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FALSE SMUT OF RICE: HISTOLOGICAL ANALYSIS OF INFECTION

FALSE SMUT OF RICE: HISTOLOGICAL ANALYSIS OF INFECTION

A thesis submitted in partial fulfilment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

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ABSTRACT

False smut of rice, caused by *Ustilaginoidea virens* (Cooke) Takahashi (Teleomorph: *Villosiclava virens*), has become a common disease in most major rice growing regions throughout the world. Considerable confusion exists regarding the infection process and the disease cycle. Therefore, a clearer understanding of pathogenesis caused by *Ustilaginoidea virens* is critical for future efforts to develop genetic and chemical tools to manage false smut in Arkansas and other regions of the world. The overall goal of this research was to clarify the infection process underlying false smut, with emphasis on comparing and contrasting the histological basis of root and foliar infections. Therefore, a green fluorescent protein (GFP)-expressing *U. virens* strain was developed to address a critical need in histological investigations of the infection process as well as future investigations in functional genomics. Transformants of *U. virens* constitutively expressed GFP at high levels throughout all developmental stages and tissues, were stable after repeated sub-culturing, and were also confirmed to be pathogenic. Thus, the GFP-expressing strain was used in root and inflorescence inoculations of rice, and infectious development was analyzed via confocal microscopy. For comparative purposes, all root inoculation experiments were performed in parallel with a GFP-expressing strain of *Magnaporthe oryzae*, a known root-infecting pathogen of rice. Injections of the flag leaf sheath at booting consistently led to the formation of spore balls in a small number of infected plants. Confocal microscopy showed no evidence of systemic infection in these experiments, but rather infection was consistent with the fungus gaining access through the gap in the lemma as reported elsewhere. In root inoculations, *U. virens* failed to penetrate the epidermis, whereas *M. oryzae* frequently colonized roots extensively. Taken together, these results indicated that systemic

infection of rice during false smut is not likely to originate from root infection, and the limited evidence for systemic infection calls into question whether it is a component of the false smut disease cycle.

This thesis is approved for recommendation
To the graduate council

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Chapter I: AN OVERVIEW OF FALSE SMUT OF RICE

INTRODUCTION

False smut: A historical overview

False smut of rice, caused by *Ustilaginoidea virens* (Cooke) Takahashi (Teleomorph: *Villosiclava virens*), has become a common disease in most major rice growing regions throughout the world, including China, India, Japan, and the Southeastern U.S. The disease was known in ancient times in China (Ou, 1972), but was first described in the scientific literature in 1878 by the noted plant pathologist M. C. Cooke (Cooke, 1878). The pathogen causing false smut was first described from the Indian city of Tirunelveli (known as Tinnevelly under British rule) as a true smut, under the name *Ustilago virens* (Cooke, 1878). A few years later, the same organism was described in Japan and named *Tilletia oryzae* (Patouillard, 1887). In following years, the fungus was successfully cultured in artificial media; macro- and microscopic analyses led to the reassignment of the fungus to the Ascomycota, with the new designation of *Ustilagenoidea oryzae* (Brefeld, 1895). One year later, Takahashi designated the fungus *Ustilagenoidea virens* (Takahaski, 1896), which is the accepted name for the anamorphic state today. The sexual stage (teleomorph) of the fungus was first observed in 1934 and assigned to the genus *Claviceps*. However, upon re-examination of various morphological and biological characteristics, Tanaka et al. (2008) concluded that the teleomorph was wholly inconsistent with any subgroup of *Claviceps*, and thus proposed the new teleomorphic genus *Villosiclava* and the simultaneous renaming of *Claviceps virens* to *Villosiclava virens* (Tanaka et al., 2008).

Early Chinese writings document an ancient, possibly pre-historic, belief that the presence of false smut in rice was a predictor of a good crop year, likely because environmental conditions

favoring the disease, particularly high rainfall, are associated with optimal rice production (Ou, 1985). Until recent decades, false smut was considered to be a disease of minor concern due to the relatively low incidence and severity in most years. As quoted by Butler in 1918, “Even if treatment [for false smut] were known, it would scarcely be worth the trouble of carrying out in most cases seen” (Butler, 1918). Scattered outbreaks periodically occurred in the first half of the 20th century, however, including Japan in the early 1900s (Akai, 1974), the Philippines in 1918 (Reinkin, 1918), and Burma in 1935 (Seth, 1945). The exact point at which false smut rapidly increased in incidence and severity is difficult to pinpoint, but the disease had become cause for much greater concern in Asia by the 1980s. To date, one of the most severe outbreaks of false smut occurred in Japan in 1988 (Yaegashi et al., 1989). In the U.S., false smut was known to be present in Louisiana since the early 1900s, but was a disease of minor concern until outbreaks were reported in the late 1990s (Rush et al., 2000). False smut was first reported to be present in Arkansas in 1997 (Cartwright and Lee, 2001), and is now presumed to be present throughout all of the rice production areas in the state. Today, false smut has become a serious rice disease throughout Asia and the Southeastern U.S. (Brooks et al., 2010; Mew et al., 2004). The ascendance of false smut is not fully understood, but may result from usage of susceptible rice varieties, heavy nitrogen fertilizer applications, and possibly even climate change that favors the disease (Fu et al., 2012).

The causal organism

U. virens, the causal agent of false smut, belongs to the division Ascomycota, subdivision Pezizomycotina, and class Sordariomycetes. The fungus grows slowly on many defined agar

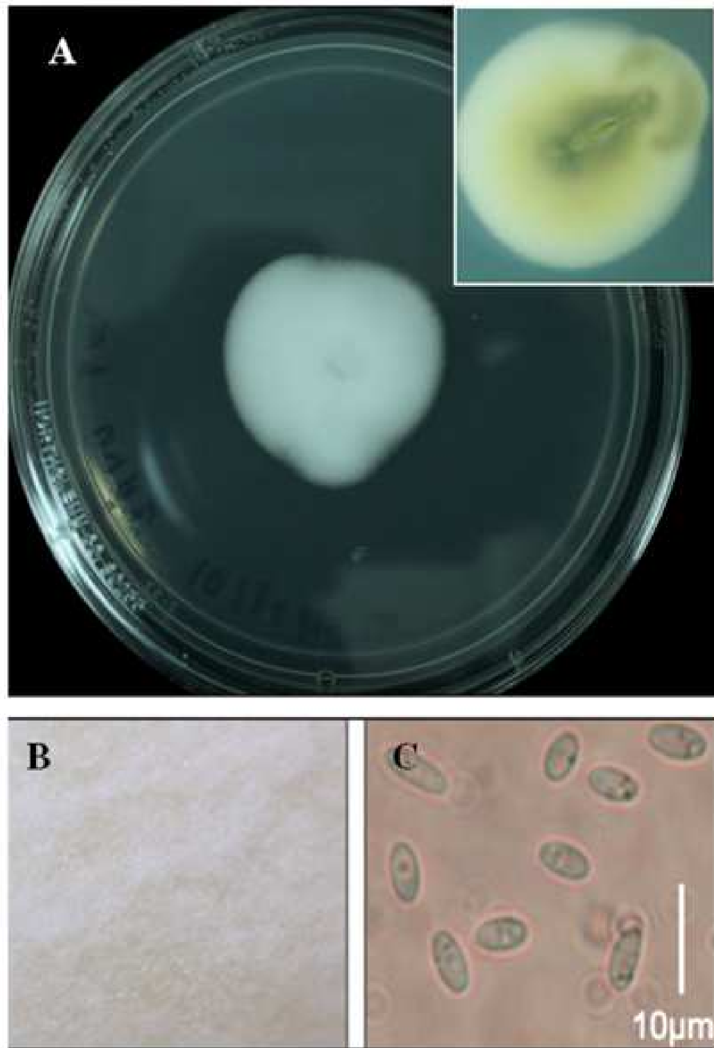


Figure 1. Phenotype of *U. virens* grown *in vitro* 14 days after inoculation. A. PDA medium uniformly inoculated with *U. virens* strain R-2. B. Flocculent mycelia on the surface of medium. C. Conidia of *U. virens.*

media, with a radial growth rate of 1 cm or less per week. Colony morphology during growth on defined agar media is generally circular, often somewhat irregular, with an even/entire margin. Colony color varies from white to greenish yellow, largely depending on the growth medium (Fig. 1) (Sharma and Joshi, 1975). In culture, the fungus often produces sclerotia as well as asexual spores (conidia). Immature sclerotia are compact and round, eventually appearing orange-yellow and ultimately olive-green and powdery due to the formation of conidia on the surface (Sharma and Joshi, 1975). Conidia are typically spherical to elliptical, olivaceous, and approximately 3.0-5.5 µm x 5.5-6.0 µm in size (Fig. 1) (Singh and Gangopadhyay, 1981; Ou, 1985). Mature conidia are globose to irregularly rounded and ornamented with prominent spines approximately 200–500 nm in length (Kim and Park, 2007). Immature conidia are orange-yellow in color, and often become olive-green to black upon maturation. Upon germination, conidia form septate germ tubes, which form conidiophores bearing large amounts of conidia (Singh and Gangopadhyay, 1981).

General environmental parameters affecting the growth of *U. virens* have been established. Not surprisingly, nutrient and sugar solutions are more favorable for the germination of *U. virens* conidia than water. The fungus grows well at 28°C, slightly at 12°C and is strongly inhibited at 36°C. The optimum pH range for growth is 6.02 to 6.72, and growth is essentially inhibited at pH extremes of 2.77 and 9.05 (Ou, 1985).

Ustilaginoidea virens is a cosmopolitan plant pathogen, capable of infecting numerous members of the Poaceae. In addition to infecting rice (*Oryzae sativa* L.), *U. virens* has been reported to infect numerous weed species associated with rice fields, including *Echinochloa crus-galli* (barnyard grass) a ubiquitous, endemic pest throughout Asia and Africa and an invasive species in North America; *Imperata cylindrical* (cogon grass), a common weed on

irrigation canals throughout Asia and Africa; and *Digitaria marginata*, a species of crabgrass common throughout much of the world (Shetty and Shetty, 1985; Atia, 2004). The fungus can also infect corn (*Zea mays* L.) (Haskell and Diehl, 1929). Typically, the fungus infects tassels, replacing flowers with galls similar in appearance to spore balls formed during infection of rice (White, 1999). In 2000, false smut of corn was observed in Mississippi in 2.5% of 1,280 commercial and inbred corn hybrids at a low level of severity; however, infection of corn is not commonly observed in the U.S. (Abbas et al., 2002).

Symptoms of false smut

Macroscopically, the hallmark symptom of false smut is the formation of spore balls in infected panicles (Fig. 2). Spore balls initially become visible two to three weeks after infected rice plants reach the flowering stage of development. Mature spore balls, which essentially replace infected rice grains, are distinctively globose and velvety (Fig. 2). Often, only a few spikelets are affected in each inflorescence, although the incidence in a given inflorescence can be high depending on cultivar susceptibility, environmental conditions, and inoculum load. Moreover, the number of plants displaying symptoms of false smut in fields can vary widely; visual symptoms can be restricted to a few plants, or can be widespread and severe.

The structure of spore balls formed by *U. virens* was thoroughly described over a century ago (Butler, 1918). Structurally, spore balls contain an inner core (sclerotium), in which a distinctive, white pseudoparenchyma replaces the grain or developing ovaries. A mass of radial hyphae that forms near the surface of the sclerotium bears conidia laterally or terminally. Within this mass of hyphae, mature conidia are formed progressively from the surface inwards; thus, the

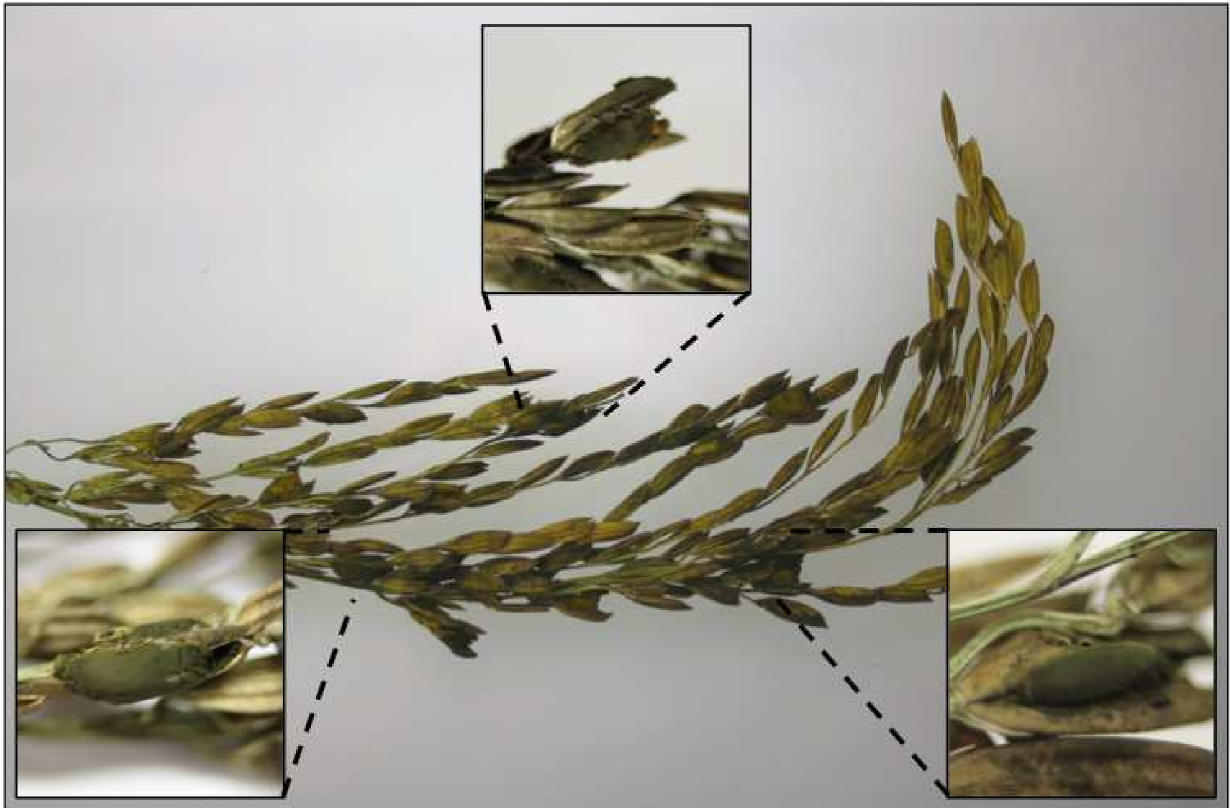


Figure 2. Rice panicle infected by *U. virens*. Inset pictures (dotted lines) highlight spore balls produced on infected spikelets.

outer layers of spore balls frequently consist of a green/olive-black layer of mature conidia, whereas inner layers contain orange-yellow, immature conidia, followed by an innermost layer of colorless, nascent conidia.

Environmental conditions favoring infection

Environmental conditions strongly affect the development of false smut. The disease is more severe in high humidity (> 90 %), temperatures between 25 and 30°C (Chen et al., 1994; Yashoda et al., 2000), frequent periods of rain, and high soil nitrogen levels resulting from rotation with soybean and/or high nitrogen fertilizer inputs (Lee and Gunnell, 1992). Furthermore, late planting also contributes to the increase in the degree of rice false smut incidence and severity (Singh and Khan, 1989).

Infection process

Over the past 100 years, a consensus has emerged regarding certain components of the infection process underlying false smut that culminates in the formation of spore balls. It has long been known that *U. virens* specifically targets ovary tissues and often replaces the developing grain (Butler, 1918). Early histological studies provided evidence for two distinct forms of infection (Raychaudhuri, 1946). In one scenario, infection occurs early in flower development, resulting in the complete destruction of the developing embryo and engulfment of the style, stigma, and anthers in the spore ball that eventually forms. In this type of infection, the fungus transforms the ovary into a mass of tightly woven hyaline hyphae, and then grows

laterally, eventually bursting through the glume tissues (Butler, 1918). A second type of infection was postulated to occur when the grain is mature or nearly so, and occurs when spores deposited on glume tissues germinate, penetrate the lemma and palea, and colonize the endosperm tissue of the grain (Raychaudhuri, 1946; Padwick, 1950). As in the other type of infection reported, the final outcome is a spore ball that replaces the grain. Recently, however, a group in China dissected over 1,000 false smut balls at different developmental stages and found, without exception, six anthers to be present within the core, which was interpreted to mean that infection occurred before anthesis and the anthers were subsequently enveloped by spore ball formation (Tang et al., 2012). Insight into the infection process was recently provided by experiments in which conidia of *U. virens* were inoculated into rice sheaths during booting, and the pathogen was demonstrated to invade spikelets through the apical gap between the lemma and palea (Ashizawa et al., 2012).

Impact of false smut on rice production

The replacement of grain with spore balls of *U. virens* has a negative impact on yield. Historically, yield reductions due to false smut were negligible in most conditions. However, in recent decades, increasing incidence and severity of false smut has led to specific examples of the disease significantly impacting yield. For example, false smut in India has been reported to cause yield losses ranging from 0 to 75% (Agrawal and Verma, 1978; Singh and Dube, 1978; Dhindsa et al., 1991). In the U.S., false smut is still regarded to have a relatively small impact on yield (Groth and Hollier, 2010). However, chronic levels of false smut commonly seen in Arkansas since the late 1990s probably have an unquantified negative impact on yield due to

direct loss of grain due to spore ball formation, increased chaffiness, and a likely reduction in 1000-grain weight commonly associated with the disease (Chib et al., 1992).

The negative impacts of false smut on harvested grain quality are significant and widely acknowledged. Toxicity associated with false smut was demonstrated as early as 1915, when Suwa reported on acute toxicoses induced in rabbits after ingestion of aqueous extracts of spore balls (Suwa, 1915). In 1992, the chemical structures of mycotoxin analogs associated with false smut were elucidated (Fig. 3) (Koiso et al., 1992). Collectively designated ustiloxins, the compounds are cyclic peptides containing 13-member rings that are closely related in structure and biological activity to phomopsin A, a mycotoxin produced by *Phomopsis leptostromiformis* (Culvenor et al., 1977) and possibly other *Phomopsis* species. *U. virens* is known to produce at least six structurally distinct ustiloxin analogs (designated ustiloxin A-F), of which ustiloxin A is the most toxic (Joullié et al., 2011) and abundantly produced during pathogenesis (Shan et al., 2012). Ustiloxin A is a potent inhibitor of mitosis in eukaryotic cells, and functions by inhibiting the assembly of the α,β-tubulin dimer into microtubules (Luduena et al., 1994). Ustiloxin A is toxic to plants and animals, and thus is designated as both a phytotoxin and a mycotoxin (Koiso et al., 1992). The full spectrum of toxicoses induced in humans and animals by ingestion of ustiloxin A is not known, although liver and kidney damage have been confirmed in mice after consumption of the mycotoxin (Nakamura et al., 1994). Currently, although the presence of ustiloxins in harvested rice grain is not subject to advisory or action limits by the U.S. Food and Drug Administration, an increasing awareness of the negative impacts of false smut on grain quality has been observed in the U.S. rice milling and processing industry (R. Cartwright, *personal communication*).

