MOLECULAR GENETIC ANALYSIS OF GERMINATION AND SPORULATION IN TWO OBLIGATE PHYTOPATHOGENS

by

LORINA G. BAKER

(Under the Direction of SARAH F. COVERT)

ABSTRACT

Two obligate fungal phytopathogens, *Cronartium quercuum* f. sp. *fusiforme* and *Ustilago maydis* were used to study how obligate pathogens sense and respond to host stimuli. Depending upon the surface where *C. q. fusiforme* basidiospores alight on, they germinate directly by sending out a long thin germ tube or indirectly by sending out a short thick germ tube and producing a secondary basidiospore. I altered surface hydrophilicities to test if this characteristic affected the germination response of *C. q. fusiforme* basidiospores. I found that altered surface hydrophilicity did have an affect on the basidiospore germination fate and that there was a critical threshold between 42% and 54% surface wettability where *C. q. fusiforme* basidiospores switched germination type. To ascertain what genes are involved in the different germination types, two expression libraries enriched for genes expressed during direct or indirect germination were made. The combined expression libraries contained one hundred and eighty unique cDNAs of which fourteen were differentially expressed between the two germination states. Proteins represented by the genes within the expression libraries may be determinants of the basidiospores' response to a favorable substrate or environment. The second part of my dissertation focuses on how obligate phytopathogens, such as *Ustilago maydis,* switch from *in planta* vegetative growth to reproductive growth. I identified three genes encoding putative

regulators of G-protein signaling *rgs1*, *rgs2*, and *rgs3* that could be involved in the switch from vegetative growth to sporulation. Deletion of *rgs1* resulted in hyphal growth on minimal medium agar, reduced pathogenicity, and reduced *in planta* sporulation. I determined that the hyphal growth phenotype was due to the Δ*rgs1* strain's inability to deactivate the Gα subunits, Gpa1 and Gpa4. This suggested that both Gpa1 or Gpa4 are negative regulators of *in planta* sporulation. This was verified using a sporulation time course experiment where I found that the deletion of *gpa1* and *gpa4* caused hyper-sporulation *in planta*. The combined data supports a model where multiple pathways control of *in planta* sporulation and that under the appropriate conditions Rgs1 regulates these pathways.

INDEX WORDS: *Cronartium quercuum* f. sp. *fusiforme*, *Ustilago maydis*, Obligate phytopathogen, fungi, Basidiomycete, surface wettabilities, basidiospore germination, *in planta* sporulation, Rgs1, Gα subunit, RGS, Gpa1, Gpa4

MOLECULAR GENETIC ANALYSIS OF GERMINATION AND SPORULATION IN TWO OBLIGATE PHYTOPATHOGENS

by

LORINA G. BAKER

B.S., UNIVERSITY OF CENTRAL OKLAHOMA, 1997

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2005

MOLECULAR GENETIC ANALYSIS OF GERMINATION AND SPORULATION IN TWO OBLIGATE PHYTOPATHOGENS

by

LORINA G. BAKER

Major Professor:	Sarah F. Covert
Committee:	Mary Bedell Jeff Dean Scott Gold Michelle Momany Pauline Spaine

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2005

DEDICATION

This dissertation is dedicated in memory of my husband,

Delbert Nathan Bennett

(1958-2003)

ACKNOWLEDGEMENTS

I want to acknowledge my advisor, Sarah Covert, for her guidance and support while I undertook and completed this research project; the members of my committee, Mary Bedell, Jeff Dean, Scott Gold, Michelle Momany, and Paula Spaine; and the members of the Fungal Molecular Biology Group at the University of Georgia, who readily gave advice and suggestions that improved me as a scientist. Most importantly, I want to acknowledge my family, Christina Corcorran and Autumn Jordan (my daughters), Vivian Baker (my mother), Bob and Nancy Long (my father and his wife), and friends who are too numerous to list for their moral support while I pursued my doctoral degree.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTER

1 Introduction and Literature Review 1

General background of *C. q. f. sp. fusiforme* 1

Substrate surface features and their effects on fungal spore germination 3

Direct and indirect germination on substrates 4

Sporulation signal transduction pathway(s) 5

Sporulation and host dependence 7

U. maydis and *in planta* sporulation 8

References 10

2 The Effect of Surface Wettability on Germination Fate and Gene Expression in *Cronartium quercuum* f. sp. *fusiforme* Basidiospores 22

Abstract 23

Introduction 24

Results and Discussion 26

Conclusion 31

Experimental Procedures....32
References....38
3 A regulator of G-protein signaling in *Ustilago maydis* promotes sporulation *in planta* and regulates filamentous growth....50
Abstract....51
Introduction....52
Results....55
Discussion....61
Experimental Procedures....67
References....74
4 Conclusions....94
References....97

LIST OF TABLES

Page

Table 2.1: Summary of cDNAs Differentially Expressed in Directly and Indirectly Germinating Basidiospores 43

Table 3.1 Pathogenicity Phenotypes 14 Days Post Inoculation.....79

LIST OF FIGURES

Page

Figure 1.1 Locations of forest inventory and analysis plots in the southeastern United States 16

Figure 1.2 *C .q. fusiforme* life cycle....18

Figure 1.3 Wavy hyphae phenotype in *S. commune thn1* mutant and. *C. q. fusiforme* culture....20

Figure 2.1 *C. q. fusiforme* direct (a) and indirect (b) basidiospore germination....44

Figure 2.2 Wettability of tested surfaces and rates of *C. q. fusiforme* basidiospore germination on them....46

Figure 2.3 Classification of cDNA contigs into categories based on BLAST search results....48

Figure 3.1 Structural comparison of RGS proteins in *A. nidulans* and *U. maydis* 80

Figure 3.2 Phenotypes of selected *U. maydis* strains on various solid media 82

Figure 3.3 Effect of *rgs1* deletion on mating....84

Figure 3.4 Effect of *rgs1* deletion on teliosporgenesis 86

Figure 3.5 Role of Gpa1 and Gpa4 in temporal patterns of *in planta* sporulation 88

Figure 3.6 Phenotypic effect and transcript abundance in *rgs1* over-expression 90

Figure 3.7 Model of Rgs1 regulatory networks in *U. maydis*....92

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

My interests are in understanding how obligate fungal phytopathogens respond to their environment or host signals. For instance, how does a fungal spore sense and "know" when it has found an appropriate host? Once the pathogen has broken dormancy, what genes are expressed leading to host infection? If germination occurs on an inappropriate surface, what genes are up-regulated for survival and movement to another surface? Lastly, how does that pathogen regulate *in planta* sporulation, a process that only occurs when the obligate pathogen is growing in association with its host? To answer these questions, I used two obligate fungal phytopathogens, *Cronartium quercuum* f. sp. *fusiforme* and *Ustilago maydis.*

General background of Cronartium quercuum f. sp. *fusiforme*

C. q. fusiforme is the causative fungal agent of fusiforme rust disease. This disease is characterized by the formation of spindle-shaped galls that weaken and reduce the value of the infected trees. Fusiform rust is the most destructive forest disease of southern pines in the southeastern United States (Powers *et al*. 1981; Schmidt 1998). As of 1997, over 13.4 million acres in the south had greater than or equal to 10 percent or mare of the slash and loblolly pines infected with *C. q. fusiforme* (Figure 1.1) (Starkey *et al*. 1997). *C. q. fusiforme* infected trees have an increased risk to damage by other fungi, insects, breakage due to high winds, and seedling mortality. Monetary losses due to decreased wood value and early tree death caused by *C. q. fusiforme* infection are estimated to cost up to $135 million

annually (Cubbage *et al*. 2000). Even with the implementation of improved silviculture, rust resistance breeding programs (Powers *et al*. 1981), and the identification of resistance genes in pine (Wilcox *et al*. 1995), *C. q. fusiforme* infection is still at epidemic proportions (Powers *et al*. 1981; Cubbage *et al*. 2000).

Compared to annual crops, the longevity of commercial pine crops (up to 35 years in the field) poses many challenges to remain disease free. Annual crops can easily be sprayed with fungicides, pesticides, and herbicides, but it is not cost effective to treat pine trees once they are lifted from the nursery beds for planting. Also, pine management practices such as rapid growing pine trees, are associated with higher rust infection rates (Boggess and Stahelin 1948; Dinus and Schmidtling 1971; Miller 1972; Hollis *et al*. 1977; Burton *et al*. 1985).

C. q. fusiforme is a heteroecious rust meaning that it alternates between two hosts, spending part of its lifecycle on pine, *Pinus spp.*, and part on oak, *Quercus spp.* Its life cycle is complex, with five types of spores being produced as it alternates between the two hosts (Figure 1.2). Two spore stages, pycniospores and aeciospores are produced on the pine host. In the fall, *C. q. fusiforme* haploid pycniospores form in droplets on the outer surface of the gall. The fungus then over-winters in the pine host as mycelium. Early in the spring large masses of dikaryotic aeciospores are produced on the surface of the gall, this is the infectious spore stage capable of infecting young succulent oak leaves.

Three spore stages in the *C. q. fusiforme* life cycle, urediniospores, teliospores, and basidiospores, are produced on oak leaves. The dikaryotic urediniospores are produced on the oak leaves. The urediniospore stage is the only spore capable of reestablishing infection on its host and is most notable in other rust fungi as the self-repeating stage involved in creating epidemics. The teliospores develop from within the same lesions caused by the urediniospores.

Within the dikaryotic teliospores, karyogamy and the start of meiosis take place. When environmental conditions such as humidity and temperature are favorable, meiotic division is completed and four basidiospores are produced from each teliospore (Mims *et al*. 1996). The basidiospores are the spore stage capable of infecting pine and starting the disease cycle again.

My main research goals have been to gain a better understanding of the relationship between *C. q. fusiforme* infection and its pine host. In particular, I wanted to know how substrate hydrophobicity influenced the germination fate of *C. q. fusiforme* basidiospores. What genes are up-regulated for infection on a hydrophobic surface? Also, what genes were being up-regulated in response to landing on a hydrophillic surface? Because obligate phytopathogens only sporulate when growing within their hosts, how is the signal transduction pathway that leads to sporulation controlled or influenced by the host?

Substrate surface features and their effects on fungal spore germination

The recognition of specific host surface features, such as topography, overall chemical composition, and hydrophilicity, are important in the successful germination and differentiation of rust fungal spores (Hoch and Staples 1991; Choi *et al*. 1998). The germ tubes of certain rust fungal species are able to sense and respond to surface topography such as depressions, cuticular ridges, and the height of stomatal openings on the epidermis of the leaf (Hoch and Staples 1991). For example, *Uromyces appendiculatus* uses the topographical signals inherent in the stomatal ridges of its host plant, *Phaseolus vulgaris*, to determine appressoria differentiation (Kwon *et al*. 1991). Plant ripening hormones, calcium, and potassium, are examples of the wide range of chemical signals to which germinating and differentiating fungi respond (Hoch and Staples 1991; Shaw and Hoch 1999). Chaky *et al* (2001) reported that in *Colletotrichum graminicola* spore

germination fate is influenced by surface hydrophilicity. Likewise, in *C. q. fusiforme* it has been observed that the basidiospores germinate "directly" or "indirectly" depending upon different substrate surfaces.

Direct and indirect germination on substrates

C. q. fusiforme basidiospores germinate when environmental conditions ,such as temperature and humidity, are favorable. Basidiospores can germinate either directly or indirectly depending upon the surface on which they land or depending upon the treatment of the spores. Direct germination is characterized by the basidiospore producing a long thin germ tube. Direct germination on the host surface can lead to infection and the establishment of disease. In contrast, a short thick germ tube characterizes indirect germination. Indirect germination can lead to the production of a secondary basidiospore in an inhospitable environment (Miller 1966). This type of germination has been shown to occur in other pathogens such as *Gymnosporangium juniperi-virginianae* (Mims and Richardson 1990).

C. q. fusiforme basidiospores that land on pine tissue can germinate directly and a majority of the time they penetrate into pine tissues without forming an appressorium (Miller 1980; Gray 1981). Once the fungus has established an infection, the mycelium grows into the wood surrounding the stem. A gall is formed and the characteristic wide, septate hyphae can be found growing intercellularly throughout the gall tissue (Jackson and Parker 1958; Jewell *et al*. 1962). A distinctive morphological feature of *C. q. fusiforme* infection in pine galls is the formation of monokaryotic haustoria (Jewell *et al*. 1962). The haustoria are specialized structures that penetrate the host cell wall while leaving the cell membrane intact. The haustoria are frequently turned toward the cell's nucleus and can be in such close proximity that the cell

nucleus becomes indented in a shape corresponding to the end of the haustorium (Jewell *et al*. 1962).

Direct and indirect germination have been mimicked in the laboratory environment on polystyrene and glass, respectively (Spaine and Kaneko 1993; Spaine *et al*. 1995; Spaine and Kaneko 1996). At the outset of this study it was unknown how the physical characteristics of different substrates affected the *C. q. fusiforme* basidiospore germination fate or the gene regulation of these events. We hypothesized that the surface hydophilicity differences between polystyrene and glass was a determinant in *C. q. fusiforme* basidiospore germination type. Therefore, I investigated how changing the surface wettability or the hydrophilicity of polystyrene and glass affected the percent total germination and percent direct germination for a range of substrate wettabilities and their gene precursors (Chapter 2). Although many studies have described the morphological characteristics typical of *C. q. fusiforme* basidiospore infection of pine, until now, genes that are involved in direct germination and thus, code for proteins implicated in host recognition and infection, have not been described. During my study I have isolated seventy-two expressed genes that are unique to directly germinating basidiospores. Likewise, this is the first molecular study that describes genes that code for indirect germination, which may include genes that are up regulated in response to germination on an unfavorable surface and genes that code for proteins involved in basidiospore formation. I have isolated eighty-seven expressed genes that are unique to indirectly germinating basidiospores (Chapter 2).

Sporulation signal transduction pathway(s)

Obligate phytopathogens depend upon their host for survival and reproduction. Because of this unique dependence, it has been proposed that obligate pathogens intercept signals from

the host to initiate different growth events (Banuett 1992). *C. q. fusiforme* only produces spores only when growing in association with its host plants, we were interested in developing a method to stimulate sporulation of *C. q. fusiforme* in culture and by-pass the need for growth within the host. Interestingly, in axenic culture, *C. q. fusiforme* hyphae display a wavy morphology similar to that of the *thn1* mutant in *Schizophyllum commune* (Figure 1.3). The *thn1* gene encodes a putative reuglator of G-protein signaling (RGS) and the mutant was identified as a spontaneous morphological change produced by an active transposon insertion (Fowler and Mitton 2000). The Thn1 protein is an ortholog of *Aspergillus nidulans* FlbA, which regulates a pathway controlling the switch from vegetative growth to asexual sporulation through the negative regulation of the Gα protein, FadA (Lee and Adams 1994; Yu *et al*. 1996; Hicks *et al*. 1997; Yu *et al*. 1999). FlbA negatively regulates FadA by increasing the hydrolysis rate of GTP to GDP, which shuts down the signaling pathway and allows asexual sporulation (Dohlman and Thorners 1997; Adams *et al*. 1998; De Vries and Farquhar 1999). Mutants carrying a recessive, loss-of-function, *flbA* mutation, exhibit vegetative growth with autolysis and no sporulation (Yu *et al*. 1996; Adams *et al*. 1998). Because *S. commune* does not sporulate in culture, the loss of Thn1 function cannot have the same sporulation phenotype as the *A. nidulans ΔflbA* phenotype (Fowler and Mitton 2000). However, homoallelic *S. commune thn1* mutants are unable to produce sexual fruiting bodies from a compatible mating (Fowler and Mitton 2000). Loss of function of *thn1* in *S. commune* also results in a reduction in mRNAs encoding hydrophobins (Wessels 1991). These hydrophobins are thought to play a role in the hyphal aggregation that initiates fruit body formation (Wessels 1997). Therefore, *thn1* may regulate a G-protein signaling pathway that is involved in regulation of hydrophobin expression and, thus, sexual spore formation (Fowler and Mitton 2000). Recently, a second RGS Gα protein pair, RgsA and

GanB, involved in the regulation of sporulation in *A. nidulans* was described (Han *et al.* 2004). This is the first evidence that a fungus may use more than one RGS-Gα protein pair to regulate sporulation.

