 3.1:** Representative images of unwounded stalks infected with *C. graminicola* or *C. sublineolum*. **A.** External lesion formed on maize stalk 4 dpi with *C. graminicola* (GFP transformant). The bleached areas (arrow) are superficial damage that was caused by the microfuge tube cap used as a moist chamber. **B.** External lesion formed at 4 dpi with *C. sublineolum* (GFP transformant). **C.** First infection hyphae (arrows) visible in epidermal cells inoculated with *C. graminicola*, 2 dpi. **D.** A typical network of hyphae formed in epidermal cells, 4 dpi with *C. graminicola*. **E.** Stromata formed in epidermal cells 4 dpi with *C. graminicola*. **F.** A closer view of a hyphae passing from one epidermal cell into another, via a very narrow connection (arrow) 5 dpi with *C. graminicola*. **G.** A view of the uppermost edge of the lesion, where the fungus could be seen progressing primarily through the fibers. 4 dpi with *C. graminicola*. **H.** A closer view of longitudinal progression through the fibers. 5 dpi with *C. graminicola*. **I.** Progression of the pathogen into the parenchyma cells, 6 dpi with *C. graminicola*. Scale bars: A and B: 0.5 cm; C: 50 µm; D, E, G, H, and I: 100 µm.
Figure 3.2 Representative images of unwounded maize stalks infected with *C. sublineolum*. A. Network of hyphae in epidermal cells, 6 dpi. B. Uppermost edge of lesion, showing fiber colonization, epidermal cell colonization, and beginnings of stromatal formation, 6 dpi. C. Closer view of stromata forming in epidermal cells, 7 dpi. Scale bars: A, B, and C: 100 µm.
Figure 3.3: Results of inoculation of leaves with *C. graminicola* and *C. sublimeolum*.  

A. Inoculation of seedling leaves with *C. sublimeolum* almost never resulted in anything other than formation of appressoria, and sometimes, small abortive penetration attempts that never extended beyond the first cell. This photograph shows a more developed infection hypha, though it is still confined to one cell, at 3 dpi, the only one I ever saw like this for this strain on maize.  

B. *C. graminicola* at 3 dpi was already forming an extensive network of hyphae that was moving from cell to cell via narrow connections (arrow).  

C. At 5 dpi, an even more extensive network of hyphae could be seen at the edges of the developing lesion.  

D. Thick-walled stromatal tissues could be seen forming in the center of the developing lesions at 5 dpi with *C. graminicola*.  

E. Colonization of the fibers associated with the bundles was common. This picture of *C. graminicola* was taken at 3 dpi and shows hyphae inside a fiber, apparently caught in the act of emerging from it (arrow) into the adjacent cells.  

F. Colonization of mature leaf tissues by *C. graminicola* proceeded similarly, but more slowly, than in the seedling leaves. *C. sublimeolum* did not germinate on the mature leaves. This photograph was taken 8 dpi with *C. graminicola*, and shows a network of hyphae proliferating in the cells adjacent to a developing lesion.  

G. An overview of a developing lesion showing the stromatal tissues forming in the center of the lesion, 8 dpi.  

H. Fiber colonization was again very common. This picture was taken at 8 dpi.  

I. Longitudinal expansion of the lesion appeared to occur by growth through fibers. This picture was taken at 8 dpi at the top leading edge of a lesion and shows hyphae moving ahead in the fibers.  

Scale Bars A-I: 100 µm.
Figure 3.4: Results of inoculation of seedling roots with *C. graminicola* and *C. sublineolum*. A. Roots 3 dpi with *C. graminicola*. Brown, water-soaked lesions are visible on the roots where they were inoculated, just above the root elongation zone (white arrows). B. Roots at 3 dpi with *C. sublineolum*. No symptoms are visible, and the roots appear comparable to C. the water control. Scale bars: A, B, and C: 0.5 cm. D. Appressorium formation on root epidermal cells, and formation of primary infection hyphae, by *C. graminicola* at 1 dpi. E. Section of root at 2 dpi with *C. graminicola*. A collapsed area can be seen in the root cortex (black arrow) and hyphae are visible inside the cortical cells (white arrow). F. Another section of root at 2 dpi with *C. graminicola*. A collapsed area of tissue (black arrow) has progressed to near the stele (S) and hyphae (white arrows) can be seen in the cortical cells quite near the stele, but no hyphae have actually entered it. G. Fluorescent hyphae of the GFP strain of *C. graminicola*, inside root cortical cells at 3 dpi. H. Appressoria (looking like small black “pepper grains”) are visible on the surface of a root 1 dpi with *C. sublineolum*, but no damage can be seen. I. Appressoria of *C. sublineolum*, 2 dpi. Most are associated with structures that might be papillae formed by the plant (white arrows), or they might be abortive penetrations. Scale bars: D, E, F, and I: 50 µm; G: 25 µm; and H: 1 mm.
CHAPTER 4
THE ROLE OF A FADA ORTHOLOGUE IN GROWTH AND DEVELOPMENT OF COLLETOTRICHUM GRAMINICOLA IN VITRO AND IN PLANTA

Introduction

Colletotrichum graminicola (Ces.) Wils. causes anthracnose stalk rot (ASR) and leaf blight (ALB) on maize (reviewed by Bergstrom and Nicholson, 1999). C. graminicola produces two types of asexual propagules. Falcate conidia are produced on the surfaces of lesions in acervuli, and play a major role in dispersal of the pathogen from plant to plant. In contrast, oval spores are produced only inside infected plant tissues, and are apparently not released to the outside (Panaccione et al., 1989). The role of these oval spores in the disease cycle is unknown, but it has been suggested that they are responsible for systemic movement of the pathogen through the vascular tissues in leaves and stalks (Panaccione et al., 1989; Bergstrom and Nicholson, 1999).

In Chapter 2 of this dissertation, I described in detail the colonization of wounded maize stalk tissues by C. graminicola. The fungus initially produced a primary lesion at the wound site, and then later it produced discontinuous secondary lesions that were always connected with the primary lesion through the vascular bundles and the stalk rind. My observations suggested that the primary means of movement of the fungus through these bundle and rind tissues was as hyphae in the associated fibers. Oval spores were produced inside of parenchyma cells in the lesions, but I could see no obvious way that these could easily enter intact vessels and be transported through them. I only observed oval spores once inside the xylem tissues, but I could always see hyphae inside fibers at the leading edges of the lesions. Nevertheless, because it is technically not possible to observe a complete hyphal connection through a single fiber between a primary lesion and a secondary lesion that may be several centimeters away, the idea that the secondary lesions actually arose from oval spores cannot be entirely discounted. In this chapter of my dissertation, I took a different approach to this question by looking at a mutant that was altered for oval spore production.

Heterotrimeric guanine nucleotide-binding (G) proteins, comprised of α, β, and γ subunits, are major players in signaling pathways that control critical aspects of fungal growth and development. The Aspergillus nidulans fadA gene encodes a G α subunit that regulates
sexual and asexual development and secondary metabolism (Hicks et al., 1997; Adams et al., 1998; Yu and Keller, 2005). The dominant \textit{fadA} mutation G42R (conversion of glycine 42 to arginine) results in constitutive activation of the GTPase activity of FADA. The mutant exhibits a “fluffy” phenotype, due to proliferation of the vegetative mycelium, and a reduction in asexual conidiation (Adams et al, 1998). Dominant activation of \textit{fadA} also decreases production of a toxic secondary metabolite called sterigmatocystin (Hicks et al, 1997). Interfering mutations in FADA have the opposite effect, namely overproduction of sterigmatocystin and precocious sporulation. The \textit{A. nidulans flbA} gene encodes a protein with a regulator of G protein signaling (RGS) domain (Hicks et al, 1997). In the current model in \textit{A. nidulans}, FADA is negatively regulated by FLBA, which allows sporulation and sterigmatocystin production to proceed (Hicks et al, 1997; Adams et al, 1998). In the absence of regulation by FLBA, FADA becomes activated, resulting in repression of these phenotypes.

Highly-conserved orthologues of \textit{fadA} have been identified in several other filamentous fungi, including \textit{Neurospora crassa, Magnaporthe grisea, Cryphonectria parasitica, Botrytis cinerea, Cochliobolus heterostrophus}, and \textit{Colletotrichum trifolii}, (Ivey et al, 1996; Liu and Dean, 1997; Gao and Nuss, 1996; Segers and Nuss, 2003; Horwitz et al, 1999; Gronover et al, 2001; Truesdell et al, 2000). In spite of the very high degree of sequence conservation among this group of fungal G proteins, disruptions of these genes often produce different results in the different species. In \textit{N. crassa}, disruption of \textit{gna-1} reduced vegetative growth and caused the production of hyphal swellings and adherent macroconidia (Ivey et al, 1996). The mutants were male-sterile, but female fertile, though the female reproductive structures were abnormal (Ivey et al, 1996). Deletion of \textit{magB} in \textit{M. grisea} caused significant reductions in vegetative growth, conidiation, and appressorium formation in \textit{vitro}, and also reduced pathogenicity to rice leaves (Liu and Dean, 1997). The \textit{magB} mutants were female-sterile, failing to form perithecia (Liu and Dean, 1997). In \textit{C. parasitica}, the \textit{Cpg1} gene is down-regulated as a result of infection by the ds-RNA virus that causes hypovirulence (Choi et al., 1995). Deletion of the \textit{Cpg1} gene reduces growth rate and colony pigmentation, and abolishes conidiation and virulence (Gao and Nuss, 1996). In \textit{B. cinerea}, deletion of the \textit{Bcg1} gene resulted in slower growth on some media, reductions in protease secretion and in the production of the phytotoxin botrydial, and a decrease in pathogenicity. However, it had no effect on spore germination, or on the ability to penetrate plant tissues (Gronover et al., 2001). In \textit{C. heterostrophus}, deletion of the \textit{Cgal} gene resulted in
reductions in appressorial development and female fertility, but had no effect on pathogenicity to maize (Horwitz et al., 1999). In C. trifolii, disruption of the Ctg1 gene resulted in mutants that produced spores with a significant reduction in the ability to germinate (Truesdell et al., 2000). The mutants were also reduced in pathogenicity, grew more slowly on agar medium, and produced longer germ tubes before producing appressoria on artificial substrates.

Given the variability in the phenotypes of fadA orthologue mutations in other fungi, it was hard to predict exactly what kind of phenotype a negative mutation in the fadA homolog in C. graminicola would produce, but by analogy with A. nidulans I hypothesized that it might result in an increase in the production of spores, and probably also a decrease in vegetative growth. If the mutant produces more oval spores in planta, and fewer hyphae, and if the oval spores rather than the hyphae are primarily responsible for formation of secondary lesions, I predicted that I would see more secondary lesion formation in stalks infected by these mutants. If, on the other hand, the mutant produced fewer spores, like mutants of M. grisea and C. parasitica, I expected I would see fewer secondary lesions. Finally, even if my predictions turned out to be wrong, I thought it would still be interesting to look at the effect of such an important developmental regulatory signaling “switch” on pathogenicity, especially since C. graminicola is a hemibiotroph and undergoes a major developmental transformation during its disease cycle from biotrophy to necrotrophy.

My colleagues at DuPont were using a high-throughput method for gene disruptions based on split-marker transformation (Fairhead et al., 1996; Fu et al., 2006). Though there are many other ways to produce gene disruptions and deletions, I decided to make disruptions in the C. graminicola fadA orthologue by using this method. My purpose was not only to produce and study the gene disruptions themselves, but also to characterize the split-marker protocol and evaluate its potential value to our lab for future high-throughput analyses.

Materials and Methods:

Fungal strains: C. graminicola strain M1.001, also known as CgM2 (Forgey et al, 1978), was used for this study. The strain was obtained from the late Dr. Robert Hanau of Purdue University. C. sublineolum isolate CgS11 was obtained from Dr. Ralph Nicholson of Purdue University. It is pathogenic on sorghum stalks and leaves, but non-pathogenic to maize in the field. It was used here as a control for maize quality. All fungal strains were routinely cultured
on potato dextrose agar (PDA, Difco) at 23°C under continuous light. For spore production, fungal strains were grown on PDA for three weeks. Falcate spores were collected by adding 10 ml of sterile water and rubbing the surface of the culture gently with a plastic mini pestle. The conidial suspension was collected, filtered though sterile glass wool, and the conidia were washed 3 times in sterile water. The concentration of conidia was adjusted to 5 x10⁶ spores per milliliter after the third wash for maize stalk inoculations, 20 spores per microliter for spore germination and in vitro development assays, or 40 spores per microliter for oval spore production assays.

**Production of “split marker” deletion constructs:** The advantage of the “split-marker” method over other means of producing gene disruptions is that it is a fast, PCR-based protocol that doesn’t involve much subcloning, and also requires minimal flanking DNA to achieve a high proportion of homologous integrants. The procedure utilizes a pair of PCR products comprised of sequence homologous to the target gene flanking overlapping truncations of a selectable marker gene. The rationale is that the selectable marker will only be expressed if the two overlapping sections are inserted together at the same site, thus reconstituting the selectable marker. This is most likely to happen when homologous integration occurs at the site of the target gene. The split-marker method has been used with great success by DuPont scientists as part of a high-throughput targeted mutagensis protocol. However, it had not been broadly applied to plant-pathogenic fungi by other researchers, and I was interested in understanding more about its advantages and potential limitations. I learned the method from Jim Sweigard during an internship at the DuPont Experimental Station in Wilmington, Delaware, in July and August of 2004.

A tBLASTp search of the DuPont *C. graminicola* genome database with the *fadA* sequence from *A. nidulans* identified a similar contig sequence of 2.1 kb. The genomic sequence was used to design primers CgFadA1 (5’-TGTCGGCGCCCACCTGTTTACC-3’) and CgFadA2 (5’-GAGGATGATGGAGGTCTTGATGAACC-3’). The 2.1 kb fragment was amplified from M1.001 genomic DNA using these primers. The polymerase chain reaction (PCR) mix contained 3.4µg of genomic DNA template, 50 pmol of each primer, and 1X QIATaq Master Mix (QIAgen Inc. Valencia CA). Thermocycling conditions were 95°C for 15 min., followed by 30 cycles consisting of 95°C for 45 sec., 63°C for 30 sec., and 72°C for 3 min. 30 sec., followed by one cycle of 72°C for 7 min. The PCR product was cloned into pCR®2.1-TOPO, using the
TOPO® TA Cloning kit (Invitrogen, Carlsbad CA), according to the manufacturers instructions. *Escherichia coli* strain DH10 was transformed by electroporation with 1 µl of the TOPO reaction mix. Bacteria were selected on kanamycin and subjected to blue-white screening, and plasmids were purified by alkaline lysis and QIAgen columns (QIAgen Inc., Valencia, CA). Plasmids containing the 2.1 kb fragment of *C. graminicola* DNA were confirmed by digesting with *EcoRI*. One of these, which I named pSM1267, was selected for transposon tagging.