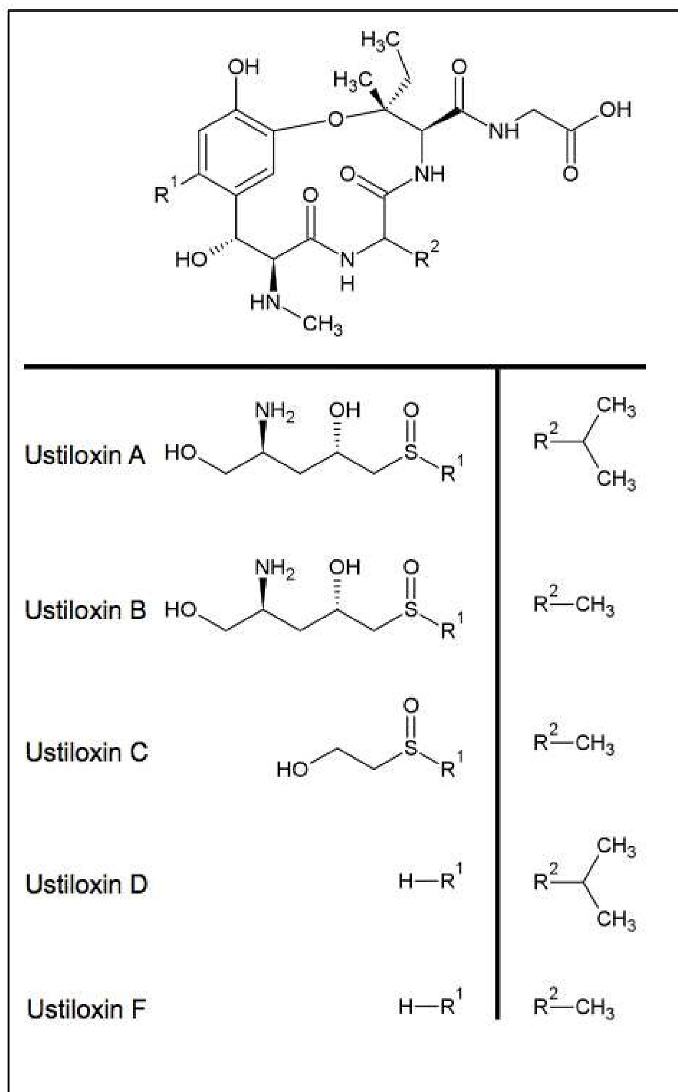


Figure 3. Chemical structure of characterized ustiloxin analogs A-F. Adapted from Joullie et al., 2011.

Management of false smut

False smut has become endemic nearly everywhere rice is grown in the world, and thus the management principle of exclusion has limited utility. Of three major strategies for disease management – genetic resistance, chemical control, and cultural control – only the latter two have produced limited measures of success regarding the management of false smut. Regarding genetic resistance, most, if not all, rice varieties commonly planted in the Southeastern U.S. are susceptible to false smut. Genetic resistance has been reported to exist in select germplasm (Li et al., 2008; S. Brooks, *personal communication*) but is not yet introgressed into commercially viable hybrids for U.S. production systems. Chemical control, i.e. fungicide application, can be effective but is often not economically viable. For example, copper oxychloride has been reported to reduce the incidence and severity of false smut when applied immediately before heading, with mixed results on improving yields (Kannaiyan and Rao, 1976; Ahonsi and Adeoti, 2003). In one study, applications of mancozeb, thiabendazole and benomyl reduced disease incidence with no effect on yield, whereas applications of iprodione had no significant effect on the disease (Ahonsi and Adeoti, 2003). From a practical standpoint, symptoms of false smut are not visible until grain fill, at which time fungicide applications are ineffective. Current chemical control recommendations in Arkansas focus on a preventative application of a propiconazole fungicide during the booting stages of rice to simultaneously prevent kernel smut and suppress false smut. When this recommendation is followed, 50-65% reductions in false smut balls have been observed in harvested rice grain, but impact on yield appears to be limited (Cartwright et al., 2000a; Cartwright et al., 2000b; Hopkins et al., 2003). Cultural control options may provide the most immediate and cost-effective remedy for false smut. Recently, field experiments in Arkansas indicated that reductions in false smut were observed as a result of continuous rice cropping (88% reduction), conservation tillage (69% reduction) and moderate nitrogen fertility

rates (34% reduction) (Brooks et al., 2009). Moreover, furrow irrigation as opposed to flood irrigation was highly effective at disease reduction (Brooks et al., 2010). In Arkansas, furrow irrigation may cause other production problems that outweigh the benefits of false smut control, such as reduced yields, increased weed control inputs, and increased risk of other diseases, particularly rice blast. However, cropping schedules, tillage practices, and maintenance of fertility rates are often reasonable parameters for growers to adjust, if necessary or desirable.

The disease cycle of false smut: confusion, contradiction, and uncertainty

There is considerable confusion and uncertainty regarding the disease cycle of false smut. The prevailing hypothesis regarding infection is that the pathogen exclusively infects rice florets via conidia dispersed by wind currents and/or rain splash at the time of before flowering (Fig. 4). In support of this hypothesis, foliar inoculations of rice plants at the booting stage of development have reliably produced symptoms for several research groups in greenhouse conditions (Kulkarni and Moniz, 1975; Zhou et al., 2003; Ashizawa and Kataoka, 2005; Tang et al., 2012). Additionally, aerial monitoring of *U. virens* spores above a rice field throughout the growing season revealed a maximum incidence of conidia during a four-week period corresponding to heading (Sreeramulu and Vittal, 1966). However, it is conceivable that foliar inoculation is excessively artificial and thus poorly represents disease etiology in field conditions, or that a combination of root and foliar infections play a role in the disease cycle of false smut.

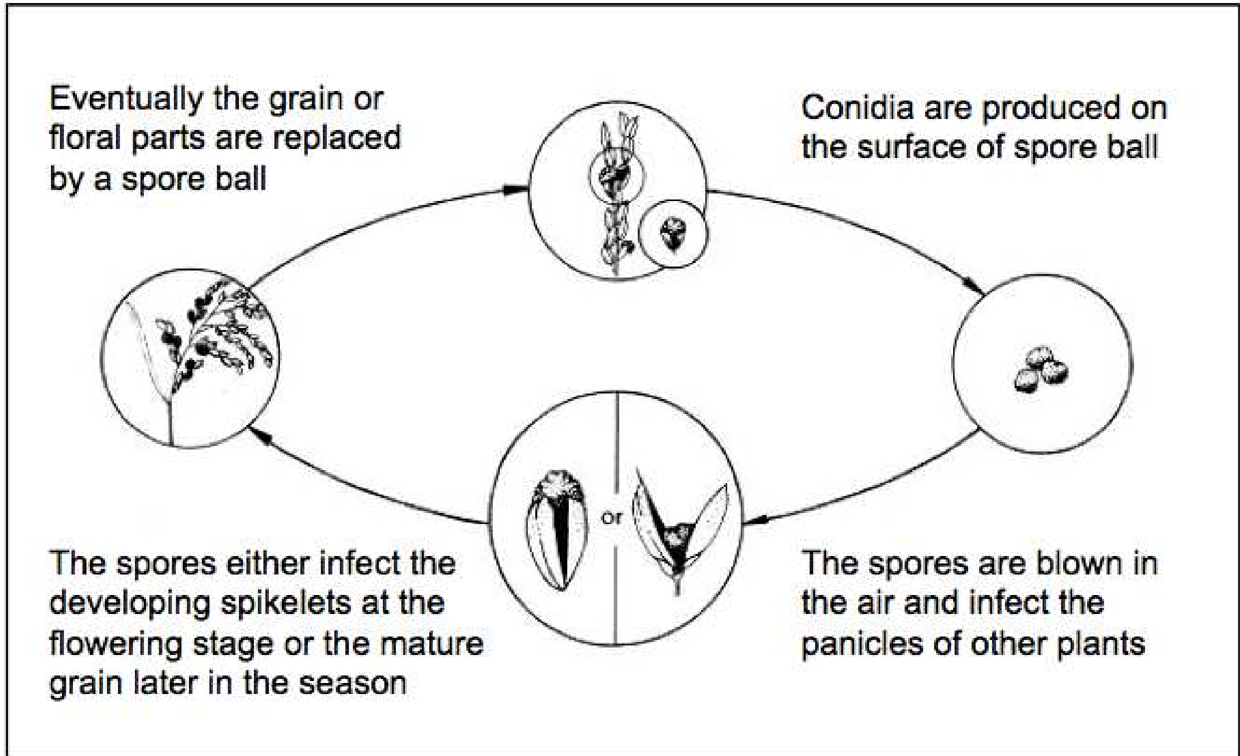


Figure 4. Hypothesized disease cycle of *U. virens* upon infection by airborne conidia. Adapted from: http://www.knowledgebanks.irri.org.

Because of the similarity between symptoms of false smut with symptoms of true smuts, and initial confusion as to whether *U. virens* was taxonomically a true smut, an alternative hypothesis that *U. virens* causes systemic infections has been explored for over 100 years (Butler, 1918). The pathogen has been reported to overwinter as either spore balls or sclerotia in soil for at least two seasons without its host (Lee and Gunnell, 1992; Anders et al., 2010), although conidia of *U. virens* become inviable much more quickly in soil (Butler, 1918). Therefore, one hypothesis is that the pathogen can infect roots of rice plants to cause a systemic infection (Fig. 5). In this scenario, the pathogen is predicted to colonize vascular and/or meristematic tissues, and subsequently colonizes the developing inflorescence from the ear primordium. The earliest experiments in support of this hypothesis were performed by Ikegami, who reported that *U. virens* spores can germinate on epidermal cells of the coleoptiles of rice seedlings in greenhouse conditions, and hyphae subsequently invaded intercellular spaces and reached the meristematic tissues of rice plants in some cases (Ikegami, 1963). In addition, microconidia of *U. virens* on infected rice seeds can germinate on and possibly invade roots of rice seedlings (Schroud and TeBeest, 2005). Furthermore, a high percentage of asymptomatic rice plants tested positive for the presence of *U. virens* when evaluated with a nested PCR assay targeting the rDNA region of the pathogen (Ditmore and TeBeest, 2005; TeBeest et al., 2010), and the same nested PCR assay indicated the presence of the pathogen in plants before booting (Ashizawa and Kataoka, 2005). In contrast to this hypothesis, however, controlled (greenhouse) experiments in which rice roots were inoculated with *U. virens* have never been reported to produce foliar symptoms such as spore balls, despite repeated attempts (Butler, 1918; Kulkarni and Moniz, 1975; D. TeBeest, *personal communication*), and Ikegami never observed the presence of *U. virens* in the ear primordium, which would presumably be required for

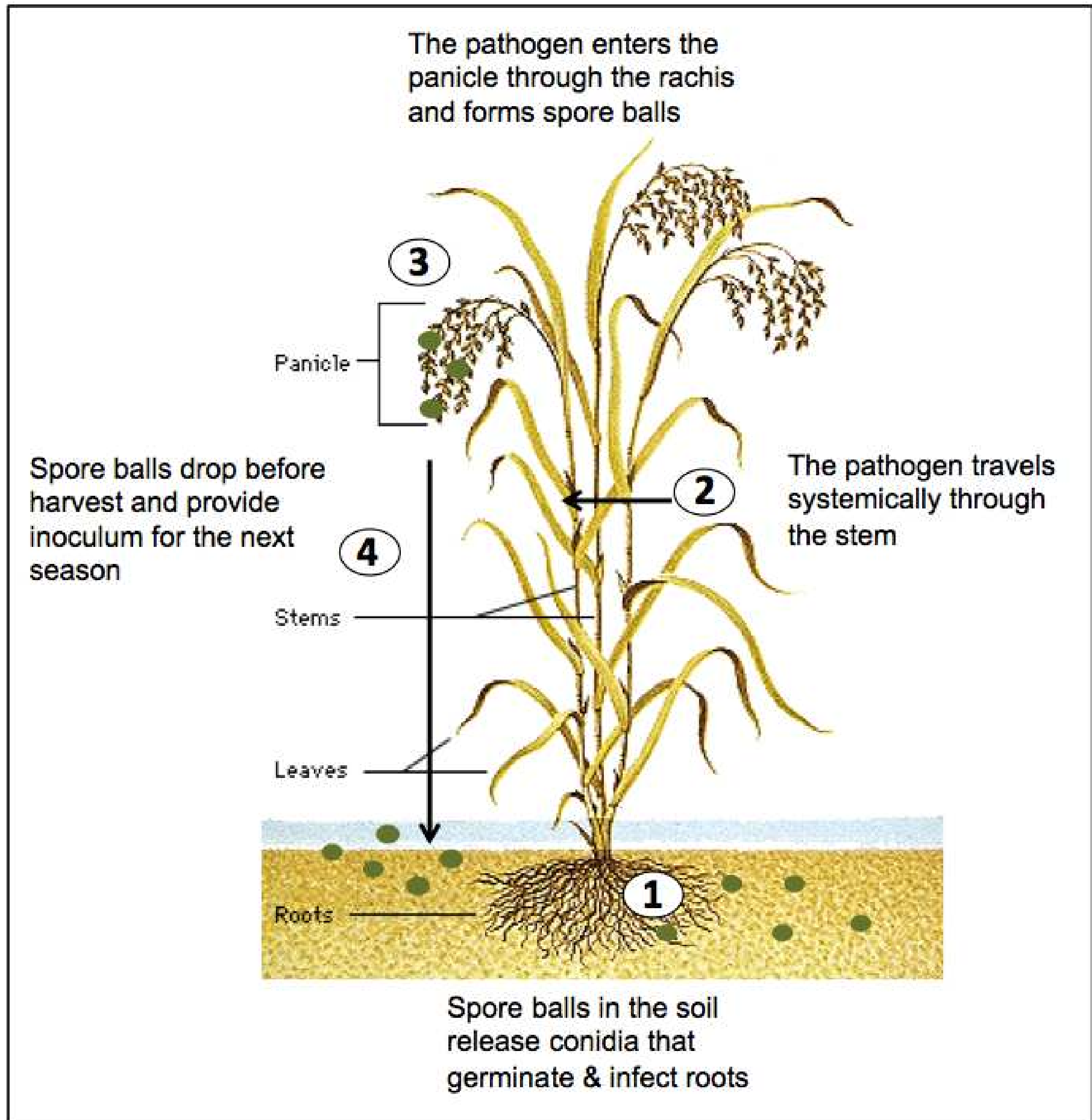


Figure 5. Hypothesized disease cycle of *U. virens* by soilborne, systemic infection. Model adapted from Ikegami, 1963, and TeBeest et al., 2010. Photo courtesy of http://science.howstuffworks.com.

systemically-initiated colonization of the inflorescence. Furthermore, nested PCR assays are notoriously problematic in detection assays, particularly because they are highly susceptible to false positive results due to PCR contamination and lack of specificity (Belák and Thoren, 2001). Perhaps most convincingly, in a recent histological examination, pedicels beneath false smut balls and stems of naturally severely infected panicles were analyzed with light microscopy and transmission electron microscopy; no hyphae of the pathogen were observed, which suggests that most if not all false smut balls arise from aerial infections at the rice booting stage (Tang et al., 2012).

In practice, the limited and often contradictory descriptions of the disease cycle and epidemiology of false smut presents a tremendous challenge for the development and implementation of control strategies for this important disease.

False smut research in the era of molecular plant pathology

U. virens has not enjoyed the rapid expansion of molecular resources witnessed for other fungal pathogens, such as *Fusarium verticillioides* and *Magnaporthe oryzae*. However, false smut research has benefitted from molecular advancements in three major areas. First, molecular detection assays have been developed for *U. virens* based on polymerase chain reaction (PCR) technologies. First, conventional PCR assays were developed to amplify ribosomal DNA (rDNA) repeats in the genome of *U. virens* utilizing direct amplification (Zhou et al., 2003) and nested PCR (Ashizawa and Kataoka, 2005). Subsequently, a real-time PCR detection assay utilizing a TaqMan probe was developed from rDNA sequences of *U. virens* (Ashizawa et al., 2010). The development of a TaqMan detection assay represents a significant advancement over

the previously published assays that relied on conventional PCR. The added specificity and sensitivity of real-time PCR has significant advantages over a conventional, direct detection assay as described by Zhou et al. (2003), and nested PCR assays are notoriously susceptible to contamination. However, increased confidence in sensitivity and specificity of PCR-based assays would result from using a TaqMan approach to target non-rDNA sequence, which is frequently difficult to differentiate from other fungi.

A second area of molecular-based research is the development of genetic transformation systems for *U. virens*. Transformation is the process through which a cell takes up and expresses exogenous DNA. A stable transformation event involves the integration of the exogenous DNA into the genome of the organism, thus making it heritable. Several approaches for transformation have proven effective for various taxa of filamentous fungi. Electroporation, the process in which a strong electric pulse is applied to an organism in order to transiently increase membrane permeability, has been commonly used to facilitate transformation of bacteria, yet is less commonly used for fungi due to low yields and unstable transformants (Utermark and Karlovsky, 2008). A second transformation strategy is to increase the permeability of fungal membranes with polyethylene glycol (PEG). In fungi, this approach generally requires enzymatic digestion of cell walls to release protoplasts, which are then exposed to exogenous DNA in the presence of PEG, and are subsequently allowed to regenerate into a viable thallus. The generation of protoplasts is often a major obstacle to this approach, since cell wall digestion parameters must often be determined empirically for individual species, or even individual strains of a given species (Fincham, 1989). An additional drawback is the low rate of successful transformants obtained for many species of fungi (Fincham, 1989; Utermark and Karlovsky, 2008). In a third approach, *Agrobacterium tumefaciens,* the cause of crown gall disease in

plants, can be engineered to transform foreign DNA into fungi, essentially utilizing the same mechanism through which galls (tumors) are induced in plants (de Groot, 1998; Mullins et al., 2001; Zhang et al., 2006). At the time this thesis research began in 2009, only one report of genetic transformation of *U. virens* had been published, in which *Agrobacterium* was utilized (Zhang et al., 2006). However, this report was in a Chinese-language journal and most of the strains & constructs were not easily accessible in the U.S. and many Western nations. Since that time, reports of transformation of *U. virens* via electroporation and PEG have been published (Tanaka et al., 2011; Ashizawa et al., 2012). Nevertheless, each protocol published to date has advantages and disadvantages when compared to other approaches, and thus a "universal" strategy to transform *U. virens* has not emerged.

A third emerging molecular focus in *U. virens* research is comparative and functional genomics. A major hindrance to this effort is the lack of publicly available genomic sequence for the pathogen. As of October 2012, searches of the National Center for Biotechnology Information (NCBI) sequence database (http://www.ncbi.nlm.nih.gov) reveal 149 publicly available DNA sequences for *U. virens*, and zero expressed sequence tags (ESTs). Of the 149 DNA sequences, few are unique: the vast majority are conserved bar-coding sequences for taxonomic identification of various strains of *U. virens*, predominantly rDNA regions. Draft sequences of the genomes of an Arkansas isolate and a Chinese isolate have been obtained (T. Mitchell, *personal communication*), but as of today, a draft genome sequence for *U. virens* is not available in the public domain. Thus, progress in understanding the molecular basis of pathogenesis has been slow. To date, only one study has been published in which a functional genomics approach was utilized to characterize a pathogenicity-associated gene in *U. virens*, a PMK1-like mitogen-associated protein (MAP) kinase (Zhang et al., 2006).