In fungi the Gα subunits of the heterotrimeric G proteins play integral roles in several signal transduction pathways, including those regulating asexual sporulation, sexual sporulation, mating, pheromone response pathways, pathogenicity, and cell wall deposition (Banuett 1992; Bölker 1998; Coca *et al*. 2000). These pathways are activated by the binding of an effector in the form of a chemical, hormone, or protein to a seven transmembrane bound receptor. This causes a conformational change in the associated heterotrimeric G-protein and GDP is exchanged for GTP on the Gα subunit. Upon disassociation and activation of the Gα subunit by the exchange of GDP for GTP, the Gα and Gγβ subunits are free to regulate the signaling cascades. The Gα subunit has an intrinsic GTP-ase activity that causes the active, GTP-bound form to hydrolyze GTP to GDP. Once GTP is hydrolyzed to GDP the Gα subunit returns to the inactive state. This intrinsic mechanism prevents the heterotrimeric G-protein from continuously signaling once it has been activated (Bölker 1998; De Vries and Farquhar 1999). Although Gα subunits display a measurable intrinsic rate of GTP hydrolysis, the turnover number found *in vitro* with purified Gα does not account for the rate at which signaling is transmitted *in vivo* (Dohlman and Thorners 1997). This finding led to the identification of RGS proteins (Dohlman and Thorners 1997).

Sporulation and host dependence

Because there are limited genetic resources available for studying *C. q. fusiforme*, we decided to study the signals/cues that are vital for the switch from vegetative to reproductive

growth in the genetically tractable basidiomycete model, *Ustilago maydis*. *U. maydis* is a semi-obligate phytopathogen of corn, *Zea mays*, which depends upon association with its host for sexual reproduction during one phase of its lifecycle. The disease caused by *U. maydi*s is known as corn smut, which is characterized by the formation of galls that can produce 2.5-6 billion teliospores/cm^3 gall tissue (Christensen 1963). An additional benefit to using *U. maydis* to study *in planta* sporulation was that a Gα protein involved in *in planta* sporulation, Gpa3, had already been described (Regenfelder *et al*. 1997; Krüger *et al*. 2000).

U. maydis and in planta sporulation

The *U. maydis* genome encodes four Gα proteins, Gpa1, Gpa2, Gpa3, and Gpa4 (Regenfelder *et al*. 1997). Mutants with separate disruptions in the *gpa1*, *gpa2*, and *gpa4* genes were reported to have no discernible *in vitro* mating or *in planta* phenotypes. In contrast, the *gpa3* deletion mutant was unable to mate and grew filamentously in liquid medium. Also, a constitutively active *gpa3* allele, *gpa3Q206L*, reduced gall formation and abolished teliospore production (Regenfelder *et al*. 1997; Krüger *et al*. 2000). In *U. maydis,* the switch from vegetative growth to reproductive growth (the production of teliospores) occurs within the gall. Teliospore formation within the gall consists of preliminary stages that include the rounding and swelling of the hyphal tips, leading to hyphal fragmentation and the release of diploid spore precursors. Functional Rum1 and Hda1 are necessary for successful hyphal swelling and rounding (Quadbeck-Seeger *et al*. 2000; Reichmann *et al*. 2002). These, along with Fuz1, which is involved in hyphal fragmentation, are essential for teliospore formation (Banuett 1996). To complete teliospore formation after hyphal fragmentation a functional Hgl1 protein is necessary (Durrenberger *et al*. 2001).

Two other pathways, the cAMP and mitogen-activated protein kinase (MAPK) cascades, are involved in *U. maydis in planta* sporulation. The cAMP pathway is implicated in sporulation, because the catalytic subunit of protein kinase A (PKA), Adr1, is necessary for *U. maydis* pathogenicity and gall formation (Durrenberger *et al*. 1998). The components of the MAPK cascade, Ubc4/Kpp4, Ubc5/Fuz7, and Ubc3/Kpp2, are necessary for successful mating and without them the infectious dikaryon or the galls cannot be formed (Kahmann and Kamper 2004). The gene product of *fuz7* is also essential for teliospore germination and tumor induction/gall formation (Banuett 1994).

It is unknown how *U. maydis* regulates the switch from vegetative growth to *in planta* sporulation or what host signals, if any, it responds to for stimulation of the switch. My research focused upon how *U. maydis* uses the RGS ortholog to *A. nidualans* FlbA, Rgs1, during *in planta* growth. I discovered that like *A. nidulans*, *U. maydis* uses more than one Gα protein to control sporulation, and that Rgs1 has the ability to regulate at least two Gα proteins, Gpa1 and Gpa4, involved in *in planta* sporulation, thus allowing the rapid switch from vegetative growth to reproductive growth.

References

Adams, T. H., J. K. Wieser, *et al*. (1998). "Asexual Sporulation in *Aspergillus nidulans*." Microbiology and Molecular Biology Review **62**(1): 35-54.

Alexopoulos, C. J., C. W. Mims, *et al*. (1996). Introductory Mycology. New York, John Wiley & Sons Inc.

Banuett, F. (1992). "*Ustilago maydis*, the delightful blight." Trends in Genetics **8**(5): 174-180.

Banuett F, H. I. (1994). "Identification of Fuz7, a *Ustilago maydis* MEK/MMAPKK homolog required for a-locus dependent and -independent steps in the fungal life cycle." Genes and Development **8**: 1367-1378.

Banuett, F. a. I. H. (1996). "Discrete developmental stages during teliospore fromation in the corn smut fungus, *Ustilago maydis*." Development **122**: 2965-2976.

Boggess, W. R. and R. Stahelin (1948). "The Incidence of Fusiform Rust in Slash Pine Plantations Receiving Cultural Treatments." Journal of Forestry **46**: 683-85.

Bölker, M. (1998). "Sex and Crime; Heterotrimeric G Proteins in Fungal Mating and Pathogenesis." Fungal Genetics and Biology **25**: 143-156.

Burton, J. D., E. Shoulders, *et al*. (1985). "Incidence and Impact of Fusiform Rust Vary with Silviculture in Slash Pine Plantations." Forest Science **31**(3): 671-80.

Chaky, J., K. Anderson, M. Moss, and L. Vallancourt (2001). "Surface Hydrophobicity and Surface Rigidity Induce Spore Germination in *Colletotrichum graminicola*." Phytpathology(91): 558-564.

Choi, W.-B., S.-H. Kang, *et al*. (1998). "Cyclic AMP restores appressorium formation inhibited by polyamines in *Magnaporthe grisea*." Phytopathology **88**(1): 58-62.

Christensen, J. J. (1963). Maize Smut Caused by *Ustilago maydis*. Monograph no. 2, Saint Paul: American Phytopathological Society.

Coca, M. A., B. Damsz, *et al*. (2000). "Heterotrimeric G-porteins of a filamentous fungus regulate cell wall composition and susceptibility to a plant PR-5 protein." The Plant Journal **22**(1): 61-69.

Cubbage, F. W., J. M. Pye, *et al*. (2000). "An Economic Evaluation of Fusiform Rust Protection Research." Southern Journal of Applied Forestry **24**(2): 77-85.

De Vries, L. and M. G. Farquhar (1999). "RGS proteins: more than just GAPS for heterotrimeric G proteins." Trends in Cell Biology **9**: 138-144.

Dinus, R. J. and R. C. Schmidtling (1971). Fusiform rust in loblolly and slash pines after cultivation and fertilization. New Orleans, LA, Southern Forest Experimental Station**:** 1-10.

Dohlman, H. G. and J. Thorners (1997). "RGS Proteins and Signaling by Heterotrimeric G Proteins." The Journal of Biological Chemistry **272**(7): 3871-3874.

Durrenberger, F., R. D. Laidlaw, *et al*. (2001). "The *hgl1* gene is required for dimorphism and teliospore formation in the fungal pathogen *Ustilago maydis*." Molecular Microbiology **41**(2): 337-348.

Durrenberger, F., K. Wong, *et al*. (1998). "Identification of a cAMP-dependent protein kinase catalytic subunit required for virulence and morphogenesis in *Ustilago maydis*." Proceedings of the National Academy of Sciences **95**(10): 5684-5689.

Fowler, T. J. and M. F. Mitton (2000). "Scooter, a new active transposon in *Schizophyllum commune*, has disrupted two genes regulating signal transduction." Genetics **156**(4): 1585-94.

Gray, D. J., H. V. Amerson, and C. G. Van Dyke (1981). "An ultrastructural comparison of monokaryotic and dikaryotic haustoria formed by the fusiform rust fungus *Cronartium quercuum* f.sp *fusiforme*." Canadian Journal of Botany **60**: 2914-2922.

Han, K.-H., Jeong-Ah Seo and Jae-Hyuk Yu (2004). "Regulators of G-protein signaling in *Aspergillus nidulans*: RgsA downregulates stress response and stimulates asexual sporulation through attenuation of GanB (Galpha) signalling." Molecular Microbiology **53**(2): 529-540.

Hicks, J. K., J.-H. Yu, *et al*. (1997). "*Aspergillus* sporulation and mycotoxin production both inactivation of the FadA G alpha protein-dependent signaling pathway." European Molecular Biology Organization Journal **16**(16): 4916-4923.

Hoch, H. C. and R. C. Staples (1991). Signaling for Infection Structure Formation in Fungi. The Fungal Spore and Disease Initiation in Plants and Animals. Hoch and Staples. New York, Plenum press**:** 555.

Hollis, C. A., J. E. Smith, *et al*. (1977). Genotype X Mineral Nutrient Interactions With Fusiform Rust Incidence in Slash Pine. 14th Southern Forest Tree Improvement Conference.

Jackson, L. W. R. and J. N. Parker (1958). "Anatomy of Fusiform Rust Galls on Loblolly Pine." Phytopathology **48**(58): 637-640.

Jewell, F. F., R. P. True, *et al*. (1962). "Histology of Cronartium fusiforme in Slash Pine Seedlings." Phytopathology **52**: 850-858.

Kahmann, R. and J. Kamper (2004). "*Ustilago maydis*: how its biology relates to pathogenic development." New Phytologist **164**(1): 31-42.

Krüger, J., Gabriel Loubradou, Gerhard Wanner, Erika Regenfelder, Michael Feldbrügge, and Regine Kahmann (2000). "Activation of the cAMP Pathway in Ustilago maydis Reduces

Fungal Proliferation and Teliospore Formation in Plant Tumors." Molecular Plant-Microbe Interactions **13**(10): 1034-1040.

Kwon, Y. H., H. C. Hoch, *et al*. (1991). "Initiation of appressorium formation in *Uromyces appendiculatus*: organization of the apex, and the responses involving microtubules and apical vesicles." Canadian Journal of Botany **69**: 2560-73.

Lee, B. N. and T. H. Adams (1994). "Overexpression of flbA, an early regulator of *Aspergillus* asexual sporulation, leads to activation of brlA and premature initiation of development." Molecular Microbiology **14**(2): 323-334.

Miller, T. (1972). "Fusiform Rust in Planted Slash Pines: Influence of Site Preparation and Spacing." Forest Science **18**: 70-75.

Miller, T., Robert F. Patton, and Harry R. Powers, Jr. (1980). "Mode of Infection and Early Colonization of Slash Pine Seedlings by *Cronartium quercuum* f.sp *fusiforme*." Phytopathology **70**(12): 1206-1208.

Miller, T. a. R. W. R. (1966). "Abjection of Secondary Sporidia of *Cronartium fusiforme*." Phytopathology **56**: 1326.

Mims, C. W., K. A. Liljebjelke, *et al*. (1996). "Ultrastructure of telia and teliospores of the rust fungus *Cronartium quercuum* f.sp. *fusiforme*." Mycologia **88**(1): 47-56.

Mims, C. W. and E. A. Richardson (1990). "Ultrastructure of secondary spore formation in the rust *Gymnosporangium Juniperi-virginianae*." Mycologia **82**(2): 236-242.

Powers, H. R., Jr., R. A. Schmidt, *et al*. (1981). "Current Status and Management of Fusiform Rust on Southern Pines." Annual Review Phytopathology **19**: 353-371.

Quadbeck-Seeger, C., G. Wanner, *et al*. (2000). "A protein with similarity to the human retinoblastoma binding protein 2 acts specifically as a repressor for genes regulated by the *b* mating type locus in *Ustilago maydis*." Molecular Microbiology **38**(1): 154-166.

Regenfelder, E., T. Spellig, *et al*. (1997). "G proteins in *Ustilago maydis*: transmission of multiple signals?" European Molecular Biology Organization Journal **16**(8): 1934-42.

Reichmann, M., A. Jamnischek, *et al*. (2002). "The histone deacetylase Hda1 from *Ustilago maydis* is essential for teliospore development." Molecular Microbiology **46**(4): 1169-1182.

Schmidt, R. A. (1998). Fusiform Rust Disease of Southern Pines: Biology, Ecology and Management. Florida, University of Florida**:** 1-85.

Shaw, B. D. and H. C. Hoch (1999). "The pycnidiospore of *Phyllosticta ampelicida*: surface properties involved in substratum attachment and germination." Mycological Research **103**(7): 915-924.

Spaine, P. C. and S. Kaneko (1993). "Spore Exudates and Other Factors Affecting Germination Type of *Cronartium quercuum* f.sp. *fusiforme* Basidiospores." Mycologia **85**(1): 51-61.

Spaine, P. C. and S. Kaneko (1996). "Nuclear Behavior During Basidiospore Germination in *Cronartium quercuum* f.sp. *fusiforme*." Mycologia **88**(6): 892-896.

Spaine, P. C., S. Kaneko, *et al*. (1995). "Fusiform Rust basidiospore and Urediniospore Germination on Host and Non-host Surfaces." In: Proceedings of the 4th IUFRO Rusts of Pine Working Party Conference, Tsukuba, Japan: 89-93.

Starkey, D. A., R. L. Anderson, *et al*. (1997). Monitoring Incidence of Fusiform Rust in the South and Change Over Time. Atlanta, GA, USDA**:** 1-29.

Wessels, J. G. H. (1991). "The *thn* mutation of *Schizophyllum commune*, which suppresses formation of aerial hyphae, affects expression of the *Sc3* hydrophobin gene." Journal of General Microbiology **137**: 2439-2445.

Wessels, J. G. H. (1997). Hydrophobins: Proteins that Change the Nature of the Fungal Surface. Advances in Microbial Physiology. R. K. Poole. San Diego, Academic Press. **38:** 1-45.

Wilcox, P. L., H. V. Amerson, *et al*. (1995). "Detection of a major gene for resistance to fusiform rust disease in loblolly pine by genomic mapping." Proceedings of the National Academy of Sciences **93**: 3859-3864.

Yu, J.-H., S. Rosén, *et al*. (1999). "Extragenic Suppressors of Loss-of Function Mutations in the *Aspergillus* FlbA Regulator of G-Protein Signaling Domain Protein." Genetics **151**: 95-107.

Yu, J.-H., J. K. Wieser, *et al*. (1996). "The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development." European Molecular Biology Organization Journal **15**(19): 5184-5190.

Figure 1.1 Locations of forest inventory and analysis plots in the southeastern United States. (a) slash pine or (b) loblolly pine forest with greater than or equal to 10% fusiform rust infection as of 1997 (Starkey *et al*. 1997).