The pMOD/Tn551 construct for transposon tagging was produced by cloning the *E. coli* hygromycin phosphotransferase (*hph*) gene, under the control of the aldolase promoter of *M. grisea* and the β-tubulin terminator of *N. crassa*, into the transposon construction vector pMOD-Tn5™ (Epicentre® Biotechnologies, Madison WI). The EZ::TN™ transposase kit (Epicentre) was used to generate the disruptions. The protocol was slightly modified from the manufacturers instructions, in that only half of the recommended amount of transposase was used in the reaction. Each reaction consisted of 0.2 µg of the target plasmid (pSM1267); 0.2 µg of the donor plasmid (pMOD/Tn551); 1 µl of 10X buffer; and 0.5 µl of transposase. Excess salt in the reaction was removed by dialysis, and 1 µl was used to transform *E. coli* by electroporation. Bacteria were selected for resistance to kanamycin and hygromycin (40 µg / ml). Plasmids were purified by alkaline lysis and QIAgen plasmid miniprep kit columns, and digested with *EcoRI*. Plasmids containing the transposed *hph* gene (hereafter known as tn/*hph*) within the 2.1 kb sequence were used as templates for PCR with the CgFadA1 and CgFadA2 primers. The PCR cycle consisted of 95°C for 15 min., followed by 25 cycles of 95°C for 45 sec., 62°C for 30 sec., and 72°C for 3 min. 30 sec. The position and orientation of the tn/*hph* fragment in the 2.1 kb sequence was determined in each case by sequencing the PCR product outward from two primers located at either end of tn/*hph*, Tn551-5’end (5’-TTCTTGCCCTTAGGTTCCAC-3’) and Tn551-3’end (5’-TAGATTCCAAGTAGGTTCACTTC-3’).

**Fungal transformations:** All fungal transformations were performed using a PEG-mediated protocol that has been described previously (Thon et al, 2000). For production of GFP-expressing transformants, I used pCT74, which contains the SGFP gene under the control of the *Pyrenophora tritici-repentis* TOX-A promoter, and the *hph* gene as a selectable marker (Lorang et al, 2001). Strains were transformed with 3-9 µg pCT74 linearized with *EcoR1*. The plasmid pAN8-1 (Punt et al, 1990), linearized with *EcoR1*, was also used for some experiments. This plasmid contains the *ble* gene that confers phleomycin resistance.
For production of split-marker transformants, pairs of overlapping truncated PCR products were produced for each disruption construct. These corresponded to opposite ends of the 2.1 kb sequence, and overlapped by about 500 bp within the inserted \textit{hph} gene sequence in each case. The \textit{hph} gene primers HygR-1 (5’-CCACGGCCTCCA GAAGAAGATG-3’) and HygR-2 (5’-GATATGTCCTGCGGGTAAATAGCTGC-3’) were used in combination with CgFadA1 or with CgFadA2 to amplify both ends of each of the disrupted sequences. The PCR conditions consisted of 94°C for 3 min; followed by 30 cycles of 94°C for 30 sec., 62°C for 30 sec., and 72°C for 3 min. The resulting PCR products were purified using the QIAgen PCR product purification kit. For each disrupted construct, 500 ng of each member of the appropriate pair of overlapping PCR products was used to transform \textit{C. graminicola} protoplasts. All transformants were single-spored and stored on silica at –80°C.

To measure the stability of the split-marker transformants in culture, the strains, including the M1.001 GFP1 transformant (see Chapter 2) as a control, were subcultured eight times on plates containing either PDA or PDA Hyg 50 µg/ml. Subcultures were made by removing a plug from the edge of a 2-week-old PDA culture and transferring it to the center of a fresh PDA plate. The cultures were finally transferred to plain PDA for spore production (spores are not produced on medium containing hygromycin, even by hygromycin-resistant strains). Falcate spores were harvested after 3 weeks, washed, and then spread onto PDA and left to germinate overnight. For each subcultured isolate, a spore-cutter device was used to transfer germinated spores into two 24-well plates containing PDA, and two containing PDA Hyg 50 µg/ml. The number of survivors in each case was counted after a week of incubation at room temperature.

\textit{Molecular methods:} DNA sequences were obtained using an ABI machine with the ABI Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City CA), according to the manufacturers instructions. Sequencing was performed in the Advanced Genetics Technology Center (AGTC), at the University of Kentucky.

Fungal genomic DNA was prepared as described in Thon et al., 2000. For Southern blotting, 1 µg of digested DNA per lane was transferred to a positively charged nylon membrane (Roche Diagnostic corporation, Indianapolis IN) using a turboblotter device (Whatman Inc, Sanford ME). Probing and detection was performed with the DIG High Prime DNA labeling and Detection Starter kit II (Roche Diagnostic corporation).
For preparation of RNA, fungal strains were grown in Fries complete medium shaking cultures for four days at 28°C, then the mycelium was harvested by vacuum filtration and flash-frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent. Each RNA sample was resuspended in 20 µl of DEPC treated water, and the concentration was determined by using a spectrophotometer. The RNA quality was assessed on a 1.2% agarose formaldehyde gel.

RT-PCR reactions were performed as follows: a mix of 6µg of total RNA, water (to a volume of 10 µl), and 1 µl of oligodT primer (P. Kachroo) was incubated for 15 minutes at 65ºC, and immediately chilled on ice. 4µl of 5X RT buffer, 2 µl of 0.1M DTT, 1 µl of 10 mM dNTPs, 1 µl RNasein (Promega), and 1 µl of Superscript II (Invitrogen) were successively added. The reaction mix was incubated for 1 h at 42ºC. The DNA/RNA complexes were denatured at 65ºC for 15 min., and then 20 µl of DEPC treated water was added. 1 µl of cDNA was used as a template for the PCR reaction. PCR cycling conditions consisted of 93ºC for 3 min, then 35 cycles of 93ºC for 30 sec., 63ºC for 30 sec., and 72ºC for 3 min., and finally one cycle of 72ºC for 1 min. Primers CgFadA18 (5’-CGCACAGCCGCAGGTTCTCTTG-3’) and CgFadA21 (5’-GCAAGGCCCGCAACGAGGAG-3’) were used. These two primers amplify nearly the complete ORF of the C. graminicola fadA orthologue.

Measurements of fungal growth and development in vitro: To measure the radial growth rate, PDA plates were inoculated with plugs taken from cultures that had been started from silica stocks. PDA cultures were incubated at 23°C under continuous light, and the colony radius was measured every two days. Each experiment consisted of two replicate plates, and the experiment was repeated eight times. The average growth rate per day for each strain was calculated.

To measure the production of falcate spores, and to control for the difference in growth rate of the cultures, a cork borer was used to remove plugs 5mm in diameter from 18-day-old, mature sections of each colony. Each plug was placed in a microcentrifuge tube, and 1 ml of sterile water was added. The falcate spores were detached by vortexing for 15 sec., and detached falcate spores were counted under the microscope with a hemacytometer. The experiment was repeated eight times for each strain.

To measure the production of oval spores, 250 ml flasks containing 100 ml of Fries complete medium were inoculated with falcate spores at a concentration of 40 spores per microliter. The flasks were shaken for 48h (28ºC, 150 rpm), and then the mycelium was homogenized thoroughly by blending. Numbers of oval spores in replicate aliquots of the
homogenates were counted with a hemacytometer. The volume of the remainder of the homogenate was measured, then it was filtered and the mycelium was dried at 65°C overnight to obtain the dry weight. Oval spore numbers were calculated on the basis of the dry weight. The experiment was repeated eight times for each strain.

To measure spore germination and development in vitro, sterile water and 0.1% yeast-casein medium were inoculated with falcate spores at a concentration of 20 spores per microliter. 100 µl drops of the spore suspensions were deposited in the bottom of empty Petri dishes (Becton Dickson, Franklin Lakes NJ). To calculate the germination rate, two plates containing four drops each were prepared for each strain. The plates were left standing at room temperature for between 1 hr. 30 min. and 2 hr. to let the spores attach to the bottom of the plates. The plates were then transferred into humidity chambers (transparent covered plastic boxes containing a layer of saturated germination paper in the base) and placed in an incubator (30°C, continuous light). Observations were made using a light microscope (Zeiss AxioSkop Microscope; Carl Zeiss, Jena Germany). Germination in water and yeast-casein was assessed at 24 hr. by determining the percentage of germinated spores within a single field observed in the center of the drop at 100X magnification. Development in yeast-casein was described at 24 hr., 48 hr., and 72hr. A minimum of 18 drops for each strain was observed at each time point.

Plant inoculations: The sweet corn hybrid Jubilee was used for this study. Plants were grown in the greenhouse in 10-inch pots in a mixture of 3 parts Pro Mix BX (Premiere Horticulture Ltd., Riviere du Loup, PQ, Canada) / 2 parts sterilized topsoil. Three seeds were planted in each pot. After germination, the seedlings were watered daily to saturation with a solution of 4.75 g of Miracle-Gro 18-18-21 formulation for tomatoes (Stern’s Miracle-Gro Products Inc., Port Washington, NY, USA) per liter. Two plants per pot were ultimately retained until they reached a late vegetative stage (V11-V12). Just prior to anthesis, the sheath tissue was stripped from the second internode above the soil line. The plants were placed on their sides, and a small wound was made in the center of the stripped internode with a sterile dissecting needle (2 mm in depth). A 10 µl drop of conidial suspension, or sterile water as a control, was applied to the wound. The wound was covered with a detached microfuge tube cap and sealed with parafilm to create a moist chamber. The plants were left overnight. The tube caps were then removed, and the plants were placed upright. The inoculated internodes from two plants were harvested each day starting at 3 days after inoculation (dpi). For each experiment, a minimum of
eight plants was inoculated with each fungal strain or with water. The experiment was repeated at least twice with the GFP strains, and four times with the non-GFP strains.

To test the stability of the split-marker transformants in planta, inoculated internodes were cut from the plants and briefly rinsed with running tap water. The internodes were split in half with a clean razor blade, and small samples of necrotic tissues were taken from the edges of the primary lesions and plated on PDA. Fungal mycelia that emerged from the tissue samples and had some resemblance to the colony form of *C. graminicola* were transferred to fresh PDA plates. One isolate per inoculated internode was ultimately retained. The isolates were later transferred to PDA containing 50 µg of hygromycin per milliliter to see if they had maintained hygromycin resistance, and genomic DNA was extracted and used for PCR and/or Southern hybridization to determine if the tn/hph insertion into the *C. graminicola* fadA orthologue was still intact.

Jubilee seedlings were used for leaf whorl inoculations. Individual seedlings were grown in conetainers in a mixture of 3 parts Pro Mix BX / 2 parts sterilized topsoil. For inoculations of 10-day-old seedling leaf whorls, the following spore dilutions were prepared in 0.01% Tween 20: 5x10⁶ spores/ml; 10⁶ spores/ml; 5x10⁵ spores/ml; 10⁵ spores/ml; 5x10⁴ spores/ml; 10⁴ spores/ml; and 5x10³ spores/ml. 200µl of each spore suspension, or 0.1% Tween 20 as a control, were deposited in the whorl of each plant. The inoculated plants were placed in a dew chamber overnight, and then returned to the greenhouse bench. Symptom development was monitored at 6 dpi. A scoring system ranging from 0 to 5 was used to estimate disease severity. A 0 was assigned when the plant exhibited no symptoms; scores of 1, 2, or 3 were given to plants that exhibited symptoms on the leaves ranging from barely noticeable (1) to heavily damaged (3); a score of 4 was given when the plant exhibited symptoms on the stalk or deep inside the whorl; and a score of 5 was given when the emerging leaf had been totally killed. Two plants per experiment were inoculated for each spore concentration, and the experiment was repeated eight times.

*Microscopy:* Fungal development in stalk tissues was monitored with a Leica TCS NT confocal microscope (Leica Microsystems Inc., Exton PA). Infected tissue sections were cut by hand with a razor blade and observed without further treatment. Green fluorescent protein (GFP) was excited at 488 nm. Plant cell walls and chloroplasts autofluoresce at this excitation wavelength, and this property was used to visualize the plant tissues. However, at lower
magnifications, the autofluorescence of the plant cell walls was usually not intense enough to be captured in the micrographs.

**Statistical analyses:** lesion length data were analyzed using the General Linear Model (GLM) procedure that is part of the SAS statistical analysis software package (SAS Institute Inc., 1997). The Tukey mean separations test was applied to the data, with a significance level of \( P < 0.01 \). The effect on lesion length of different strains (also including the water control) was analyzed. For data other than lesion lengths, SAS ANOVA and the Waller Duncan multivariate analysis were used, with a significance level of \( P < 0.01 \).

**Results**

*Cloning of a fadA orthologue from Colletotrichum graminicola: A C. graminicola contig with significant similarity to the fadA gene of A. nidulans (#AAC49476.1) was identified after tBLASTp analyses of a proprietary DuPont genomic sequence database. The putative fadA orthologue of C. graminicola was named Cgg1. The A. nidulans FADA protein is 353 amino acids (aa) in length, while the predicted Cgg1 protein sequence was apparently incomplete, consisting of only the first 291 aa. The contig sequence was used to design primers, and a 2.1 kb fragment containing the partial Cgg1 gene was amplified from the genomic DNA of C. graminicola, and cloned into the pCR®2.1-TOPO vector to create plasmid pSM1267. This plasmid was used for transposon tagging to produce disrupted clones for the split-marker transformation protocol (see below). Meanwhile, DuPont continued to expand their C. graminicola genomic database, and eventually they were able to identify a larger contig that contained the full length Cgg1 gene. Jim Sweigard of DuPont designed primers to amplify a 3.2 kb fragment containing the entire Cgg1 gene, which he cloned into the pCR®2.1-TOPO vector using the TOPO cloning kit to produce plasmid pCgFadA. I sequenced the entire 3.2 kb fragment in pCgFadA to confirm the low-pass sequence of DuPont (Figure 4.1). The Cgg1 gene is predicted to encode a protein of 353 aa, which is more than 99% identical to C. trifolii CTG1, and 95% identical to A. nidulans FADA (Figure 4.2). There are three predicted introns (Figure 4.1). I have confirmed the first two of these by RT-PCR and sequence analysis (data not shown), but the third one has not yet been confirmed. There are several CAAT box motifs upstream of the putative start codon, but no obvious TATA box (Gurr et al., 1987). The putative start codon is embedded in a near-consensus Kozak motif (Kozak, 1986). There is also an interesting
GAGA repeat upstream of the ORF, similar to the one reported just upstream of the C. graminicola Cpr1 gene (Thon et al., 2002)

Production of Cgg1 disruptant strains. Three different disruption constructs containing single tn/hph insertions were recovered after transposon tagging of pSM1267. One had an insertion just upstream of the predicted Cgg1 ORF, and two others were within the ORF itself (Figure 4.3). I confirmed all of these by sequencing the DNA flanking the insertions. All three constructs were used in split-marker transformation experiments. The resulting transformants could be divided into obvious “fast-growing” versus “slow-growing” groups (Figure 4.4A). Several representatives of each group were chosen for PCR analysis in order to detect the presence of homologous integrations. PCR indicated that only strains in the slow-growing group had homologous integrations of the tn/hph DNA (Figure 4.4B). One or two single-spored disruptant strains resulting from transformation with each of the three constructs were retained for further analysis (Table 4.1). In addition, three single-spored strains with ectopic integrations were kept for use as controls (Table 4.1).