Pressing needs in false smut research

Currently, the confusion over the disease cycle poses one of the single biggest obstacles to developing effective management for false smut. Without this information, it is very difficult to develop effective chemical control, or to efficiently identify sources of genetic resistance other than in field trials relying on natural inoculum. Thus, a detailed histological investigation documenting the infection process would be of great benefit to the false smut research community. However, limitations with conventional light microscopy often lead to ambiguity in interpretations of micrographs. Thus, a molecular approach to histology (e.g., a genetically modified strain of the pathogen expressing a specific fluorescent reporter) would be of great value. Before this approach can be taken, however, a robust, efficient, and simple genetic transformation protocol is required for the pathogen.

PROJECT RATIONALE AND LONG-TERM GOALS

Currently, confusion about the false smut disease cycle means that satisfactory methods to control this disease are unavailable. Therefore, a clearer understanding of pathogenesis caused by *Ustilaginoidea virens* is critical for future efforts to develop genetic and chemical tools to manage false smut in Arkansas and other regions of the world. The overall goal of this research was to clarify the infection process underlying false smut, with emphasis on comparing and contrasting the histological basis of root and foliar infections. This project was designed to provide new molecular protocols to the broader rice research community as well as a more detailed understanding of false smut etiology.

OBJECTIVES

The specific objectives of this research were to:

1. Devise a robust transformation protocol for *U. virens* and create an infectious strain expressing green fluorescent protein (GFP).
2. Compare and contrast disease development via molecular histopathology (confocal microscopy) in root inoculations vs. foliar inoculations of rice plants.

Chapter 2 of this thesis describes the experiments performed to address the first objective, whereas Chapter 3 describes experiments performed to address the second objective. Chapter 4 provides an integrated summary of all of the research, as well as perspectives on future research directions that would build upon the current results.

Chapter II: TRANSFORMATION OF THE FALSE SMUT PATHOGEN

SYNOPSIS

False smut of rice, caused by *Ustilaginoidea virens* (Cooke) Takahashi (Teleomorph: *Villosiclava virens*) has become a common disease in most major rice growing regions throughout the world. Considerable confusion exists regarding the infection process and the disease cycle. Therefore, the generation of a green fluorescent protein (GFP)-expressing *U. virens* strain would address a critical need in histological investigations of the infection process as well as future investigations in functional genomics. Here, three methodologies were evaluated for transformation of *U. virens*: electroporation, polyethelyene glycol (PEG)-mediated transformation, and *Agrobacterium*-mediated transformation. Electroporation did not yield transformants, despite adjusting parameters such as spore concentration, cuvette gap width, and electroporation voltage. For PEG-mediated transformation, a protocol was devised to produce abundant amounts of protoplasts, but transformants were not obtained after repeated transformation attempts, despite adjusting parameters such as protoplast concentration, incubation time in PEG, and DNA concentration. Ultimately, *Agrobacterium*-mediated transformation proved successful, and a protocol for transformation was developed. Transformants of *U. virens* constitutively expressed GFP at high levels throughout all developmental stages and tissues. GFP-expressing strains of *U. virens* were stable after repeated sub-culturing, and were also confirmed to be pathogenic, since false smut balls were formed in repeated tests of inflorescence inoculations on rice plants in the greenhouse. Moreover, the production of ustiloxin A was confirmed in the GFP-expressing strain.

INTRODUCTION

The fungus *Ustilaginoidea virens* (Cooke) Takahashi (Teleomorph: *Villosiclava virens*; is the causal agent of rice false smut, a common disease of rice reported to affect virtually all rice-growing regions throughout the world (Gupta and O'Toole, 1986; Tanaka et al., 2008). The disease culminates in the formation of spore balls that replace individual rice grains on infected panicles. In addition to concerns about yield losses caused by *U. virens*, the fungus also produces ustiloxin mycotoxins, anti-mitotic tetrapeptides containing a 13-membered ring with an ether linkage, that are harmful to humans and animals (Koiso et al., 1992). The incidence of false smut has increased dramatically throughout much of the world's rice growing regions in recent decades, and has become a disease of increasing concern to growers in Arkansas since the late 1990s.

The disease cycle of false smut has not been fully resolved. Specifically, it is not clear whether a systemic phase of the disease, initiated by root infection, plays a role in the rice false smut disease cycle as is the case for many of the true smuts. Because of this confusion, management guidelines and effective chemical controls for false smut in rice have been stymied. To address this problem, a strain of *U. virens* expressing a fluorescent reporter is needed to visualize the interactions between the fungus and its host, specifically in the context of soilborne vs. airborne sources of inoculum. Green fluorescent protein (GFP), first isolated from the jellyfish *Aequorea victoria*, fluoresces under blue light without substrates or cofactors. Thus, the GFP gene has been cloned and widely used as a reporter as well as a vital marker in prokaryotic and eukaryotic cells (Chalfie et al., 1994). However, the native GFP gene from *A. victoria* does not express well in many filamentous fungi (Maor et al., 1998) due to codon bias; therefore, optimization of the codon usage of the GFP gene was necessary to enhance GFP expression in

plants, many animals, and fungi (Sheen et al., 1995; Pang et al., 1996). Specifically, a of GFP variant known as "superfolder" GFP (sGFP) was developed with a serine to threonine substitution at amino acid 65, which enhanced GFP expression and improved spectral properties such that the gene could be used widely in many eukaryotes, including fungi (Chiu et al., 1996; Spellig et al., 1996). For example, the sGFP gene has been used with great success in filamentous plant pathogenic fungi, including species of *Aspergillus* (Rajasekaran et al., 2008), *Cochliobolus* (Maor et al., 1998), *Mycosphaerella* (Skinner et al., 1998), *Magnaporthe* (Kershaw et al., 1998; Liu and Kolattukudy, 1999; Campos-Soriano and Segundo, 2009), *Trichoderma* (Bae and Knudson, 2000), *Phaeomoniella* (Landi et al., 2012), and *Colletotrichum* (Dumas et al., 1999; Horowitz et al., 2002).

Several approaches for transformation have proven effective for filamentous fungi. Electroporation, the process in which a strong electric pulse is applied to an organism in order to transiently increase membrane permeability, has the advantages of being rapid and inexpensive, yet has the drawbacks of a relatively low efficiency in many fungi and a tendency for transformants to be unstable (probably due to a reduced efficiency of integration of exogenous DNA into the genome) (Utermark and Karlovsky, 2008). Transforming fungi with polyethylene glycol (PEG)-mediated approaches is typically more efficient than electroporation and generally yields a higher percentage of stable transformants, but generally requires enzymatic digestion of cell walls to release protoplasts, a costly and time-consuming process, and protoplasts of some fungi are less amenable to transformation than others. In a third approach, *Agrobacterium tumefaciens* can be harnessed to transform foreign DNA into fungi, essentially utilizing the same mechanism through which galls (tumors) are induced in plants (de Groot, 1998; Mullins et al., 2001; Zhang et al., 2006). *Agrobacterium*-mediated transformation is a fast and easy way to

transfer foreign DNA into fungal cells because of the reduction of factors reducing the efficiency of transformation and the increased stability of transformants (de Groot, 1998; Mullins et al., 2001; Zhang et al., 2006). Many species of plant pathogenic fungi have been transformed with *Agrobacterium*, including *Magnaporthe oryzae* (Rho et al., 2001; Khang et al., 2005; Campos-Soriano and Segundo, 2009), *Fusarium oxysporum* (Mullins et al., 2001; Mullins and Kang, 2001), *Botrytis cinerea* (Rolland et al., 2003), *Leptosphaeria maculans* (Gardiner and Howlett, 2004), and *Verticillium dahlia* (Dobinson et al., 2003). Transformation of *U. virens* with *Agrobacterium* has been reported in a Chinese-language journal (Zhang et al., 2006), but the plasmids and strains are not easily accessible in the U.S. Because the efficiency of each technique is dependant on the fungal strain being studied and the GFP expression constructs being used, additional transformation protocols would be of wide benefit to the false smut research community.

The objective of this research was to devise a robust transformation protocol for *U. virens* and create an infectious strain expressing sGFP. To this end, attempts were made to transform *U. virens* via electroporation, PEG-mediated transformation, and with *Agrobacterium*. Expression and stability of sGFP was evaluated in selected transformants, which were then assayed for pathogenesis in greenhouse experiments.

MATERIALS AND METHODS

Fungal strain and growth conditions

Ustilaginoidea virens wild-type strain R-2, stored in 30% glycerol at - 80°C, is a part of the fungal collection in the Bluhm lab at the University of Arkansas. This strain was originally

isolated from spore balls collected from Arkansas County, Arkansas in 2009. The fungus was routinely maintained on 0.2X strength potato dextrose agar (0.2X PDA) in a 12-hour photocycle at 28°C. To provide sufficient conidia for transformation experiments and to provide inoculum for liquid cultures, conidia were first collected from three-week-old 0.2X PDA cultures of *U. virens* by adding sterile distilled-deionized water (1 ml) to the plate surface and gently scraping mycelia with a sterile L-spreader. The suspensions of conidia were transferred to 15-ml conical tubes and concentrations were adjusted to 1x 10^6 spores per ml as determined by counting conidia with a hemocytometer. Then, conidia (1ml) were transferred to a 250 ml sterile flask containing 50 ml of YEPD liquid broth medium (per L: 5g yeast extract, 10g peptone, and 30g dextrose) at 25°C with rotary shaking at 170 RPM. After incubation for seven days, conidia were collected by filtering flasks' contents through a sterile spectramesh filter and centrifuging the filtrate for 10 min at 4°C and 3220 × g. The filtrate was washed twice with distilled water and centrifugation was repeated as described above. To provide sufficient tissue for enzymatic digestion of cell walls, as required to release protoplasts, *U. virens* was grown in YEPD liquid broth medium. Erlenmeyer flasks (250 ml) containing YEPD (50 ml) were inoculated with 10^6 conidia of *U. virens* and were incubated at 28°C with shaking (200 RPM) for 24-72 hours.

Agrobacterium **strain and growth conditions**

Agrobacterium tumefaciens strain AGL1 stored in 30% glycerol at -80°C is part of the permanent bacterial collection in the Bluhm lab at the University of Arkansas. Originally, this strain was kindly provided by Dr. Steven Marek, Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, Oklahoma. *Agrobacterium tumefaciens* strain

AGL1 containing vector pBHt2-sGFP was streaked out from the stock and incubated on a Petri dish containing LB solid medium (per L: 10 g NaCl, 10g Tryptone, 5 g Yeast extract and 20 g agar) supplemented with carbenicillin (50 µg/ml) kanamycin (50 µg/ml) at 28°C for three days to obtain a single, isolated colony.

Transformation vector

Transformation vector pBHt2-sGFP used in this research was kindly provided by Dr. Thomas Mitchell, Department of Plant Pathology, Ohio State University, Columbus OH. This vector contains the sGFP gene with expression driven by the *Aspergillus nidulans* Pgpd promoter to provide a visual reporter, as well as the hygromycin resistance gene (hph) with expression driven by the trpC promoter (Horowitz et al., 2002; Kamenidou et al., 2006) to provide a selectable marker. This plasmid is a binary vector, originally derived from pBHt2, which was created by cloning the hygromycin resistance cassette described above into pCAMBIA 1300 (CAMBIA, Canberra, Australia) after removal of the existing *hph* cassette driven by the *Cauliflower mosaic virus* 35S (CaMV35S) promoter (Mullins et al., 2001).

Identification of a suitable selectable marker for transformation of *U. virens* and determination of a kill curve

A conidial suspension of 1x 10^5 spores per ml as determined with a hemocytometer was obtained as described above. Then, conidia (200 µl) were plated on 0.2X PDA amended with 0, 25, 50, 75, 100, or 150 µg/ml hygromycin B. After seven days of incubation at 25°C in a 12-

hour photocycle, the colonies that emerged were counted. The lowest concentration of hygromycin B at which the growth of *Ustilaginoidea virens* wild-type strain R-2 was completely inhibited over the seven-day period was determined and utilized for selection of transformants in subsequent experiments. These experiments were performed three times with three replications per experiment to determine the proper concentration of hygromycin B for efficient selection of transformants.

Electroporation of *U. virens*

Conidia for electroporation were obtained as described above, with concentrations varying from 10^7 - 10^9. For electroporation, conidia of *U. virens* (40 µl) were transferred into a 2 ml sterile Eppendorf tube containing 1µg of linear transformation vector pBHt2-sGFP (generated by enzymatic digestion consisting of: 10 µl of vector pBHt2-sGFP, 5 µl of BglII, 10 µl of buffer 3 (New England Biolabs), and 75 µl of H_2O) overnight at 37°C. This mixture was transferred to a cold electroporation cuvette that was then incubated on ice for five minutes before being subjected to a high-voltage electric pulse in an Eppendorf 2510 series electroporator. After inserting the electroporation cuvette into integrated cuvette holder, the 'Set Voltage' key was pressed to select a voltage range of 1.5kV to 1.75 kV. Then, the discharge of electricity was initiated by pressing the 'Pulse' key twice. After electroporation was complete, electroporation cuvette was removed from the machine, cold 1M Sorbitol (1 ml) was instantly added to the electroporation mixture, and then the cuvette containing the diluted conidia was incubated on ice for 10 minutes. To plate the transformation, the suspension of conidia was dispersed from the electroporation cuvette into Petri dishes and suspended in molten YT medium (50°C when

poured; 10 ml per plate) amended with carbenicillin (75 µg/ml). The plates were incubated at 25°C overnight before being overlaid with 10mL of molten YT medium amended with 75 µg/ml of carbenicillin and 150 µg/ml of hygromycin B. Plates were then incubated at 25°C for four weeks and were checked daily for the emergence of hygromycin-resistant colonies. These experiments were repeated at least seven times with various empirical adjustments to voltage, age and concentration of conidia, and DNA concentration.

PEG-mediated transformation of *U. virens*

To produce sufficient tissue for enzymatic digestion/release of protoplasts, 50 ml of PDB medium (per L: 24 g PDB powder; Difco) in a 250 ml sterile flask was inoculated with a 1 ml suspension of *U. virens* conidia (1 x 10^{6} spores per ml) collected from three-week-old 0.2X PDA cultures. The flasks were incubated on a rotary shaker at 170 RPM, 25°C, for 5 days. After incubation, the contents of each flask were poured into a 50 ml conical tube, and centrifuged for 5 min at 4°C and 3220 × *g*. The supernatant was removed with a pipette, and the tissue was washed with approximately 10 ml protoplasting buffer (1.2 M $MgSO_4$, 10 mM KH_2PO_4, pH 5.8) by inverting the tube several times. After centrifugation to discard the supernatant, the tissue was transferred to a 125 ml sterile flask containing 25 ml of protoplasting buffer (10 mg/ml lysing enzyme (Sigma), 2 mg/ml driselase (Sigma), and 50 µl of β-glucuronidase; filter sterilized). This flask was then incubated on a rotary shaker at 80 RPM at 28°C for 10 hours. After incubation, the solution was assessed with a microscope to confirm the release of protoplasts; a successful digest contained a mixture of protoplasts, cellular debris, ungerminated microconidia, and partially digested tissue. After confirming digestion of cell walls and release

of protoplasts, approximately 10 ml of the solution was transferred to a 15 ml conical tube with a pipette. Then, 4 ml of separation buffer A (0.6 M Sorbitol and 100mM Tris-Cl, pH 7.0) was gently overlaid on the top of the digestion solution by dripping slowly down the sidewall of the tube, which was tilted at a 45° angle. To separate protoplasts from debris, microconidia and partially digested mycelia, the tube was centrifuged in a clinical centrifuge at 3220 × *g* at 4°C for 10 min; protoplasts accumulated as a visible, cloudy band at the interface of buffer A and the digestion solution. The protoplasts were removed from the interface with a sterile Pasteur pipette and were transferred to a new 15 ml conical tube. Then, protoplasts were rinsed with sterile separation buffer B (1.2 M Sorbitol and 10 mMTris-Cl, pH 7.5) and centrifuged at 3220 × *g* at 4°C for 10 min; in these conditions, the protoplasts formed a pellet at the bottom of the centrifuge tube. After removing the supernatant, protoplasts were resuspended in separation buffer B and centrifuged as described above. The supernatant was discarded and the protoplasts were finally suspended in re-suspension buffer (2 M sorbitol and 10mM $CaCl_2$) and stored in 2 ml screw-top centrifuge tubes (100 µL per tube) at -80°C for future experiments.

In transformation reactions, protoplasts (100 µl) were added to a sterile centrifuge tube (2 ml) containing 5 µg of the linearized transformation vector pBHt2-sGFP generated by incubation of a mixture (10 µl of vector pBHt2-sGFP, 5 µl of BglII, 10 µl of Buffer 3, and 75 µl of H_2O) overnight at 37°C. The contents of the tube were gently mixed and then the tube was incubated on ice for 30 min. Then, 100 µl of PEG solution (1.2 M Sorbitol, 50 mM $CaCl_2$, 10mM Tris pH 8.0, 40% PEG 4000) was gently mixed with the protoplast suspension containing pBHt2-sGFP DNA and incubated at room temperature for 30 min. Immediately following the 30 min incubation, YT medium (per L: 1 g yeast extract, 1 g tryptone, and 10 g glucose) was added to each tube such that sufficient airspace remained to facilitate homogenization (assessed visually

by the appearance of a small bubble travelling across the tube when incubated on a rocking shaker). Tubes at this stage were incubated at room temperature with rocking for at least one night to facilitate regeneration of cell walls by protoplasts. After visual confirmation that cell walls were forming, tube contents were dispersed into the center of 10 x 150 mm sterile plastic Petri dishes and were then overlaid with 10 ml of molten (50°C) YT medium amended with hygromycin B (100 µg/ml). These plates were incubated at 25°C under a 12:12 hour light: dark cycle and inspected each day for regeneration and growth.

PEG-mediated transformation was repeated three times with parametric adjustments. In the first experiment, the time of cell wall digestion and regeneration was 10 hours and overnight, respectively. In the second experiment, the time for digestion was increased to 12 hours, with two days for regeneration. In the third experiment, tissue was digested for 16 hours. All other parameters were consistent throughout the three experiments.