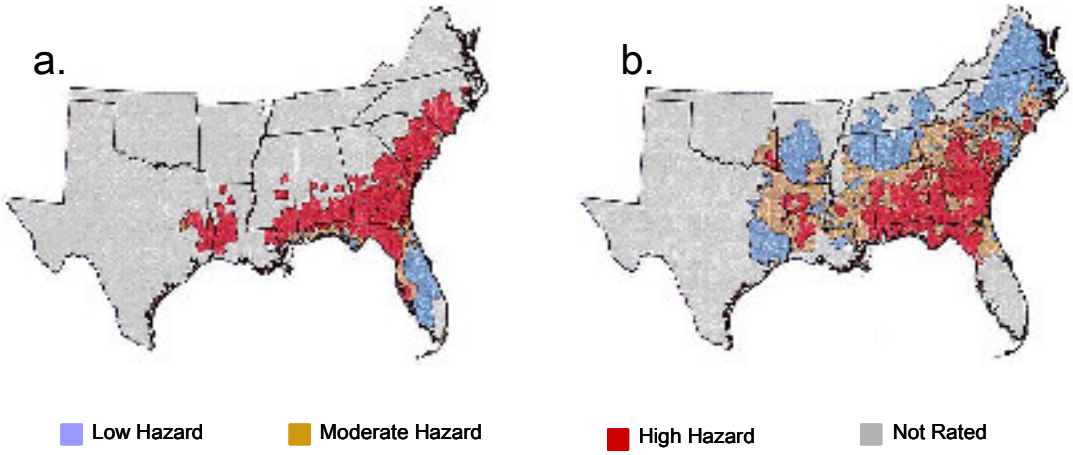

a.
b.
Low Hazard
Moderate Hazard
High Hazard
Not Rated

Figure 1.2 *C .q. fusiforme* life cycle. *C .q. fusiforme* is a basidiomycete, in the order *Uredinales*. Basidiomycete fungi exist primarily in the haploid (N) or dikaryotic (N+N) state and have only a short diploid (2N) phase immediately before meiosis (Alexopoulos *et al*. 1996). *C. q. fusiforme* is an obligate, biotrophic fungus that requires two living hosts for its complete life cycle. It has five distinct sporulation stages, of which 2 occur on pine, *Pinus spp*., and three occur on the oak, *Quercus spp*.

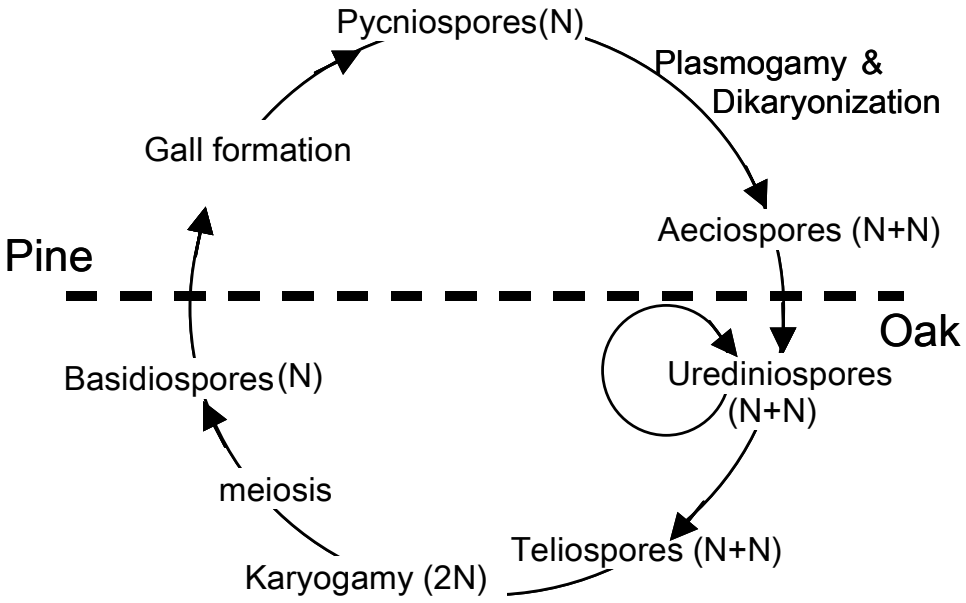
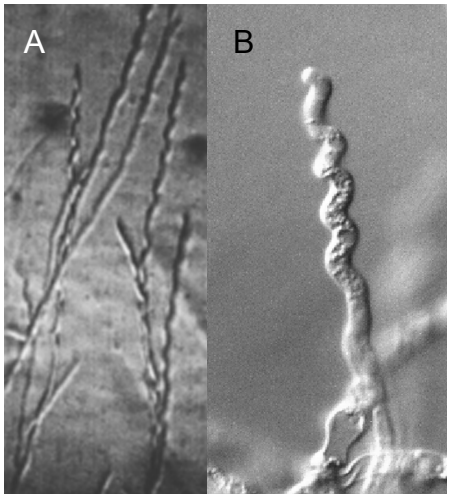

Pycniospores(N)
Plasmogamy &
Dikaryonization
Gall formation
Aeciospores (N+N)
Pine
Oak
Basidiospores(N)
Urediniospores
(N+N)
meiosis
Teliospores (N+N)
Karyogamy (2N)

Figure 1.3 Wavy hyphae phenotype in (A) *S. commune thn1* mutant and. (B) *C. q. fusiforme* culture.


A
B

CHAPTER 2

THE EFFECT OF SURFACE WETTABILITY ON GERMINATION FATE AND GENE EXPRESSION IN *CRONARTIUM QUERCUUM* F. SP. *FUSIFORME* BASIDIOSPORES[1]

[1] Baker, L. G., P. Spaine, and S. F. Covert. To be submitted to *Molecular Plant Pathology*

Abstract

Cronartium quercuum f. sp. *fusiforme* is an obligate biotrophic pathogen of pine. Of its five distinct spore types, the basidiospores are the only spore stage that is capable of establishing infection on pine. We are interested in understanding how obligate fungal phytopathogens recognize their unique host surface. One surface characteristic we chose to investigate was that of hydrophilicity or wettability. Depending on its environment, *C. q. fusiforme* basidiospores can undergo two different types of germination, direct or indirect. In direct germination, the basidiospore sends out a long thin germ tube that can lead to infection of the pine host. In contrast, indirect germination is characterized by the emergence of a short thick germ tube often with the subsequent formation of a secondary basidiospore. When we decreased the substrate hydrophilicity, we found that the percent total germination and percent direct germination of *C. q. fusiforme* basidiospores increased. This demonstrated that substrate hydrophilicity could affect the germination fate of *C. q. fusiforme* basidiospores. We also found that there is a critical threshold for substrate wettability, between 42% and 54%, at which the basidiospores switched from direct to indirect germination. In the second part of this study we took a genomic approach to identify genes that are up-regulated in *C. q. fusiforme* basidiospores germinating directly or indirectly. We made two suppression subtraction hybridization (SSH) libraries enriched for each germination type. A total of one hundred and eighty unique gene sequences were recovered from the two expression libraries and 14 cDNAs that were differentially expressed between them were identified. It is likely that genes within the expression libraries are determinants of the basidiospores' response to a substrate and/or environment.

Introduction

The heteroecious rust fungus *Cronartium quercuum* f. sp. *fusiforme* is the causative agent of fusiform rust on pine. This disease is characterized by the formation of woody, spindle-shaped galls that reduce the value and weaken the wood of loblolly pine (*Pinus taeda*) and slash pine (*Pinus elliottii*) (Powers *et al*. 1981; Cubbage *et al*. 2000). In the last 50 years, fusiform rust has become a serious threat to commercial forestry in the southeastern United States (Schmidt 1998). It is credited with causing between $25 million and $135 million in losses annually, depending on how the trees might have been utilized (Cubbage *et al*. 2000). Improved silviculture and breeding (Powers *et al*. 1981) have aided in the reduction of losses due to fusiform rust. Identification of resistance genes in pine (Wilcox *et al*. 1995) has been started, but it is not yet clear if these methods will allow for durable control of fusiform rust throughout the pathogen's range. Furthermore, intensive pine plantation management practices, such as weed control and fertilization, are increasingly used in the southeast, but they are also associated with contributing to elevated rates of fusiform rust infection (Boggess and Stahelin 1948; Dinus and Schmidtling 1971; Miller 1972; Hollis, Smith *et al*. 1977; Rowan 1978; Burton *et al*. 1985; Schmidt *et al*. 1995; Schmidt 1998). There is a need for more basic research that could support the development of novel *C. q. fusiforme* control methods.

C. q. fusiforme basidiospores are the infectious agents of fusiform rust disease on pine. Basidiospores form on the teliospore stage found on the oak leaves and are transported from oak to pine by the wind or rain. As in other pathogenic fungi, successful spore germination in *C. q. fusiforme* requires a combination of specific environmental signals, such as correct humidity and temperature (Powers *et al*. 1981; Spaine and Kaneko 1993; Spaine *et al*. 1995; Spaine and Kaneko 1996). Furthermore, depending on the surface upon which *C. q. fusiforme* basidiospores

land, they undergo one of two germination fates (Spaine and Kaneko 1993; Spaine and Kaneko 1996). If they land on a pine needle or on polystyrene, they send out long, thin germ tubes, and thus are said to germinate "directly" (Figure 2.1) (Spaine and Kaneko 1993; Spaine *et al*. 1995; Spaine and Kaneko 1996). In nature, this type of germination is a precursor to eventual infection of pine. However, if the basidiospores land on glass, they produce short, thick germ tubes often with secondary basidiospores at their tips (Spaine and Kaneko 1993; Spaine *et al*. 1995; Spaine and Kaneko 1996; Figure 2.1). The contents of the primary basidiospores empty into the secondary basidiospores, which can germinate again if the environment is favorable (Spaine and Kaneko 1993; Spaine *et al*. 1995; Spaine and Kaneko 1996). This type of germination is known as "indirect" germination. Depending upon substrate and water availability, the basidiospores of other rust fungi may also germinate directly or indirectly (Metzler 1982; Bauer 1986; Bauer 1987; Bauer and Oberwinkler 1988; Fretag *et al*. 1988; Mims and Richardson 1990). Therefore, the production of secondary basidiospores during indirect germination appears to be an adaptation that provides rust fungi with a second opportunity to land in an environment hospitable to germination and subsequent infection (Sacadura and Saville 2003).

The processes of germination and host surface recognition by rust fungi have not been widely studied. The purpose of this project therefore, was to investigate how surface hydrophilicity influences *C. q. fusiforme* basidiospore germination fate and gene expression during germination. In this study polystyrene and glass substrates were manipulated to generate surfaces with extreme and intermediate wettability characteristics. The total germination frequency and direct germination frequency of *C. q. fusiforme* basidiospores decreased as the substrate hydrophilicity increased. To better understand how surface hydrophilicity affects gene expression during basidiospore germination, two suppression subtraction hybridization (SSH)

libraries enriched for cDNAs expressed in either directly or indirectly germinating basidiospores were made. A total of one hundred and eighty unique cDNA sequences were recovered from the two SSH libraries and macro-array hybridization experiments identified 14 cDNAs that were differentially expressed during direct or indirect germination. It is likely that particular genes are key determinants in the ability of a spore to respond to a favorable substrate or environment. Understanding the molecular regulation of developmental events like basidiospore germination and sporulation will provide a foundation for novel methods directed at fusiform rust control.

Results and Discussion

Effect of surface wettability on C. q. fusiforme germination

A majority of *C. q. fusiforme* basidiospores will germinate directly on polystyrene and indirectly on glass (Spaine and Kaneko 1996; Figure 2.1). One of the physical differences between these two substrates is their hydrophilicity or percent "wettability". Hydrophobic surfaces have a low wettability score, whereas hydrophilic surfaces have a high wettability score (Terhune and Hoch 1993). The wettability of the polystyrene and glass surfaces used in this study were determined to be 21% and 54%, respectively, and each caused basidiospores to germinate as previously reported (Figure 1). To test the importance of surface hydrophilicity in determining the germination fate of *C. q. fusiforme* basidiospores, we altered the surface wettability of glass by coating it with two different silanes, dimethyldichlorosilane (DMS) or diphenyldichlorosilane (DPS). We also purchased Δpolystyrene®, a relatively hydrophilic polystyrene that is sold for certain tissue culture applications. The wettability of the silane-treated glass was comparable to that of unmanipulated polystyrene, while the wettability of Δpolystryrene was close to that of unmanipulated glass (Figure 2.2).

To examine how surface wettability influenced *C. q. fusiforme* germinating basidiospores hydrated basidiospores were cast on various surfaces and allowed to germinate for up to 10 hours. The highest frequencies of germination (50% - 55%) occurred on the three hydrophobic surfaces: i.e. on both types of silane-treated glass, as well as on polystyrene (Figure 2.2). In contrast, only 3% of the spores germinated on glass, which was the most hydrophilic surface tested (Figure 2.2). The frequency of germination on Δpolystyrene was intermediate between the hydrophobic and hydrophilic surfaces, in keeping with its intermediate wettability (Figure 2.2). Furthermore, as the hydrophilicity of the surfaces increased, the proportion of germinated spores that germinated directly decreased from 100% to 18%, indicating that substrate wettability is not only a factor in *C. q. fusiforme* basidiospore germination, but also in germination fate (Figure 2.2).

Similarly, the pycnidiospores of the causal agent of black rot of grape, *Phyllosticta ampelicida,* require proper surface hydrophilicity and substratum attachment to germinate (Shaw and Hoch 1999). *P. ampelicida* spores do not attach to hydrophilic surfaces unless they are treated with various cations (Shaw and Hoch 1999). Even after attachment is acheived, *P. ampelicida* spore germination is not stimulated until the spores are coated with Ca^{2+} (Shaw and Hoch 1999). Therefore, in *P. ampelicida*, surface hydrophilicity and spore surface properties both affect spore attachment and germination. Unlike *P. ampelicida*, *C. q. fusiforme* basidiospores can germinate on a hydrophilic glass surface, but the frequency of early germination was greatly reduced compared to that on hydrophobic polystyrene over short periods (Figure 2.2). Over longer periods, *C. q. fusiforme* basidiospores cast on glass germinated indirectly (data not shown).

The percent overall germination and the percent direct germination decreased dramatically between 42% and 54% wettability suggesting that there is a critical cut-off after 42% that is recognized by *C. q. fusiforme* basidiospores (Figure 2.2). A critical wettability switch point for germination is not unprecedented for fungal spores. Chaky *et al* (2001) reported that in *Colletotrichum graminicola* spore germination dropped from 74% to 12% when wettability decreased from 58^{o} to 45^{o} advancing contact angle. Because Chaky *et al* (2001) used advancing contact angles to measure wettability, it is difficult to make direct comparisons between the wettability of the substrate surfaces in this study (Figure 2.2 ethanol dilution method; Terhune and Hoch 1993) with those reported by Chaky *et al.* However, it does suggest that *C. graminicola*, like *C. q. fusiforme*, has a critical cut-off point where germination type switches due to surface hydrophilicity (Chaky *et al.* 2001).

Sequence Analysis of Genes Expressed in Directly and Indirectly Germinating Basidiospores

To gain a better understanding of how polystyrene and glass surfaces affect gene expression in germinating *C. q. fusiforme* basidiospores, two SSH libraries enriched for sequences expressed in either directly or indirectly germinating basidiospores were made. Nine hundred sixty clones from each library were sequenced and assembled into overlapping contigs. Of the 180 different cDNA contigs identified in the libraries, seventy-two were unique to the direct library, eighty-seven were unique to the indirect library, and twenty-one were common to both libraries. Based on BLAST search results producing E-values less than $10e^{-5}$, 41% of these sequences were homologous to entries in the NCBI database: 17% could be placed into functional groups based upon their best hit to a gene product of known function and 23% were similar to ESTs or hypothetical proteins (Figure 2.3).

Of the genes that encoded putative defense or stress-related proteins, three of five were found only in the direct library, one was common to both libraries and the other was found only in the indirect library. Two examples of the putative stress-related proteins were homologous to Rds1 (NCBI AL670011) and glutathione peroxidase (NCBI 2711788) from *Neurospora crassa*. Glutathione peroxidases reduce reactive oxygen species (Inoue 1999), which typically are viewed as harmful by-products of aerobic respiration, but they also are elevated in association with cellular differentiation in microbial eukaryotes (Aguirre *et al.* 2005). Of the genes that coded for putative metabolic proteins, 59 % were found in the indirectly germinating library, 35% were found only in the direct library, and the remaining 6% were common to both libraries. These putative proteins had a variety of functions including sphingolipid metabolism, protein degradation, and carbohydrate catabolism. One *C. q. fusiforme* cDNA found in the direct library was similar to a virulence-related surface glycoprotein, Epa5p, from *Candida glabrata* (De Las Penas *et al*. 2003).

The cDNAs of unknown function contained in the direct library of unknown function as well as those showing no homology to other genes in the NCBI database remain potential virulence candidates and their protein products could be potential targets for pathogen control. It is possible that some of the unknown genes from the direct library code for host recognition and infection proteins that determine the ability of the basidiospores to respond to a favorable substrate or environment. In contrast, the genes contained within the indirect library may encode environmental recognition factors that activate the secondary sporulation pathway instead of initiating host infection.