Molecular characterization of disruptant strains: Southern analysis confirmed that all of the disruptant and ectopic strains contained the hph gene, and that all but one of the disruptant strains had a homologous integration of a single copy of the 2 kb tn/hph fragment (Figure 4.5, 4.6). One disruptant mutant, produced with the pSM1270 construct, appears to contain at least three copies of tn/hph inserted in tandem (Figure 4.5, lane 10).

Many of the mutants were “leaky” to a greater or lesser degree, as evidenced by my RT-PCR experiments (Figure 4.7). The amplified transcripts from the mutants containing insertions of tn/hph in the ORF were the same size as those in the wild-type strain. This suggested that the leakiness might have been due to the loss of the integrated DNA in some percentage of the nuclei, resulting in a heterokaryotic mycelium. The reason for leakiness of the mutants with insertions upstream of the ORF was not confirmed, but could also be due to loss of the integrating DNA, or alternatively to the fact that the ORF is still intact in these strains.

Phenotype of disruptant strains in vitro. The leakiness of the mutants in the RT-PCR, and the occasional production of fast-growing sectors by the slow-growing mutants (Figure 4.4A), suggested the possibility that the tn/hph insertions might be unstable. One experiment I did to address this question was to conduct PCR analysis of four fast-growing sectors that arose from several of the slow-growing disruptant strains. However, all of the sectors still gave rise to a
major band that suggested that most, if not all, of the nuclei still contained the disruption (Figure 4.4B). The presence of some fainter bands in a few of the sectors hinted at a minor degree of instability. To test this further, I subcultured several mutant and ectopic strains eight times in a row, either in the presence or absence of selection (50 mM hygromycin) and then analyzed the results by Southern hybridization and by evaluating hygromycin resistance of 48 single-spored progeny from the last subculture (each spore of C. graminicola has only one nucleus). These results are reported in Table 4.2, and showed that the mutants were mostly stable even in the absence of selection, but that a few nuclei did lose the integration since a very small percentage of progeny were recovered from some of the strains that were no longer hygromycin resistant. In Southern hybridizations, all the progeny that had lost resistance also appeared to have lost the integrated tn/hph DNA, whereas none of the progeny that retained hygromycin resistance had apparently lost the insertion (data not shown).

As already mentioned, the disruption mutants grew more slowly than the wild type on PDA (Figure 4.8A). Some of the mutants produced significantly more falcate spores than the wild type, and all of them produced significantly fewer oval spores (Figure 4.8B, C). Falcate spore germination rates in vitro appeared to vary among the different strains, but this variation was not obviously connected to the mutation (Figure 4.8D). Rates of appressorial formation in vitro after 24 hours were similar, but the mutants typically produced appressoria on longer germ tubes, rather than producing them directly from the spore, as the wild-type did (Figure 4.8E, Figure 4.9).

The ability of the mutants to germinate and develop in vitro in drops of yeast-casein liquid medium was evaluated. One interesting observation was that the spores produced by the mutants were fusiform in shape, rather than falcate (Figure 4.9A, B, C). Approximately 75% of the spores of the wild-type strain were falcate, whereas for the mutants the percentages of falcate spores ranged from 0 to 40%, with an average of only 9% (Table 4.3). By 24 hr. after inoculation (hpi), the wild-type had developed unevenly distributed clumps of mycelium, or “colonies”, and was already producing oval spores (Figure 4.9D). By 48 hpi, the wild-type colonies were usually well developed, and numerous hyphopodia were visible (Figure 4.9G). Falcate spore production was first observed at 48 hpi (Figure 4.9J; Table 4.4). By 72 hpi, all the wild-type colonies were producing falcate spores, usually from clusters of hyphae that were all producing conidiogenous cells (Figure 4.10C). The Cgg1 mutants BH70 2-4, BH703-2,
BH71 2-2, and BH72 3-15 also developed colonies by 24 hpi (Figure 4.9E), but these were usually smaller than the wild-type colonies, suggesting that germination and/or vegetative growth was delayed (Figure 4.10A; Table 4.4). By 48 hpi, the mutants were producing oval spores (Figure 4.10B). The mutant colonies seemed to develop fewer hyphopodia than the wild-type (Figure 4.9H). Falcate spore production was first observed at 48 hpi (Table 4.4). By 72 hpi, falcate spore production appeared to have replaced oval spore production in about 50% of the drops containing the mutant strains. The mutant colonies continued to lag behind the wild-type colonies in size (Figure 4.9F). Colonies that were producing oval spores as well as falcate spores appeared to be larger than those producing only falcate spores (Figure 4.9L). In the colonies producing both types, falcate spores were produced only at the edges. Conidiogenous cells were observed all along the hyphae that were producing falcate spores (Figure 4.10D,E).

Strain BH71 2-10 behaved differently from the other mutants in vitro. This mutant did not germinate very well in water (Figure 4.8D) but it germinated even better than the wild-type in yeast-casein (40% germination versus 10% for the wild-type). At 24hpi, BH71 2-10 had developed small colonies like the other mutants (Figure 4.9F, Table 4.4) and by 48hpi oval spores were observed in all of the drops (Table 4.4). In contrast to the other mutant strains, BH71 2-10 was only rarely observed to produce falcate spores (Table 4.4). By 72hpi, the colonies were usually quite large, and oval spore production was very abundant (Figure 4.9M).

Phenotype of disruptant strains in vivo. I evaluated the pathogenicity of the mutant, wild-type, and ectopic control strains on maize stalks and leaves. Because I was concerned that the tn/hph insertions might be unstable in planta, I made several isolations from the leading edges of stalk lesions caused by the mutant strains and analyzed them for hygromycin resistance (Table 4.5). I also conducted PCR and Southern analysis in order to confirm that they still contained the disruptions. In all cases, these isolates were still hygromycin resistant, and still had typical mutant phenotypes in culture, including production of fusiform spores and slow growth. Results of PCR assays and Southern indicated that they were all still disruptants (data not shown).

Pathogenicity assays: All of the mutants were significantly less pathogenic than the wild-type or ectopic control strains, both on unwounded maize seedling leaves and on wounded mature stalks (Figure 4.11, Table 4.6). In stalks, there were no obvious differences in the ability of the mutants to form secondary lesions, or to move beyond the wounded internode, in comparison to the wild type or ectopic strains, with the exception of the mutant BH71 2-10,
which appeared to be less capable of doing these things than the other strains (Table 4.7). This strain also had an abnormally low rate of germination in water (Figure 4.8D).

**Complementation of mutant strain BH72 3-15, and analysis of development in planta.** One mutant, BH72 3-15, was chosen for a more detailed cytological analysis of pathogenic development in maize stalk tissues. The mutant was co-transformed with pAN8-1, conferring phleomycin resistance, and pCT74, which contains the SGFP gene driven by the TOXA promoter. In a second transformation, the plasmid pCgFadA, containing a full-length copy of the \( C_{gg}l \) gene, was also included with the other two. Green fluorescent phleomycin-resistant transformants were identified in each case. Two GFP transformants of the mutant strain were obtained. Both were similar to the original mutant strain in their growth rate on agar and both produced fusiform spores like the mutant. BH72 3-15 GFP2 was tested by RT-PCR and, like BH72 3-15, it produced no detectable \( C_{gg}l \) transcript (Figure 4.7B). BH72 3-15 GFP1 has not been tested by RT-PCR.

Only one highly fluorescent transformant was recovered from the “three-way” complementation transformation experiment. I named this transformant BH72 3-15 C1 GFP. Southern hybridizations suggested that this strain contained the wild-type copy of the \( C_{gg}l \) gene inserted in a position adjacent to the original disrupted copy, but the data were insufficient to be certain of its exact orientation or position (Figure 4.12). Growth rate on agar plates, and oval and falcate conidiation, was restored in this complemented strain to levels that were not significantly different from the original wild-type strain (data not shown). Spore shape reverted to the normal falcate type (Table 4.3). Lesion sizes in inoculated maize stalks (Table 4.6), and apparent levels of transcript in the RT-PCR (Figure 4.7, lane 5, compare to lane 4), were also similar to the wild-type.

Wounded maize stalks were inoculated with GFP-expressing mutant and complemented strains and also, as a control, with the GFP1 wild-type strain that was used for previous studies (see chapters 2 and 3). Primary lesions produced by the mutant and complemented strains at the inoculation site developed in a manner similar to the wild-type (Chapter 2). A cavity developed under the rind that was surrounded by darkly discolored parenchyma tissues (Figure 4.13A, B, C). By 4 dpi, all the strains were established as mycelium in these discolored tissues at the margin of the primary lesion (Table 4.8). I observed a few things about the growth and development of the mutant strains that seemed to be very unusual, based on the prior extensive
observations I made of the wild-type in maize tissues, which are described in Chapter 2 of this dissertation. As was typical, M.1001 GFP1 was colonizing new parenchyma cells at the margins of the primary lesion by producing relatively few invasive hyphae (Figure 4.13D). The complemented strain appeared similar (Figure 4.13E). Invading hyphae passed through the cell wall via extremely narrow connections, as described in Chapter 2. In contrast with the wild-type and complemented strains, BH72 3-15 GFP1 produced an unusually large number of invasive hyphae as it colonized new cells (Figure 14.3F, G). Passage through the cell walls still occurred via narrow connections, as described for the wild-type. BH72 3-15 GFP2 did not seem to over-produce invading hyphae to the same extent as BH72 3-15 GFP1, but each strain has been observed in planta only once and so these observations need to be repeated.

The two mutant strains, and the complemented strain, produced oval spores inside the plant tissues (Table 4.13; Figure 4.13H, J), and they also produced falcate spores in acervuli in the primary lesions as described for the wild-type in Chapter 2 (not shown, Table 4.8). The mutant strains appeared to produce oval and falcate spores more abundantly in the tissues than the wild-type or complemented strains (Table 4.8). The two mutant strains, and the complemented strain, colonized the epidermal cells and produced stromata, apparently normally (Figure 4.13 I, K). Secondary lesions containing fluorescent mycelium were observed for all the strains, both in the inoculated internode, and in the internodes above and occasionally below. The secondary lesions seemed normal, and there were no obvious differences among the strains in their ability to produce them. Additional observations of the mutant and complemented strains in planta will be necessary before it will be possible to confirm any differences that are due to the mutation in the \( Cgg1 \) gene.

**Discussion**

The \( fadA \) family of G\( \alpha \) proteins is very highly conserved among filamentous fungi. Orthologues of \( fadA \) have so far been identified and characterized in *Neurospora crassa*, *Magnaporthe grisea*, *Cryphonectria parasitica*, *Botrytis cinerea*, *Cochliobolus heterostrophus*, and *Colletotrichum trifolii* (Ivey et al, 1996; Liu and Dean, 1997; Gao and Nuss, 1996; Segers and Nuss, 2003; Horwitz et al, 1999; Gronover et al, 2001; Truesdell et al, 2000). These proteins play critical roles in the regulation of fungal development and secondary metabolism in response to various environmental stimuli. The G\( \alpha \) proteins function at the apex of signaling cascades,
interacting with membrane-bound receptors and with Gβγ subunits to switch downstream pathways “on” and “off”. Activation of the Gα by receptor stimulation results in release of the Gβγ, turning the pathway “on”. The pathway is turned “off” again when the GTPase activity of Gα hydrolyzes GTP to GDP, causing re-formation of the inactive heterotrimer Gα/βγ. Dissociated Gα or Gβγ can affect a range of secondary messengers, including cAMP, Ca2+, and inositol 1,4,5-triphosphate. Except in A. nidulans, relatively little is known about the downstream signaling pathways of the fadA orthologues. In A. nidulans, fadA acts to regulate the cAMP-dependent protein kinase A (PKA) (Yu and Keller, 2005). Deletion of PKA in A. nidulans produces a phenotype that is the opposite of a FADA activating mutation, i.e. an increase in conidiation, and decreased vegetative growth. There is some evidence that fadA orthologues in other fungi also operate by regulating cAMP and PKA. For example, disruptions in the C. parasitica fadA orthologue Cpg1 have elevated levels of cAMP (Gao and Nuss, 1996), and C. trifolii strains disrupted in the catalytic subunit of PKA exhibited precocious sporulation (Yang and Dickman, 1999). In turn, cAMP and PKA can also affect multiple downstream targets, and this suggests a reason why perturbation of fadA and other G-proteins results in pleomorphic effects on development and metabolism.

In my study, strains of C. graminicola with disruptions in the fadA orthologue Cgg1 generally produced more falcate conidia and fewer oval spores in vitro, grew more slowly on PDA, and were less pathogenic to maize. These phenotypes are comparable to those of other fungi in which fadA orthologues have also been disrupted. For example, all fungal fadA-orthologue disruptants reported to date grow more slowly in culture, at least on some media. Growth of B. cinerea and C. parasitica was only inhibited on starvation media: on rich medium there was not much difference from the wild-type. In C. parasitica, it was suggested that the CPG1 protein was important for dealing with stress, including starvation. This could be one explanation for the reduced pathogenicity phenotype that most of the fadA-orthologue mutants, including C. graminicola, display. During pathogenesis, fungi may be exposed to reactive oxygen species, and starvation for carbon and nitrogen, among other stresses. Only the Cga1 mutant of C. heterostrophus appeared to be as pathogenic as the wild-type (Horwitz et al., 1999). It may be relevant to mention here that I did not see much of a difference in pathogenicity between the Cgg1 mutant and wild-type strains of C. graminicola on maize leaves if I used very
high levels of inoculum (5X10^6 spores/ml) (data not shown). It is possible that inoculating with fewer *C. heterostrophus* spores might reveal quantitative differences in pathogenicity.