Agrobacterium-mediated transformation of *U. virens*

Agrobacterium tumefaciens strain AGL1 containing vector pBHt2-sGFP was reactivated from a 30% glycerol stock stored at -80°C by streaking to isolation on a Petri dish containing LB agar medium (per L: 10 g NaCl, 10 g Tryptone, 5 g Yeast extract and 20 g agar) supplemented with carbenicillin (50 µg/ml) kanamycin (50 µg/ml) at 28°C for 3 days. A single bacterial colony was transferred into a 15 conical tube containing 10 ml of LB medium and incubated at 28°C on a rotary shaker at 250 RPM for three days or until visible opalescent growth appeared. The OD_{600} of the culture was measured periodically until it reached a value of 0.2, and then cultures were centrifuged at 4°C, 3220 × *g*, for 10 min to collect the cell pellet. To induce the

expression of virulence genes in *Agrobacterium tumerfaciens* (thus increasing the efficiency of transformation), pelleted cells were suspended in *Agrobacterium*-inducing minimal medium (IMM, per L: 2.05 g K_2HPO_4, 1.45 g KH_2PO_4, 0.5 g NH_4NO_3, 0.15 g NaCl, 0.0025 g $FeSO_4$, 0.01 g $CaCl_2$, 0.25 g $MgSO_4$, 0.9 g glucose, 5.33g MES-H_20, 5 ml glycerol, 20 µl Vogel trace elements) supplemented with acetosyringone (200 µl of a 1M stock), carbenicillin (50 µg/ml), and kanamycin (50 µg/ml). The OD_{600} of the resuspension was adjusted to a value of 0.2 with liquid IMM, followed by overnight incubation at 28^oC on a rotary shaker at 250 RPM. The following day, the OD_{600} of the culture was adjusted to 0.2 by dilution with liquid IMM and stored at -4^oC for up to four days.

For transformation, conidia of *U. virens* were prepared as described above with the concentration adjusted to 2.0 x 10^6 cells/ml. An induced *A. tumefaciens* culture (OD_{600} value of 0.2 in liquid IMM, as described above) was mixed with *U. virens* microconidia (1:1/ v: v) in a 15 ml conical centrifuge tube. The mixture (200 µl) was equally dispersed on sterile filter paper pieces (1 cm x 2 cm) overlaid on Petri dishes containing IMM amended with 1 M acetosyringone (200 µl 1M stock), carbenicillin (50 µg/ml), and kanamycin (50 µg/ml). The plates were incubated at 24^oC in a 12:12 hour light: dark cycle for 4 days.

To select transformants, the filter papers were flipped and transferred to the selection YPS amended with hygromycin B (100 µg/ml) and cefotaxime (50 µg/ml) with a sterile forceps. The plates were incubated at 25^oC under 12:12 hour light:dark cycle for at least 4 days or until visible colonies were present. Hygromycin-resistant colonies were maintained in 24-well plates containing 0.2x PDA amended with hygromycin B (100 µg/ml).

Phenotypic evaluation of *U. virens* transformants

One week old cultures of hygromycin-resistant colonies were assessed for GFP expression with the Dual Fluorescent Protein Flashlight (NightSea). For microscopic analyses, all tissues (*e.g*., hyphae, conidia) of GFP-expressing isolates were assessed with confocal microscopy (excitation wavelength: 488 nm). Transgene stability was evaluated by assessing levels of GFP expression after every week of repeated sub-culturing of transformants on 0.2X PDA in the presence and absence of hygromycin B.

Pathogenicity assays with *U. virens* transformants on rice plants

Three rice cultivars commonly grown in Arkansas and reported to be susceptible to false smut (Wells, Jupiter, and Francis; seed kindly provided by Dr. James Correll) were used in this study. Rice seeds were allowed to germinate on a moist filter paper in Petri dishes at room temperature and five of the most vigorous seedlings were transplanted to 7.5 cm square plastic pots containing field soil. Six pots were placed into a large plastic tub and rice seedlings were sub-flooded and fertilized with N-P-K (24-8-16) and an iron supplement (X-Xtra Iron 6-0-0; 9% Fe and 6% N) every week until the booting stage. Under greenhouse conditions (24°C, 70% RH, and 18 hour photocycle), the emerging tillers of all plants were cut to maintain only the main culm.

One week before heading, the leaf sheath of each rice plant was injected with 2 ml of inoculum containing *U. virens* conidia (1x10^{6} spores/ml) collected from one-week-old 0.2X PDA cultures of GFP-expressing strain UVLN. The inoculated plants were then covered with plastic bags and placed under shade clothes in the greenhouse for over one week before moving

to an original place (24^oC, 70% RH, and 18 h photocycle). After inoculation, plants were evaluated daily for signs of the pathogen with the Dual Fluorescent Protein Flashlight and spore balls were allowed to mature for 2-3 weeks after initial observation.

Extraction and analysis of ustiloxin A from *U. virens* sporeballs

Spore balls were collected in 2010 from growers' fields in Arkansas and Desha Counties, Arkansas, by Extension Plant Pathology faculty. Extraction and analysis of ustiloxin A from spore balls was adapted from the method published by (Miyazaki et al., 2009). Two grams of false smut balls are placed in a 50 ml conical tube containing 20 ml of water. For smaller samples, such as analysis of an individual spore ball, the same ratio is maintained (1 ml water used to extract 0.1 g of spore ball material). Each extraction was shaken mechanically for 1 hour and then centrifuged at 3220 × *g* for 10 min. The supernatant was separated by filtration through filter paper (Whatman No.1). Then, a 15 ml aliquot of the clarified supernatant was transferred into a Sep-Pak Vac 35cc C18 cartridge preconditioned with 3 ml of methanol followed by 3 ml of water. After the clarified supernatant eluted completely, the cartridge was washed with 3 ml of water. The fraction containing ustiloxin A was then eluted with 2 ml of 20% (v/v) methanol. Finally, one ml of the eluted aliquot was transferred to a 2 ml autosampler vial for HPLC.

Chromatographic isolation and spectrophotometric analysis of ustiloxin A was performed essentially as described by Miyazaki et al. (2009) with a high-performance liquid chromatography (HPLC) system with photo diode array detection at 220 nm (Shimadzu, Columbia, MD, U.S.A.) and a 4.6-U ODS column (250 × 4.6 mm; Phenomenex, Torrance, CA, U.S.A). Compounds were eluted with an isocratic

gradient, a mobile phase consisting of water:methanol:phosphoric acid (400:100:1), and a flow rate of 1.0 ml/min. In the absence of a commercially available standard, the distinctive UV absorption spectrum of ustiloxin A (distinct peaks at 201 nm, 254 nm, and 290 nm) was used in conjunction with retention time to identify the molecule.

RESULTS

Hygromycin is a suitable selectable marker for transformant selection in *U. virens*

Kill curves were performed, in which *U. virens* conidia were plated on PDA medium containing varying concentrations of hygromycin B. Experiments focused on hygromycin B as a selectable marker because it is useful in transformation systems for other fungal species, and the Bluhm lab has multiple plasmids available that convey fungal resistance to this antibiotic. Dilute concentrations of spores were plated to allow calculations of percent survival rates, and data represent the results from triplicate platings. Growth of *U. virens* was uninhibited at 25 µg/ml, partially inhibited at 50 µg/ml, and almost completely inhibited at 75 µg/ml. However, growth was completely inhibited at 100 µg/ml and 150 µg/ml of hygromycin B, a result that was consistent across multiple experiments. Thus, since 100 µg/ml was the lowest concentration of hygromycin B that consistently inhibited growth of *U. virens*, this concentration was used for selection of transformants in all subsequent experiments. These findings correspond closely with recently-published results describing a hygromycin B kill curve in a Japanese isolate of *U. virens* (Tanaka et al., 2011).

Transformation of *U. virens* by electroporation

Electroporation (also known as electropermeabilization) is the process of applying electricity to solutions of cells or protoplasts to increase membrane solubility. The increase in membrane solubility often facilitates the transfer of exogenous DNA into cells, which can lead to transient expression or stable integration into the host genome. Seven transformation experiments were performed, in which several parameters were adjusted, including electrode gap, age of conidia, and concentration of conidia (Table 1). However, no transformants were obtained in any of the experiments (Table 1). Thus, electroporation does not appear to provide an easy or straightforward technique for transformation of strain R-2 of *U. virens*.

Table 1. Electroporation parameters adjusted and transformants obtained

Experiment number	Electrode gap (mm)	Age of conidia (days)	Concentration of conidia	Electroporation voltage (kV)	Number of plates evaluated	Number of transformants obtained
1	1	7	10^7	1.5 - 1.75	10	0
2	1	7	10^7	1.5 - 1.75	10	0
3	1	5	10^8	1.5 - 1.75	10	0
4	1	5	10^8	1.5 - 1.75	10	0
5	1	5	10^9	1.5 - 1.75	10	0
6	2	5	10^9	1.5 - 1.75	10	0
7	2	5	10^9	1.5 - 1.75	10	0

Polyethylene-glycol (PEG)-mediated transformation

In this approach, PEG increases membrane solubility and facilitates the uptake of exogenous DNA. However, for PEG-mediated transformation to work, fungal cell walls must be removed first to create protoplasts. Adaptation of a protoplasting protocol developed by the Bluhm lab for *Fusarium verticillioides*(Ridenour et al., 2012)was used to obtain protoplasts from *U. virens*. Enzymatic digestion of *U. virens* tissue was allowed to proceed for 10, 12, or 16 hours; all three of these conditions yielded 10^7 – 10^8 protoplasts per gram of tissue digested (Fig. 6). However, after three attempts of following the protocol published by Ridenour et al. (2012) for PEG-mediated transformation of fungal protoplasts, no transformants were observed. Thus, the time of regeneration was increased to 48 – 72 hours in subsequent experiments. However no transformants were obtained under any conditions using this approach.

Agrobacterium- mediated transformation

Agrobacterium tumefaciens is a plant pathogenic bacterium that causes tumors (galling) in plants by transferring bacterial DNA into the host genome. This unique ability has been utilized in laboratory conditions to transform many species of plants and fungi for research purposes. To apply *Agrobacterium*-mediated transformation to *U. virens*, *A. tumefaciens* strain AGL1 (widely used for fungal transformation) was used in conjunction with a binary transformation vector, pBHt2-sGFP, that contains a constitutive expression cassette for GFP in fungi through the use of the trpC promoter from *Aspergillus*. Transformation of *U. virens* with *A. tumefaciens* was first attempted on conidia (2 x 10^5 spores) collected from five-day-old YEPD liquid cultures. A total of 51 transformants that were resistant to hygromycin and strongly expressed GFP were obtained. Interestingly, in a second transformation with seven-day-old

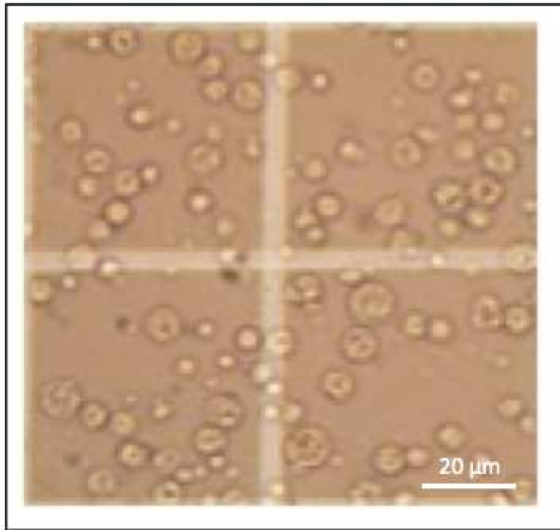


Figure 6. Typical protoplasts of *U. virens* obtained after 10-16 hours by enzymatic digestion of cell walls.

conidia, only 16 transformants were obtained; when nine-day-old conidia were transformed, only 5 transformants were obtained. From these results, it could be inferred that age of conidia has a substantial influence on the transformation efficiency of *U. virens* when using *Agrobacterium*-mediated transformation.

Screening, selecting, and purifying transformants

After several successful *Agrobacterium*-mediated transformation experiments, thirty transformants strongly expressing GFP were chosen for further analysis. Based on macroscopic analysis with the Dual Fluorescent Protein Flashlight (NightSea, Bedford, MA), ten isolates were chosen that exhibited substantially higher visual levels of GFP expression than observed for a "typical" transformant. These 10 isolates were then single-spored and further evaluated for GFP expression. All 10 isolates were visually indistinguishable from the wild type in growth and development on 0.2X PDA, and showed high levels of GFP expression when evaluated with the Dual Fluorescent Protein Flashlight or compound fluorescence microscopy. No sectoring or any visually obvious indicator of genomic instability was observed among these isolates. Ultimately, one GFP-expressing strain (designated UVLN) was selected arbitrarily from this group for further experimentation.

Evaluation of transgene expression and stability in *U. virens* strain UVLN

GFP expression in a *U. virens* transformant must be strong, constitutive, and durable in the absence of antibiotic selection to be of maximum utility in histopathological experiments. Consistent GFP expression in the absence of a selectable marker (hygromycin B, in this study) indicates stable integration of the plasmid into the genome, which is required to monitor

expression of GFP *in planta*. Thus, for strain UVLN, GFP expression was evaluated with confocal microscopy throughout all stages of growth and development (e.g., hyphae and conidia of various ages). Expression of GFP was strong and consistent throughout hyphae and conidia (Fig. 7). Transgene stability was evaluated by assessing levels of GFP expression after repeated sub-culturing of transformants, including in the absence of antibiotic selection. After being sub-cultured 10 times on 0.2x PDA in the absence of antibiotic selection, expression of GFP was unchanged (Fig. 7), and thus the genomic integration in the GFP-labeled strain UVLN of *U. virens* was determined to stable.

Evaluation of pathogenicity of *U. virens* strain UVLN

To determine whether strain UVLN retained pathogenicity, inoculations of the inflorescence at booting were performed. This approach was chosen because several groups have reported the ability to reliably induce symptoms observed in the field (e.g., sporeball formation) in greenhouse conditions with this technique. In each experiment, six – ten individual plants of cultivars Wells, Jupiter, and/or Francis were inoculated essentially as described by (Zhou et al., 2003; Ashizawa et al., 2011) with strain R-2 or UVLN. A small yet consistent number of false smut balls were formed in infected rice panicles two to three weeks after inoculation. A possibility to consider is that spore balls observed in the greenhouse could result from systemic (seed-borne) infections, and thus provide a false positive assessment of pathogenicity. However, 100% of the spore balls obtained after inoculation with strain UVLN displayed GFP expression, as visualized with the Dual Fluorescent Protein Flashlight (Fig. 8) and confocal microscopy. These results confirm that strain UVLN has retained pathogenicity.

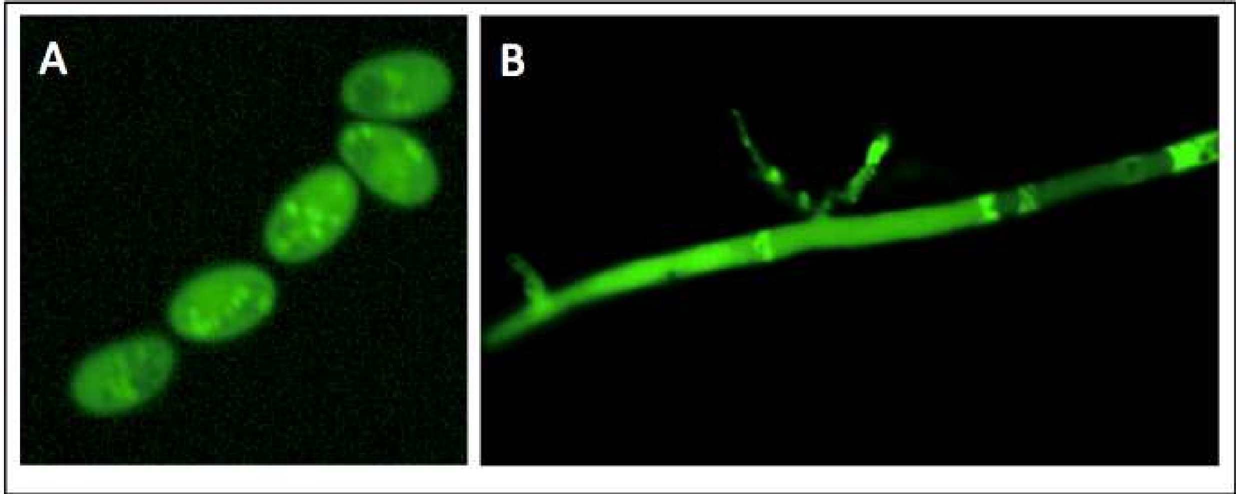


Figure 7. Constitutive GFP expression in *U. virens* strain UVLN. GFP expression was consistently observed in conidia (A) and hyphae (B) after repeated sub-culturing.

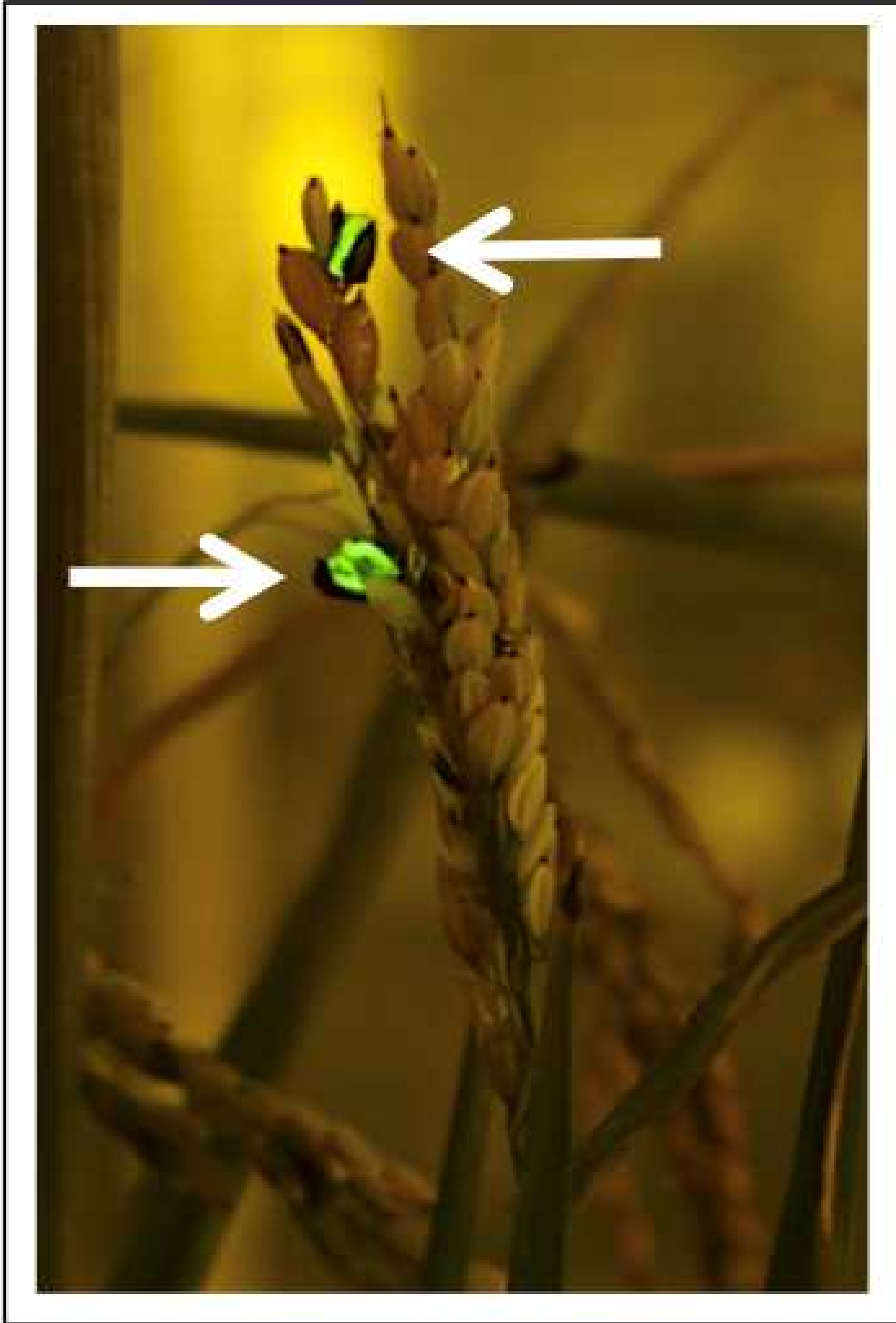


Figure 8. False smut spore balls (white arrows) produced by strain UVLN of *U. virens* in greenhouse conditions.