Analysis of Differential Gene Expression

To confirm and quantify the differential expression of genes in the direct and indirect SSH libraries, nylon macro-arrays containing duplicate spots of a representative clone for each unique contig were probed with first strand cDNA from directly and indirectly germinating basidospores. Significant quantities of indirectly germinated spores were generated by allowing them to germinate for 30-36 hours. After subtracting background and normalizing the hybridization data, we used $\log_2$ spot signal intensity ratios to determine the difference in expression levels for each cDNA on the arrays. The mean and standard deviation of all the $\log_2$(D/I) values were calculated and all clones whose $\log_2$(D/I) was greater than or equal to 1.9 standard deviations from the mean were judged to be differentially expressed (Quackenbush 2002). This cut-off corresponded to a 1.9-to 5.2-fold difference in gene expression (Table 2.1). Using this criterion, 8% of the genes spotted on the arrays had significantly different hybridization signals between the direct and indirect probes (Table 2.1). Of the fourteen cDNAs that had z-scores greater than or equal to 1.90, only five had homologs in the NCBI database. Four of the five were homologous to genes in other fungi and three were assigned putative functions (Table 2.1). The majority of the genes present on the arrays did not appear to be differentially expressed during the different types of germination, despite the fact that they were isolated from SSH libraries that had few sequences in common. It is possible, therefore, that the number of clones sequenced from the two libraries was insufficient to fully sample library diversity. Alternatively, the subtraction procedure may have been very sensitive and enriched even those genes whose difference in expression under the two conditions was judged insignificant in the array study.

We hypothesized that some of the genes involved in the formation of secondary basidiospores during indirect germination would be expressed during the development of other spore stages in the *C. q. fusiforme* life cycle. To test this idea, cDNA probes were made from RNA extracted from galled pine phloem actively producing either *C. q. fusiforme* pycniospores or aeciospores. The positive control spots of *C. q. fusiforme* genomic DNA and the internal reverse transcription controls showed significant hybridization signals in these experiments, but three independent probes from each of the pycniospores or aeciospores samples failed to bind significantly to any of the sequences on the arrays (data not shown). This suggests that the genes contained in the basidiospore libraries may be limited to the basidiospore stage of *C. q. fusiforme's* life cycle.

Conclusion

The data presented here strongly suggests that surface hydrophilicity influences not only whether *C. q. fusiforme* basidiospores germinate, but also what type of germination development occurs. In particular, *C. q. fusiforme* infectious development is impeded by hydrophilic surfaces and promoted by hydrophobic surfaces, a finding that would appear consistent with the naturally waxy surface of young pine shoots. To control fusiform rust disease in nurseries, perhaps seedlings could be treated with a substance that increases their surface wettability and thus blocks direct germination. Proteins encoded by the genes found in the SSH libraries of the directly and indirectly germinating basidiospores may be targets for novel control methods, such as intentional induction of indirect germination to reduce infection or interruption of sporulation to limit the spread of the disease.

Experimental Procedures

Measurement of Surface Hydrophilicity

The percent wettability of the polystyrene (Fisher Bio Sciences), Δpolystyrene® (NUNC), and glass surfaces was determined by averaging the diameter of spreading drops from a series of methanol dilutions as described elsewhere (Terhune and Hoch 1993). Glass slides were coated with dimethyldichlorosilane (DMS) and diphenyldichlorosilane (DPS) as described by Terhune and Hoch (1993), except the glass slides were cleaned with detergent and then rinsed at least 3X with DI water instead of being baked at 400 ºC for one hour. For ease of use, polystyrene and Δpolystyrene® petri-plates were cut into approximately 3" X 1" sections.

Basidiospore Production

Leaves of *Quercus rubra L.* were inoculated with *C. q. fusiforme* aeciospores (mixed gall collection, Asheville North Carolina and Clarke County, Georgia) at the USDA Forest Service Resistance Screening Center (Asheville, North Carolina) according to the method of Knighten *et al* (1988). Oak leaves bearing telia were collected approximately 3 weeks after inoculation and hydrated in a humidity chamber (made from a polystyrene petri dish) for 36 hours at 20 C with no light. For the wettability tests, basidiospores from the hydrated leaves were cast directly onto three to four randomly placed surfaces for 5 hours at 20 ºC with no light. The oak leaves were removed from the hydration chambers, which were resealed. The basidiospores were allowed to germinate for an additional 5 hours at 20 ºC, with no light. Two hundred and fifty spores per surface were scored in thirteen replicate experiments for germination and germination type. For the gene expression studies, oak leaves with telia were hydrated for 30-36 hours while suspended over polystyrene or glass petri dishes at 20 ºC with no light. Because basidiospores from

different leaves germinated at different rates, the dishes were classified into one of three germination categories: “most spores non-germinating”, denoted by a lack of germ tubes; “most spores germinating”, denoted by the presence of germ tubes at least 1-2 microns in length; or “almost all spores germinated”, denoted by the production of hyphal masses on polystyrene and the development of secondary basidiospores on glass. To ensure that the full suite of developmental stages from each surface were represented in the subsequent RNA samples, representatives of all 3 germination categories were pooled together in equal amounts in each final sample. A rubber policeman and 200µl of DEPC treated DI water was used to loosen the basidiospores from the surfaces. The DEPC water containing the basidiospores was aspirated from the plates and flash frozen in liquid nitrogen. Samples were stored in –80 ºC until RNA extraction.

RNA extraction

RNA for library construction and array probes was extracted from pools of *C. q. fusiforme* basidiospores ground using micro-plastic pestle and tube (Kontes Glass Co.; Vineland, NJ) and 450-600 micron acid washed glass beads (Sigma; St Louis, MO), as previously described (Warren and Covert 2004). For array probes, two independent biological pools were made for each of the directly germinating and indirectly germinating samples.

For the RNA from pycniospores and aeciospores, juvenile *Pinus taeda* trees were inoculated with a mixed basidiospore inoculum at the USDA Forest Service Resistance Screening Center as described elsewhere (Knighten *et al*. 1988). In October of each year, pycniospores produced on pine seedlings were harvested. Five galls were combined to make one biological sample. The bark and phloem tissues, where *C. q. fusiforme* was actively growing and

sporulating, were stripped from the galls and flash frozen in liquid nitrogen. Samples were stored at –80 ºC until RNA was extracted. In March, galls producing aeciospores were harvested and prepared as described for the pycniospore galls. RNA was extracted as described by Chang *et al* (1993), except that isoamyl alcohol was not used in the chloroform extractions.

All RNA samples were DNAse treated, cleaned with Phenol:Chloroform (3:1), and quantified using the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR). RNA quality was determined using formaldehyde denaturing gels.

SSH Library Construction

Two SSH cDNA libraries (direct and indirect) were each made from 1µg of total pooled RNA using the Smart PCR cDNA Synthesis Kit and the PCR-Select cDNA Subtraction Kit (CloneTech Laboratories Inc; Palo Alto, CA) following the manufacture's directions. The cDNAs were ligated into the TOPO TA pCRII vector (Invitrogen; Carlsbad, CA) and 960 clones were picked from each library. Plasmid DNA was extracted from all clones using Rapid Extraction Alkaline Lysis (R.E.A.L.) Prep 96 (Qiagen; Valencia, CA).

DNA Sequence Analysis

The SSH libraries were sequenced using a 96-well format ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1 or 2 (Applied Biosystems; Foster City, CA). Unincorporated dyes were removed from the samples using QIAquick 96 PCR Purification Kit (Qiagen; Valencia, CA). The libraries were sequenced at the University of Georgia, Genome Analysis Facility. The sequences from each SSH library were assembled into contigs using

SeqMan (DNAStar; Madison, WI). The contigs were used for BLAST searches (blastx and tblastx) against the NCBI nr and EST databases, using the BLOSUM62 matrix.

Putative gene functions were assigned based on the best homologous hit (BLAST E value $<10^{-5}$) to a protein of known function. These were run through the BRENDA database (http://www.brenda.uni-koeln.de) (Schomburg *et al*. 2004). When the BRENDA database did not have a function for the protein, the following databases were searched: *Saccharomyce*s Genome database (http://www.yeastgenome.org) (Balakrishnan *et al* 2005); Munich Information center for Protein Sequences (MIPS) database (http://mips.gsf.de); *S. Pombe* gene database (http://www.genedb.org); bimolecular interaction network (BIND) database (http://bind.ca) (Bader *et al* 2003); and the NCBI database.

Macro-array Hybridization Experiments

Inserts from plasmids containing unique gene sequences from the subtracted libraries were amplified using M13 forward and M13 reverse primers. Aliquots were run on a 1% agarose electrophoresis gel to verify amplification of a single product. Unincorporated dNTPs and primers were removed using QIAquick 96 PCR Purification kit (Qiagen; Valencia, CA). Approximately 1µl of DNA was stamped onto Hybond™-XL nylon membranes (Amersham Biosciences, Piscataway, NJ) using a MULTI -BLOT™ Replicator (V&P Scientific, Inc., San Diego, CA). Each insert was stamped twice on each array. DNA on arrays was denatured, neutralized, and rinsed using a solution containing, 1.5 M NaCl and 0.5 M NaOH, 0.5 M TRIS (pH 7.4) and 0.5 M NaCl, and 2X SSC with 0.05% SDS, respectively. Dry membranes were spotted with calibration controls 1 through 4 (7500, 2500, 750, and 250 pg, respectively) from the Lucidia Universal Scorecard kit (Amersham Biosciences, Piscataway, NJ) in 6 different

locations and with genomic DNA from *C. q. fusiforme* and loblolly pine (3 spots each of 10, 5, 1, 0.5, and 0.1 ng). Each array also contained negative hybridization controls of 12 randomly placed spots of vector and a mouse gene, *Steel* (Rajaraman *et al.* 2002), which encodes a growth factor that has not been found in fungal genomes (kindly provided by Dr. Mary Bedell, University of Georgia). Array membranes were UV cross-linked for 30 seconds.

Total RNA was extracted from 2 independent biological samples of basidiospores germinating directly or indirectly. Three independent biological samples were used to isolate RNA from pycniospores or aeciospores. Each bio-replicate was divided into two technical replicates. For first strand cDNA synthesis, 17µg of total RNA was added to a 50µl reaction containing 0.5µl Lucidia Universal Scorecard reference spike mix, 5µl primers (equal mix of 0.5mM *Hind*III-T11G/A/C oligo-dT primers), 10µl-5X first strand buffer (Promega, Madison WI), 2.5µl 20mM dATP/dGTP/dTTP (Fisher Bio Sciences), and 0.83µl RNAsin (Promega, Madison WI). The mixture was heat denatured at 65°C for 10 minutes and cooled to 37 °C for 5 minutes. 10µl of ^{32}P dCTP and 3.5µl MMLV-RT (Promega, Madison WI) were added to the mixture, which was then incubated for 2 hours at 37 °C. First stand synthesis was terminated by the addition of 10µl 2 M NaOH and reactions were cleaned on NICK columns (Amersham Biosciences, Piscataway, NJ).

Membranes were prehybridized for 3 hours at 65 °C in Churches modified buffer (Sambrook *et al.* 1989). The buffer was changed when the probe was added. The membranes were hybridized overnight at 65 °C, then washed twice in 2x SSC, 0.2% SDS at 65 °C, and once in 1x SSC, and 0.2% SDS at 65 °C, for 15 minutes each wash. Hybridization signals were captured on PhosphoImager Screens and quantified with ImageQuant Version 5 software (Molecular Dynamics, Amersham Pharmacia Biotech; Piscataway, NJ).

The background value for each array was computed as the average signal intensity of 45 blank spots randomly distributed throughout the array grid. The background value was subtracted from each signal, then each spot was normalized using the average signal intensity of six 750 pg Lucida calibration control spots. Normalized replicate spot signal intensities for each array were averaged together. The signal intensities were converted to $\log_2$ and then the two technical replicates for each biological sample were compared to each other. Spots having a technical replication ratio ($\log_{2a}/\log_{2b}$), where (a) and (b) refer to the two technical replicates, greater than one standard deviation from the mean were excluded from further analysis. The hybridizing spots that made it through the technical replicate screen were averaged together (total of 4 signal intensities, i.e. 2 bio-replications x 2 technical replications) and converted into $\log_2$ values for further analysis. The $\log_2$ ratios were used to determine gene expression differences between the treatments, as described by Quakenbush (2002). The mean and standard deviation for the distribution was calculated and the z-score for each $\log_2$ spot ratio was computed to determine its distance (in standard deviations) from the mean. Log_2 ratios were converted to z-scores to determine how many standard deviations from the population mean each $\log_2$ ratio fell. A z-score value of 1.90 or greater was used to identify differentially expressed genes.

References

Aguirre, J., Mauricio Rýos-Momberg, David Hewitt, and Wilhelm Hansberg (2005). "Reactive oxygen species and development in microbial eukaryotes." Trends in Microbiology **13**(3): 111-118.

Bader, G., D. Betel, C.W. Hogue (2003). "BIND: the Biomolecular Interaction Network Database." Nucleic Acids Research. **31**(1): 248-250.

Balakrishnan, R., K. R. Christie, M. C. Costanzo, K. Dolinski, S. S. Dwight, S. R. Engel, D. G. Fisk, J. E. Hirschman, E. L. Hong, R. Nash, R. Oughtred, M. Skrzypek, C. L. Theesfeld, G. Binkley, C. Lane, M. Schroeder, A. Sethuraman, S. Dong, S. Weng, S. Miyasato, R. Andrada, D. Botstein, and J. M Cherry. (2005). "*Saccharomyces* Genome Database.".

Bauer, R. (1986). "Basidiosporenentwicklung und -keimung bei Heterobasidiomyceten." Berichte Der Deutschen Botanischen Gesellschaft. **99**: 67-81.

Bauer, R. (1987). "Uredinales-germination of basidiospores and pycniospores." Studies in Mycology **30**: 111-125.

Bauer, R. and F. Oberwinkler (1988). "Nuclear degeneration during ballistospore formation of *Cronartium asclepiadeum* (Uredinales)." Acta Botanica Croatica **101**: 272-282.

Boggess, W. R. and R. Stahelin (1948). "The Incidence of fusiform rust in slash pine plantations receiving cultural treatments." Journal of Forestry **46**: 683-85.

Burton, J. D., E. Shoulders, *et al*. (1985). "Incidence and impact of fusiform rust vary with silviculture in slash pine plantations." Forest Science **31**(3): 671-80.

Chaky, J., K. Anderson, M. Moss, and L. Vallancourt (2001). "Surface hydrophobicity and surface rigidity induce spore germination in *Colletotrichum graminicola*." Phytpathology (91): 558-564.

Chang, P., and Cairney (1993). “A simple and efficient method for isolating RNA from pine trees.” Plant Molecular Biology Reporter **11**(2): 113-116.

Cubbage, F. W., J. M. Pye, *et al*. (2000). “An economic evaluation of fusiform rust protection research.” Southern Journal of Applied Forestry **24**(2): 77-85.

De Las Penas, A., S.J. Pan, *et al*. (2003). “Virulence-related surface glycoproteins in the yeast pathogen *Candida glabrata* are encoded in subtelomeric clusters and subject to RAP1- and SIR-dependent transcriptional silencing.” Genes and Development **17**(18): 2245-2258.

Dinus, R. J. and R. C. Schmidtling (1971). Fusiform rust in loblolly and slash pines after cultivation and fertilization. New Orleans, LA, Southern Forest Experimental Station**:** 1-10.

Fretag, S., L. Bruscaglioni, *et al*. (1988). “Basidiospores of rust fungi (*Uromyces* species) differentiate infection structures *in vitro*.” Journal of Experimental Mycology **12**: 275-283.

Hollis, C. A., J. E. Smith, *et al*. (1977). Genotype X mineral nutrient interactions with fusiform rust incidence in slash pine. 14th Southern Forest Tree Improvement Conference.

Inoue, Y., T. Matoshifumi, K. Sugiyama, S. Izawa, and A. Kimura (1999). “Genetic analysis of glutathione peroxidase in oxidative stress response of *Saccharomyces cerevisiae*.” Biological Chemistry **274**(38): 27002-27009.