Although it is generally true that mutation of *fadA* orthologues in pathogenic fungi results in reduced pathogenicity, most researchers have not gone very much farther in investigating the basis for this reduction. The *M. grisea magB* mutant produced fewer appressoria *in vitro*, and the *C. trifolii Ctg1* mutant produced spores that germinated poorly *in vitro*, so in those cases the observed reductions in pathogenicity on leaves could be an indirect result of these phenotypes. In *B. cinerea*, staining of inoculated tissues revealed that the pathogen was able to penetrate the tissues but that lesion expansion was blocked. Reductions in production of pectinase and phytoxins, caused by the *Bcg1* mutation, might explain this phenotype. In *C. parasitica*, deleting the *Cpg1* gene results in down-regulation of the laccase genes that are important for lignin digestion, and this may interfere with the ability of this pathogen to colonize its woody host (Segers and Nuss, 2003). In *C. graminicola*, it appears that a reduction in pathogenicity to leaves is not due to decreases in the percentages of spore germination or appressorium formation, since these appear to be comparable to the wild type, at least *in vitro*. However, germ tubes formed by *Cgg1* mutant strains *in vitro* were longer than the wild type, and this suggests that the production of infection structures is delayed. This might give the plant more time to respond defensively. Inoculations of wounded stalks removed the necessity for penetration via appressoria, and yet pathogenicity of the *Cgg1* mutants was also reduced in stalks. It appeared from my preliminary cytological observations that the mutant fungus was not “controlling itself” and may have been spending too much of its energy making spores and infection hyphae, leaving less energy for expansion through the host tissues.

*C. graminicola* produces two types of asexual spore, falcate and oval. We do not know much about the developmental relationship between these two types, but we do know that their production is stimulated by different environmental conditions, both *in vitro* and *in planta* (Panaccione et al., 1989). Thus, *in vitro*, oval spores are produced in submerged cultures whereas falcate spores are produced at an air interface; production of oval spores appears to be light-independent while production of falcate spores requires light; and *in planta*, oval spores are produced only within the host tissues while falcate spores are produced primarily on the surface. *C. graminicola* mutants disrupted in the *Cgg1* gene produced more falcate conidia than the wild-type, but production of oval spores appeared to be unaffected *in vitro*. This suggests that falcate
conidia, but not oval conidia, are subject to negative regulation in vitro by Cgg1. However, both types of conidia appeared to be over-produced in planta. This suggests that production of both types of conidia is normally negatively regulated during the early stages of pathogenesis by the activity of Cgg1. Other than in A. nidulans, an increase in conidiation has not been reported in any of the other fungi in which a fadA orthologue has been disrupted. Instead, if conidiation is affected, it usually seems to be decreased. However, it should be noted that many studies do not compensate for the slower growth of the cultures when counting spores. This does not apply to C. parasitica, in which deletion of the Cpg1 gene completely abolishes conidiation. This suggests that there may be differences in the roles of the fadA orthologues in sporulation in different species, and under different environmental conditions.

It may be quite complicated to pin down the exact role of Cgg1 in causing the observed phenotypic alterations in C. graminicola. For example, studies have shown that removal of Gα transcript causes changes in levels of the interacting Gβγ also, and these subunits have their own roles to play (reviewed in Yu and Keller, 2005). Thus, manipulating components of signaling pathways is like perturbing one part of a spider’s web: the resulting vibrations are felt throughout and can cause a cascade of direct and indirect effects. One aid in teasing these effects apart is use of “knock-down” rather than “knock-out” strains. Reducing the amount of the transcript to varying degrees can provide subtler clues to the roles that the proteins play.

Differences in phenotype for the Cgg1 mutant strains may relate to the relative “leakiness” of the mutants. The reason for this leakiness remains unknown, but given the fact that the RT-PCR amplicon produced from the leaky mutants was the same size as that from the wild-type, the possibility of expression from a gene containing the tn/hph integration can be eliminated. Instead, it seems most likely that the expression resulted after loss of the integrated DNA in some nuclei. Evidence for a small degree of instability was obtained from my PCR experiments with fast-growing sectors, and from my subculturing experiments. The instability could have been related to the use of the transposon tagging approach for the production of disruption constructs. Transposon tagging is not essential for the split marker protocol, because split marker constructs can be produced by other means, e.g. overlap PCR (Fu et al., 2006). However, the use of the transposon tagging system is advantageous in a high-throughput protocol. An alternative explanation for the “leakiness” could be that the mutant phenotypes observed for at least some of these mutant strains were due to gene silencing rather then gene
disruption. I had both PCR and Southern hybridization evidence that the mutants actually contained homologous integrations in the \textit{Cgg1} gene, but the Southerns were not high-quality, and so it is possible that some or all of the nuclei did not actually contain the homologous insertion, and that the expression of the gene was being down-regulated by silencing as a result of expression of an ectopically inserted gene. This explanation seems rather unlikely, but more data are needed before it can be eliminated as a possibility. In the future I think it will be important to confirm my results by producing a deletion of the \textit{Cgg1} by a gene replacement method.

The two mutants that were produced in my study from the pSM1271 construct, in which the insertion was just upstream of the \textit{Cgg1} ORF, produced intermediate phenotypes in many cases between the wild-type and the other mutants that had insertions in the ORF itself. My RT-PCR results, though not quantitative, suggest that the two pSM1271 mutants might produce more transcript than the other mutants. There is evidence that relative expression of the \textit{Cpg1} gene in \textit{C. parasitica} can produce a range of severities in the resulting mutant phenotype. Transgenic co-suppression of \textit{Cpg1}, in which the presence of a transgenic sense transcript silences, to varying degrees, the endogenous gene, resulted in a reduction in production of aerial hyphae, slower growth on PDA, and reduced pathogenicity. Phenotypes of different mutants were qualitatively similar but varied in severity. The degree of expression of the CPG1 protein, measured by Western blots, correlated with the severity of the phenotype (Segers an Nuss, 2003). If this is what is going on with the \textit{Cgg1} mutants, then we can surmise that some mutant phenotypes are less affected by the relative levels of transcript than others. For example, growth rate was similar for all the mutants, but the relative number of fusiform versus falcate conidia was intermediate in the pSM1271 mutants, as was the production of appressoria on long germ tubes. It would be interesting to test this hypothesis by quantifying the amount of transcript and protein in these \textit{Cgg1} mutants and correlating that with these phenotypes.

The results of my cytological studies of the mutant and wild type strains must be treated as preliminary, but I did observe some unusual things that should be explored further. I observed an apparent increase in the production of falcate and oval spores in the diseased tissues, but more spores did not seem to lead to production of more secondary necroses. Thus, these experiments did not provide evidence supporting the idea that spores are primarily responsible for systemic movement of the pathogen in maize stalks. A most interesting observation was “hyper-
"infectivity" of the GFP2 mutant, which appeared to be producing a very large number of primary infection hyphae as it moved cell to cell. This implies that primary hyphal formation in planta is negatively regulated by Cgg1. Perhaps it is more efficient to minimize the number of new infection points in order to direct most of the organism’s energy into lesion expansion. Or perhaps there is less risk of activating defense responses. It will be interesting if primary hyphae really are negatively regulated, because hyphae in culture appear to be the opposite. This would fit with other evidences that Colletotrichum primary hyphae are distinct from secondary necrotic hyphae (Perfect et al., 1999). Indeed, it suggests that developmentally they may be more like spores. This observation definitely needs to be investigated further, and many more observations and measurements will be necessary before it can be confirmed.

Disruption experiments in a range of fungi, now including C. graminicola, have shown that the fadA orthologues, though highly conserved and presumably part of a signaling pathway that is also conserved, have varying effects on pathogenicity. The nature of these effects may depend on the pathogenic lifestyle of the particular fungus in its particular host. It will be particularly exciting to study the role of this protein in C. graminicola, which is a hemibiotroph and undergoes a dramatic developmental shift from biotrophy to necrotrophy. Analysis of the role of fadA in pathogenic fungi gives us important insights into pathogenesis as a developmental process. It demonstrates that the production of pathogenicity structures, including appressoria and primary hyphae, and the production of secondary metabolites that aid in pathogenesis, such as toxins, utilize the same signaling pathways, and ultimately share evolutionary and functional roots, with other non-pathogenicity related development in fungi.
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<th>Type of Integration</th>
<th>In the ORF?</th>
<th>Comments</th>
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<tr>
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<td>homologous</td>
<td>yes</td>
<td>GFP and phleomycin resistant, and complemented with a wild-type copy of the Cgg1 gene</td>
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Table 4.2. Survival of progeny from mutant and wild-type strains subcultured on PDA, or PDA containing 50µg/ml hygromycin.

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<tr>
<td></td>
<td>PDA HygB 50µg/ml</td>
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<td>NT</td>
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<td>98</td>
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<td>100</td>
<td></td>
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<tr>
<td></td>
<td>PDA HygB 50µg/ml</td>
<td>NT</td>
<td>NT</td>
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* “NT” means not tested because the spores failed to germinate for those treatments.
Table 4.3. Spore shape in the wild-type and various transformant strains.

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<td>3-15</td>
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<td>Falcate</td>
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<td>Fusiform</td>
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Table 4.4. Development of strains in drops of yeast Casamino acid medium. The data are expressed as percentages of drops in which each event was observed.
### Table 4.5. Stability of mutant strains *in planta.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total number of isolates</th>
<th>Grow on PDA</th>
<th>Grow on PDA HygB 50 µg/ml</th>
<th>Confirmed by PCR</th>
<th>Confirmed by Southern</th>
</tr>
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<tbody>
<tr>
<td>M.1001BH</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>BH70 2-4</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>2*</td>
</tr>
<tr>
<td>BH71 2-2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>BH71 2-10</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>BH70 3-2</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5**</td>
</tr>
<tr>
<td>BH72 3-15</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2*</td>
</tr>
</tbody>
</table>

* The digestion did not work for the missing isolates

** Not enough DNA to run a Southern for one isolate

The isolates have been saved on silica (no single-sporing)
Table 4.6. Analysis of lesion lengths on inoculated wounded maize stalks

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total number of samples</th>
<th>LS Mean (cm)</th>
<th>Class</th>
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<tbody>
<tr>
<td>M.1001BH</td>
<td>28</td>
<td>4.8</td>
<td>a</td>
</tr>
<tr>
<td>BH72 3-11</td>
<td>23</td>
<td>4.6</td>
<td>a</td>
</tr>
<tr>
<td>BH72 3-15 C1 GFP</td>
<td>17</td>
<td>4.5</td>
<td>a</td>
</tr>
<tr>
<td>BH70 2-2</td>
<td>13</td>
<td>3.9</td>
<td>a,b</td>
</tr>
<tr>
<td>BH71 2-3</td>
<td>12</td>
<td>3.3</td>
<td>b</td>
</tr>
<tr>
<td>BH70 2-4</td>
<td>12</td>
<td>2.7</td>
<td>b,c</td>
</tr>
<tr>
<td>BH71 2-2</td>
<td>12</td>
<td>3.2</td>
<td>b,c</td>
</tr>
<tr>
<td>BH72 3-15</td>
<td>28</td>
<td>3.1</td>
<td>b,c</td>
</tr>
<tr>
<td>BH71 2-10</td>
<td>12</td>
<td>2.0</td>
<td>c,d</td>
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<td>BH70 3-2</td>
<td>12</td>
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<td>c,d</td>
</tr>
<tr>
<td>CgSl1</td>
<td>13</td>
<td>1.0</td>
<td>d,e</td>
</tr>
<tr>
<td>water</td>
<td>17</td>
<td>0.3</td>
<td>e</td>
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</table>
Table 4.7: Percentages of wounded maize stalk samples observed that displayed each of these important phenotypes, including production of acervuli with spores in the wound; secondary lesion production; and movement into the internode above, or below, the wounded internode.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total number of samples</th>
<th>acervuli</th>
<th>secondary lesions</th>
<th>upper internode</th>
<th>lower internode</th>
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</thead>
<tbody>
<tr>
<td>M.1001BH</td>
<td>28</td>
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<td>46</td>
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<td>BH70 2-4</td>
<td>12</td>
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<tr>
<td>BH71 2-2</td>
<td>12</td>
<td>83</td>
<td>8</td>
<td>0</td>
<td>0</td>
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<tr>
<td>BH71 2-10</td>
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<td>25</td>
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<td>BH70 3-2</td>
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<td>83</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BH72 3-15</td>
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<tr>
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<td>83</td>
<td>67</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>BH71 2-2</td>
<td>12</td>
<td>75</td>
<td>33</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>BH72 3-15</td>
<td>27</td>
<td>56</td>
<td>41</td>
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<td>4</td>
</tr>
<tr>
<td>BH72 3-15C1GFP</td>
<td>22</td>
<td>36</td>
<td>23</td>
<td>4.5</td>
<td>4.5</td>
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<table>
<thead>
<tr>
<th>Phenophases, see the text</th>
<th>Phenophases, see the text</th>
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<td>The results are expressed as percentages of samples for which the phenophases observed. For explanations of these</td>
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<tr>
<td>Phenophases, see the text</td>
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<td>The results are expressed as percentages of samples for which the phenophases observed. For explanations of these</td>
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<table>
<thead>
<tr>
<th>TABLE 4.8: Numbers of observations of various important phenophases in wounded tissue slices inoculated with Heterosolan Lignal</th>
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<tr>
<td>0.1% 0.2% 0.3% 0.4% 0.5% 0.6% 0.7% 0.8% 0.9% 1.0% 1.1% 1.2% 1.3% 1.4% 1.5% 1.6% 1.7% 1.8% 1.9% 2.0% 2.1% 2.2% 2.3% 2.4% 2.5% 2.6% 2.7% 2.8% 2.9% 3.0% 3.1% 3.2% 3.3% 3.4% 3.5% 3.6% 3.7% 3.8% 3.9% 4.0% 4.1% 4.2% 4.3% 4.4% 4.5% 4.6% 4.7% 4.8% 4.9% 5.0% 5.1% 5.2% 5.3% 5.4% 5.5% 5.6% 5.7% 5.8% 5.9% 6.0% 6.1% 6.2% 6.3% 6.4% 6.5% 6.6% 6.7% 6.8% 6.9% 7.0% 7.1% 7.2% 7.3% 7.4% 7.5% 7.6% 7.7% 7.8% 7.9% 8.0% 8.1% 8.2% 8.3% 8.4% 8.5% 8.6% 8.7% 8.8% 8.9% 9.0% 9.1% 9.2% 9.3% 9.4% 9.5% 9.6% 9.7% 9.8% 9.9% 10.0%</td>
</tr>
<tr>
<td>0.1% 0.2% 0.3% 0.4% 0.5% 0.6% 0.7% 0.8% 0.9% 1.0% 1.1% 1.2% 1.3% 1.4% 1.5% 1.6% 1.7% 1.8% 1.9% 2.0% 2.1% 2.2% 2.3% 2.4% 2.5% 2.6% 2.7% 2.8% 2.9% 3.0% 3.1% 3.2% 3.3% 3.4% 3.5% 3.6% 3.7% 3.8% 3.9% 4.0% 4.1% 4.2% 4.3% 4.4% 4.5% 4.6% 4.7% 4.8% 4.9% 5.0% 5.1% 5.2% 5.3% 5.4% 5.5% 5.6% 5.7% 5.8% 5.9% 6.0% 6.1% 6.2% 6.3% 6.4% 6.5% 6.6% 6.7% 6.8% 6.9% 7.0% 7.1% 7.2% 7.3% 7.4% 7.5% 7.6% 7.7% 7.8% 7.9% 8.0% 8.1% 8.2% 8.3% 8.4% 8.5% 8.6% 8.7% 8.8% 8.9% 9.0% 9.1% 9.2% 9.3% 9.4% 9.5% 9.6% 9.7% 9.8% 9.9% 10.0%</td>
</tr>
</tbody>
</table>
**Figure 4.1** Nucleotide sequence, and predicted amino acid sequence, of Cgg1. The section in blue and green is the 2.1 kb fragment containing the partial gene that was cloned in plasmid pSM1272 for transposon tagging. The green part overlaps with the 3.2 kb fragment that is cloned in plasmid pCgFADA, which contains the whole gene. Notable features are highlighted in bold, including upstream CAAT boxes and GAGA repeat element, intron splice junctions and lariat sequences associated with predicted introns (in red), a potential downstream polyadenylation signal, and Kozak’s consensus sequence surrounding the putative start codon. The pink box is the single NheI restriction site: the green boxes are AvaI sites, and the blue boxes are XmnI sites. The forward and reverse primers used to produce the Cgg1 probe for the Southern shown in figures 4.5 and 4.12 are highlighted in gray.
TTCCGTTTCTCCCTAAACC GTTAGCTGCTGGTGAATCCGAAAATCGACAAT
---A---G---E---S---G---K---S---T---I