However, the low number of spore balls obtained from experiment to experiment does not provide enough statistical power to accurately assess whether strain UVLN is equally as virulent as its progenitor, wild-type strain R-2.

Evaluation of ustiloxin A biosynthesis by the GFP-expressing strain UVLN of *U. virens*

At least some strains of *U. virens* are known to produce ustiloxin mycotoxins (Miyazaki et al., 2009), although the extent to which ustiloxins potentially contaminate Arkansas-grown rice is not clear (Abbas et al., 2002). A commercial standard is not available for any of the ustiloxin analogs, and therefore chromatographic analyses can only provide a yes/no answer regarding the presence of the toxin and a qualitative assessment of the amount in one sample compared to another. To assess whether ustiloxin could be detected in spore balls collected in Arkansas, the method of (Miyazaki et al., 2009) was adapted for extraction and analysis via HPLC-DAD. Ustiloxin A was detected in spore balls produced in the greenhouse by strain UVLN (Fig. 9A, B). Moreover, among the five samples of spore balls collected from Arkansas County and Desha County, all five contained ustiloxin A as determined by the UV spectral signature of the aqueous extracts (Fig. 9C, D). This is the first report of ustiloxin A in rice infected by *U. virens* in Arkansas, and suggests that contamination is not because of a single, isolated strain. Moreover, this finding demonstrates that strain UVLN is fully pathogenic, including production of ustiloxin A, which makes it an excellent experimental model for future molecular dissection of pathogenesis and mycotoxigenesis in *U. virens*.

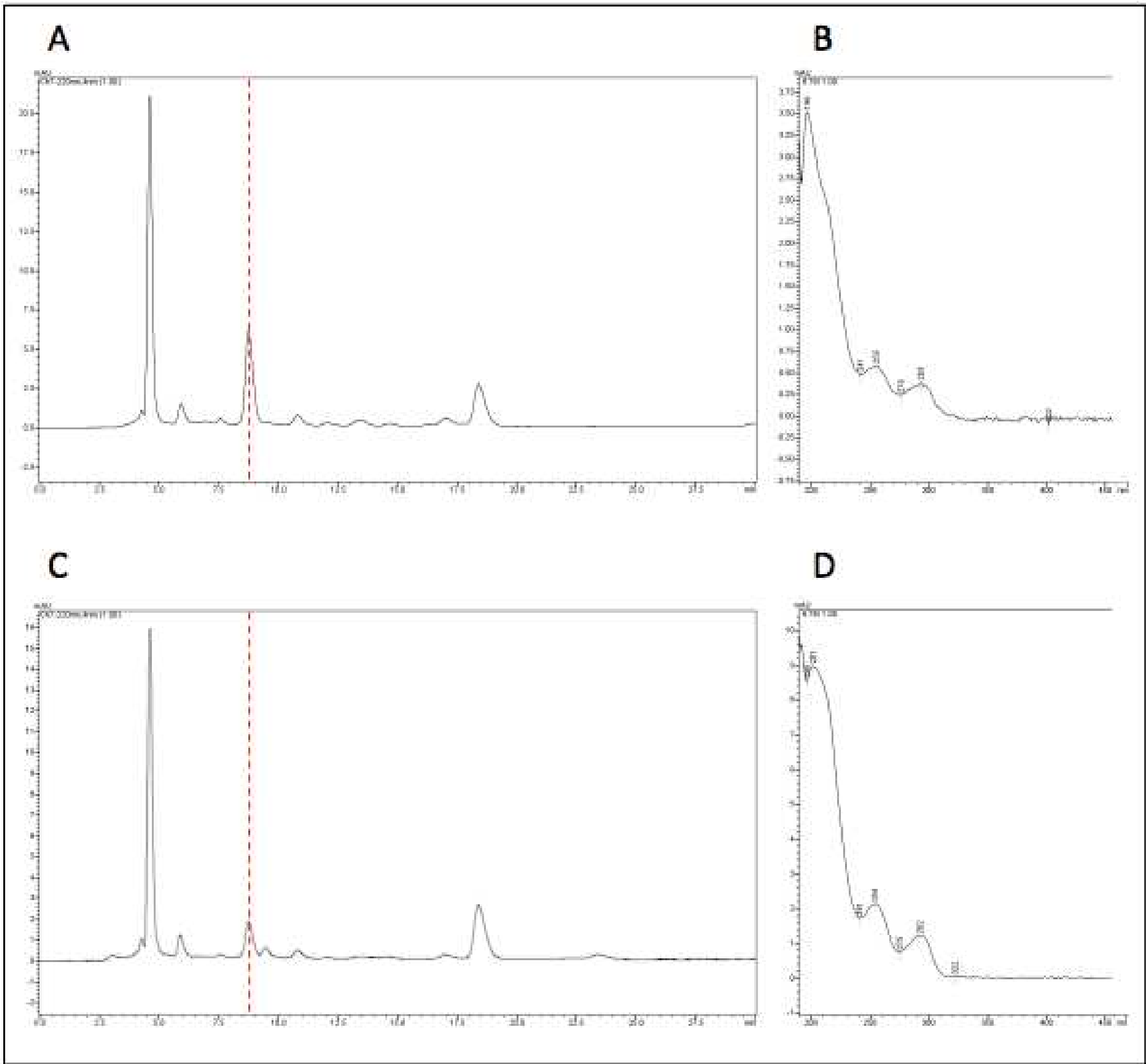


Figure 9. HPLC analysis of ustiloxin A biosynthesis in false smut balls. A. Chromatographic separation of ustiloxin A (red dotted line) in greenhouse produced spore balls from *U. virens* strain UVLN. B. UV spectral profile of ustiloxin A in greenhouse produced spore balls from strain UVLN. C. Chromatographic separation of ustiloxin A (dotted red line) in field-collected spore calls from Arkansas County, AR. D. UV spectral profile of ustiloxin A in in field-collected spore balls.

DISCUSSION

In this study, three distinct approaches for transformation were evaluated in *U. virens*: electroporation, PEG-mediated protoplast transformation, and *Agrobacterium*-mediated transformation. Despite repeated attempts and empirical adjustment of many parameters, only *Agrobacterium*-mediated transformation was successful. Since the initiation of the research described in this thesis, successful transformation of *U. virens* has been reported by electroporation and PEG-mediated protoplast transformation (Tanaka et al., 2011; Ashizawa et al., 2012). A careful analysis of these protocols reveals subtle differences in methodologies that may explain the negative results presented in this thesis. For example, the electroporation protocol reported by Tanaka et al. (2011) utilized essentially the same buffers, DNA concentrations, and electroporation voltages that were tested in the experiments presented in this thesis. An important difference, however, was the GFP expression vector. Tanaka et al. (2011) used a 6.1 kb plasmid derived from pCB1004, a widely used fungal transformation vector (Carroll et al., 1994) that is substantially smaller than pBHt2-sGFP. Irregardless, the transformation efficiency via electroporation reported by Tanaka et al. (2011) was 3.8 ± 2.3 transformants/μg of plasmid DNA, which is nearly two orders of magnitude lower than what is reported for electroporation in other fungi (Sanchez and Aguirre, 1996), and PEG-mediated transformation of other many other fungal species (Irie et al., 2001). When comparing the recently-published protocol used for successful PEG-mediated protoplast transformation of *U. virens* (Ashizawa et al., 2012) with the protocol described in this thesis, several potentially significant differences stand out. First of all, a much shorter time of enzymatic digestion of cell walls was utilized; 3.5 hours by Ashizawa et al. (2012) compared to 10-16 hours in our experiments. The longer period of digestion used in our experiments could have reduced the

protoplasts' amenability for transformation by decreasing viability or allowing some level of cell wall reformation to occur during the digestion step. Secondly, Ashizawa et al. (2012) used plasmid pMK412 to convey hygromycin resistance and constitutive GFP expression (Watanabe et al., 2007). Plasmid pMK412 is 7.1 kb in size (Watanabe et al., 2007), which is considerably smaller than pBHt2-sGFP (10 kb); smaller plasmids generally have higher transformation efficiencies. During the transformation step, Ashizawa et al. (2012) used a 60% PEG solution to induce membrane solubility, as opposed to the 40% PEG solution tested in this study. It is possible that the higher concentration of PEG dramatically increases transformation efficiency. Interestingly, Ashizawa et al. (2012) report using a very large amount of linearized plasmid in transformation reactions (200 µg), yet only obtained three transformants. The unusually low transformation efficiency obtained by both Tanaka et al. (2011) and Ashizawa et al. (2012) suggests either that the transformation efficiency for *U. virens* is unusually low, or that there is room for further optimization of both protocols. In relation to the experiments presented in this thesis, if the low transformation efficiency observed for two other strains of *U. virens* is indicative of most strains, it is possible that the wild type used in this study (R-2) is simply difficult to transform even under the most ideal conditions.

When the research presented in this thesis began, there was a single report in a Chinese-language journal regarding *Agrobacterium*-mediated transformation of *U. virens*. This situation posed two significant problems for utilization of the protocol by the international false smut research community: first, translation of the article into English caused some confusion about the details of the protocol (B. Bluhm, *personal communication*), and second, neither the *Agrobacterium* strain used nor the vector harboring GFP are readily available in the U.S. Therefore, we investigated transformation of *U. virens* with an *Agrobacterium* strain (AGL1) and

GFP expression vector (pBHt2-sGFP) that are publicly available throughout most of the world. This effort has resulted in the development of a reliable and robust *Agrobacterium*-mediated transformation protocol for *U. virens*. This approach will facilitate a variety of molecular approaches for the increasing number of U.S. research groups studying false smut.

Pathogenicity assays confirmed that the GFP-expressing strain UVLN is capable of infecting rice and causing typical false smut symptoms (e.g., emergence of spore balls on infected heads). However, greenhouse inoculations with *U. virens* are notoriously problematic, and often result in a much lower incidence of disease than what is observed in field conditions (D.O. TeBeest, *personal communication*). In this study, we consistently observed spore balls formed by the GFP-expressing UVLN strain across multiple experiments, but the low incidence of symptoms (1-3 spore balls formed among 20 inoculated plants, regardless of fungal strain or rice cultivar) made it impossible to make meaningful statistical comparisons between the GFP-expressing strain UVLN and the wild-type strain R-2 to determine if transformation conveyed a loss of virulence. However, the spore balls observed after infection with strain UVLN were morphologically indistinguishable from those formed by strain R-2 (other than the observation of GFP when excited by blue light), and the similar (albeit low) incidence of infection observed between the two strains led us to conclude virulence was not significantly impacted.

The apparent ability of the Arkansas isolate R-2 of *U. virens* to produce the mycotoxin, ustiloxin A, along with the presence of ustiloxin A detected in a small number of field-collected spore balls from Arkansas, raises a serious concern as to whether ustiloxins are contaminating Arkansas-grown rice. Before this finding can be published, however, additional validation is required. First, current published methodologies to detect ustiloxin A (as used in this research project) rely on HPLC-DAD, which isolates compounds in the aqueous extract of rice, and

identifies them based on a UV spectral scan (200 - 400 nm). While this provides strong evidence for the presence of ustiloxin A in a substrate, it is not as definitive as making comparisons against a purified ustiloxin A standard (which is not currently available from a commercial vendor), or detecting the compound based on accurate mass or masses of fragmentation products (as provided by time-of-flight mass spectrometry or tandem mass spectrometry-mass spectrometry, respectively). Because of the potential impact on the Arkansas rice industry, a definitive identification of ustiloxinA needs to be established to provide the highest level of certainty the compound is potentially present in rice grown within the state. Earlier reports suggest that ustiloxins may not be produced by Arkansas isolates of U. virens, although an exhaustive study was not performed (Abbas et al., 2002). If the potential for ustiloxins to be present in Arkansas does exist, however, it will be important to determine the frequency, distribution, and amounts of ustiloxin A that are found within fields affected by false smut within Arkansas.

The results of this study demonstrate that *Agrobacterium tumefaciens* provides a robust, reliable, and simple way to transform *U. virens*. This, in turn, opens many doors for future molecular research into false smut. For example, *Agrobacterium*-mediated transformation is a powerful tool for molecular genetics and functional genomics in plant pathogenic fungi; this approach can be used for targeted gene disruption (reverse genetics) as well random mutagenesis to identify mutants impaired in pathogenesis (forward genetics). Most immediately, the creation of a GFP-expressing, pathogenic strain will be invaluable in the histopathological dissection of the infection process underlying soil borne versus air borne infection of rice plants.

Chapter III: COMPARISON OF INFECTIOUS DEVELOPMENT IN ROOT VERSUS INFLORESCENCE INOCULATIONS

SYNOPSIS

Ustilaginoidea virens (Teleomorph: *Villosiclava virens*) causes false smut of rice, a disease of widespread and growing concern throughout the world's rice producing regions. There is considerable uncertainty regarding the false smut disease cycle, specifically whether or not systemic infections initiated in young rice plants lead to meristematic infection and subsequent colonization of inflorescence tissues. In this study, root and inflorescence inoculations were performed with GFP-expressing strain UVLN of *U. virens*, and infectious development was analyzed via confocal microscopy. For comparative purposes, all root inoculation experiments were performed in parallel with a GFP-expressing strain of *Magnaporthe oryzae*, a known root-infecting pathogen of rice. Injections of the flag leaf sheath at booting consistently led to the formation of spore balls in a small number of infected plants. Confocal microscopy showed no evidence of systemic infection in these experiments, but rather infection was consistent with the fungus gaining access through the gap in the lemma as reported elsewhere. In root inoculations, *U. virens* failed to penetrate the epidermis, whereas *M. oryzae* frequently colonized roots extensively. To determine whether previous reports of systemic infection based on a nested-PCR assay could be premature due to technical issues, the specificity of the assay was tested against a variety of non-target organisms. The assay consistently yielded false positives for the presence of *U. virens*, which calls into question previous reports relying on

the technique and highlights the need for additional molecular tools to study false smut. Taken together, the results presented in this chapter indicate that systemic infection of rice during false smut is not likely to originate from root infection, and the limited evidence for systemic infection calls into question whether it is a component of the false smut disease cycle.

INTRODUCTION

Ustilaginoidea virens (Cooke) Takahashi (Teleomorph: *Villosiclava virens*) is the causal agent of false smut of rice, a common disease in most major rice- growing regions throughout the world including China, India, Japan, and the Southeastern U.S (Ashizawa et al., 2010). The disease was first reported in Arkansas in 1997, and is now presumed to be present throughout all of the rice production areas of the state. The disease is noted for the formation of spore balls, masses of hyphae and spores that replace individual rice grains on infected panicles. In addition to yield loss, a major concern associated with false smut is the accumulation of ustiloxin, mycotoxins in spore balls, which are retained with grain during harvesting and pose a significant risk of entering the human food supply.

Unlike the "true" smut fungi that belong to the Basidiomycota (Order: Ustilaginales), *U. virens* belongs to the Ascomycota (Order: Hypocreales). A systemic phase of infection is common among the true smuts, with disease culminating in the production of masses of black, powdery spores (teliospores) that often develop in place of the grain in cereal crops or in place of organs such as the anthers of some flowering plants. Among true smut diseases, the site of initial infection varies considerably depending on the pathosystem; systemic infections can be initiated via infection of roots, stems, or foliar tissue, whereas direct infection of inflorescences without

systemic infection can occur as well. In false smut of rice, the disease cycle has not been fully resolved. Specifically, it is not clear whether the fungus undergoes a systemic phase of infection, or if direct infection of developing florets is the primary (or even exclusive) means through which the disease is initiated.

Molecular tools for histopathological investigation of the false smut disease cycle would greatly accelerate efforts to resolve uncertainty regarding the infection process. To date, three PCR-based protocols have been published for the detection of *U. virens* (Zhou et al., 2003; Zhou et al., 2008; Ashizawa et al., 2012), all of which target the rDNA sequences in the pathogen's genome. Several studies have relied on the nested PCR approach described by (Zhou et al., 2003) to analyze the possibility of systemic infections (Ditmore and TeBeest, 2005; TeBeest et al., 2010). However, the specificity of the nested PCR assay has only been tested against a relatively limited number of organisms. Thus further evaluation of the technique is warranted

Magnaporthe oryzae B.C. Couch & L.M. Kohn (anamorph, *Pyricularia oryzae*) causes blast disease, one of the most widespread and devastating diseases affecting rice production worldwide. Rice blast is most noted for the necrotic foliar lesions associated with the disease, which can cause severe reductions in photosynthetic capacity and premature plant death in severe cases (Campos-Soriano and Segundo, 2009). Intriguingly, *M. oryzae* can also infect rice roots, possibly causing systemic infections that culminate in foliar lesions (Sesma and Osbourn, 2004). Recently, progress has been made in understanding how *M. oryzae* interacts with rice roots, and evidence suggests that the fungus adapts a biotrophic infection strategy with roots compared to hemibiotrophic infection of leaves (Marcel et al., 2010). Since both *M. oryzae* and *U. virens* are Sordariomycete fungi, a comparison of how these two pathogens interact with rice

roots would provide an interesting perspective on potentially shared mechanisms of pathogenesis.

Currently, satisfactory methods to manage false smut are unavailable. Therefore, a clearer understanding of pathogenesis in *Ustilaginoidea virens* is critical for future efforts to develop genetic and chemical tools to manage false smut in Arkansas and other regions of the world. To clarify the infection process underlying false smut of rice, a GFP-expressing strain (UVLN) of *U. virens* was inoculated on roots and foliar tissues of rice. A GFP-expressing strain of *Magnaporthe oryzae*, a known root-infecting pathogen of rice, was examined in parallel so that infectious development could be compared between the two pathogens. Additionally, the specificity of the nested PCR technique described by (Zhou et al., 2003) was evaluated against other fungi and non-inoculated rice plants.

MATERIALS AND METHODS

Fungal strains and growth conditions

Wild type and GFP-expressing *U. virens* strains (R-2 and UVLN, respectively) were cultured on 0.2X PDA plates at 25°C for 7-21 days in a 12:12 h light –dark cycle to produce conidia. Conidia were collected from *U. virens* cultures by adding sterile water (3 ml) and scraping the culture surface with a sterile "L" spreader. The microconidia suspension was transferred to a 15- ml conical tube and adjusted to the appropriate concentration with a hemocytometer.