Knighten, J. L., C. H. Young, *et al*. (1988). Resistance Screening Center Procedures Manual: A Step-by-Step Guide Used in the Operational Screening of Southern Pines for Resistance to Fusiform Rust. USDA Forest Service, USDA Forest Service**:** 23-25.

Metzler, B. (1982). "Untersuchungen an Herterobasidiomyceten (23): Basidiosporenkeimung und infectionsvorgang beim brinengitterrost." Phytopathologische Zeitschrift. Journal of Phytopathology. **103**: 126-138.

Miller, T. (1972). "Fusiform rust in planted slash pines: influence of site preparation and spacing." Forest Science **18**: 70-75.

Mims, C. W. and E. A. Richardson (1990). "Ultrastructure of secondary spore formation in the rust *Gymnosporangium Juniperi-virginianae*." Mycologia **82**(2): 236-242.

Powers, H. R., Jr., R. A. Schmidt, *et al*. (1981). "Current status and management of fusiform rust on southern pines." Annual Review Phytopathology **19**: 353-371.

Quackenbush, J. (2002). "Microarray data normalization and transformation." Nature Genetics **32**: 496-500.

Rajaraman, S., W. S. Davis, A. Mahakali-Zama, H. K. Evans, L. B. Russell, and M.A. Bedell. (2002). "An allelic series of mutations in the *Kit ligand* gene of mice. I. Identification of point mutations in seven ethylnitrosourea-induced $Kitl^{Steel}$ alleles". Genetics. **162**(1): 331-340.

Rowan, S. J. (1978). Susceptibility to fusiform rust in slash pine seedlings depends upon fertilization and cumulative inoculum density in multiple inoculations. Asheville N.C., Southern Forest Experiment Station**:** 5.

Sacadura, N. T. a. B. J. S. (2003). "Gene expression and EST analyses of *Ustilago maydis* germinating teliospores." Fungal Genetics and Biology **40**(1): 47-64.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: A Laboratory Manual. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.

Schmidt, R. A. (1998). Fusiform Rust Disease of Southern Pines: Biology, Ecology and Management. Florida, University of Florida**:** 1-85.

Schmidt, R. A., J. E. Allen, *et al*. (1995). "Influence of oak control and pine growth on fusiform rust incidence in young slash and loblolly pine plantations." Southern Journal of Applied Forestry **19**(4): 151-156.

Schomburg, I., A. Chang , C. Ebeling, M. Gremse, C. Heldt, G. Huhn, and D. Schomburg (2004). "BRENDA, the enzyme database: updates and major new developments." Nucleic Acids Research **1**(32 Database issue): D431-433.

Shaw, B. D. and H. C. Hoch (1999). "The pycnidiospore of *Phyllosticta ampelicida*: surface properties involved in substratum attachment and germination." Mycological Research **103**(7): 915-924.

Spaine, P. C. and S. Kaneko (1993). "Spore exudates and other factors affecting germination type of *Cronartium quercuum* f.sp. *fusiforme* basidiospores." Mycologia **85**(1): 51-61.

Spaine, P. C. and S. Kaneko (1996). "Nuclear Behavior During Basidiospore Germination in *Cronartium quercuum* f.sp. *fusiforme*." Mycologia **88**(6): 892-896.

Spaine, P. C., S. Kaneko, *et al*. (1995). "Fusiform rust basidiospore and urediniospore germination on host and non-host surfaces." In: Proceedings of the 4th IUFRO Rusts of Pine Working Party Conference, Tsukuba, Japan: 89-93.

Terhune, B. T. and H. C. Hoch (1993). "Substrate hydrophobicity and adhesion of *Uromyces* urediospores and germlings." Experimental Mycology **17**: 241-52.

Warren, J. M. and S. F. Covert (2004). "Differential expression of pine and *Cronartium quercuum* f. sp. *fusiforme* genes in fusiform rust galls." Applied and Environmental Microbiology: 441-451.

Wilcox, P. L., H. V. Amerson, *et al*. (1995). "Detection of a major gene for resistance to fusiform rust disease in loblolly pine by genomic mapping." Proceedings of the National Academy of Sciences **93**: 3859-3864.

Table 2.1 Summary of cDNAs Differentially Expressed in Directly and Indirectly Germinating Basidiospores

cDNA[1]	bp	Homology, if any	BLAST E-value	%ID[2]	log2(D/I)[3]	log2(D/I)[4] z-score
C676	569	EST from fusiform rust infected pine	9e-93	93	-0.9	-1.9
C711	319				-1.4	-2.4
C724	648	*Cryptococcus* putative cytoplasm protein	1e-11	72	2.5	2.3
C735	393				-2.0	-3.3
D808	394				2.3	2.0
D817	109				2.6	2.4
D822	368				2.2	1.9
D858	358	Mouse genome sequence	4e-6	57	2.1	1.9
D885	280				2.5	2.3
I685	383				-1.8	-3.0
I691	377				-1.6	-2.7
I710	174	*Cryptococcus* putative ABC transporter	3e-6	89	-2.3	-3.7
I727	114				-1.8	-3.0
I752	480	*Cryptococcus* putative H3 histone	1e-37	93	-1.1	-2.2

[1]cDNA found in; C, both libraries; D, direct library; I, indirect library

[2]Percent identity based on predicted amino acid sequence

[3]log2(mean Direct germination signal intensity/mean indirect germination signal intensity)

[4]A measure of the standard deviations of the log2(D/I) value from the mean log2(D/I) value of the entire data set

z score= $[(x - \bar{x})/s]$, (population value "x"- population mean "x")/ population standard deviation "s"

Figure 2.1 *C. q. fusiforme* direct (a) and indirect (b) basidiospore germination. The primary spore and secondary spore, which is made during indirect germination, are indicated by arrows.

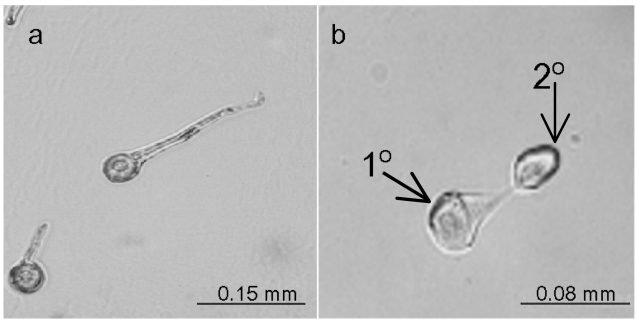

a
0.15 mm
b
2°
1°
0.08 mm

Figure 2.2 Wettability of tested surfaces and rates of *C. q. fusiforme* basidiospore germination on them. a. Proportion of basidiospores that germinated on the different surfaces. b. Proportion of germinated basidiospores that germinated directly on different surfaces. Treatments were as follows: glass coated with dimethyldichlorosilane (+DMS), glass coated with diphenyldichlorosilane (+DPS), polystyrene (PS), Δ-polystyrene (ΔPS), and untreated glass. Mean $\pm$ 1.6-2.3 (n=3250/treatment)

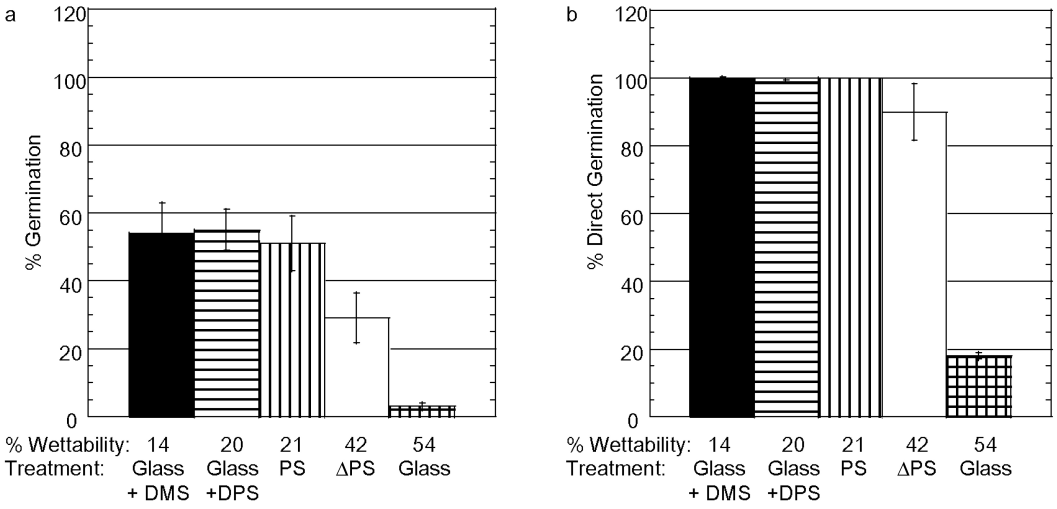
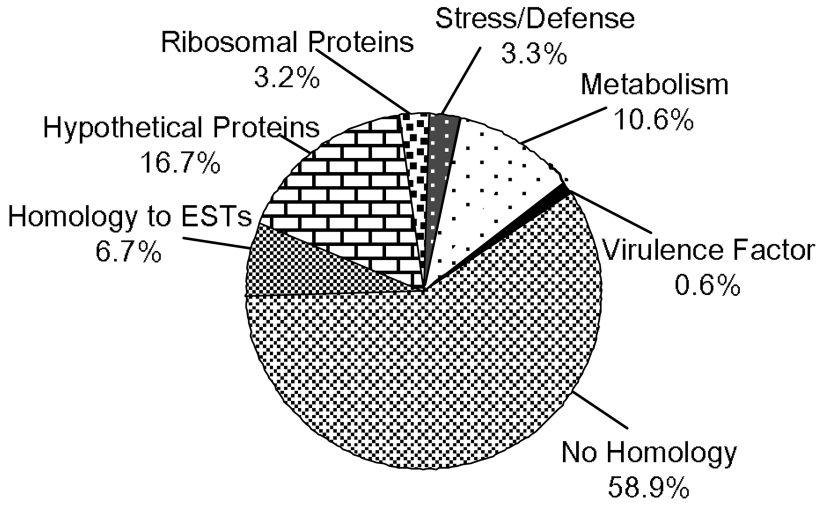

a
120
100
80
60
40
20
0
% Germination
% Wettability: 14 20 21 42 54
Treatment: Glass + DMS Glass +DPS PS ΔPS Glass
b
120
100
80
60
40
20
0
% Direct Germination
% Wettability: 14 20 21 42 54
Treatment: Glass + DMS Glass +DPS PS ΔPS Glass

Figure 2.3 Classification of cDNA contigs into categories based on BLAST search results. The 180 non-redundant sequences were sorted according to best Blast score for each sequence (E-value < 10^{-5}). The proportion of contigs in each category is shown.


Stress/Defense
3.3%
Ribosomal Proteins
3.2%
Metabolism
10.6%
Hypothetical Proteins
16.7%
Homology to ESTs
6.7%
Virulence Factor
0.6%
No Homology
58.9%

CHAPTER 3

A REGULATOR OF G-PROTEIN SIGNALING IN *USTILAGO MAYDIS* PROMOTES SPORULATION *IN PLANTA* AND REGULATES FILAMENTOUS GROWTH[1]

[1] Baker, L. G., S. Gold, and S. F. Covert. To be submitted to *Molecular Microbiology*

Abstract

Ustilago maydis only produces teliospores only when growing within its host plant, *Zea mays*. *U. maydis* has three growth phases: haploid yeast-like growth, infectious vegetative growth, and *in planta* reproductive growth. Our focus is the identification of the regulator(s) of G-protein signaling (RGS) involved in pathway(s) that *U. maydis* uses *in planta* to switch from vegetative growth to reproductive growth. We identified three genes (*rgs1*, *rgs2*, and *rgs3*) encoding putative RGS proteins in the *U. maydis* genome. We selected *rgs1* for further characterization based on its homology to other RGS proteins involved in the regulation of sporulation in other fungi. The deletion of *rgs1* resulted in hyphal growth on minimal medium agar, as opposed to the normal yeast-like growth of the wild type, and reduced pathogenicity and *in planta* sporulation. We determined that the hyphal growth phenotype was due to Δ*rgs1* strain's inability to deactivate the Gα subunits, Gpa1 and Gpa4. In contrast to wild type, Δ*gpa1* and Δ*gpa4* strains do not filament on low nutrient medium. These observed phenotypes for Δ*gpa1* and Δ*gpa4* were not previously described. This suggests that both Gpa1 and Gpa4 are involved and are non-redundant in activating hyphal growth on low nutrients. Double deletion mutants, Δ*gpa1* Δ*rgs1* and Δ*gpa4* Δ*rgs1*, did not maintain the filamentous growth of the Δ*rgs1* mutant, supporting the idea that Rgs1 regulates Gpa1 and Gpa4. Moreover, the combined data implied that Gpa1 and Gpa4 could be negative regulators of *in planta* sporulation. Using a sporulation time course experiment we found that the deletion of *gpa1* or *gpa4* caused hyper-sporulation *in planta*. The over- expression of *rgs1* was expected to have a budding phenotype similar to the individual deletions of *gpa1* and *gpa4*, but instead it caused a filamentous phenotype reminiscent of Δ*gpa3*. When *rgs1* was over-expressed in the $gpa3_{Q206L}$ background, the constitutive activation of Gpa3 restored wild type budding growth. This suggested that Rgs1 is capable of

regulating Gpa3. The combined data supports a model where multiple pathways are used for the control of *in planta* sporulation and under the appropriate conditions Rgs1 is used to regulate these pathways.

Introduction

Fungi from the orders *Ustilaginales* and *Uredinales*, which contain the smuts and the rusts, are of global economic importance causing disease losses in a variety of crops (Cubbage *et al*. 2000; Martinez-Espinoza *et al*. 2002; Williams-Woodward 2003). Obligate fungal phytopathogens must grow within their host plants in order to gain their nutrition and complete their life cycles. Therefore, they only sporulate when growing in association with their host plants. Thus, both their reproduction and the dissemination of the diseases they cause appear to be dependent on physical or chemical cues from the host. *Ustilago maydis* is a semi-obligate phytopathogen of maize, *Zea mays*. Although it can grow vegetatively in axenic culture, *U. maydis* must grow within maize in order to complete its sexual development. This suggests that maize provides *U. maydis* with vital signals that trigger the switch from vegetative growth to reproductive growth (Banuett 1992). *U. maydis* causes corn smut, a disease characterized by the formation of galls that produce 2.5-6 billion teliospores per cm^3 gall tissue (Christensen 1963).

In *U. maydis* only a few genes are known to code for proteins that effect teliospore production. One of the four Gα subunits, Gpa3, negatively regulates *in planta* sporulation (Regenfelder *et al*. 1997). The constitutively active Gpa3 mutant, $gpa3_{Q206L}$, causes reduced gall formation and abolishes teliospore production at the hyphal tips (Krüger *et al*. 2000; Regenfelder *et al*. 1997) and instead of wild type haploid yeast-like growth, the disruption of *gpa3* results in a filamentous haploid phenotype that is unable to mate or infect the host plant (Regenfelder *et al*.

1997). Disruptions of the other Gα subunit genes in *U. maydis*, Δ*gpa1*, Δ*gpa2*, and Δ*gpa4* did not have a phenotype implied (Regenfelder *et al*. 1997). In *U. maydis* the switch from vegetative growth to reproductive growth, and subsequent production of teliospores, occurs within the gall tumor. Teliospore formation consists of preliminary stages that include the rounding and swelling of hyphal tips, hyphal fragmentation, and the release of diploid spore precursors (Banuett and Herskowitz 1996). The *rum1* and *hda1* gene products are essential for hyphal swelling and rounding, while the *fuz1* gene product is necessary for hyphal fragmentation. In the absence of these genes teliospores do not form (Banuett and Herskowitz 1996; Krüger *et al*. 2000; Reichmann *et al*. 2002). Also, the protein encoded by *hgl1* is important for teliospore formation at a step after hyphal fragmentation (Durrenberger *et al.* 2001).