TCTGAAGCAGATGAAGCTCATCCACGAGGTTGGCTACTCGCCTCGATGAACGGCAAA

TCTTTCAAGGAAATTATCTTCCAGAACACACAGTGCAATCGATGCGGGTGATCT

GCCATGGGATCATGGGAACCTGCCCTTGAGGACCAACGCATGGGAGTACCTACCAGT

CCAGACCACATCTTCATGGCAACCCGCTCAGATTGAAAGGGATGTCTGCGCCGCC

GTCGGAAGTGGCATTGGACGCGTCGTGGAAGGATCTGGCTGCAAGATTGTGCTCA

AGCGCTCTCGGCAATACCGACTCAATGACTCTGCGAGATAGTACTTGATGC

CCGGGCTGACACAGGCCCGTTTGGCTAACTAGTAGTACCAGTTGGCTGCC
CTAACAGTAGCATCTTTTGGCAAACATCGACCTCGCGCCTCGGATCCCGTG

ATGTTGACGCTCGGTGCAAGCAMPCTCCTCTCCTCGTGCACCACGCCATCGC
AGTTTAACTCTCTCCGACACTGATCCTGCGATCTGCGGCT

AGAACGTCACCAATCCTCCTCTCCTCGTGCCATCCACGACCGATCTGGCTG

GTTCGAGACGCACTGTCAATCGCATGCAGGGAAGTGGGATCTGATCCCGTG ACTCC

ATCTGAAAGGATGCTTATGGTTCATCAAGACCTCCTCATCACTCCTCTCCGA
CAACATCAAGACCTCCTCCCAAGACATCCCTGCCTCCGACTTGACTTTG

TCGATCGGGATTGGAAGCTCCCTAGGCGCCGATCCGAGGACTATATTTCCCGA

CTATGAGGGGGCCGATGACTATGCGGCGGGCGTGTGAGTACCATCTCTG
GAACCGATTC
GTCAGCCTGAAACAGCATGAAACCAAGCAAATCTACACGCACTTCACTTGCGCGA
-V--S--L--N--Q--H--E--T--K--Q--I--Y--T--H--F--T--C--A--

CAGACACGACCCAGATCCCTCGCTCATGCGCAGTGACGTGAGTTAAAAACC
T--D--T--T--Q--I--R--F--V--M--A--A--V--N-----------------

CCCATCAGACCATGACCGGCTAAAGATGACAAAGGACGGTGGCTAA

CAGACACGATCATCATTCAGAGAAGACCTGCCGGCTGTCGGCCTTGAGTTTAAAGAAACATC
---D---T---I---Q---E---N---L---R---L---C---G---L---I---*

AAAACGTTTTGCTTTTTTCCAACAATACCTTTCTTTACTTTAATTGACGACACAAG
CCAGAAACGTCATGGGAGGCTGGCTCATGAAACAAACTTTTCTCCCGATGAAACAG
ACGGACGCCGATGTTTTCCCAAGATTTCCCTGATTACCGGACTGTATTTTCC
CCACTCTCGACCACCCTGGATTATTTCTCTCCCGGACTTGGGCGTCAACTTT
TTGTGGTTCTACTCCTATTGTACACGCTGAAATGAGAGGAATGGAGCGG
CACGCATCTACAAACTTTGACGTGCAGCAGCAGACGACGGCGGATCGCAGGGA
TGAGGCCCTGCCGATGCCGGGCAGCTAGCCAGGCGAGGAGAGGAGAGGAA
AGGGGTGGAATTGAAATCCCGGATGGATGGATGAGAAGAGAAGAACAA

TGAAACTTGAAAGCATTTTCAGAGAAAGCCCGATGGATGGAAGGTGGTTAAATATCC
GTCTAAATCAGAGAATTCTCCTTTATTACACGAGCTGAGCTGTGACATTAGCGGCT
ATACCAGCTGTGCCCCGTTTCTTTCAACAGGCTTATAAACACATCGCTTTGACGTGC
CTTTGCGCAATGGCCCGAGTTTGACGGGCTGCTTAAGAGCGGATGAGATCCTG
ATTTTCTTGACCCTTCGAAAAACAGCAAGCAGCTACGCCCCATGACACTCTGCT
GCCGATGTGACAGAATTAAAAATCTTTTGAGATATCTGCACATCACTCCGACA
GGATCTGATAGGCTCTGGGCCGGCAAGCTTCCTTTTCCCGCTCTGCGCGTTCAGCG
CGTCAAGGTCGCTGACTCGCCCGCCTCCCGGCCCTGGGATGGACCATTTCT
CACAAAGCAGCGGCGCTGGTGAAGAAAGGGCGAATTCTGCAGATATCC
TCACACTGGCCGCGCAGCTCGACACGACATCAGAGGCCCAATTCGGCCT
ATAGTGACTTCATATCAATACGTCCGCTGCTTTTACACGTTCTGA
CTGGGAAACCCTGCGTTACCCAACTTAAATCGCCTTGAGCAGCATCC
CTTTCGAGACTGCGCTGTAATAGCCAGAGGCGCGAACCCTGCGCCCTTC
CAACAGGTGCGC
Figure 4.2 Alignment (BLAST algorithm) of the predicted amino acid sequences of FADA from *Aspergillus nidulans*, CTG1 from *C. trifolii* (AF044894), and CGG1. Residues shared only by the two *Colletotrichum* species are highlighted in red. Residues shared by *C. graminicola* and *A. nidulans* are highlighted in blue. The green highlighted residue is one that is shared by *C. trifolii* and *A. nidulans*, but not by *C. graminicola*.

<table>
<thead>
<tr>
<th></th>
<th>A. nidulans</th>
<th>C. trifolii</th>
<th>C. graminicola</th>
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<td>MGCGMSTEDKEGKARNEIENQLKRDKMMQRNEIKMLLLGAGES</td>
<td>MGCGMSTEDKEGKARNEIENQLKRDKMMQRNEIKMLLLGAGES</td>
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<tr>
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<td>GKSTILKQMKLIHEGGYSRDERESFKEIIYSNTVQSMRVICEAM</td>
<td>GKSTILKQMKLIHEGGYSRDERESFKEIIYSNTVQSMRVICEAM</td>
<td>GKSTILKQMKLIHEGGYSRDERESFKEIIYSNTVQSMRVICEAM</td>
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<tr>
<td><strong>C. graminicola</strong></td>
<td>GKSTILKQMKLIHEGGYSRDERESFKEIIYSNTVQSMRVICEAM</td>
<td>GKSTILKQMKLIHEGGYSRDERESFKEIIYSNTVQSMRVICEAM</td>
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**Figure 4.3.** Maps of the three deletion constructs produced by transposon tagging of plasmid pSM1267. The 2.1 kb fragment containing the partial *Cgg1* gene is shown in green. The position of the start codon (ATG) is shown by the red triangle, and the number refers to its relative position on the 2.1 kb fragment. The position of the *Nhe1* site relevant for the Southern in figure 4.5 is also shown. The triangles at the ends of the bars indicate the orientation of the genes. Transposon inverted repeats are blue, and the *hph* gene is orange. The approximate position of the probe used for the Southerns in figures 4.5 and 4.12 is shown as a red bar. The introns are shown in magenta.
Figure 4.4 Transformants were either slow-growing or fast-growing. Panel A shows several slow–growing types compared at the same age on PDA with the wild type, which was equivalent to members of the fast-growing group. I did PCR to test for integration of the 2 kb tn/hph sequence using primers from either end of the 2.1 kb fragment of CggI that was cloned in pSM1267. Thus, the PCR product resulting from amplification of the endogenous CggI gene was 2.1 kb but if there was a homologous integration, I expected to see a PCR product of approximately 4 kb. Results of the PCR are shown in Panel B. L = ladder. Template DNAs in each lane were: (1) pSM1267 (2) M1.001 wild type (3) BH70 2-4, (4) BH71 2-2 (5) BH71 2-10 (6) BH70 3-2 (7) BH72 3-15 (8) BH70 2-1 (9) BH70 2-2 (10) BH70 3-6 (11) BH703-12 (12) BH71 1 2-1 (13) BH71 2-3 (14) BH71 2-8 (15) BH72 3-1 (16) BH72 3-11. Lanes 3-7 were strains that were “slow-growing”, while lanes 8-16 represented “fast-growing” strains. I noticed the presence of occasional faster growing sectors arising from some of the slow-growing strains (black arrow), and wanted to test the possibility that these represented loss of the integrated DNA and restoration of the wild type CggI sequence. I cultured four such sectors and isolated DNA from them for PCR analysis. The results appear in lanes 17-20. All appeared to retain the homologous tn/hph integrations, though there was also some evidence for instability especially in the sectors in lanes 19 and 20. Lane 21 was a negative PCR control (no template DNA)
**Figure 4.5:** Panel A: Southern hybridization analysis of the mutant and control strains. Genomic DNA was cut with *Nhe*I, and probed with the *CggI* probe (see figure 4.1). The approximate MW of the major bands is indicated on the left of the picture. A photograph of the ethidium-stained agarose gel used for this Southern is shown in panel B. Lane (1) (pink) is the untransformed M1.001 wild-type strain. (2) BH70 2-4. (3) BH72 3-15. (4) BH72 3-15 C1GFP. (5) BH72 3-15 GFP1. (6) BH72 3-15 GFP2. (7) BH70 2-2. (8) BH72 3-1. (9) BH70 3-2. (10) BH71 2-2. (11) BH71 2-10. (12) BH71 2-3. A single homologous insertion of the *tn/hph* fragment would be expected to increase the size of the hybridizing band by 2kb. The mutant in lane 10, BH71 2-2, apparently has a tandem insertion of at least three copies of the sequence. Otherwise, each mutant appears to have the expected size band for a single homologous insertion. The strains in the three lanes labeled in blue, 7, 8, and 12, have one or more ectopic insertions of the fragment.
Figure 4.6. Panel A: Southern hybridization analysis of the mutant and control strains. The genomic DNA was cut with XmnI, and probed with a portion of the hph gene that is not cut by this restriction enzyme. The ethidium-stained gel used for this Southern is shown in panel B. (1) = ladder. Lane (2) is the untransformed M1.001 wild-type strain. (3) BH70 2-4. (4) BH72 3-15. (5) BH72 3-15 C1GFP. (6) BH72 3-15 GFP1. (7) BH72 3-15 GFP2. (8) BH70 2-2. (9) BH72 3-1. (10) BH70 3-2. (11) BH71 2-2. (12) BH71 2-10. (13) BH71 2-3. There is one XmnI site just downstream of the end of the 2.1 kb genomic fragment in pSM1267 (Figure 4.1). Homologous integration of a single copy of tn/hph produces a band of approximately 8 kb, suggesting that there is a second XmnI site about 6 kb upstream from the first in the genomic sequence. The mutant that appears to have at least three integrations of tn/hph has a single band of about 12kb, as expected. The GFP transformants of mutant BH72 3-15 have additional bands at various positions, also expected since the pCT74 plasmid contains the hph gene as well as SGFP. However, these strains and the complemented strain still retain the 8kb band, indicating that they all still contain the original tn/hph integration. The ectopically integrated strains in lanes 8 and 12 each have several bands, none of which are 8 kb in size, consistent with insertion of the tn/hph randomly at other sites (the ectopic strain BH72 3-1 in lane 9 does not show a band in this Southern, but did in other hybridization experiments).
Figure 4.7. RT-PCR of mutant and control strains. Panel A. Primers from each end of the *Cgg1* ORF were used for this experiment. The amplicon from the cDNA should encompass the regions containing the three introns, and so should be smaller than the genomic DNA amplicon by about 175 base pairs. Lane 1 is amplified genomic DNA from the wild-type strain. In lane 2, the template was cDNA prepared from the wild-type strain. Lane (3) BH70 2-4 cDNA. (4) BH72 3-15 cDNA. (5) BH 72 3-15 C1GFP cDNA. (6) BH72 3-15GFP2 cDNA. (7) BH70 2-2 cDNA. (8) BH72 3-11 cDNA. (9) BH70 3-2 cDNA. (10) BH71 2-2 cDNA. (11) BH71 2-10 cDNA. (12) negative control, no template DNA. The strains in lanes 7 and 8 have ectopic integrations, while the others have homologous integrations in *Cgg1*. The strains in lanes 10 and 11 have integrations in the region upstream of the *Cgg1* ORF, whereas the other mutants all have integrations in the ORF itself. Panel B: the same templates in the same order as in Panel A, but amplified with primers for the *C. graminicola* β-tubulin gene, as a control for cDNA quality.
**Figure 4.8:** A: Radial growth rate (measured as average millimeters per day) of various strains on PDA. Panel B. Falcate spore production by various strains on PDA. Panel C. Oval spore production by various strains in PDB. Panel D. Germination rate of falcate spores in water drops. Panel E. Percentage of spores producing appressoria at the ends of longer germ tubes in water drops, instead of producing sessile appressoria immediately from the spore. In each case, bars labeled with the same letter are not significantly different according to a Waller-Duncan multiple range test, $P < 0.01$. 
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growth rate on PDA 

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B  

falcate spore production 

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C  oval spore production (*10^6/g dry mycelium)