A GFP-expressing strain of *Magnaporthe oryzae* derived from the rice-infecting wild-type strain Guy 11 (kindly provided by Dr. Jin-RongXu, Department of Botany and Plant

Pathology, Purdue University, West Lafayette, Indiana) was cultured on rice bran medium (1L: 16 g defatted rice bran and 20 g agar) at 25°C for 7-14 days in a 12:12 hour light:dark cycle. Conidia were collected by adding 3 ml of sterile water and scraping the culture surface with a sterile "L" spreader. Conidia were counted and diluted to appropriate concentrations as described above for *U. virens*.

Rice plants and growth conditions

All experiments (greenhouse, growth chamber, and laboratory) were conducted in the Rosen Center for Alternative Pest Control Research on the University of Arkansas main campus. Rice varieties Wells, Jupiter, and Francis were used in this study. For inflorescence inoculation experiments, rice seeds were germinated on moistened #1 Whatman filter paper in Petri dishes at room temperature for one week. Five of the most vigorous seedlings were transplanted to 7.5 cm square plastic pots containing autoclaved field soil and six pots were placed into a large plastic tub. Seedlings were sub-flooded irrigated and were fertilized with N-P-K (24-8-16) and an iron supplement (X-Xtra Iron 6-0-0; 9% Fe and 6% N) every week until the booting stage. Rice plants were grown in a greenhouse at 24°C, 70% relative humidity, with a 18:6 hour light: dark photoperiod. The main culms were maintained until the flowering stage by cutting all the emerging tillers. Details of how rice seedlings were grown are included in following sections describing inoculation techniques.

Confocal microscopy

Observations of GFP expression by confocal laser scanning microscopy were performed with a Nikon Eclipse 90i microscope outfitted with a D-eclipse C1 confocal system purchased by the Arkansas P3 program and located in the Bluhm lab.

Inflorescence inoculations with *U. virens*

The protocol for inoculating spikelets before the booting stage as described by Zhou et al. (2003) was adapted with minor changes. Briefly, the leaf sheath of each rice plant was injected with 2 ml of inoculum consisting of microconidia (1 x 10^6 spores/ml) of either the wild type or strain UVLN. Injections of sterile distilled water served as a negative control. The inoculated plants were then covered with plastic bags and placed under shade cloth in the greenhouse for one week after inoculation to promote disease development. After inoculation, plants were evaluated daily for signs of the pathogen, and **s**pore balls were allowed to mature for 2 - 3 weeks after initial observation. When harvesting inflorescences containing spore balls, the rachis, pedicel, and lemma in the vicinity of mature spore balls were carefully dissected with a sterile scalpel. Confocal microscopy was used to assess the extent of fungal colonization of each tissue. The experiment was repeated more than five times with three replications per rice cultivar in order to obtain as many spore balls as possible in each experiment (minimum of three). In each experiment, the final number of infected plants analyzed depended on the efficacy of greenhouse inoculations. Data collected included: number of plants infected, percent of infections that were systemic (rachis and/or pedicel underlying spore ball was colonized), and descriptive, qualitative comparisons of infection in each tissue type (representative confocal microscopy images).

Root-dip inoculations with *U. virens* and *M. oryzae*

Root-dip inoculations were performed by immersing roots of young, hydroponically-grown rice plants or germinated rice seedlings in spore suspensions of either the GFP-expressing *U. virens* strain (UVLN) or the GFP-expressing *M. oryzae* strain contained in a sterile Petri dish. After inoculating for over 10 min, inoculated rice seedlings were transferred to 7.5 cm square plastic pots containing sterilefield soil. Plants were tended in the greenhouse as described above until termination of experiments (3, 7, or 11 days). At each time interval, roots were cleaned by gentle washing first in tap water and then in distilled water to dislodge all soil and debris. The roots were then dissected with a sterile sharp blade and placed on glass slides under cover- slips. Confocal microscopy was used to analyze the colonization of root tissues by the pathogens. The experiment was conducted three times with four plants evaluated per treatment. Data collected included the number of plants infected, the percent of infections that was superficial vs. invasive (i.e., the fungus penetrated the epidermis), and descriptive, qualitative comparisons of infection (representative confocal microscopy images).

***In vitro* root inoculation assays**

In this approach, cultures of *U. virens* or *M. oryzae* were started on agar growth media in a Petri dish. Once the fungal colony was established, surface-sterilized and germinated rice seeds were placed in the plates, which were sealed in parafilm, turned to a vertical orientation, and incubated at 25°C under a 12:12 hour light:dark cycle until termination (3, 7, and 11 days). Roots of the young rice plants eventually encountered the fungal mycelium, thus providing the pathogens an opportunity to infect. At each time interval, rice seedlings were removed from Petri dishes and cleaned by washing in sterilized water to eliminate all agar. The cleaned roots of rice seedlings were dissected by a sterile sharp blade and placed on a glass slide containing a

drop of sterilized water under a cover slip. Confocal microscopy was used to identify the colonization of the pathogen in root tissues.

To assess the effects of root infection on plant viability, 16-day-old rice seedlings inoculated as described above were transplanted to 7.5 cm square plastic pots containing sterile field soil and sub-irrigated and fertilized with N-P-K (24-8-16) and an iron supplement (X-Xtra Iron 6-0-0; 9% Fe and 6% N). Plants were grown in greenhouse conditions as described above and roots were collected at 4, 9, and 12 days, after transplantation. At each time interval, roots of rice seedlings were removed from their pots and cleaned by washing first in tap water and then in distilled water to eliminate all soil and debris. The cleaned roots of rice seedlings were dissected by a sterile sharp blade and placed on a glass slide added one drop of sterilized water under cover- slips. Confocal microscopy was used to identify the colonization of the pathogen in root tissues.

In vitro inoculations were conducted three times with four plants evaluated per each treatment. Data collected included the number of plants infected, the percent of infections that was superficial vs. invasive (i.e., the fungus reached the inner cortex), and descriptive, qualitative comparisons of infection (representative confocal microscopy images).

Soil infestation assays

Rice seedlings were transplanted to 7.5 cm square plastic pots containing artificially infested field soil as described above. Greenhouse conditions, fertilization, and irrigation was performed as described above. They were grown under controlled greenhouse conditions (24°C, 70% relative humidity, and 18 hour photoperiod) until the terminal experiments (3, 5, 7, and 11 days). At each time interval, rice seedling roots were cleaned by washing in tap water followed

by distilled water to eliminate soil and debris. The cleaned roots of rice seedlings were dissected with a sterile sharp blade and placed on glass slides under cover-slips. Confocal microscopy was used to identify the colonization of the pathogen in root tissues. The adjustment of parameters included conidium concentration, germinating rice in infested soil vs. transplanting seedlings into infested soil, and the time course of root evaluation after inoculation. The experiment was conducted three times with four plants evaluated per each treatment. As above, data collected included the number of plants infected, the percent of infections that was superficial vs. invasive (the fungus penetrated the epidermis), and descriptive, qualitative comparisons of infection (representative confocal microscopy images).

Evaluation of nested PCR as a tool for histological analyses

The nested PCR technique for the detection of *U. virens* was used as described by Zhou et al. (2003) without modification from the published protocol. Purified genomic DNA from *Fusarium verticillioides*, *F. graminearum*, and *Alternaria alternata* was prepared as described by Sambrook et al., (1989). Rice plants 'spiked' with *U. virens* were prepared by soaking seeds in a conidial suspension for 1 hour before analysis or planting. PCR reactions were in a 1% agarose-TAE gel containing GelSafe dye and were visualized on a UV transilluminator(Sambrook et al., 1989). The analysis was repeated extensively, with DNA extracted from samples at least three times, and multiple PCRs were performed on each template.

RESULTS

Colonization of the rice inflorescence by *U. virens* after boot inoculation

To assess which tissues are colonized after inoculation of the rice inflorescence with *U. virens*, we performed a series of inoculations with strain UVLN, monitored macroscopic signs of that pathogen, and visualized fungal growth with confocal microscopy in dissected inflorescence tissues. Macroscopically, we consistently observed that a low percentage of spikelets on inoculated inflorescences formed spore balls that closely resembled spore balls formed in field conditions, albeit slightly smaller (Fig. 9). The rachis, primary branches, and pedicels immediately underlying spore balls were inspected visually in five inflorescence inoculation experiments, each of which yielded 1 – 5 spore balls; no obvious necrosis or discoloration was observed (e.g., Fig. 10). For 10 spore balls collected across six different experiments, GFP expression was visualized with the NightSea Light to confirm that strain UVLN was the causal agent. Then, a piece of the rachis was removed, spanning 3 cm in either direction of the point of attachment of the pedicel bearing the spore ball. This section of the rachis was hand sectioned into approximately 10 sections/cm, each of which was observed via confocal microscopy. In parallel, rachis tissue collected from visually healthy inflorescences was collected from uninoculated plants as a negative control. Among all of these samples, no visual evidence of strain UVLN (as assessed by the absence of GFP expression) was observed (Fig. 11). Additionally, the pedicels underlying the same 10 spore balls were then carefully sectioned into approximately 20 slides, each of which was observed with fluorescence microscopy and scored for the presence or absence of strain UVLN (as determined by GFP expression). In parallel, pedicels collected from visually healthy spikelets were collected from uninoculated plants as a negative control. Among all of these samples, there was no evidence for the presence of strain UVLN (Fig. 11). However, analysis of the lemma tissue from the same 10 spore balls consistently revealed the presence of strain UVLN (Fig. 11). The presence on the pathogen on

Figure 10. Typical visual symptoms associated with spore ball formation. Note the lack of necrosis on the rachis and pedicel.

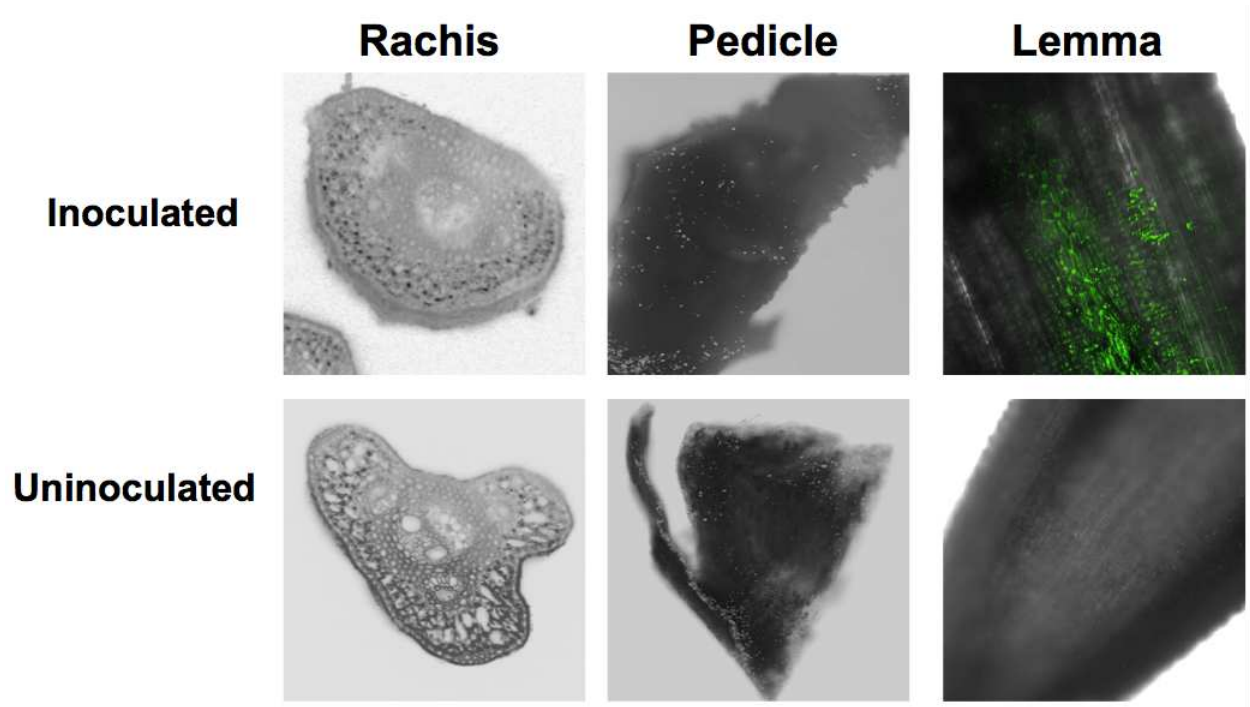
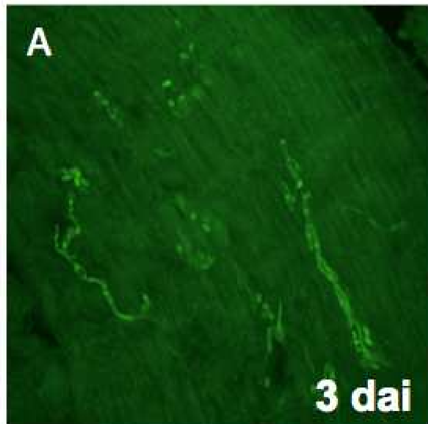


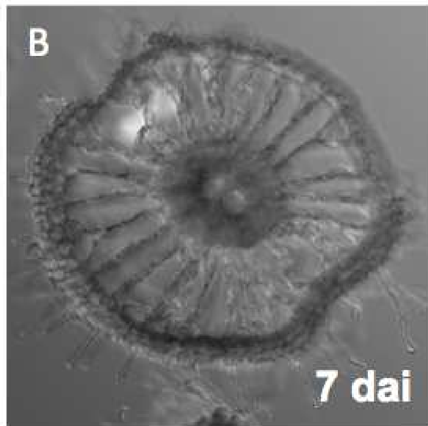
Figure 11. Representative confocal microscope images of rice inflorescence tissue after inoculation with *U. virens* strain UVLN. Fungal growth is visualized via GFP expression.

lemma tissues would be expected if infection occurred via either direct penetration of the spikelet or opportunistic entry through natural openings (a non-systemic infection). A systemic infection would seemingly require the pathogen to be observed at some frequency in inflorescence tissues giving rise to the spikelet; however, no colonization of such tissues was observed in this study.

Comparative histology of root infections by *U. virens* and *M. oryzae*: root dip inoculations

A series of experiments were performed to 1) determine if *U. virens* was capable of infecting rice roots, and 2) how the infection process, if it occurred, compared to the more established root infection process exhibited by *M. oryzae*. To provide a straightforward, although somewhat artificial, assay for root infection, young rice seedlings were grown in conditions such that soil or hydroponic medium could be easily washed from intact roots of living plants. Then, roots were soaked in solutions of conidia of either *U. virens* or *M. oryzae*. By three days after inoculation, superficial growth of *U. virens* was often observed on root surfaces (Fig. 12A); confocal microscopy was used to create a three-dimensional image with a Z-stack, which confirmed that the fungus did not penetrate the epidermis. Interestingly, in repeated experiments in which at least three roots were sectioned and analyzed from each replicate plant within the treatment, penetration of the root epidermis by *U. virens* was never observed up to 11 days after inoculation (Fig. 12 B, C). As with *U. virens*, superficial growth of *M. oryzae* on root surfaces was commonly observed by three days after inoculation (Fig. 13A). In contrast, *M. oryzae* frequently penetrated the epidermis by seven days after inoculation (Fig. 13B), and often appeared to colonize root cortex tissues by 11 days after inoculation (Fig. 13C). By 11 days after inoculation, *M. oryzae* infected approximately 70% of the roots analyzed, whereas *U. virens* was not observed in any of the roots analyzed (Fig. 14). Based on these observations, it appeared





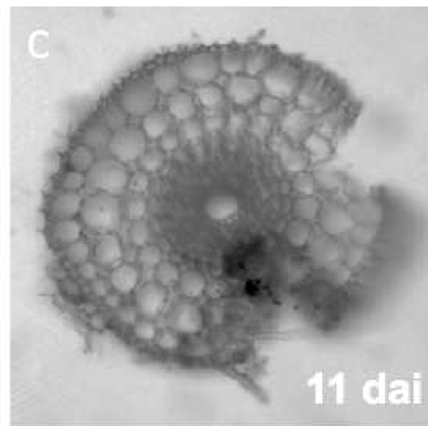
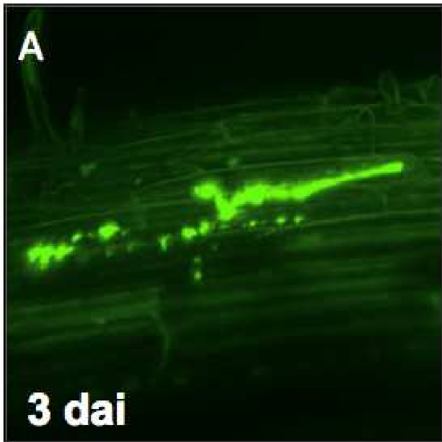
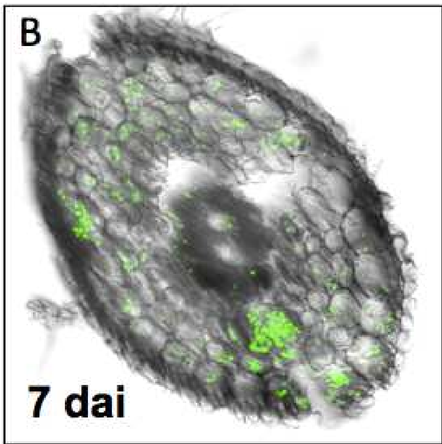


Figure 12. Representative confocal microscopy images of rice roots (cross sections) after root-dip inoculation with *U. virens* strain UVLN.





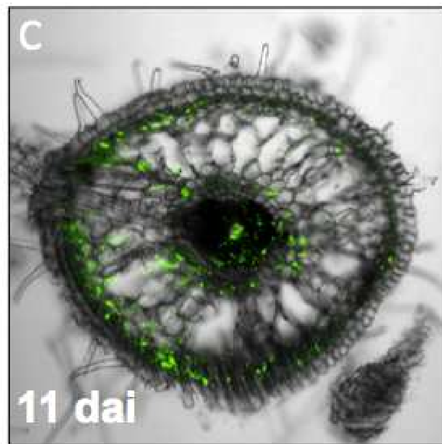


Figure 13. Representative confocal microscopy images of rice roots (cross sections) after root-dip inoculation with *M. oryzae* strain Guy11-GFP.

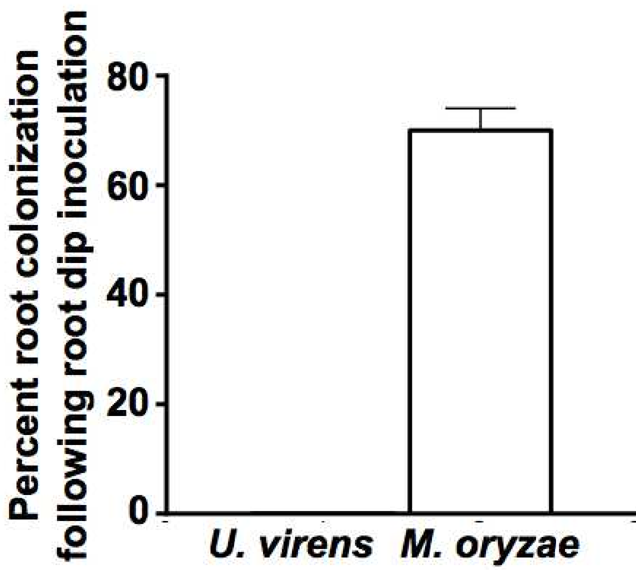


Figure 14. Quantification of root infection at 11 days after inoculation.

either that *U. virens* was not capable of infecting rice roots, or that the assay conditions were not favorable for infection by *U. virens*. Thus, other inoculation techniques were explored with the goal of corroborating these findings.