We do not know how *U. maydis* regulates the switch from vegetative growth to *in planta* sporulation or what host signals, if any, stimulate this switch. We predict that *U. maydis* uses a system similar to that used by other filamentous fungi, such as *Aspergillus nidulans* and *Schizophyllum commune*, to regulate their sporulation pathways. Both *A. nidulans* and *S. commune* use regulator of G-protein signaling (RGS) proteins to control the switch from filamentous growth to reproductive growth. RGS proteins regulate heterotrimeric G-protein signal transduction pathways by stimulating the hydrolysis of the GTP bound to activated Gα subunits, which in turn deactivates the G-protein signaling. The *A. nidulans* genome encodes four RGS proteins, FlbA, RgsA, RgsB, and RgsC (Han 2004; Lee and Adams 1994) and three Gα proteins, FadA, GanA, and GanB (Han 2004 *et al*.; Yu *et al.* 1996). Two of the Gα subunits, FadA and GanB, and their associated RGS proteins, FlbA and RgsA, are involved in sporulation signal trandsuction pathways (Han *et al*. 2004; Hicks *et al*. 1997; Yu *et al.* 1996). FadA in the active GTP-bound form suppresses sporulation and is deactivated by FlbA to allow conidiophore

development, i.e. asexual sporulation (Adams *et al.* 1998). The over-expression of *flbA* causes precocious sporulation with little to no vegetative growth (Lee and Adams 1994). In contrast, an *A. nidulans* mutant carrying a recessive loss-of-function *flbA* mutation, exhibits vegetative growth with autolysis and no sporulation (Adams *et al.* 1998; Yu *et al.* 1996). Similar to FadA, GanB, in the active GTP bound form suppresses sporulation (Chang *et al*. 2004; Han *et al*. 2004). The over-expression of *rgsA*, which regulates GanB, causes inappropriate sporulation (Chang 2004 *et al*.; Han *et al*. 2004). In *S. commune*, the *thn1* gene encodes a putative RGS protein and homo-allelic *thn1* mutants are unable to produce sexual fruiting bodies from an otherwise compatible mating (Fowler and Mitton 2000). However, RGS proteins and their role, if any, in *in planta* sporulation have not been studied in fungi.

We chose *U. maydis* to study *in planta* sporulation and its regulation by an RGS protein because it is a genetically tractable organism in which to study "obligate" phytopathogenic developmental processes that depend on growth within the host. We used *A. nidulans* RGS protein sequences to search the *U. maydis* genome and found three putative RGS proteins (Rgs1, Rgs2, and Rgs3). The deletion of *rgs1* resulted in hyphal growth on minimal medium. We hypothesized that the hyphal growth phenotype was due to Δ*rgs1*'s inability to deactivate a Gα protein involved in the negative regulation of hyphal growth on high or moderate nutrients. Therefore, we tested the ability of the individual Gα disruption mutants, Δ*gpa1*, Δ*gpa2*, Δ*gpa3*, and Δ*gpa4* to produce filaments on low or no nutrient agar. Both the Δ*gpa1*and Δ*gpa4* mutants lost the ability to grow filamentously on the water agar. Double deletion mutants, Δ*gpa1* Δ*rgs1* and Δ*gpa4* Δ*rgs1*, did not maintain the filamentous growth of the Δ*rgs1* mutant, which indicated that Rgs1 regulates the same signal transduction pathways as Gpa1 and Gpa4. Deletion of *rgs1* did not affect mating but it did reduce pathogenicity and *in planta* sporulation. Because Rgs1 is

involved in sporulation and the double mutant data suggested that it acts in the same pathways as Gpa1 and Gpa4, we took a closer look at the *in planta* phenotypes of Δ*gpa1* and Δ*gpa4*. Using an *in planta* sporulation time course experiment we found that the disruption of *gpa1* or *gpa4* caused hyper-sporulation as compared to wild-type. The over- expression of *rgs1* was expected to have a budding phenotype similar to the individual deletions of *gpa1* and *gpa4* (Regenfelder *et al*. 1997), but instead it caused an unexpected filamentous phenotype reminiscent of Δ*gpa3* (Regenfelder *et al*. 1997). Interestingly, when *rgs1* was over-expressed in the $gpa3_{Q206L}$ background, the constitutive activation of Gpa3 restored wild type budding growth. This suggested that under the correct conditions Rgs1 is capable of regulating Gpa3. The combined data supports a model where multiple pathways are used for the control of *in planta* sporulation and that under the appropriate conditions Rgs1 is used to regulate these pathways. We can use the knowledge gained from this study of *U. maydis* and how it uses Rgs1 to regulate *in planta* sporulation to make inferences into how other obligate fungal phytopathogen sporulation pathways are regulated.

Results

Identification of Ustilago maydis rgs genes

To identify RGS proteins in *U. maydis*, sequences of the four *A. nidulans* RGS proteins, FlbA, RgsA, RgsB, and RgsC (Lee and Adams, 1994; Han *et al*., 2004; XP410030, AN5755.2, AN3622.2, and AN1377.2, respectively) were used as BLAST queries against the *U. maydis* genome (Broad Institute). Three putative *U. maydis* RGS proteins (Rgs1, Rgs2, and Rgs3) were identified in this manner. Striking differences in the overall length and conserved domains in these proteins made it possible to match them with their putative orthologs in *A. nidulans* with a

high degree of confidence despite their low % identities: Rgs1 31% to FlbA, Rgs2 35% to RgsB, and Rgs3 31% to RgsC (Figure 1 A). The RGS domain in Rgs1 has 133 more amino acids than that of FlbA, and Rgs3 lacks the PXA domain found in RgsC, but otherwise the domains in these orthologous pairs are well conserved (Figure 1 A).

Initial BLAST searches of the *U. maydis* genome failed to identify an ortholog to *A. nidulans* RgsA. To determine if the absence of this gene might reflect a common difference between ascomycetes and basidiomycetes, we searched the available fungal databases for sequences homologous to RgsA. Three other basidiomycete genomes *Cryptococcus neoformans*, *Coprinus cinereus*, and *Phanerochaete chrysosporium*, contained clear orthologs to FlbA, RgsB, and RgsC, but they lacked an RgsA-like protein (data not shown). In contrast, RgsA orthologs were identified in ten out of twelve ascomycete genomes and the one zygomycete genome, *Rhizopus oryzae*, but none of these proteins were similar to any proteins encoded by the *U. maydis* genome (data not shown). Of the four RGS proteins in *A. nidulans* two of them, FlbA and RgsA, are involved in the regulation of sporulation (Han 2004 *et al.*; Yu *et al.* 1996). FlbA negatively regulates the Gα protein FadA, and RgsA negatively regulates the Gα protein, GanB (Fig. 1B; Han *et al.*, 2004; Yu *et al.*, 1996). Based on amino acid similarity, *A. nidulans* FadA is orthologous to *U. maydis* Gpa1 and *A. nidulans* GanB is orthologous to *U. maydis* Gpa3. These conclusions were based on the fact that the putative orthologs were more closely related to each other (69-75% similarity) than to the other Gα subunits (31-48% similarity). Thus, the comparison of the orthologous and regulatory relationships between the *A. nidulans* and *U. maydis* components of these pathways raised the possibility that *U. maydis* Rgs1 regulates Gpa1 and not Gpa3 (Figure 1 B) as we had expected based upon Gpa3's role as a negative regulator of sporulation (Regenfelder *et al.* 1997). Furthermore, the absence of an RgsA ortholog in the *U.*

maydis genome made it impossible to predict which RGS protein, if any, modulates the activity of Gpa3.

At the outset of this project, neither the *A. nidulans* nor the *U. maydis* genome had been sequenced and the only *A. nidulans* RGS protein known to regulate sporulation was FlbA (Yu *et al.* 1996). Therefore, to identify a *U. maydis* RGS protein involved in this process, we designed degenerate primers from the conserved RGS domains of *A. nidulans* FlbA and the *S. commune* Thn1 ortholog. The primers amplified a 268 bp fragment from *U. maydis* genomic DNA and this fragment appeared to encode an RGS protein based on similarity to *A. nidulans* FlbA. When this fragment was used to screen a *U. maydis* genomic library (Barrett *et al.*, 1993) the probe hybridized to one cosmid. The *rgs* gene on this cosmid corresponds to Rgs1, and its later sequence proved to be identical to the *rgs1* mRNA predicted by the whole genome sequence project (NCBI accession: XM_399719).

Deletion of rgs1 causes hyphal growth on minimal medium

To study the function of the *U. maydis* Rgs1, we deleted the gene from compatible, *a1 b1* and *a2 b2*, haploid strains. This was accomplished by replacing the entire predicted open reading frame with a hygromycin resistance cassette. Over seventy-five hygromycin resistant transformants from each mating type were screened by Southern blotting to identify single *rgs1* gene replacement events (data not shown). Only two homologous transformants, one from each mating type, were recovered.

If Rgs1 negatively regulates the Gα subunit, Gpa3, then the phenotype of the *rgs1* deletion mutant should have been similar to that of the constitutively active Gpa3 mutant, $gpa3^{Q206L}$. On potato dextrose agar (PDA) supplemented with 1% active charcoal, the *Δrgs1*

mutant was indistinguishable from wild type, yeast-like growth, while the *gpa3*$_{Q206L}$ mutant displayed the glossy colony morphology previously described (Regenfelder *et al.*, 1997; Figure 2 A). Furthermore, when strains were grown on *U. maydis* minimal medium agar (MMA), the Δ*rgs1* mutant produced filaments, a phenotype in strong contrast to the yeast-like growth of the wild type and *gpa3*$_{Q206L}$ mutant (Figure 2 B). Thus, the MMA data suggested that Rgs1 negatively regulates a Gα other than Gpa3, which stimulates hyphal growth.

The filamentous growth of the Δ*rgs1* mutant is a characteristic displayed by other yeast and yeast-like fungi when searching their environment for nutrients. If *U. maydis* utilizes a Gα protein to activate a nutrient-sensing signal transduction pathway, then deletion of that Gα would disable *U. maydis*' ability to stimulate filamentous growth under low nutrient conditions. To determine, which, if any, of the remaining Gα proteins might be involved in such nutrient sensing pathway(s), Δ*gpa1,* Δ*gpa2*, and Δ*gpa4* strains (Regenfelder *et al*. 1997) were grown on 2% water agar (WA). The Δ*gpa1* and Δ*gpa4* mutants lost the ability to produce filaments in low nutrient environments (Figure 2 C), suggesting that in haploids both Gpa1 and Gpa4 are negatively regulated by Rgs1 under high nutrient conditions. However, the Δ*gpa1* mutant displayed a stronger smooth edged colony morphology than the Δ*gpa4* mutant (Figure 2 C). To determine if Rgs1 regulates Gpa1 and Gpa4 under nutrient deprivation conditions, Δ*gpa1* Δ*rgs1* and Δ*gpa4* Δ*rgs1* double mutants were grown on 2% WA. If Rgs1 is deactivating a particular Gα subunit then the smooth colony phenotype of Gα deletion mutant should be epistatic to the filamentous phenotype of the Δ*rgs1* on WA. Both of the double mutants failed to produce filaments on WA, suggesting that Rgs1 regulates both Gpa1 and Gpa4 under nutrient limiting conditions (Figure 2 C).

Deletion of rgs1 reduces virulence and sporulation in U. maydis

In *U. maydis*, mating is a pre-requisite for the formation of the filamentous infectious dikaryon. Therefore, plate-mating assays were used to determine if Δ*rgs1* strains could mate with compatible mating partners. The Δ*rgs1* x Δ*rgs1* cross behaved like a wild-type cross and formed the white filaments characteristic of the dikaryon. This results indicated that functional Rgs1 is not necessary for mating or the formation of dikaryotic filaments in *U. maydis* (Figure 3). The Δ*rgs1* strains were also able to mate with the compatible wild-type strain (Figure 3).

To determine if Rgs1 has a role in pathogenicity, we inoculated 7 to 10 day old corn plants with wild-type or compatible Δ*rgs1* mutants. At 14 days post inoculation (DPI) the Δ*rgs1* x Δ*rgs1* crosses displayed a reduced ability to cause disease compared to the wild-type crosses (Table 1). Notably, virulence was also reduced in compatible *wt* x Δ*rgs1* crosses as compared to the wild-type (Table 1).

In planta data also indicated that Rgs1 was essential for production of viable teliospores. By seventeen DPI the Δ*rgs1* x Δ*rgs1* galls produced only a small fraction of the teliospores made in wild type galls (Figure 4). Although the few spores that were produced by Δ*rgs1* x Δ*rgs1* galls were normal in appearance, they were not viable as compared to wild type (data not shown). The Δ*rgs1* x Δ*rgs1* results suggested that the sporulation defect in this cross was due to the over-activation of a Gα subunit involved in the negative regulation of sporulation. Notably, Gpa3 is the only Gα subunit reported to negatively regulate sporulation in *U. maydis* (Regenfelder *et al*. 1997).

Deletion of gpa1 or gpa4 leads to precocious sporulation

Because the data from *in vitro* cultures suggested that Rgs1 negatively regulates Gpa1 and Gpa4, and the *in planta* experiments indicated that Rgs1 is necessary for prolific spore formation, we predicted that Gpa1 and Gpa4 might be negative regulators of sporulation. To test this, we carefully monitored the temporal pattern of teliospore formation by Δ*gpa1* or Δ*gpa4* mutants. If *gpa1* or *gpa4* were negative regulators of this process, mutants carrying deletions of either gene would sporulate more quickly than wild-type *U. maydis*. Plants infected with Δ*gpa1* or Δ*gpa4*, had visible teliospores produced 2 to 3 days before wild-type galls (Figure 5). Furthermore, by 14 DPI, virtually all of the plants inoculated with Δ*gpa1* or Δ*gpa4* were producing copious amounts of externally visible teliospores, while only about 50% of the plants inoculated with wild-type *U. maydis* were producing externally visible spores (Figure 5). The effect of *gpa2* deletion on the timing of sporulation was also examined; sporulation by this mutant was similar to that of wild type (data not shown). The Δ*gpa3* mutant was not re-examined because it does not mate and thus, does not infect corn (Regenfelder *et al*. 1997).

Over expression of rgs1 stimulates hyphal growth in rich medium

The *rgs1* open reading frame was fused to the *U. maydis* glyceraldehyde 3-phosphatase dehydrogenase promoter, P*gap*, and ectopically integrated into the *U. maydis* genome. Northern blots verified that *rgs1* was more highly expressed in two representative P*gap*::*rgs1* strains than in wild-type *U. maydis* (Figure 6 C). Because our other experiments with the Δ*rgs1*, Δ*gpa1*, and Δ*gpa4* mutants all indicated that Rgs1 negatively regulates Gpa1 and Gpa4, but not Gpa3, we expected that P*gap*::*rgs1* strains would display budding growth in culture similar to that of *Δgpa1* or *Δgpa4* strains (Regenfelder *et al*. 1997). However, all 80 P*gap*::*rgs1* transformants

tested had a highly filamentous phenotype reminiscent of the Δ*gpa3* phenotype in complete liquid or on complete solid medium (Figure 6 A & B), suggesting that Rgs1 negatively regulates Gpa3, at least under high nutrient conditions. Additionally, the P*gap::rgs1* strains also grew much more slowly than wild-type *U. maydis* (data not shown).

To test the idea that Rgs1 negatively regulates Gpa3, we transformed the Gpa3 constitutively active mutant, *gpa3*$_{Q206L}$, with the P*gap::rgs1* construct on an autonomously replicating vector (Kojic and Holloman, 2000). Northern blot analyses confirmed that *rgs1* was highly expressed in two transformants (Figure 6 D) whose morphology was representative of forty other *gpa3*$_{Q206L}$ p*gap::rgs1* strains. The constitutively active form of Gpa3 displays budding growth in culture (Regenfelder *et al*. 1997). In the double mutants, this phenotype was epistatic to the filamentous phenotype of P*gap::rgs1*(Figure 6 A & B).

Discussion

This study allowed us to assign functions to three *U. maydis* signal transduction proteins. Using Δ*gpa1* and Δ*gpa4* that were created previously (Regenfelder *et al*. 1997), we determined that both *gpa1* and *gpa4* are essential for haploid filamentation on low nutrients and that they serve as negative regulators of teliospore formation *in planta*. These are the first recorded functions for *gpa1* or *gpa4* in *U. maydis*. In addition, we determined that Rgs1 suppresses haploid filamentation on defined medium, probably via deactivation of Gpa1 and Gpa4, and that Rgs1 is essential for normal teliospore production *in planta*.