D  germination rate in water
E germ tubes versus sessile appressoria

% germ tubes

strains

M1001BH BH70 2-4 BH71 2-2 BH71 2-10 BH70 3-2 BH72 3-15

a b c d e f
Figure 4.9. Development of wild-type and mutant strains in yeast-casein medium. Panels A, B, and C: germination in water. A: M.1001 spores developed sessile appressoria. B: BH72 3-15 germinated spores developed long germ tubes that did not always form appressoria. C: closer view of BH72 3-15 GFP2 germinated spores. The mutant spores have lost the normal falcate shape and appear fusiform. Panels D to M: development in 0.1% yeast-casein medium. Panels D, G, and J: M.1001 development at 24 hr., 48 hr., and 72 hr., respectively. In panel J, the superimposed ovals indicate regions where falcate spores are being produced from clusters of conidiogenous hyphae. Panels E, H, and K and L: mutant development at 24 hr., 48 hr., and 72 hr., respectively. E: BH70 2-4 colony at 24 hr. H: BH70 2-4 colony at 48 hr. K: BH72 3-15 colony that is producing only falcate spores, compare with L: BH72 3-15 , a colony that is producing both types of spores, at 72 hr. Panels F, I, and M: BH71 2-10 development at 24 hr., 48 hr., and 72 hr., respectively. Scale bars: A, and C: 50 µm; B, D-M: 100µm.
Figure 4.10. Development in yeast casein, details. A: BH72 3-15 GFP2 germinated spores at 24 hr. B: short hypha producing oval spores in a BH70 2-4 colony, at 48 hr. C: M.1001 conidiogenous cells producing falcate spores. The hyphae bearing conidiogenous cells were grouped together in a cluster. Compare with panels D and E. D: a closer view of hyphae in a BH72 3-15 colony that is producing only falcate spores, at 72 hr. E: a detail of the spore production by this type of hyphae. Conidiogenous cells are produced all along the hypal length. The picture was taken on a BH72 3-15 GFP2 colony, at 72h. Scale bars: A and D: 100 μm; B, C, and D: 50 μm
Figure 4.11. Results of leaf-whorl inoculation assay.
Figure 4.12. Panel A: Southern hybridization analysis of the BH72 3-15 mutant strain (Lane 1), and the BH72 3-15C1GFP complemented strain (Lane 2). The genomic DNA was cut with \textit{AvaI}, and probed with a portion of the \textit{CggI} gene (see figure 4.1). The ethidium-stained gel used for this Southern is shown in panel B. There is one \textit{AvaI} site just downstream of the \textit{tn/hph} insertion in Bh72 3-15 (see figure 4.1). There is one hybridizing band of approximately 4 kb in this strain, suggesting that a second \textit{AvaI} site is located about 2 kb upstream of the first, just beyond the edge of the 2.1 kb sequence. The complemented strain retains the 4 kb band, indicating that insertion of the complementing \textit{CggI} DNA has not occurred within these \textit{AvaI} sites, and that the original disruption is still intact. The complemented strain also retained the 8 kb \textit{XmnI} band when probed with the \textit{hph} gene probe (Figure 4.6), so the integration of the complementing DNA has also not occurred between these \textit{XmnI} sites. In contrast, the 8kb \textit{NheI} band was shifted (Figure 4.5) to between 9 and 10kb, suggesting that all or part of the complementing \textit{CggI} gene sequence has integrated between the flanking \textit{NheI} sites. The fact that there are two \textit{AvaI} bands in the complemented strain means that the part of the sequence of the \textit{CggI} DNA that contains the probe is present. But the size difference (<2 kb) is not adequate to accommodate the entire 3.2kb sequence, suggesting that only part of it has been integrated, or that endogenous DNA has been replaced. Unfortunately I do not have sufficient data to resolve this question at this time. My interpretation of the Southern data is illustrated by the diagram in part C of this figure. The 3.2 kb sequence is shown in green, and the \textit{tn/hph} sequences are orange. The probe sequence is in red. The yellow circles represent the approximate positions of the start and stop codons. The figure is drawn to scale.
A

B

C

5 kb

4 kb

4 kb

6 kb

6 kb

XmnI

AvaI

NheI

XmnI

tn/hph

AvaI

AvaI

AvaI

AvaI

?
**Figure 4.13**: Development of GFP strains *in planta*. Panels A, B, and C: inoculated internodes split in half with a razor blade 12 days post inoculation. The upper internode are oriented to the right. A: internodes inoculated with M.1001 (WT), *C. sublineolum* (CgS11), and water. B: internodes inoculated with the mutant strains. C: internodes inoculated with M.1001, BH72 3-15 and one of its GFP transformant, and with the complemented strain. Panels D to M: cytological observations. D and E: M.1001 GFP1 and BH72 3-15 C1 GFP, respectively, invading parenchyma cells at the very edge of the necrotic tissues. D was taken at 4 dpi, E at 7 dpi. F and G: BH72 3-15 GFP1 invading parenchyma cells at the edge of the necrotic tissues. G was taken at the very edge. Both photographs were taken at 4 dpi. H: BH72 3-15 GFP1 short hypha producing oval spores in a colonized parenchyma cell. The photograph was taken at 4 dpi. I: epidermal cells colonized by BH72 3-15 GFP1. The photograph was taken at 4 dpi. J: BH72 3-15 GFP1 mycelial network in the necrotic tissues, with oval spore production in the colonized parenchyma cells. The photograph was taken at 4 dpi. K: epidermal cells colonized by BH72 3-15 C1 GFP. The photograph was taken 8 dpi. L: bundle fiber colonization by M.1001 GFP1. The photograph was taken at 4 dpi. M: bundle fiber colonization by BH72 3-15 GFP1. The photograph was taken 8 dpi. Scale bars: A-C: 5 cm; D-M: 100 µm.
CHAPTER 5
CONCLUDING REMARKS

My Ph.D. dissertation work was undertaken with the encouragement of our collaborators at DuPont Nemours, Inc. who generously sponsored my research assistantship for three years here at the University of Kentucky. Pioneer Hi-Bred International, a DuPont company, is the biggest producer of hybrid seed corn in the world, and they consider ASR to be one of their greatest disease challenges. A major problem when they began their research program on ASR in the late 1990s was a lack of information about the biology of the disease, and so they sponsored my research in part to begin to address this deficit.

The goal of my Ph.D. project was to contribute to a better understanding of the biology of the interaction between \textit{C. graminicola} and maize. In particular, it was to understand the role of the oval spores in this interaction. Oval spores have always been a bit of a mystery in \textit{C. graminicola}. They appear at first glance to be entirely different from the falcate spores, including their shape, nuclear condition, wall chemistry, and time and place of development. My work has suggested that there is actually less difference between the two types of conidia than we thought: it turns out that multiple spores of both types are produced sequentially from a single conidiogenous cell (Appendix A), and that both can actually be produced within infected plant tissues, as well as in submerged culture (Chapter 2, Appendix A). But I never saw oval spores produced on the surface either \textit{in planta} or in culture, and my studies with \textit{Cgg1} (Chapter 4) suggest that they might not be regulated by the same signaling pathways as the falcate spores, at least \textit{in vitro}. There was speculation in the literature that the oval spores were important for systemic spread of the pathogen through the vascular tissues, but there was no published evidence supporting this hypothesis. In fact, no one had published observations of \textit{C. graminicola}, or of any other stalk rot fungus, growing in maize stalks before. I used green fluorescent protein as a marker to visualize \textit{C. graminicola} inside the plant tissues. My observations revealed that this aggressive pathogen of maize rapidly establishes in and macerates the parenchyma tissues, and it produces quantities of apparently dormant oval spores within these lesions. However, I found that the primary way the fungus moved through the stalks was not as oval spores in the xylem, but apparently through the fibers associated with the bundles and rind. This particular way of moving has not, to my knowledge, been described for any other
pathogen, so I do not know if it is unique to *C. graminicola*. It would be interesting to study the development *in vivo* for other stalk rot fungi, and to compare this with *C. graminicola*.

My observations did not support the hypothesis that oval spores played a significant role in systemic movement, or that *C. graminicola* was a vascular wilt pathogen, at least in the early stages of the ASR disease. Oval spores were not produced in the vessels in intact stalks. Instead, I observed them only in colonized parenchyma cells, where they mostly seemed to stay dormant. Given their location and dormancy, a more likely hypothesis is that the oval spores are acting as resting propagules of some sort, but when and how they might be released to germinate and re-infect their host is still a mystery.

In Chapter 3, I addressed the question of how *C. graminicola* penetrates inside the host stalk tissues in the absence of a wound. Again, there has been much speculation about this in the literature, but very little actual data. I found that *C. graminicola* was pretty efficient at colonizing the epidermal rind tissues of Jubilee sweet corn in the greenhouse, and, given enough time, could pass through the rind fibers to colonize the parenchyma tissues below. It was interesting that the pathogen did not seem to digest the fibers to any significant extent, but instead passed through them via some type of narrow opening that was either natural or produced by the fungus itself, I couldn’t tell which. To my knowledge, mine is the first study to show the whole process of infection of intact maize stalks by *C. graminicola* in the absence of a wound. Surprisingly to me, the sorghum pathogenic fungus *C. sublineolum* also colonized maize stalk epidermal cells very efficiently, almost as well as *C. graminicola* did. However, this same strain did not colonize parenchyma cells from a wound nearly as well as *C. graminicola* (described in Chapter 2). This suggested that the plant defense mechanisms might be less efficient in the epidermis versus the pith. *C. sublineolum* also did not appear to pass through the rind fibers as easily as *C. graminicola*, suggesting that the rind fibers might be doing more than just acting as a physical barrier to these fungi. I tested the ability of the strain to infect leaves and roots, and again, found that *C. sublineolum* was significantly inhibited from infecting those tissues. So there is something different about the stalk epidermal cells. What is most interesting to me about this finding is that it shows that nonhost resistance to *C. sublineolum* is conditional. As an aside, I believe that my observations of root infection and colonization are the first with this pathogen, though it had been known that *C. graminicola* can rot roots of susceptible seedlings.
In chapter 4, I took a molecular approach to address the question of the role of spore development in pathogenicity of \textit{C. graminicola} to maize. \textit{FadA} is an important regulator of sporulation in \textit{A. nidulans}. I was able to clone and characterize an orthologue of \textit{fadA} from \textit{C. graminicola}, which I named \textit{Cgg1}. Disrupting \textit{Cgg1} had a pleiomorphic effect on the phenotype \textit{in vivo}. The phenotype of the mutants was reminiscent of interfering mutations in \textit{fadA} in \textit{A. nidulans}, namely, vegetative growth was inhibited while sporulation (at least of falcate spores) was increased. This suggests that these processes in \textit{C. graminicola} are controlled by a signaling pathway involving \textit{Cgg1}, and that there may be similarities with \textit{A. nidulans} in the regulation and function of that pathway. The \textit{Cgg1} mutants were less pathogenic than the wild-type to wounded stalks, and though my cytology is very preliminary, this seemed to be associated with increases in production of spores in the lesions and also possibly in the number of primary infection hyphae. This suggests that the \textit{Cgg1} signaling pathway has a role to play in maximizing colonization of host tissues, and that this apparently involves negative regulation of sporulation and primary hyphae production. It will be important to follow up on these observations \textit{in planta}. In particular, it would be interesting to know more about the signals that \textit{Cgg1} is responding to, both \textit{in vitro} and \textit{in planta}, and about the other components of the signaling pathway that regulate \textit{Cgg1} activity.

I think that my work has been able to answer some important questions about the way that \textit{Colletotrichum graminicola} infects and colonizes stalks. However, I also think it has created many more questions. I hope that my research, reported in this dissertation, will be a useful foundation for future researchers as they continue to work to understand and manage this very important disease.
APPENDIX A
PRELIMINARY INVESTIGATIONS OF FOUR REMI MUTANTS
DEFICIENT IN PATHOGENICITY AND OVAL SPORE PRODUCTION

Introduction

Anthracnose, caused by the filamentous ascomycete Colletotrichum graminicola (Ces.) G. Wils., is a disease of worldwide importance on maize (Zea mays). The disease affects all parts of the plant and can be found throughout the growing season. It is observed most frequently in the form of anthracnose leaf blight or anthracnose stalk rot (Figure A.1).

C. graminicola produces two distinct types of asexual conidia (Figure A.2). One type is falcate and is produced from morphologically distinct conidiogenous cells. The second type is oval to elliptic and is produced from hyphae that lack distinct conidiogenous cells (Panaccione et al., 1989; Bergstrom and Nicholson, 1999).

The role of the oval spores in the fungal life cycle has been unclear. Oval conidia are produced only inside the plant tissues. If they have a role in disease, it is probably not related to plant-to-plant dispersal, a function that is associated with the falcate conidia that are produced on the surface of infected tissues. It has been suggested that the oval conidia are involved in lesion expansion, and in movement of the pathogen systematically within the stalk through the vascular system (Panaccione et al., 1989; Bergstrom and Nicholson, 1999).

A restriction enzyme insertional mutagenesis (REMI) system was used to produce quasi-random insertional mutants of C. graminicola (strain M1.001) (Thon et al., 2000). More than 1200 of these were tested for pathogenicity in maize leaf and stalk assays (Figure A.3). Of the nine pathogenicity mutants identified, four, 80-37, 84-6, 83-45, and 87-7, also appeared to be deficient, to a greater or lesser degree, in the ability to produce oval spores in culture (Figure A.4).

I originally wanted to test the hypothesis that reductions in pathogenicity were directly related to decreased production of oval spores by these mutants. The objective of this preliminary study was to compare the development of the mutants with the wild-type both in vitro and in planta. These were some of the very first experiments I did, and they helped me to become familiar with the pathogen and with many of the microscopy and culturing techniques I would be using. The confocal microscopy in this appendix was all done in 2003 during an
internship of several weeks at DuPont Nemours Co. in Wilmington, DE, working together with Dr. A. Conceicao under the direction of Dr. R. Howard.

Materials and Methods

_Fungal strains and culture:_ C. graminicola strain M1.001, also known as CgM2 (Forgey et al, 1978), was used for this study. The REMI mutant strains 84-6, 83-45, 80-37, and 87-7, are all derived from M1.001, and were identified after a mutagenesis screen described in Thon et al., 2000. These strains are all either non-pathogenic, or reduced in pathogenicity, on maize leaves and detached stalks (Figure A.3). I transformed each strain to express ZsGreen green fluorescent proteins, as described in more detail in Appendix B. All strains were routinely cultured on potato dextrose agar (PDA, Difco) at 23°C under continuous lights.

For spore production, strains were grown on PDA for two weeks. Falcate spores were collected by adding 10 ml of sterile water and rubbing the surface of the culture gently with a plastic mini pestle. The conidial suspension was collected, filtered through sterile glass wool, and the conidia were washed three times in sterile water. The concentration of conidia was adjusted to 5 x10^6/ml. after the third wash for plant inoculations, or 2X10^4/ml. for _in vitro_ sporulation assays.