Comparative histology of root infections by *U. virens* and *M. oryzae*: *in vitro* inoculations in agar medium

In an attempt to provide inoculation conditions more representative of field conditions, yet without factors that could potentially confound the analysis of results, an *in vitro* inoculation system in agar medium was devised. In this approach, agar medium was inoculated with either *U. virens* or *M. oryzae* and incubated for sufficient time for a mycelium to form. Then, rice seeds just barely germinating were surface sterilized and transferred to the plates containing the fungi. This approach allowed the roots to either passively encounter the fungal pathogens through normal growth and elongation, or for the fungal pathogens to grow towards and infect the roots (Fig. 15). In this assay, five seeds were transferred to a Petri dish containing either *U. virens* or *M. oryzae*. For each fungus, three replicate plates were analyzed, and the entire experiment was repeated three times. In each experiment, the same design was followed, but seeds were transferred to sterile medium (neither fungus) to provide a negative control.

For each plant exposed to either *U. virens* or *M. oryzae*, roots were assessed visually for necrosis/discoloration and sections from three individual roots were analyzed via confocal microscopy. For roots exposed to *U. virens*, very minimal levels of visual discoloration were observed, and discoloration levels were similar to what was observed in negative controls. Additionally, among all roots analyzed across all experiments, no penetration of the root epidermis by *U. virens* was observed (Fig. 16). In contrast, although *M. oryzae* was not observed

U. virens **M. oryzae**

Diurnal incubation

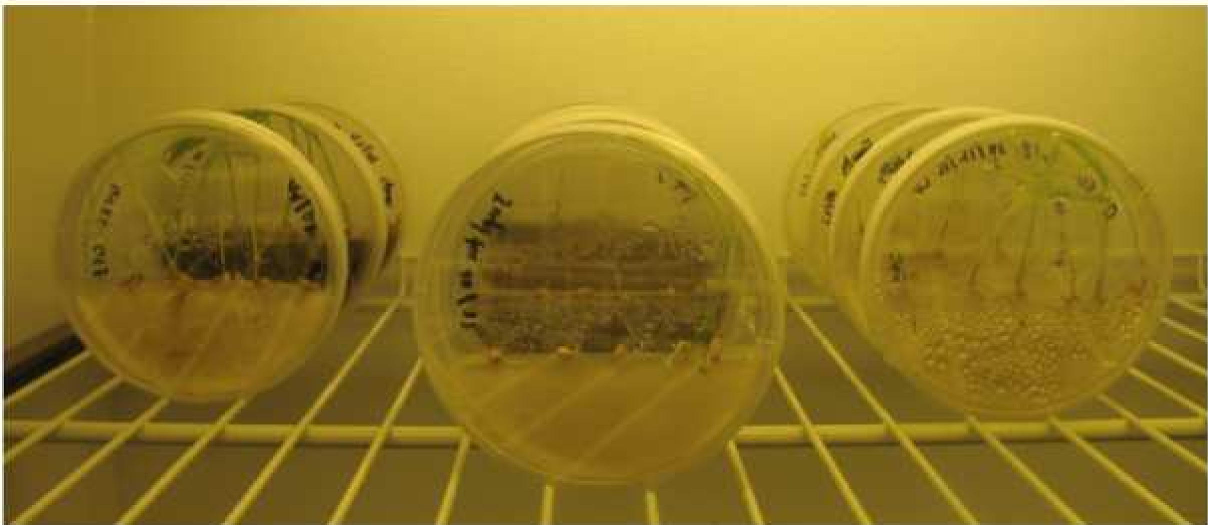


Figure 15. Technique for *in vitro* inoculations of rice seedlings with *U. virens* or *M. oryzae* in agar medium.

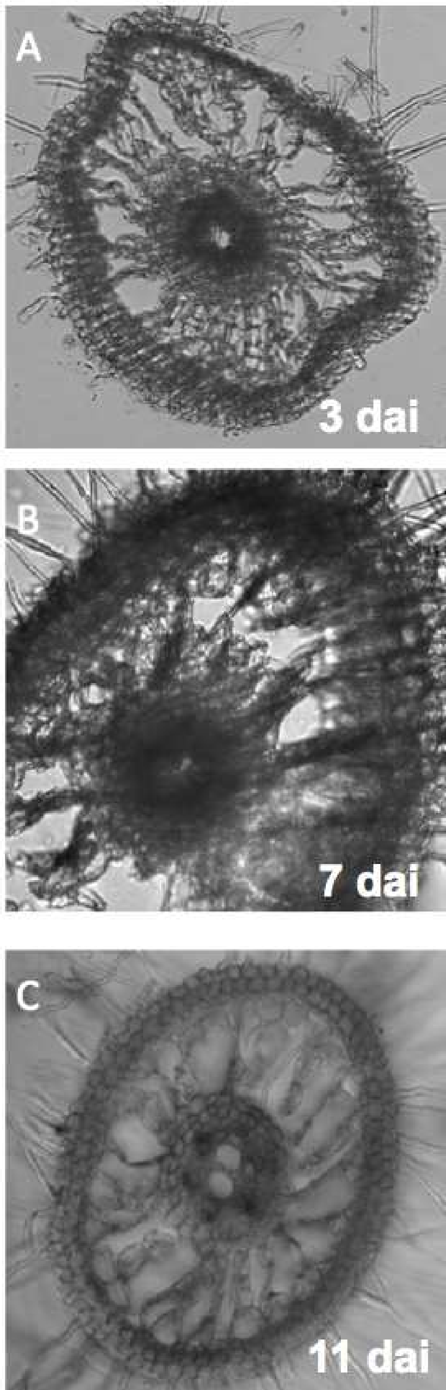
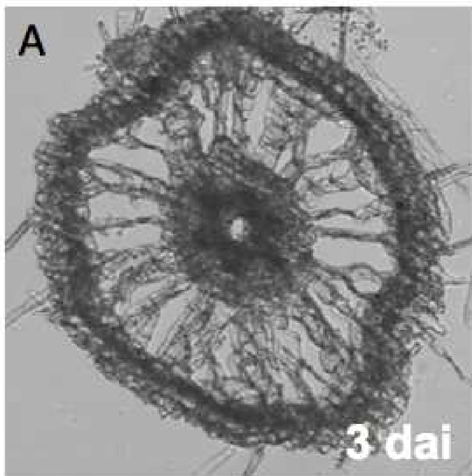


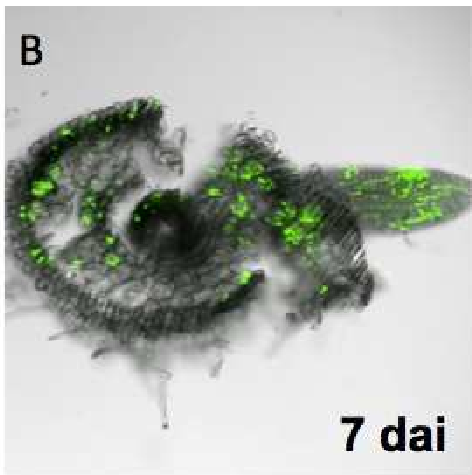
Figure 16. Representative confocal microscopy images of rice roots (cross sections) from the *in vitro* inoculation assay with *U. virens* strain UVLN.

to infect roots three days after inoculation (Fig. 17 A), the pathogen extensively colonized roots 7 - 11 days after inoculation (Fig. 17 B-C). By 11 days after inoculation, approximately 80% of roots exposed to *M. oryzae* showed signs of infection (e.g., penetration of the root epidermis), where as 0% of roots exposed to *U. virens* were infected (Fig. 18). The apparent inability of *U. virens* to infect roots of rice was consistent with results observed after root dip inoculations, described above.

Comparative histology of root infections by *U. virens* and *M. oryzae*: infested soil

In a third approach for root inoculations, autoclaved soil was infested with conidia of either *U. virens* or *M.oryzae*, and young rice seedlings were transferred into the soil. While this was projected to be the most "realistic" simulation of natural, field inoculations, a concern was that the conidia would be excessively dilute to initiate infection. This concern did not turn out to be valid, as roots analyzed from soil infested with *U. virens* showed a low, yet consistent, level of superficial fungal colonization as early as three to five days after transplantation (Fig. 19A, B). However, even as late as 11 days after transplantation, no roots were observed to be infected internally by *U. virens* (Fig. 19C). In soil infested with *M. oryzae*, root infection occurred at some point between three and seven days after transplantation (Fig. 20A, B), and colonization was extensive by 11 days after transplantation (Fig. 20C). In an analysis of three plants per treatment (five individual roots per plant), approximately 40% of roots exposed to *M. oryzae* were infected, as compared to 0% of roots exposed to *U. virens* (Fig. 21). These findings provided a third line of evidence that *U. virens* does not readily infect rice roots, if at all.





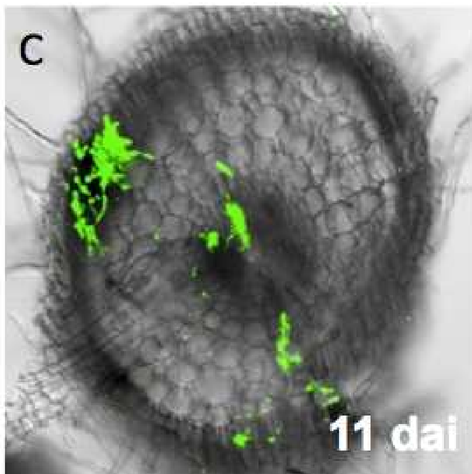


Figure 17. Representative confocal microscopy images of rice roots (cross sections) from the in *vitro* inoculation assay with *M. oryzae* strain GUY11-GFP.

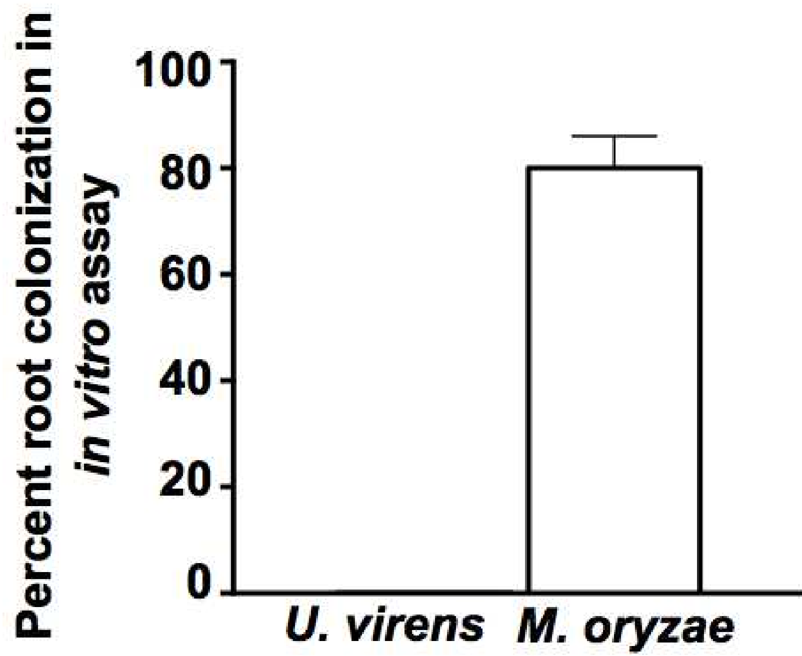


Figure 18. Quantification of root infection at 11 days after inoculation.

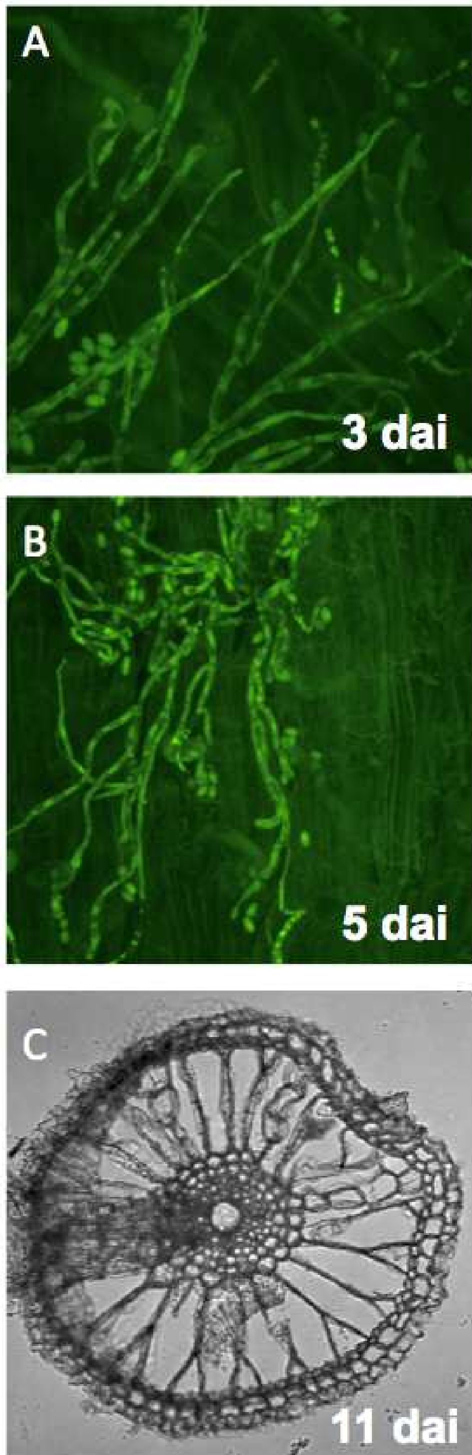
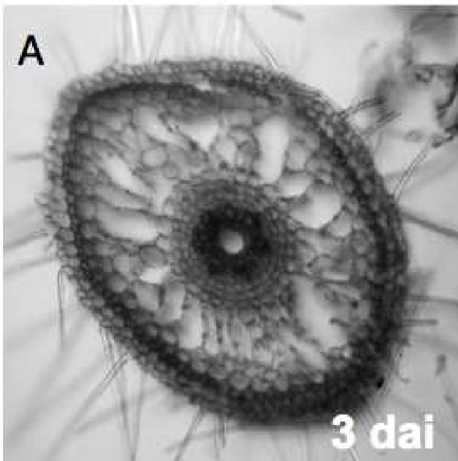
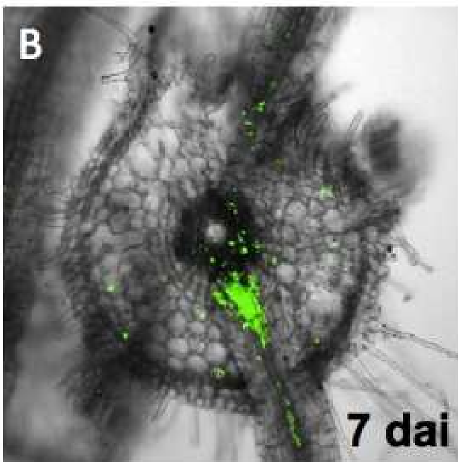


Figure 19. Representative confocal microscopy images of rice roots (cross sections) after transplantation into soil infested with *U. virens* strain UVLN.





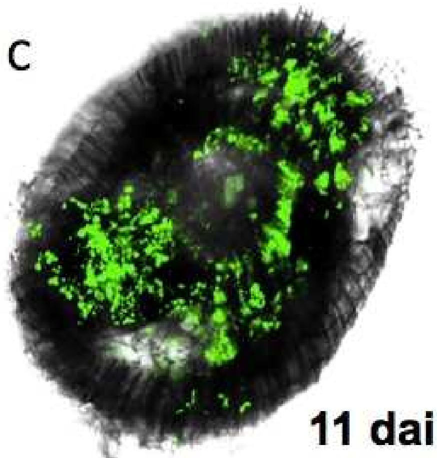


Figure 20. Representative confocal microscopy images of rice roots (cross sections) after transplantation into soil infested with *M. oryzae* strain Guy11-GFP.

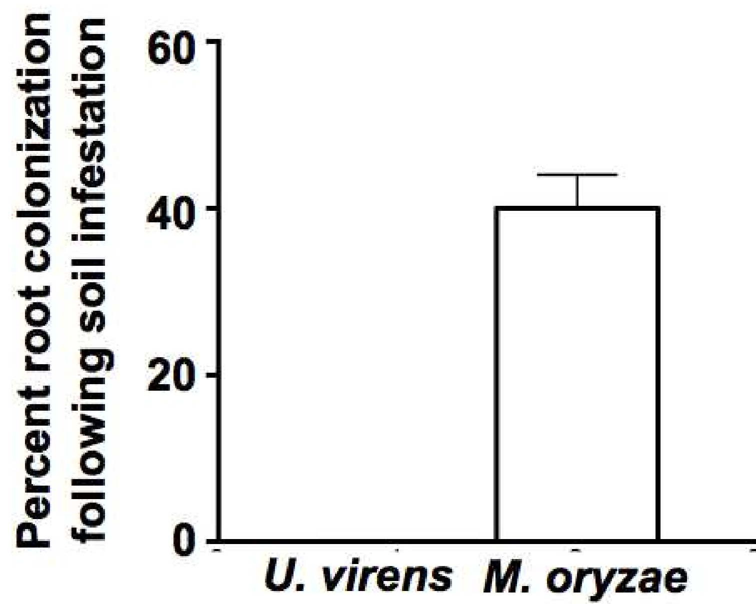


Figure 21. Quantification of root infection at 11 days after inoculation (dai).

Evaluation of the specificity of the nested PCR assay for *U. virens*

The results obtained above called into doubt whether *U. virens* is capable of initiating a systemic infection via root infection. A lingering question was whether or not the nested PCR assay heavily relied upon to detect *U. virens* in infected tissue was truly species-specific. In repeated assessments of specificity by amplifying known non-target DNA, the nested PCR assay did not provide sufficient specificity to be used for histopathological investigations of the presence of *U. virens* in rice tissue. Specifically, the assay produced false positive results on rice seeds and stems collected from greenhouse-grown rice regardless of whether or not the seeds had been treated with *U. virens* conidia before analysis (Fig. 22). Of even greater concern was the amplification of purified genomic DNA from non-target fungi that belong to the Sordariomycets (*Fusarium graminearum* and *F. verticillioides*) and the ubiquitous saprophyte *Alternaria alternata* (Fig. 22). No amplification was observed in negative control reactions (water template), which indicated that the lack of specificity arose from factors other than pipetting error or aerosol contamination of neighboring reactions (Fig. 22).