U. maydis Rgs1 and Gα proteins Involved in Nutrient Sensing

The deletion of Rgs1 caused a filamentous phenotype when the haploid was grown on MMA. This filamentous phenotype indicated that Rgs1 could regulate a Gα protein involved in a nutrient-dependant hyphal process, such as pseudohyphal growth or conjugation tube formation. In diploid *Saccharomyces cerevisiae* starved for nitrogen, a Gα protein, Gpa2, is activated in coordination with Ras2 to stimulate pseudohyphal growth (Lorenz and Heitman 1998; Lorenz and Heitman 1997). In response to nitrogen limitation and an abundant fermentable carbon source, diploid *S. cerevisiae* cells switch from budding growth to pseudohyphal growth (Lengeler *et al*. 2000; Lorenz and Heitman 1997). This is mediated through a cAMP pathway (Lengeler *et al*. 2000; Lorenz and Heitman 1997) regulated by Rgs2, which regulates the Gα protein, Gpa2 (Versele *et al*. 1999). Cullen *et al* (2000) found that haploid *S. cerevisiae* starved for glucose grow invasively, a type of growth form associated with filamentation, but different from diploid pseudohyphal growth. Addition of glucose suppressed filamentation in hyper-invasive growth mutants (Cullen *et al*. 2000). Similarly, the human pathogen *Candida albicans* regulates hyphal growth and morphogenesis in a glucose-dependent manner through a Gα protein pathway that regulates cAMP levels (Miwa *et al*. 2004). The fission yeast, *Schizosaccharomyces pombe,* uses a Gα-protein, Gpa2, which responds to environmental glucose levels by modulating adenylate cyclase activity. This results in the inhibition of conjugation tube formation and meiosis (Welton and Hoffman 2000). Therefore, it is possible that the filamentous phenotype of the Δ*rgs1* mutants in *U. maydis* is caused by the improper regulation of a nutrient-sensing Gα protein.

In response to a low-nutrient environment, such as water agar, wild type haploid *U. maydis* sporidia grow filamentously (Figure 2 C). If the filamentous response under starvation

conditions in *U. maydis* is mediated through a Gα protein pathway, then disruption of the Gα would result in the inability of the mutant to grow filamentously. Both the *gpa1* and *gpa4* disruption mutants were unable to filament under starvation conditions (Figure 2 C), indicating that both Gαs regulate pathways responsive to low nutrients. The non-filamentous phenotype of Δ*gpa1*, a smooth edge colony phenotype, appears to be more severe than that of the Δ*gpa4*, which has an irregular colony edge (Figure 2 C). Furthermore, the addition of glucose or nitrogen to WA alleviates the non-filamentous phenotype of the Δ*gpa4* mutant, but not of the *Δgpa1* (data not shown). This suggests that functional Gpa1 may compensate in some way for the loss of Gpa4. Because both Δ*gpa1* and Δ*gpa4* strains had a non-filamentous phenotype on WA, we could not rule out the possibility that under starvation conditions Rgs1 could regulate both Gα proteins. To better understand which of the Gα proteins were negatively regulated by Rgs1, we prepared double deletion mutants, Δ*gpa1* Δ*rgs1* and Δ*gpa4* Δ*rgs1*. If the Gα protein and RGS are in the same signaling pathway, then the phenotype of the Gα disruption mutant should be epistatic to that of the *rgs1* deletion. When the double mutants were streaked on WA both the Δ*gpa1* Δ*rgs1* and Δ*gpa4 Δrgs1* mutants remained unable to form filaments (Figure 2 C). This suggests that Rgs1 negatively regulates both Gpa1 and Gpa4 under high nutrient conditions, thus suppressing filamentation.

Roles of Rgs1, Gpa1, and Gpa4 in Pathogenicity and Sporulation

The deletion of *rgs1* decreased the severity of disease and amount of sporulation caused by infection of *U maydis*. The disease index and the disease severity in the Δ*rgs1* x Δ*rgs1* crosses were slightly less than those of wild type crosses (Table 1 and data not shown). This suggests that Rgs1 regulates at least one pathway contributing to pathogenicity, although it is

possible that these minor defects were due to an undetected and small reduction in *Δrgs1* mating efficiency. In the mutant crosses sporulation was not completely abolished, but it was greatly reduced (Figure 4). The few teliospores produced in the Δ*rgs1* x Δ*rgs1* galls could have been due to the Gα pathway(s) being deactivated by the Gα intrinsic GTPase activity, which could lead to small pockets of sporulation in the galls. Previous results have shown that of the four Gα proteins in *U. maydis*, only Gpa3 is involved in pathogenicity and *in planta* sporulation (Regenfelder *et al*. 1997). Because *U. maydis* lacks an RGS protein that would be predicted to regulate Gpa3 (when compared to *A. nidulans* orthologous functional pairs, Figure 1), and because Gpa3 and Rgs1 are both involved in pathogenicity, either Rgs1 regulates Gpa3 *in planta* or one or more of the other Gαs are involved in pathogenicity or sporulation. Intriguingly, upon closer examination of the *in planta* phenotypes of the other Gα deletion mutants we found that the deletion of *gpa1* or *gpa4* led to hyper-sporulation (Figure 5). This suggested that *U. maydis* uses not one, but three Gαs to negatively regulate *in planta* sporulation. Having more than one heterotrimeric G-protein pathway that negatively regulate sporulation has been demonstrated in *A. nidulans* (Han 2004; Yu *et al.* 1996). Therefore, it is conceivable that *U. maydis* uses more than one Gα protein for the tight control of *in planta* teliospore formation.

U. maydis Rgs1 regulates Gpa3

The first direct indication that Rgs1 might regulate Gpa3 came from the over-expression of *rgs1*, which caused a hyphal phenotype similar to the disruption of *gpa3* (Figure 6; Regenfelder *et al.*, 1997). We considered that if the filamentous phenotype of the P*gap::rgs1* mutant was actually due to the regulation of Gpa3, then the budding phenotype of the constitutively active Gpa3 mutant should be epistatic to that of the P*gap::rgs1* filamentous

phenotype. Because the double mutant *gpa3*$_{Q206L}$ p*gap::rgs1* grew by budding in liquid DCM, the filamentous phenotype of the P*gap::rgs1* mutant appeared to be due to the negative regulation of Gpa3 (Figure 6). Interestingly, the double mutant, *gpa3*$_{Q206L}$ p*gap::rgs1*, did not have the same glossy phenotype of the single mutant, *gpa3*$_{Q206L}$, (compare Figure 6 to Figure 2) indicating that the Rgs1 over-expression may also be affecting Gpa1 and or Gpa4 pathways. Both the P*gap::rgs1* and *gpa3*$_{Q206L}$ p*gap::rgs1* mutants grew poorly in liquid medium and on PDA, and required growth in a nutrient rich medium, such as DCM (data not shown). It is possible that the filamentous phenotype of P*gap::rgs1* is due to the negative regulation of Gpa3 and the poor growth phenotype is due to the negative regulation of Gpa1 and Gpa4. Although, the over-expression phenotype of the P*gap::rgs1* mutant suggested that Rgs1 can regulate Gpa3, this data should be viewed with caution because increased expression of *rgs1* might lead to Rgs1 interacting with an alternate Gα, i.e. Gpa3.

U. maydis Rgs1 Regulation of More Than One Gα protein

We have demonstrated that Rgs1 can regulate Gpa1 and Gpa4 in the appropriate environmental conditions and when over-expressed it can regulate Gpa3. Therefore, the over-expression of *rgs1* should result in the simultaneous shutdown of three Gα pathways. Different combinations of Gα deletions in *U. maydis* have not been performed. It would be interesting to investigate how a double or triple Gα deletion compares in phenotype to the p*gap::rgs1* mutant. A study performed in *N. crassa* showed that a combination of Gα deletions caused synergistic effects (Kays and Borkovich 2004). If Rgs1 in *U. maydis* regulates all three pathways, then the triple Gα knock-out would cause a similar hyphal growth phenotype, as well as have a severe growth defect as displayed by the P*gap::rgs1* over-expression mutant. Therefore, a new model

for Gα regulation suggests that Rgs1 is capable of regulating not just one, but possibly three Gα proteins, Gpa1, Gpa3, and Gpa4 (Figure 7). This model could be further tested by decoupling Rgs1's ability to bind with the individual Gα's. Point mutations could be introduced into the individual Gα subunits to abolish the ability of RGS protein to bind to activated Gα subunits. DiBello *et al* (1998) demonstrated that a mutation at G302S in the *S. cerevisiae* Gα subunit, Gpa1, interfered with the RGS protein's ability to bind the activated Gα.

We are interested in how pathogens respond to signals from the host to initiate developmental changes (i.e. switch from vegetative growth to reproductive growth) and what those host signals may be. Establishing that Rgs1 is involved in signal transduction leading to *in planta* sporulation is the first step in working up the signal transduction pathway to discover how fungal pathogens interpret host signals. The data presented here argues that *in planta* sporulation and its regulation is more complex than previously believed. The addition of two more Gα subunits to the sporulation pathway gives rise to additional questions. Is one pathway more important than the others or is a combination of the pathways needed for the proper timing and control of *in planta* sporulation? Do three pathways represent a safeguard (i.e. redundancy in the sporulation pathway)? Is negative regulation of all three Gα protein pathways by Rgs1 a global mechanism for the simultaneous shut down of Gα proteins involved in sporulation or does it occur on (in) a spatial-or-temporal specific manor? Do the pathways have different functions in the haploid vs. the dikaryon form of the fungus?

Experimental Procedures

Identification of U. maydis RGS and Gα orthologs

The sequences of four *A. nidulans* RGS proteins, FlbA, RgsA, RgsB, and RgsC (Lee and Adams, 1994; Han, 2004; XP410030, AN5755.2, AN3622.2, and AN1377.2, respectively) were used to perform BLAST searches (tblastn, BLOSUM45, no filter), against the *U. maydis* genome (Broad Institute). Three putative *U. maydis* RGS proteins (Rgs1, Rgs2, and Rgs3) were identified. We used NCBI bl2seq program to produce pair-wise protein alignments to determine which of the *U. maydis* Gα subunits (NCBI: Gpa1, P87032; Gpa2, P87033; Gpa3, P87034; and Gpa4, P87035) were orthologous to *A. nidulans* Gα subunits (NCBI: FadA, S71965; GanA, AAD34893; and GanB, AAF12813).

Strains, growth conditions, and DNA manipulations

The following strains were used in this study: wt 1/2 and 2/9 (Strain 521; Gold *et al.*, 1997), and both *a1 b1*or *a2 b2* mating types of strains *gpa3*$_{Q206L}$, *Δgpa1*, *Δgpa2*, *Δgpa3*, and *Δgpa4* (Regenfelder *et al*. 1997). *U. maydis* strains were grown or maintained at 29^{o}C in liquid YEPS (1% yeast extract, 2% peptone, 2% sucrose), liquid double complete medium (DCM, Holliday, 1974), on solid DCM agar, in liquid potato dextrose broth (PDB, Difco), or on solid potato dextrose agar (PDA). When appropriate, 30μg ml^{-1} of hygromycin B was added to liquid media and 200μg ml^{-1} was added to solid media. Transformed *U. maydis* were plated on solid DCM containing sorbitol and 300 μg ml^{-1} hygromycin B, as previously described (Barrett *et al*. 1993). For nutrient deprivation experiments strains were grown on *U. maydis* minimal medium agar (MMA, Holliday, 1974) or 2% water agar (WA).

All PCR products were gel-purified using the Freeze-N-Squeeze Kit (Bio-Rad), and cloned into the pCRII-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). All subsequent, restriction digestions, ligations, and *Escherichia coli* transformations (into strain DH5α) used standard procedures (Sambrook *et al.*, 1989). DNA sequencing was done at the University of Georgia Molecular Genetics Instrumentation Facility or Genome Analysis Facility.

Cloning of rgs1

Degenerate primers (5'-TGRTCTTGTTIARYTCRCA-3' and TGYGARGARAAYYTIACITT) were designed from the RGS domains in *A. nidulans* FlbA (NCBI XP410030) and *S. commune* Thn1 (NCBI AF267870). PCR reactions were carried out in a total volume of 50µl and contained 5µl taq polymerase reaction buffer, 4µl 25mM MgCl2, 8µl dNTPs (10mM each dNTP), 30 pmole of each primer, 100 ng of genomic DNA from *U. maydis* strain 1/2, and 0.05U taq polymerase enzyme (Fisher Scientific). Amplification was achieved in 35 cycles of 1 minute each at 94 ^{o}C, 41 ^{o}C, 72 ^{o}C, and a final extension for 10 minutes at 72 ^{o}C. The resulting PCR product was used to probe a *U. maydis*, strain 521, genomic library (Barrett *et al*. 1993). The cosmid 7-5-B2 hybridized to the fragment and was used as a template for sequencing the entire *rgs1* gene by primer walking. Sequencing reactions were done on the ABI 310 at the Genome Analysis Facility (Miller Plant Sciences, University of Georgia, Athens, GA).

Construction of Δrgs1

1.2kb of the *rgs1* 5'UTR was amplified by PCR using the cosmid 7-5-B2 as template and the oligonucleotides 5'UTR-5, (5'-TCGGGTACCCGTCCCGCTCCCCTTTGTTTCTC-3') and 5'UTR-3Not1BglII (5'-TCGGCGGCCGCAGATCTTCGCGTGGAGGTGCTTGAAT-3') as

primers. 0.8kb of the *rgs1* 3'UTR was amplified by PCR using the oligonucleotides 3RGSKpn1Bgl2, (5'-TCGGGTACCAGATCTCTCGTGTCTTGAGTTTGT-3') and 3RGSNot1 (5'-TCGGCGGCCGCAGTGAGGCGATGAAGCGA-3') as primers. Separate reactions carried out in a total volume of 25µl and contained 2.5µl taq polymerase reaction buffer, 2µl 25mM MgCl2, 4µl dNTPs (10mM each dNTP), 15 pmole of each primer, approximately 400 ng cosmid 7-5-B2, and 0.05U taq polymerase enzyme (Fisher Scientific). Amplification was performed as described above.

The 5' and 3'UTRs were removed from their respective vectors by digestion with *Kpn*I and *Not*I and cloned separately into pCR2.1-BB digested with the same enzymes. pCR2.1-BB is a modified form of pCR2.1 that has *Bgl*II and *Bam*HI sites destroyed, (personnel communication, John Egan, University of Georgia). The *rgs1* 5'UTR was shuttled into the vector containing the 3'UTR by double digesting both plasmids with *Hind*III and *Bgl*II. The resulting vector contained both the 5'UTR and 3'UTR of *rgs1* separated by a unique *Bgl*II restriction enzyme site. The hygromycin cassette was digested from the vector pIC19R+HL (Mayorga *et al.* 2001) with *Bam*HI and *Bgl*II. This fragment was inserted into the *Bgl*II site between the 5'UTR and 3'UTR to create the *rgs1* gene replacement construct. *U. maydis* 1/2 and 2/9 strains were transformed with the linearized *rgs1* gene replacement construct. Replacement of the native *rgs1* gene by homologous recombination was confirmed by PCR and Southern blot analysis. All restriction enzymes and their buffers were from New England Biolabs (Beverly, MA).