_Development of an in vitro sporulation assay:_ A prerequisite for my studies was an assay system that would allow me to observe and compare the development of the fungal strains _in vitro_. I tested various assay systems, using both solid (agar) and liquid media. The primary advantage of the solid medium was that it did not dry out as quickly and so the experiments could run longer and did not require constant tending. However, the disadvantages, including an inability to take high-resolution photographs, outweighed the advantages. Thus, I ended up using a liquid medium assay system for most of my experiments. In general, the process of sporulation in solid and liquid medium was similar. However, two interesting things observed in the solid medium assay system are worth mentioning here. First, both oval and falcate spores accumulated in “piles” (Figure A.5). This was very interesting because it had not been realized previously that the oval spores were being produced continuously from the same spot on the hypha, in the same way that multiple falcate spores are produced by the same conidiogenous cell. Second, falcate spores were formed within the agar in a completely submerged position; prior studies had suggested that only oval spores were produced in a submerged condition in solid...
media, whereas falcate spores were produced only at the air-surface interface (Panaccione et al., 1989).

For the liquid medium sporulation assay, falcate spores of the mutant and wild type strains were suspended at a rate of $2 \times 10^4$/ml in 0.1% yeast extract/casein hydrolysate medium (yeast-casein). I found that the medium should be freshly made on the day of the experiment for this to work reliably. 100 µl drops of the spore suspension were placed in empty Petri dishes, and the dishes were incubated in a humidity chamber at 23°C for 72 hours. Observations were made every 12 hours by placing cover slips on the drops and observing under the microscope.

*Development of fungi in detached, inoculated maize stalk pieces:* Inoculations of detached stalk pieces were done as described in Thon et al., 2000. The plants used were proprietary DuPont maize lines that were either resistant or susceptible to *C. graminicola*. Fungal development in detached stalk tissues was monitored by using a Zeiss laser scanning microscope model 410 (Carl Zeiss Inc., Thornwood NY). Infected tissue sections were cut by hand with a razor blade and observed without further treatment. Green fluorescent protein (ZsGreen) was excited at 488nm. Plant cell walls and chloroplasts autofluoresce at this excitation wavelength, and this property was used to visualize the plant tissues.

**Results**

*Oval spore production by the mutants in a liquid culture assay system.* Development of the wild-type strain in yeast-casein is also described in detail in Chapter 4 of this dissertation. Figure A.6 presents representative microscopy views of each strain at different time points. By **12 hours** after the spores were deposited, conidia of all the strains had germinated. Unlike the other strains, 87-7 did not produce appressoria directly from the spores, and instead produced long, unbranched germ tubes (note that panel C is at approximately 1/2 the magnification of panels A-E). By **24 hours**, all the strains except 87-7 had produced mycelium from secondary germ tubes. Strain 87-7 had produced much more mycelium than the other strains, and numerous “swellings” were visible (arrow, panel H). By **36 hours**, strain 87-7 had begun to produce a few oval and falcate spores (arrows, panel M). By **48 hours**, the wild type was also producing both kinds of spores (panel P), and strain 84-6 had begun to produce a few falcate spores (not shown). By **60 hours**, all of the strains except 83-45 and 80-37 were producing both types of spores (panels U-W). Strains 83-45 and 80-37 only began to produce oval and falcate
spores between 60 and **72 hours** (not shown). By 72 hours, the wild type was producing huge quantities of both types of spore, which could easily be seen in the medium surrounding the mycelium. In contrast, the mutants were producing relatively few spores of either type. Thus, the mutants appeared to differ from the wild type both in the quantity of oval spores produced, and in the timing of their production. However, they also differed in other respects including growth rate and production of falcate spores.

*Development in detached stalk pieces:* The wild type fungal strain was compared with two of the mutants, 87-7 and 83-45, in plant tissues. It is very important to point out that these experiments were not replicated very many times, particularly for the mutants. My internship at DuPont was only for a few weeks, and the resistant and susceptible maize lines were not available to me at the University of Kentucky. Therefore, the following observations must be treated as very preliminary.

By 24hpi, the wild-type strain had colonized the parenchyma cells surrounding the wound site, and had begun to produce oval spores in both the resistant and the susceptible maize genotypes (**Figure A.7A, B, C, I**). Progression through the parenchyma cells was also very similar in both maize genotypes (**Figure A.7B,H, F, J**). Massive production of oval spores was observed behind the hyphal progression front (**Figure A.7A, I**). Maceration of the parenchyma tissues sometimes resulted in the formation of a cavity in which only the lignified bundles remained (**Figure A.7D**). These bundles eventually were colonized by the fungus (**Figure A.7D**). Hyphae were often observed inside the xylem vessels (**Figure A.7E, G**). Oval spores were also observed being produced inside the xylem vessels (**Figure A.7K, L**). There was no obvious difference in fungal development in the resistant and susceptible maize genotypes, and the lesion sizes produced on both were equivalent.

The development of the mutants inside the maize tissues was qualitatively similar to the wild-type. Both mutants colonized the plant tissues from the wound similarly in both the susceptible and resistant phenotypes (**Figures A.8A, B, C, D; Figure A.9A, B, C, E**). They both produced falcate spores in colonized parenchyma cells (**Figure A.8E, F; Figure A.9D, E**). Both strains also colonized xylem vessels and fibers (**Figure A.8G, H, I; Figure A.9G, H, I**). The only difference between the mutants and the wild type was the fact that the mutants progressed more slowly through the tissues.
Discussion

A technique for inoculating detached pieces of maize stalks was developed by Thon et al. (2000) because it was not possible to test more than 1200 REMI mutant strains in living stalks in a reasonable time frame in the greenhouse, due to a lack of space. Use of detached tissues for pathogenicity assays is not uncommon, even though the physiology of the tissues is likely to be different from that of attached tissues. In the case of the maize stalk assay, the stalk pieces are extensively washed in water to remove contaminating fungi and bacteria, and so they tend to be very saturated when they are inoculated. Nonetheless, the detached stalk assay proved to be useful as a screening tool, and yielded nine pathogenicity mutants that were also reduced in pathogenicity to attached seedling leaves (Thon et al., 2000). Therefore, I didn’t have a lot of concerns initially about using it for my cytological analyses.

In the course of this early work, however, I discovered that detached stalk pieces do not express resistance normally. I found that the mutant strain 6-2 does not colonize living stalks to any great extent, but it can actually grow in detached stalk pieces rather well, though it doesn’t always produce normal browning symptoms on those pieces (data not shown). The same is true for the sorghum pathogen *C. sublineolum* (data not shown). Furthermore, I found during my study at DuPont that maize germplasm that expresses strong major-gene resistance in living stalks can be colonized by *C. graminicola* to the same extent as near-isogenic susceptible germplasm. Thus, even though stalk pieces have significant advantages in an assay (speed, ability to replicate extensively, and better control of environmental conditions), I decided to do the remainder of my cytological work, which is reported in Chapters 2-4 of this dissertation, in living stalks.

A starting hypothesis for my work was that oval spores were produced in and moved through the xylem, and were responsible for systemic colonization of maize stalks. Though I ultimately found no evidence for this in living stalks, it is interesting to note that in the detached stalk pieces we see extraordinarily large numbers of oval spores in parenchyma cells, and we also see them often in the vessels. This suggests that oval spores may not form in vessels until the stalk tissues are senescent, and/or become saturated and anerobic.

My original thought was to study the role of oval spores in development by looking at some of the existing REMI mutants that produced fewer oval spores *in vitro*. The preliminary
studies reported in this appendix were done to find out if the mutants also produced fewer oval spores \textit{in vivo}, but otherwise developed fairly normally in the tissues. If so, it would suggest that the reduction in pathogenicity of these strains was connected to oval spore production. In fact, the two mutants that I looked at \textit{in planta} appeared to produce fewer spores than the wild type, but this seemed to be related to a general reduction in growth in the plant tissues. The mutants still produced some spores, and I felt that any results ultimately would be difficult to interpret. Ideally one would inoculate with a strain that produces no spores at all, but otherwise grows well. Despite many efforts over the years in the lab of the late R. Hanau at Purdue, such a strain was never obtained. A further complication was the fact that the disrupted genes in these REMI strains have not been identified. A few of them actually have more than one insertion. I ultimately abandoned the idea of using the existing REMI mutants, therefore, in favor of attempting to produce a strain with a targeted mutation in a well-known gene that might alter spore production, as described in Chapter 4 of this dissertation.

It will still be very interesting for someone to study the REMI mutants further, and the preliminary work presented here should be useful for future researchers. In particular, fluorescent strains of all nine mutants were produced in the course of my work, and these are listed in Appendix B.
**Figure A.1**: A: symptoms of stalk rot, B: longitudinal cut of rotten stalk, and C: lodging caused by stalk rot (these photographs were kindly provided by Dr. William Dolezal, Pioneer Hi-Bred International, a DuPont Company).

![Figure A.1](image1)

**Figure A.2**: A: oval conidia produced by mycelium, and B: falcate conidium produced by a conidiogenous cell (arrow) (culture in yeast-casein medium, at 29h and 53h, respectively).

![Figure A.2](image2)
Figure A.3: Pathogenicity phenotypes of the REMI mutants in a leaf bioassay (A-F) and a detached maize stalk pith bioassay (G-L). Biossays were performed as described in Thon et al., 2000, and Dr. Thon actually took these photographs. The wild-type, M1.001, served as a positive control in each experiment. Negative controls were treated with the related fungus *C. sublineolium*, which is pathogenic to sorghum leaves but not to maize leaves. One representative leaf blade, 6 days after inoculation, and three representative pith sections, 3 days after inoculation, are shown.
**Figure A.4:** Relative production of oval spores by the wild type and four REMI transformants in potato dextrose broth (PDB). Transformants 83-45, 84-6, and 87-7 were substantially inhibited in production of oval spores. For this experiment, a single plug was removed from the growing edge of a 2-week-old plate culture of each strain, any falcate conidia on the surface of the plug were washed away, and the plug was used to inoculate 5 mls of PDB in a test tube. The culture was shaken for 72 hours at 30°C, and then the oval spores were counted using a hemacytometer.
**Figure A.5:** Development of “piles” of oval spores (panel A) or falcate spores (panel B) in a solid medium assay system. The medium was 0.4% agarose (*ultra*Pure Agarose, Life Technologies Inc, Gaithersburg MD) in double distilled sterile water. The temperature of the medium was lowered to 45ºC before the spores were added. **A:** REMI mutant 65-21 (a non-pathogenic derivative of the wild type M1.001 that does NOT have a sporulation defect); **B:** WT strain M1.001. Similar results were observed for the wild-type and for all of the REMI mutant strains; the best photographs of the phenomenon were chosen to present here. Taking really nice photographs through agar proved to be very difficult.
Figure A.6: Representative microscopy views of germinating spores from wild type and various REMI mutants. The first row of images was taken at 12h. The second row was taken at 24h. The third row was taken at 36h. The fourth row was taken at 48h. The bottom row was taken at 60h.
Figure A.7: Confocal micrographs of the development of M1.001 fluorescent transformant strain 615-2 (see appendix B for more detail) in detached stalks of susceptible and resistance maize genotypes. Panel A: production of masses of oval spores in parenchyma cells at 47 hours after inoculation (hpi), in the susceptible (S) genotype. B: Mycelial movement through parenchyma cells at 47hpi in S line. C: Masses of oval spores, and some hyphae visible in bundle tissues, at 67hpi in the S line. D. Possible spores in the xylem at 67hpi of the S line, close to the puncture wound. E. Mycelium in the xylem, 25hpi of the S line. F. Hyphae proliferating in parenchyma cells 25hpi of the resistance (R) genotype. G. What appears to be spores in the xylem 75hpi of the R line. H. Hyphae growing from cell to cell through the parenchyma, 75hpi of the R line. I. Oval spore production in parenchyma cells 49hpi of the R line. The bundle that is visible is in the inner tissues, the wound was located off to the right in this photograph. J. Growth of hyphae through parenchyma 24hpi of the R line. K and L: two views, taken very close together, of oval spore production in vessels 24hpi of the R line.
Figure A.8: Confocal views of the mutant 83-45, fluorescent transformants 611-4 or 611-1 (see appendix B for more details) in detached stalks of susceptible and resistant maize genotypes. Panel A: View of 83-45 strain 611-4 proliferating through cells surrounding the wound site, 41hpi of the R maize genotype. B. Strain 84-35 611-1 growing through pith parenchyma cells 91hpi of the R line. C. Closer view of hyphae of strain 83-45 611-4 growing cell to cell through the parenchyma just below the epidermis (which is beyond the left edge of this photomicrograph) 89hpi of the R line. D. Colonization of epidermal cells surrounding the wound site 47hpi of the S line with 84-35 611-4. E. Mutant 83-45 did produce oval spores in parenchyma cells, here is a close view of that 70hpi of the S line with strain 611-4. F. Tissue destruction, sporulation, and colonization at the wound site, 74hpi of the R line with strain 611-1. G. Possible intracellular growth of the fungus near a bundle. 91hpi of the R line with 611-1. H and I. Two views of colonization of xylem and bundle fibers. Image H is 23 hpi, and I is 89hpi, of the R line with 611-4.
Figure A.9: Confocal views of the mutant 87-7, fluorescent transformant 611-1B (see Appendix B for more details) colonizing detached stalks of resistant and susceptible maize genotypes.
Panel A. Colonization of tissues around the wound 73hpi of the R line. B. Colonization of epidermal cells, 47hpi of the S line. Note that the thinner hyphae are the abnormally elongated germ tubes of this mutant, which are on the surface, while the thicker primary infection hyphae are inside the epidermal cells. C. More epidermal cell colonization, 73hpi of the R line. D. Oval spore production in parenchyma cells, 45hpi of the R line. E. A broader overview of oval spore production, 92hpi of the R line. F. Falcate spore production in the area near the puncture wound, 69hpi of the S line. G. Colonization of xylem vessels, 45hpi of the R line. H. Growth of fungal hyphae through fibers beneath the rind, 69hpi of the R line. I. More extensive growth through the rind fibers, 94hpi of the R line.
APPENDIX B

LIST OF FLUORESCENT COLLETOTRICHUM TRANSFORMANTS

In the course of my dissertation work I produced and stored a large number of fluorescent transformants of both mutant and wild-type strains, only some of which actually appear in the work in this dissertation. The transformants were produced by using several different fluorescent genes, and several different transformation constructs. A few were produced by co-transformation and contain multiple vector sequences. All the transformations of C. graminicola strain M.1001 and its derivatives were done using the PEG method (Thon et al., 2000), but C. sublineolum CgS11 could not be transformed this way and for that strain I used a Agrobacterium transformation protocol developed by J. Flowers for germinating spores of C. graminicola (Flowers and Vaillancourt, 2005).