DISCUSSION

In previous work, a GFP-expressing strain of *U. virens* was created for molecular histology purposes (strain UVLN). Combined with confocal microscopy, this strain represented a significant technological advancement for the histopathological analysis of the infection process underlying false smut. In root infection assays, background fluorescence from rice root tissues minimally overlapped with GFP (e.g., Fig. 13, 17, 20), and the technique clearly – and unambiguously – facilitated analyses of individual hyphae of *U. virens* (e.g., Fig. 19). In the tissues of the inflorescence, background fluorescence was substantially higher than in roots, but the strong GFP signal easily facilitated unambiguous differentiation of the fungus from the host

M = DNA size marker

1. Wells seed (inoculated with *U. virens*)
2. Jupiter seed (inoculated with *U. virens*)
3. Francis seed (inoculated with *U. virens*)
4. Wells stem (inoculated with *U. virens*)
5. Jupiter stem (inoculated with *U. virens*)
6. Francis stem (inoculated with *U. virens*)

7. Wells seed (uninoculated)
8. Jupiter seed (uninoculated)
9. Francis seed (uninoculated)
10. Wells stem (uninoculated)
11. Jupiter stem (uninoculated)
12. Francis stem (uninoculated)

13. *U. virens* (genomic DNA)
14. *Alternaria alternata* (genomic DNA)
15. *Fusarium graminearum* (genomic DNA)
16. *Fusarium verticillioides* (genomic DNA)
17. negative control (water template)

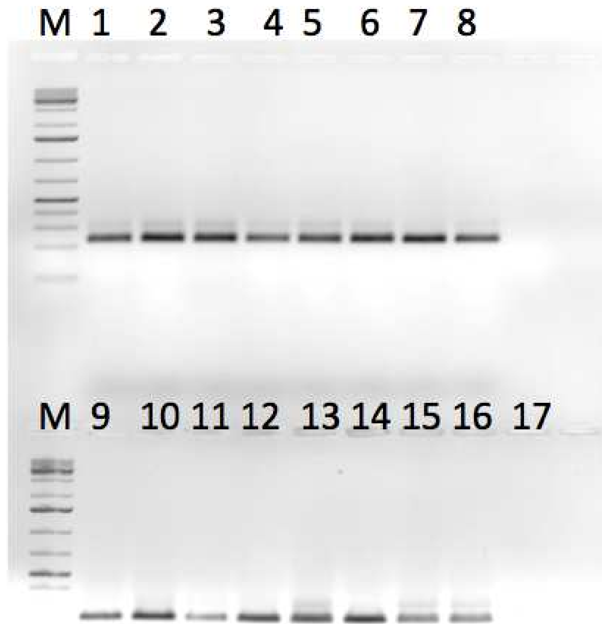


Figure 22. Evaluation of the specificity of the nested-PCR assay to detect *U. virens*, as described by Zhou et al., 2003.

in almost all cases. When analyzing tissues inoculated with strain UVLN, confocal microscopy provided much clearer and more highly resolved images than fluorescence microscopy with a simple compound microscope. Confocal microscopy was particularly useful for the three-dimensional resolution of fungal colonization, and provided much more convincing evidence for superficial versus invasive growth than provided by light microscopy or fluorescence microscopy with a compound microscope. Thus, the combination of a GFP-expressing strain of *U. virens* with confocal microscopy facilitated a thorough histopathological investigation of false smut in rice.

Careful consideration of how the rice inflorescence develops provides insight into the dynamics underlying how aerially dispersed conidia most likely initiate disease. The rice inflorescence is a compound raceme (panicle), on which individual flowers (spikelets) are attached by pedicels to primary branches, which in turn are attached to the central axis of the inflorescence (rachis). The staging of inflorescence development can be divided into nine distinct stages, as summarized in Table 2 (Itoh et al., 2005). Conceivably, a systemic infection initiating in the vegetative stage of development would require *U. virens* to be present in the SAM, and therefore, the pathogen would infect the nascent inflorescence at the stage of initiation (Stage In1: Establishment of rachis meristem). It would follow, then, that the pathogen would most likely be present throughout the entire rachis, all primary branches, all higher-order branches (Stages In2 – In5), and would certainly be present in floret meristems, long before formation of floral organs (Stage In7). Stages In1 – In8 of inflorescence development occur throughout the overlapping stages of jointing and booting in rice development. Jointing is a stage of internode elongation that begins concomitantly with panicle initiation (PI; also called ‘green ring’) and continues until full plant height is reached, whereas booting is loosely defined

as the stage at which the flag leaf sheath swells due to the increasing size of the panicle. Thus, booting overlaps with, and can be considered a sub-stage of jointing. In this study, the developing inflorescences of rice plants were inoculated at booting by direct injection of conidia through the sheath of the flag leaf with a hyperdermic needle. One possible consequence of this approach is that conidia might be inadvertently injected directly into the rachis, thus mimicking a systemic infection, rather than beingdepositedexclusively on the surface of developing spikelets. However, in repeated experiments, including heads of plants displaying one or more spore balls of strain UVLN (confirmed by monitoring GFP expression), no colonization of the rachis, primary branches, or pedicels was observed. The colonization of the glume tissue observed in this study is more consistent with the outward expansion of the spore ball from tissues of the ovary than a systemic infection initiating from the rachis. Moreover, all spore balls produced by strain UVLN throughout this study contained six stamens, which suggets spore ball development, and presumably infection, occurred after Stage In7 of inflorescence development. This lack of evidence for systemic infection is consistent with recently published observations, in which >1000 spore balls from field conditions (natural inoculum) were dissected & no evidence of the fungus was ever observed in the pedicel (Tang et al., 2012). At the very least, these experiments indicate that *U. virens* either infects or initiates spore ball formation in spikelets at a late stage of development, and that inoculation of the developing inflorescence is sufficient to cause normal symptom expression in the absence of systemic infection.

Table 2. Staging of the rice inflorescence (adapted from Itoh et al., 2005).

Symbol	Name	Defining physiological events
In1	Establishment of rachis meristem	Conversion of vegetative meristem to rachis meristem.
In2	Formation of primary branches I	Rachis meristem reaches maximum size. First primary branch primordia are formed.
In3	Formation of primary branches III	Primary branch primordial form spiral arrangement. Rachis meristem aborts at the end of this stage.
In4	Elongation of primary branches	Simultaneous elongation of primary branches
In5	Formation of higher-order branches	Formation of secondary and tertiary branches. Onset of uppermost stem elongation.
In6	Differentiation of glumes	Differentiation of two rudimentary glumes initiated.
In7	Differentiation of floral organs	Floral organs formed: two lodicules, six stamens, and carpel in a whorl.
In8	Elongation of rachis and branches	Rapid elongation of rachis and branches. Completion of anther and ovule development
In9	Heading and flowering	Inflorescence emerges from the sheath of flag leaf. Flowering typically occurs around noon.

Infection of roots by conidia has been postulated to be a possible mechanism through which *U. virens* initiates a systemic infection (Schroud and TeBeest, 2005). Many plant pathogenic fungi are capable of infecting roots of a compatible host and initiating a systemic infection (Agrios, 2003). Intriguingly, numerous species within the fungal order Sordariomycetes initiate systemic infections via root entry, including *Colletotrichum graminicola* (Sukno et al., 2008) and *M. oryzae* (Marcel et al., 2010). In these examples, neither fungus is known to readily infect floral tissue, instead causing leaf and stem necrosis. Additionally, both have high rates of infection in greenhouse and growth chamber conditions, along with distinct morphological adaptations to root infection, which implies that root infections in these two species are not experimental artifacts. In this study, the infectious development on

roots was compared extensively between *U. virens* and *M. oryzae*. Regardless of root inoculation technique, *U. virens* only formed superficial associations with roots, primarily growing on the root epidermis and root hairs without penetrating the epidermal layer. In contrast, *M. oryzae* readily penetrated the epidermis, colonized cortex tissues, and grew extensively along the endodermis in all of the inoculation strategies tested. Moreover, no specialized infection structures were observed to be formed by *U. virens*, whereas *M. oryzae* forms abundant hyphopodia consistent with published descriptions of root infection (Marcel et al., 2010). The lack of root penetration by *U. virens* observed in this study, despite repeated attempts with multiple inoculation techniques, suggests that the fungus is unlikely to colonize roots aggressively enough to reach the SAM via hyphal penetration or translocation of microconidia.

The lack of specificity observed for the nested PCR assay presented by Zhou et al. (2003) calls into question the conclusions of other studies that have relied heavily on this technique. There are at least two possible explanations for the lack of specificity observed for the assay. One explanation is that the assay was improperly performed, with some source of contamination or operator error, sloppiness, etc. This scenario is possible, but unlikely, since similar results were obtained after multiple trials, when performed in blind tests with other researchers in the Bluhm lab, and negative control (water template) reactions consistently failed to amplify, which suggests an absence of wide-spread PCR contamination. A second explanation is that the assay is not sufficiently specific to accurately detect *U. virens*. This is a valid concern because the assay employs a nested PCR technique to amplify rDNA regions of the *U. virens* genome. Nested PCR operates on the principle that a first round of amplification will either increase a rare template or selectively amplify a subset of target sequences, and then specificity is obtained in a

second PCR step that utilizes dilute primary PCR product as template (Zhou et al., 2003). Nested PCR detection assays are notoriously problematic, and frequently provide false positive results due to lack of specificity in one or both rounds of amplification, and an increased likelihood of false positive results from template contamination due to exponential amplification of the target amplicon in positive reactions. The rDNA of *U. virens* is highly similar to analogous sequences from other Sordariomycetes, including many species of *Neotyphodium*. *Neotyphodium* species are ubiquitous endophytes commonly associated with members of the Poaceae; *Neotyphodium* species are commonly associated with rice as arbuscular mycorrhizal (AM) fungi (Vallino et al., 2009). Regardless of whether operator error or lack of specificity is the cause of the false positive results obtained with the nested PCR assay in this study, the development of a more robust, species-specific detection assay for *U. virens* – preferably, one that did not target rDNA sequences – would be highly beneficial to the false smut research community. Upon development of a more robust detection assay, it would be highly worthwhile to revisit experiments in which systemic colonization of rice was predicted based on results from the nested PCR assay.

In light of the results obtained in this study, ergot diseases may provide the most representative model for the false smut disease cycle. Taxonomically, ergot fungi (Order: Sordariomycetes, Genus: Claviceps) are closely related to *U. virens*, and the teleomorph of *U. virens* was *Claviceps virens* until 2008 (Tanaka et al., 2008). Ergot fungi typically initiate disease with sexual spores (ascospores) formed after sclerotia in soil germinate in spring or early summer and produce stroma containing perithecia. Ascospores are carried from the soil surface to nearby inflorescences of susceptible species of Poaceae, germinate, and replace the developing grain with a sclerotium (analogous to a false smut spore ball). Then, abundant microconidia are

produced, which are either carried by wind or rain splash to adjacent plants, or are trapped in a sugar-rich secretion termed ‘honeydew,’ which attracts insects that subsequently serve as vectors of spore dissemination. As with false smut of rice, most ergots only affect a few spikelets per infected ear. At the end of the growing season, many ergot sclerotia dislodge from ears and are deposited on the ground (as is also the case for false smut balls), where they overwinter and become metabolically active again in spring or early summer, thus initiating a subsequent season’s disease cycle.

In summary, this study is the first in which confocal microscopy was applied to compare and contrast infection of the inflorescence vs. roots in the context of false smut of rice. The apparent inability of *U. virens* to penetrate roots or colonize the SAM after direct injection in the coleoptile, as well as the absence of the pathogen from rachis or pedicel tissue in rice heads bearing spore balls, suggests that systemic infections are rare and/or play a secondary role in the disease cycle of false smut, or that systemic infection simply does not exist in this pathosystem. The findings in this study will guide future experiments to explore the possibility of systemic infection in false smut, and highlight the need for more robust molecular detection assays for *U. virens*.

Chapter IV: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The research presented in this thesis has provided a valuable first step towards understanding fundamental aspects of the false smut disease cycle, and making future progress towards a molecular-level understanding of the disease. Anecdotally, false smut has been labeled as a difficult disease to study due to the slow growth of the pathogen in culture and the lack of a straightforward inoculation technique in controlled conditions, such as the greenhouse. This project was designed to provide new molecular resources to study false smut, as well as novel insight into the infection process. Both of those goals were achieved, and major findings are discussed below:

1. *U. virens* has unrecognized potential as a tractable model for molecular genetics of pathogenesis. With the *Agrobacterium*-mediated transformation protocol developed in this thesis, the fungus shows a high transformation frequency. *U. virens* can be grown in culture, although slowly compared to many other fungi. Importantly, it is possible to get full expression of false smut symptoms in greenhouse conditions with a GFP-expressing isolate. Moreover, strain UVLN appears to produce ustiloxin A in greenhouse inoculations, and at a high enough level that it can be detected in a single spore ball. With these resources, significant progress can be made to understand the molecular interactions involved in pathogenesis and host resistance. What is needed most immediately is a draft genome sequence of the pathogen and genetic resources such as collections of tagged, insertional mutants. Considering the decreasing cost of sequencing and the high transformation frequency with *A. tumefaciens*, both of these needs can realistically be met in the relatively near future.

2. Whether *U. virens* is capable of infecting root tissues is highly questionable. Although many Ascomycete plant pathogenic fungi can infect roots of Graminae crops to initiate systemic infections, there is no published evidence of *U. virens* being able to do so, and no evidence in support of this idea was obtained during the course of the research described in this thesis. Therefore, it is questionable whether root infection is a plausible mechanism through which a systemic false smut infection could be initiated. Based on this information, if systemic infections are indeed associated with false smut of rice, they are more plausibly initiated by infection of asymptomatic, healthy seed, infection of the coleoptile shortly after germination, or possibly an unexplored mechanism such as insect transmission (Wandeler et al., 2008). It is important to note that negative results can support a hypothesis but cannot disprove an alternative hypothesis; therefore, it is impossible to say that root infections never occur during false smut. However, it is clear that when given several reasonable opportunities to infect roots, *U. virens* declined, whereas the known root-infecting pathogen *M. oryzae* did not hesitate.

3. The nested PCR assay widely used to explore systemic infections by *U. virens* appears to have serious flaws regarding specificity. The source of the apparent lack of specificity does not matter as much as the implications. If false positives are possible, or even common, as this study would suggest, then the interpretation of results using the nested PCR assay must be called into question. A lack of specificity of the nested PCR assay could explain some of the conflicting results recently published in the literature, especially the lack of histopathological evidence for systemic infection in greenhouse- or naturally-inoculated rice plants showing clear symptoms of false smut (spore balls), yet the consistent detection of the pathogen in asymptomatic, vegetative tissues of rice plants that may or may not develop spore balls upon flowering. This discrepancy highlights the need for more sensitive and specific molecular

detection assays for *U. virens*, and also indicates that multiple, corroborating techniques to detect the pathogen in rice tissues should be employed whenever possible to overcome concerns about error and to provide a convincing, definitive answer regarding the cycle of infection.

4. Irrespective of whether or not systemic infections are a component of the false smut disease cycle, spikelet infections from airborne conidia are likely to play a much more significant role. The results presented in this thesis demonstrate that 1) inoculation of the developing inflorescence with a tagged (GFP-expressing) isolate can produce spore balls than are unambiguously associated with the inoculating strain, and 2) there is no evidence of systemic infection e.g., demonstrable fungal colonization of the rachis or pedicel bearing a spikelet containing a spore ball. The lack of evidence for systemic infection is consistent with the findings of other groups investigating false smut (Kulkarni and Moniz, 1975; Tang et al., 2012). Aerial infection of developing spikelets is supported by epidemiological studies monitoring the airborne *U. virens* spore load over rice fields in India, which reported highest seasonal spore counts during heading (Sreeramulu and Vittal, 1966). Thus, future management efforts will likely be most effective if focused on reducing aerial infections during heading through the use and application of effective chemical controls, the implementation of cultural controls that reduce the aerial spore load, and the increased deployment of resistant cultivars and hybrids as they become available.

5. Ustiloxin mycotoxins may be an underappreciated threat to rice production in Arkansas. Although the research in this thesis did not provide an exhaustive survey of ustiloxin production in spore balls and in defined media by Arkansas isolates, detectable levels were observed in spore balls collected within the state. Additionally, the GFP-expressing strain UVLN is capable of making ustiloxinA during greenhouse inoculations of rice. Whether or not

ustiloxin contamination of rice is a widespread issue in Arkansas bears further research in the future.

Future directions

Looking forward, areas of immediate need in false smut research include a more robust molecular detection assay and a more thorough assessment of the risks associated with ustiloxin contamination of Arkansas rice. In the longer term, a more thorough and replicated analysis of systemic vs. spikelet infection will help to clarify the false smut disease cycle and thus guide future disease management research and recommendations. Ultimately, sustainable control of false smut will benefit greatly from a more fundamental understanding of the plant-fungal interactions underlying the disease, which will come from functional genomic studies of pathogenicity in *U. virens* and resistance in rice.

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APPENDIX

Table 1. Data from root-dip inoculation experiments.

Experiment number	Cultivars	Time observed (dai)	Number of plants inoculated	% Plants infected		
				Root dip	*In vitro* inoculation	Infested soil
1	Wells	3	10	20	0	10
2	Wells	7	10	60	60	20
3	Wells	11	10	70	80	40

Table 2. Data from inflorescence inoculation experiments.

Experiment number	Cultivars	Number of plants inoculated	*U. virens* strain	Number of spore balls
1	Wells	25	WT	0
	Francis	25	WT	2
	Jupiter	15	WT	0
2	Wells	15	WT	2
	Francis	15	WT	0
	Jupiter	15	WT	0
3	Wells	15	WT	0
	Francis	15	WT	0
	Jupiter	15	WT	0
4	Wells	15	UVLN	3
	Francis	10	UVLN	2
	Jupiter	10	UVLN	0
5	Wells	20	UVLN	0
	Francis	20	UVLN	0
	Jupiter	20	UVLN	0
6	Wells	15	UVLN	2
	Francis	0	UVLN	0
	Jupiter	25	UVLN	0
7	Wells	20	UVLN	2
	Francis	0	UVLN	0
	Jupiter	0	UVLN	0
8	Wells	20	UVLN	1
	Francis	0	UVLN	0
	Jupiter	15	UVLN	0

Table 3. Data from nested PCR experiments.

Template	Number of repeated experiments	Nested PCR	Sequencing results
Rice inoculated with *U. virens*			
Wells seed	4	+	*U. virens*
Jupiter seed	4	+	*U. virens*
Francis seed	4	+	*U. virens*
Wells stem	4	+	*U. virens*
Jupiter stem	4	+	*U. virens*
Francis stem	4	+	*U. virens*
Rice uninoculated with *U. virens*			
Wells seed	4	+	*U. virens*
Jupiter seed	4	+	*U. virens*
Francis seed	4	+	*U. virens*
Wells stem	4	+	*U. virens*
Jupiter stem	4	+	*U. virens*
Francis stem	4	+	*U. virens*
Fungal genomic DNA			
*U. viren*s	*4*	+	
Alternaria alternata	*4*	+	*U. virens*
Fusarium graminearum	4	+	*U. virens*
Fusarium verticillioides	4	+	*U. virens*
Negative control			
water template	4	-	