The following tables contain all the strains that I created, the plasmid(s) I used to produce them, and the type of resistance(s) they contain. All the strains were single-spored and are stored on silica at –80°C where they are available to future researchers. It is particularly worth noting that I was able to transform nine REMI pathogenicity mutants (Thon et al., 2000) to express GFP and/or ZsGreen, and these may be very interesting for someone to look at in intact maize leaves and stalks in the future.
### GFP transformants:

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<tr>
<th>Transformant</th>
<th>Original strain</th>
<th>Plasmid</th>
<th>Amount of DNA</th>
<th>Linearized</th>
<th>Selection on Regeneration medium</th>
<th>Total</th>
<th>Comment</th>
</tr>
</thead>
</table>
| M.1001BH GFP2-1 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH GFP2-2 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH GFP2-3 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH GFP2-6 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH GFP2-7 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH GFP2-14 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH GFP2-17 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH GFP2-18 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH GFP2-19 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH GFP2-20 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH GFP2-25 | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH 2Ac | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH 2Ad | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH 2Ac | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH 3a | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH 3b | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH 3c | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH 3d | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| M.1001BH 3e | M.1001BH | pCT74 | 3.5µg | Yes | Hygromycin B 250µg/ml | 47* | Very good level of expression brave_pants
| 87-7 GFP1 | 87-7 | pCT74 | 3µg | Yes | Hygromycin B 250µg/ml | NC | Low to medium level of expression in vitro brave_pants
| 87-7 GFP2 | 87-7 | pCT74 | 3µg | Yes | Hygromycin B 250µg/ml | NC | Low to medium level of expression in vitro brave_pants
| 87-7 GFP3 | 87-7 | pCT74 | 3µg | Yes | Hygromycin B 250µg/ml | NC | Low to medium level of expression in vitro brave_pants
| 83-45 GFP1 | 83-45 | pCT74 | 3µg | Yes | Hygromycin B 250µg/ml | NC | Low to medium level of expression in vitro brave_pants
| 83-45 GFP2 | 83-45 | pCT74 | 3µg | Yes | Hygromycin B 250µg/ml | NC | Low to medium level of expression in vitro brave_pants
| 83-45 GFP3 | 83-45 | pCT74 | 3µg | Yes | Hygromycin B 250µg/ml | NC | Low to medium level of expression in vitro brave_pants
| 83-45 GFP4 | 83-45 | pCT74 | 3µg | Yes | Hygromycin B 250µg/ml | NC | Low to medium level of expression in vitro brave_pants
| 83-45 GFP5 | 83-45 | pCT74 | 3µg | Yes | Hygromycin B 250µg/ml | NC | Low to medium level of expression in vitro brave_pants
| 83-45 GFP6 | 83-45 | pCT74 | 3µg | Yes | Hygromycin B 250µg/ml | NC | Low to medium level of expression in vitro brave_pants
| 83-45 GFP7 | 83-45 | pCT74 | 3µg | Yes | Hygromycin B 250µg/ml | NC | Low to medium level of expression in vitro brave_pants
| 80-37 a | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 b | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 c | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 d | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 GFP1 | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 GFP2 | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 GFP3 | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 GFP4 | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 GFP5 | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 GFP6 | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 GFP7 | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 a | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
| 80-37 b | 80-37 | pCT74 | 3.9µg | Yes | Phleomycin 50µg/ml | 80 | Good level of expression brave_pants
## GFP transformants (table continued)

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<td></td>
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<td>150</td>
<td>3,4,7: very good level of expression; 5,6: good level; 1,2,8: low level</td>
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<td></td>
</tr>
<tr>
<td>84-6 GFP4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CgSl1 pJF1-4</td>
<td>CgSl1 pJF1</td>
<td>pJF1</td>
<td>Agrobacterium transformation</td>
<td>PDA HygB 150µg/ml</td>
<td>NC</td>
<td>Good level of expression</td>
</tr>
<tr>
<td>CgSl1 pJF1-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td></td>
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</tr>
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<td>CgSl1 pRAN2-1</td>
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<tr>
<td>CgSl1 pRAN2-2</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CgSl1 pRAN2-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The level of expression was a little better than pJF1 transformants.
## ZsGreen transformants:

<table>
<thead>
<tr>
<th>Transformant</th>
<th>Original strain</th>
<th>Plasmid</th>
<th>Amount of DNA</th>
<th>Linearized</th>
<th>Selection on Regeneration medium</th>
<th>Efficiency</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.1001BH 615-1</td>
<td>M.1001BH</td>
<td>pSM615</td>
<td>3-6µg</td>
<td>Yes with EcoR1</td>
<td>Hygromycin B 250µg/ml</td>
<td>2</td>
<td>Very good level of expression. M.1001 615-2 was used in the <em>in vivo</em> studies (“M.1001 GFP2”)</td>
</tr>
<tr>
<td>M.1001BH 615-2</td>
<td>M.1001BH</td>
<td>pSM615</td>
<td>3-6µg</td>
<td>Yes with EcoR1</td>
<td>Hygromycin B 250µg/ml</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>M.1001BH 615-3</td>
<td>M.1001BH</td>
<td>pSM615</td>
<td>3-6µg</td>
<td>Yes with EcoR1</td>
<td>Hygromycin B 250µg/ml</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>87-7 611-1A</td>
<td>87-7</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>NC</td>
<td>Very good level of expression</td>
</tr>
<tr>
<td>87-7 611-1B</td>
<td>87-7</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>87-7 611-1C</td>
<td>87-7</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>84-6 611-1</td>
<td>84-6</td>
<td>pSM611</td>
<td>3µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>NC</td>
<td>611-2: very good level of expression; 611-1: low level</td>
</tr>
<tr>
<td>84-6 611-2</td>
<td>84-6</td>
<td>pSM611</td>
<td>3µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>84-6 611-3</td>
<td>84-6</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>4</td>
<td>Very good level of expression</td>
</tr>
<tr>
<td>84-6 611-4</td>
<td>84-6</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>84-6 611-5</td>
<td>84-6</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>80-37 611-1</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>2</td>
<td>Good level of expression</td>
</tr>
<tr>
<td>80-37 611-2</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>80-37 611-3</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>36</td>
<td>Very good level of expression</td>
</tr>
<tr>
<td>80-37 611-4</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>80-37 611-5</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>80-37 611-6</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>36</td>
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</tr>
<tr>
<td>80-37 611-7</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>80-37 611-8</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>80-37 611-9</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>80-37 611-10</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>80-37 611-11</td>
<td>80-37</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>83-45 611-1</td>
<td>83-45</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>NC</td>
<td>Very good level of expression</td>
</tr>
<tr>
<td>83-45 611-2</td>
<td>83-45</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>83-45 611-3</td>
<td>83-45</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>83-45 611-4</td>
<td>83-45</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>83-45 611-5</td>
<td>83-45</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>83-45 611-6</td>
<td>83-45</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em> and EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>6-2 611-1</td>
<td>6-2</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>NC</td>
<td>Very good level of expression</td>
</tr>
<tr>
<td>6-2 611-2</td>
<td>6-2</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>6-2 611-3</td>
<td>6-2</td>
<td>pSM611</td>
<td>6µg</td>
<td>Yes with <em>BamHI</em></td>
<td>Geneticin 850µg/ml</td>
<td>NC</td>
<td></td>
</tr>
</tbody>
</table>

133
### fadA orthologous gene disruption project:

<table>
<thead>
<tr>
<th>Transformant</th>
<th>Original strain</th>
<th>Plasmid</th>
<th>Amount of DNA</th>
<th>Linearized</th>
<th>Selection on Regeneration medium</th>
<th>Efficiency</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH70 2-4</td>
<td>M.1001BH</td>
<td>500ng of each PCR product obtained with pSM1270</td>
<td>PCR product</td>
<td>Hygromycin 250µg/ml</td>
<td>2 transformants, BH70 2-4 and BH70 3-2, on 12 were disrupted</td>
<td>2</td>
<td>This method generated a reduced number of transformants with ectopic insertions.</td>
</tr>
<tr>
<td>BH70 2-2</td>
<td></td>
<td>500ng of each PCR product obtained with pSM1271</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH70 2-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
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</tr>
<tr>
<td>BH70 3-2</td>
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<td>1</td>
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<td>BH70 3-6</td>
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<td></td>
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<td>1</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>BH71 2-2</td>
<td></td>
<td>500ng of each PCR product obtained with pSM1272</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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<td></td>
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<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BH72 3-15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BH72 3-11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BH72 3-15 GFP1</td>
<td>BH72 3-15</td>
<td>pCT74 pAN8-1</td>
<td>3µg 1µg</td>
<td>Yes, both with EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>47</td>
<td>Very good level of expression</td>
</tr>
<tr>
<td>BH72 3-15 GFP2</td>
<td>BH72 3-15</td>
<td>pCgFadAJ3 pCT74 pAN8-1</td>
<td>1.2µg 2µg 1µg</td>
<td>ApaI EcoR1 EcoR1</td>
<td>Phleomycin 50µg/ml</td>
<td>100</td>
<td>Only one, the complemented strain, had a good level of expression</td>
</tr>
</tbody>
</table>

*Total number of transformants obtained in the experiment. NC= not counted*
APPENDIX C

DIFFERENCES BETWEEN SPORES OF COLLETOTRICHUM GRAMINICOLA
HARVESTED FROM POTATO DEXTROSE AGAR VERSUS OATMEAL AGAR

All of my early work, when I had been developing my in vitro spore germination assays (Appendix A), had been done with falcate spores harvested from potato dextrose agar (PDA). However, when I started inoculating living plants I switched to oatmeal agar (OMA) because it produced a larger number of spores. All of my plant inoculations in chapters 2 and 3 are done with spores from OMA. When I wanted to test the development of my Cgg1 mutants in vitro, I used spores from OMA plates for both mutant and wild type, but found that the spores were not germinating in the yeast-casein medium as they had in my earlier experiments. I compared the germination of spores from PDA and from OMA side-by-side in both water and yeast-casein. I discovered that the spores germinated with the same efficiency in water, but spores from OMA germinated at a rate only about 10% of that of the spores from PDA in yeast-casein (Figure C.1).

I decided to use PDA for my experiments involving the Cgg1 mutants, and I wanted to test if there was also an effect of the medium on pathogenicity. I did just a single experiment consisting of side-by-side leaf whorl inoculations of Jubilee seedlings with varying dilutions of mutant and wild-type spores, harvested either from OMA or PDA, and found that the spores from PDA appeared to be considerably more aggressive than the spores from OMA (Table C.1, Figure C.2). I used spores harvested from PDA for all of my experiments for Chapter 4. I think this is a very curious observation, but have no explanation for it at this point, except to suppose that it has something to do with the different nutritional value of PDA versus OMA. I don’t think it makes a difference to my experiments because in all cases I included controls and these were always grown on the same medium as the strains being tested. Interestingly, the average lesion length caused by the wild-type strain on wounded Jubilee stalks was the same in Chapter 2, when the inoculum came from OMA, and in Chapter 4, when the inoculum came from PDA.
Figure C.1. Falcate spores from 3-week-old cultures on either PDA or OMA were harvested in sterile water, filtered through glass wool, and rinsed 2 times in water. Spore concentration was adjusted to 20 spores per microliter and 100µl drops were deposited on the bottom of Petri dishes. The plates were left standing at room temperature for 1 hr. to 1hr. and half before being transferred to an incubator (30°C-continuous light) in humidity chambers, as described in Chapter 4. The germination rates were calculated at 24 hr., also as described in Chapter 4.
Table C.1: Disease ratings (0-5) resulting from inoculation of Jubilee seedlings with varying concentrations of spores from either PDA or OMA. Ratings were taken 8 dpi. Each number represents just one single plant.

<table>
<thead>
<tr>
<th>Strain</th>
<th>growth medium</th>
<th>5 x10⁶</th>
<th>1x10⁶</th>
<th>5 x10⁵</th>
<th>1 x10⁵</th>
<th>5x10⁴</th>
<th>1x10⁴</th>
<th>5 x10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1001BH</td>
<td>PDA</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>BH70 2-4</td>
<td>PDA</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BH71 2-2</td>
<td>PDA</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
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<td>BH71 2-10</td>
<td>PDA</td>
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<td>1</td>
<td>1</td>
<td>0</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>PDA</td>
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<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M1001BH</td>
<td>1/2 OMA</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>BH70 2-4</td>
<td>1/2 OMA</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BH71 2-2</td>
<td>1/2 OMA</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BH71 2-10</td>
<td>1/2 OMA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>BH70 3-2</td>
<td>1/2 OMA</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BH72 3-15</td>
<td>1/2 OMA</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure C.2: Data from the table, above, for M1.001 spores from PDA (upper dark blue line) and from OMA (lower green line).
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   M.S.: Biology, Health, and Biotechnology
   Major: Plant Science and Biotechnology
1999-2000:
   University of Burgundy, Dijon, France
   M.S.: Cellular Biology and Physiology
   Major: Plant Science and Biotechnology
1997-1999:
   University of Nantes, France
   B.S.: Life Science
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PROFESSIONAL POSITIONS HELD
2002 to present:
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August 2004:
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February – July 2002:
   Research Assistant at DOW Agroscience, Dijon, France
January – June 2001:
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June – September 2000:
   Research Assistant, Unite de Pathologie Vegetale et Mycologie, Departement Sante des Plantes et Environment, INRA, Clermont-Ferrand, France
July – August 1999:
   Undergraduate Summer Assistant, ADVANTA France, Mulsans, France
SCHOLASTIC AND PROFESSIONAL HONORS
2005-2006:
  Dissertation Year Fellowship, University of Kentucky
2005 to present:
  Gamma Sigma Delta, The Honor Society of Agriculture, University of Kentucky Chapter
2005:
  American Phytopathology Society foundation named Graduate Student Travel Award in honor of Dr Zahir Eyal
2003 and 2005:
  Graduate Student Support Grant from the Graduate School to fund travel to professional meetings, University of Kentucky

PROFESSIONAL PUBLICATIONS
Refereed Journals:

Manuscript Accepted with Revision:
  Venard, C.M., Vaillancourt, L.J. Colonization of fiber cells by Colletotrichum graminicola in wounded maize stalks. Phytopathology.

Abstracts:
  Venard C., and Vaillancourt L. 2005 Growth and colonization of Colletotrichum graminicola inside maize stalk tissues Phytopathology 95:S107
  Venard C., and Vaillancourt L. 2003 Developmental biology of spore dimorphism in Colletotrichum graminicola Phytopathology 93:S87

PROFESSIONAL MEETING PRESENTATIONS
Poster Presentations:
  Aug. 2005:
    Growth and colonization of Colletotrichum graminicola inside corn stalk tissues
    American Phytopathological Society Annual Meeting, Austin, Texas
  Mar. 2005:
    Growth and colonization of Colletotrichum graminicola inside corn stalk tissues
    23rd Fungal Genomics Conference, Asilomar, California
  Aug. 2003:
    Developmental biology of spore dimorphism in Colletotrichum graminicola
    American Phytopathology Society Annual Meeting, Charlotte, North Carolina