Construction of P*gap::rgs1 over-expression vectors*

The entire predicted open reading frame of *rgs1* was ligated into pCM768, which contains an ARS sequence and a hygromycin cassette (Kojic and Holloman 2000). Briefly, the

predicted open reading frame of *rgs1*, 2.25 kb, was long distance-PCR amplified from the cosmid 7-5-B2 using oligonucleotides UMOE5-1, (5'-TCGGGATCCATGCCTCTCTCCGACAGC-3') and UMOE3-1, (5'-TCGAAGCTTTTCAAGGATTGCTAGGAGAAGG-3') as primers. The PCR reaction was carried out in a total volume of 50µl and contained: 5.0µl 10X BD Advantage 2 PCR Buffer, 1µl 50X dNTP Mix (10 mM of each dNTP), 15 pmole of each primer, approximately 400 ng cosmid 7-5-B2, and 2µl 50X BD Advantage 2 Polymerase Mix (BD Biosciences, Palo Alto, CA). Amplification was achieved in 35 cycles of 1 minute at 94 °C, 1 minute at 66 °C, 2.5 minutes at 72 °C, and a final extension for 10 minutes at 72 °C. The PCR product was cloned into TOPO TA vector PCRII and sequenced on the ABI 310 at the Genome Analysis Facility (Miller Plant Sciences, University of Georgia, Athens, GA) to insure that the PCR reaction did not introduce mutations. The *rgs1* gene was shuttled from the TOPO-TA vector by digesting with *Bam*HI and *Hind*III and then ligated into the vector pCM768. For random ectopic integration of P*gap::rgs1* into the *U. maydis* genome the 3.4 KB section containing the ARS sequence was released using *Hind*III and *Cla*I before transformation. The remaining portion of the vector containing the hygromycin gene, P*gap* promoter, and the *rgs1* gene was gel purified, as described previously, and transformed into both the 1/2 and 2/9 strains. Forty hygromycin-resistant transformants from each strain were grown in liquid YEPS or DCM for 24 hours and observed by phase-contrast microscopy. Four transformants from each strain were selected for further analysis. Because over-expression strains grew poorly in liquid medium or on PDA the strains were each streaked onto 2-4 DCMC plates and incubated at room temperature for 6 days to acquire enough material for RNA extraction. RNA was extracted as described below. Total RNA (15 µg) from a representative of each strain and a wild type control was electrophoresed on a formaldehyde

denaturing gel and blotted overnight onto a Hybond-N nylon membrane (Ambion, Austin, TX). Ethidium bromide stained ribosomal bands were used as loading controls. The over-expression of *rgs1* by transformants was confirmed by northern blots and probed with the P^{32}-labeled 268 bp *rgs1* fragment, previously used to isolate the *rgs1* containing cosmid from the genomic library.

Double mutants

Progeny were collected from Δ*gpa1* x Δ*rgs1* or Δ*gpa4* x Δ*rgs1* crosses as described by Holliday (1974; see plant inoculations below). Double mutants (Δ*gpa1* Δ*rgs1* and Δ*gpa4* Δ*rgs1*) were identified by Southern blots probed with a 400bp P^{32}-labeled hygromycin gene fragment.

The double mutant, *gpa3*$_{Q206L}$ p*gap::rgs1*, was made by transforming *gpa3*$_{Q206L}$ with the undigested vector containing *rgs1* in pCM768 (Kojic and Holloman 2000). Ten hygromycin-resistant putative transformants from each mating type were screened by PCR for possession of the plasmid. Two transformants from each mating type were selected for further analysis and grown 24 hours in liquid DCM or on DCMC agar. RNA was extracted from transformants grown 4 days on DCM containing 200 ug ml^{-1} as described below. Expression of *rgs1* of transformants was confirmed by northern blot analysis as described for P*gap::rgs1* strains.

MMA, WA, and plate mating

Strains were streaked onto MMA (Holliday 1974) plates, wrapped with parafilm, and incubated at room temperature. Filamentous growth of the Δ*rgs1* mutants was observed between four to eight days after strains were streaked. To determine which of the Gα deletion strains could not respond to nutrient deprivation, they were streaked onto WA plates, wrapped with parafilm, and incubated at room temperature for 2 days.

Plate mating assays were used to determine the effect of *rgs1* deletion on the filamentous phenotype associated with a positive mating reaction. Strains were grown overnight in PDB and then spotted on PDA containing 1% activated charcoal at room temperature. Compatible mating types were either spotted singly or co-spotted sequentially, and observed after 24 hours for the production of filaments indicative of successful mating. All assays were photographed.

Plant inoculations

Golden Bantam (Territorial Seed Co., Cottage Grove, OR) seedlings were grown in UGA soil mix (Gold *et al*. 1997), and at 10 days were injected in the culms, just above the soil line, with 10^6 cells ml^{-1} of each of the mating strains. Plants were maintained in the natural greenhouse environment. This condition was preferred over the controlled growth chambers because plants were healthier and those infected with wild-type crosses survived to produce teliospores. Disease symptom data was collected at 7, 10, and 14 days post inoculation. Disease ratings were as previously described (Gold *et al*. 1997). Briefly, the ratings were: 0, no disease; 1, chlorosis or anthocyanin; 2, leaf gall; 3, large stem gall; 4, basal gall; 5, plant death due to disease. Experiments to analyze pathogenicity were carried out a minimum of three times.

Sporulation time course

For each experimental cross 20 plants were maintained and inoculated as described above with either compatible mating combinations of *wt* x *wt*, Δ*gpa1* x Δ*gpa1*, or Δ*gpa4* x Δ*gpa4*. Plants were checked in the morning each day for production of teliospores. The Δ*gpa2* x Δ*gpa2* cross was not performed because previous observations indicated that it did not differ from wild type crosses. The experiment was replicated twice. Sporulation was scored both in a normal and

blind assay format. Results for the blind assay were recorded for day 8-through day 11-day post inoculation (instead of days 8-14 of the normal assay) and did not differ from the normal assay (data not shown).

DNA and RNA procedures

All DNA extractions were performed as previously described (Hoffman -Kosack and Jones 1987), treated with RNase Cocktail (Ambion, Austin, TX), extracted once with phenol:chloroform (3:1), then ethanol precipitated. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), treated with RNasin (Ambion, Austin, TX) and DNase, extracted once with phenol:chloroform (3:1), then ethanol precipitated. DNA and RNA concentrations were determined using a Beckman Coulter Spectrophotometer, model DU 640. All other molecular techniques followed standard procedures (Benson-Chanda 1998; Sambrook *et al*. 1989).

The probe for the northerns and Southerns was made from the 268bp fragment originally isolated from the *rgs1* gene and was radio-labeled using P^{32} and DECA Prime II Radio-labeling Kit (Ambion, Austin, TX).

Gall sections and viability of teliospores

Basal galls were sliced horizontally and pictures were taken on an Olympus Dissection microscope with an Olympus CAMEDIA C-4040ZOOM camera. Each gall picture represents greater than 20 individual galls. It should be noted that the amount of "brown" spots in the *Δrgs1* x *Δrgs1* galls varied and the picture in Figure 4 is a representative of the majority of galls from this cross. Teliospore viability was carried out as previously described (Holliday 1974).

References

Adams, Thomas H., Jenny K. Wieser, and Jae-Hyuk Yu. (1998). "Asexual sporulation in Aspergillus nidulans". Microbiology and Molecular Biology Review **62** (1):35-54.

Banuett, Flora. (1992). "*Ustilago maydis*, the delightful blight". Trends in Genetics **8** (5):174-180.

Banuett, Flora and Ira Herskowitz. (1996). "Discrete developmental stages during teliospore formation in the corn smut fungus, *Ustilago maydis*". Development **122**:2965-2976.

Barrett, K.J., S. E. Gold, and J. W. Kronstad. (1993). "Identification and complementation of a mutation to constitutive filamentous growth in *Ustilago maydis*". Molecular Plant-Microbe Interactions. **6**:274-283.

Benson-Chanda, Virginia. (1998). *Current Protocols in Molecular Biology*. Edited by V. Benson-Chanda: John Wiley & Sons Inc.

Broad Institute. (2003). *Ustilago maydis* Sequencing Project. *Broad Institute of MIT and Harvard*.

Chang, Mi-Hee Chae, Keon-Sang Han, Dong-Min Jahng, Kwang-Yeop. (2004). "The GanB Gα-protein negatively regulates asexual sporulation and plays a positive role in conidial germination in *Aspergillus nidulans*". Genetics **167** (3):1305-1315.

Christensen, J.J. (1963). Maize Smut Caused by *Ustilago maydis*. Monograph no. 2: Saint Paul: American Phytopathological Society.

Cubbage, Frederick W., John M. Pye, Thomas P. Holmes, and John E. Wagner. (2000). "An economic evaluation of fusiform rust protection research". Southern Journal of Applied Forestry **24** (2):77-85.

Cullen, Paul J. and George F. Sprague, Jr. (2000). "Glucose depletion causes haploid invasive growth in yeast". Proceedings of the National Academy of Sciences **97**(25):13619-13624.

DiBello, Paul R., Tiffany Runyan Garrison, Donald M. Apanovitch, Ginger Hoffman, David J. Shuey, Kimberly Mason, Mark I. Cockett, and Henrik G. Dohlman. (1998). "Selective uncoupling of RGS action by a single point mutation in the G-protein alpha -subunit". Journal of Biological Chemistry **273** (10):5780-5784.

Durrenberger, Franz, R. David Laidlaw, and James W. Kronstad. (2001). "The *hgl1* gene is required for dimorphism and teliospore formation in the fungal pathogen *Ustilago maydis*". Molecular Microbiology **41** (2):337-348.

Fowler, T.J., and M.F. Mitton. (2000). "Scooter, a new active transposon in *Schizophyllum commune*, has disrupted two genes regulating signal transduction". Genetics **156** (4):1585-94.

Gold, S.E., S.M. Brogdon, M.E. Mayorga, and J.W. Kronstad. (1997). "The *Ustilago maydis* regulatory subunit of a cAMP-dependent protein kinase is required for gall formation in maize". Plant Cell **9**:1585-94.

Han, Kap-Hoon, Jeong-Ah Seo and Jae-Hyuk Yu. (2004). "Regulators of G-protein signaling in *Aspergillus nidulans*: RgsA down-regulates stress response and stimulates asexual sporulation through attenuation of GanB (G alpha) signaling". Molecular Microbiology **53** (2):529-540.

Hicks, Julie K., Jae-Hyuk Yu, Nancy P. Keller, and Thomas H. Adams. (1997). "*Aspergillus* sporulation and mycotoxin production both inactivation of the FadA G alpha protein-dependent signaling pathway". European Molecular Biology Organization Journal **16** (16):4916-4923.

Hoffman, Charles S., and Fred Winston. (1987). "A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*". Gene:267-272.

Holliday, Robin. (1974). *Ustilago maydis. In Handbook of Genetics*. Edited by R. C. King. Vol. 1. New York: Plenum Press.

Kays, Ann M., and Katherine A. Borkovich. (2004). "Severe impairment of growth and differentiation in a *Neurospora crassa* mutant lacking all heterotrimeric Gα proteins". Genetics **166** (3):1229-1240.

Kojic, Milorad and William K. Holloman. (2000). "Shuttle vectors for genetic manipulations in *Ustilago maydis*". Canadian Journal of Microbiology (46):333-338.

Krüger, Julia, Gabriel Loubradou, Gerhard Wanner, Erika Regenfelder, Michael Feldbrügge, and Regine Kahmann. (2000). "Activation of the cAMP pathway in *Ustilago maydis* reduces fungal proliferation and teliospore formation in plant tumors". Molecular Plant-Microbe Interactions **13** (10):1034-1040.

Lee, Bee Na, and Thomas H. Adams. (1994). "Overexpression of *flbA*, an early regulator of *Aspergillus* asexual sporulation, leads to activation of *brlA* and premature initiation of development". Molecular Microbiology **14** (2):323-334.

Lengeler, Klaus B., Robert C. Davidson, Cletus D'souza, Toshiaki Harashima, Wei-Chiang Shen, Ping Wang, Xuewen Pan, Michael Waugh, and Joseph Heitman. (2000). "Signal transduction cascades regulating fungal development and virulence". Microbiology and Molecular Biology Review **64** (4):746-785.

Letunic, Ivica, Copley, Richard R., Schmidt, Steffen, Ciccarelli, Francesca D., Doerks, Tobias, Schultz, Jorg, Ponting, Chris P., Bork, Peer. (2004). "SMART 4.0:towards genomic data integration". Nucleic Acids Research (32):D142-144.

Lorenz, Michael C., and Joseph Heitman. (1998). "The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*". European Molecular Biology Organization Journal. **17** (5):1236-1247.

Lorenz, Michael C. and Joseph Heitman. (1997). "Yeast pseudohyphal growth is regulated by GPA2, a G protein homolog". European Molecular Biology Organization Journal (16):7008-7018.

Martinez-Espinoza, A. D., M. D. Garcia-Pedrajas, and S. E. Gold. (2002). "The *Ustilaginales* as plant pests and model systems". Fungal Genetics and Biology 35:1-20.

Miwa, Takuya, Yukinobu Takagi, Makiko Shinozaki, Cheol-Won Yun, Wiley A. Schell, John R. Perfect, Hidehiko Kumagai, and Hisanori Tamaki. (2004). "Gpr1, a putative G-protein-coupled receptor, regulates morphogenesis and hypha formation in the pathogenic fungus *Candida albicans*". Eukaryotic Cell **3** (4):919-931.

Regenfelder, Erika, Tilman Spellig, Andreas Hartmann, Stephanie Lauenstein, Michael Bolker, and Regine Kahmann. (1997). "G proteins in *Ustilago maydis*: transmission of multiple signals?" European Molecular Biology Organization Journal **16** (8):1934-42.

Reichmann, Michael, Alexander Jamnischek, Gerhard Weinzierl, Oliver Ladendorf, Sieglinde Huber, Regine Kahmann, and Jorg Kamper. (2002). "The histone deacetylase Hda1 from *Ustilago maydis* is essential for teliospore development". Molecular Microbiology **46** (4):1169-1182.

Sambrook, J., E.F. Fritsch, and T. Maniatis. (1989). *Molecular cloning: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Versele, Matthias, Johannes H.de Winde and Johan M. Thevelein. (1999). "A novel regulator of G-protein signaling in yeast, Rgs2, down-regulates glucose-activation of cAMP pathway through direct inhibition of Gpa2". European Molecular Biology Organization Journal **18** (20):5577-5591.

Welton, Robert M., and Charles S. Hoffman. (2000). "Glucose monitoring in fission yeast via the gpa2 Gα, the git5 Gβ and the git3 putative glucose receptor". Genetics **156** (2):513-521.

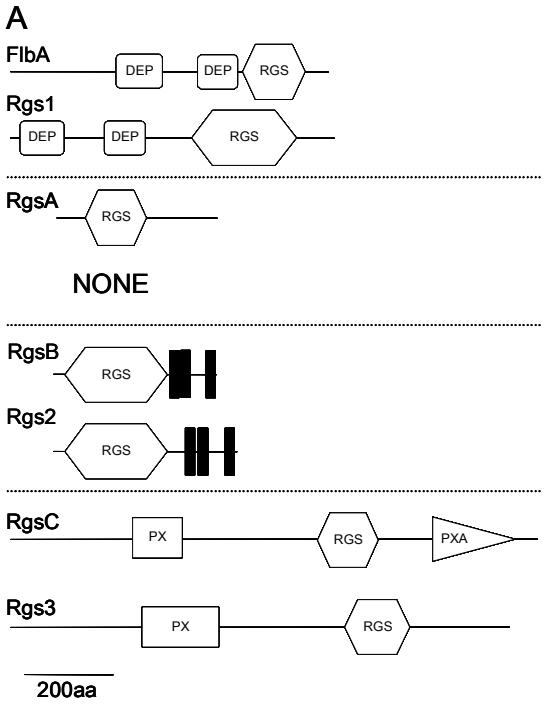
Williams-Woodward, Jean L. (2003) Georgia Plant Disease Loss Estimates. Athens: University of Georgia.

Yu, Jae-Hyuk, Jenny K. Wieser, and Thomas H. Adams. (1996). "The *Aspergillus* FlbA RGS domain protein antagonizes G-protein signaling to block proliferation and allow development". European Molecular Biology Organization Journal **15** (19):5184-5190.

Table 3.1. Pathogenicity Phenotypes 14 Days Post Inoculation

Cross *a1 b1* x *a2 b2*	#Plants tested	Disease Index	% Galls
wt x *wt*	190	3.22	87.4
wt x Δ*rgs1*	100	2.24	67.0
Δ*rgs1* x *wt*	100	2.47	71.0
Δ*rgs1* x Δ*rgs1*	120	2.28	69.2
Δ*gpa1* x Δ*gpa1*	50	3.80	100.0
Δ*gpa2* x Δ*gpa2*	60	3.63	100.0
Δ*gpa3* x Δ*gpa3*	10	0.40	0.0
Δ*gpa4* x Δ*gpa4*	60	3.87	98.3

Figure 3.1 A. Structural comparison of RGS proteins in *A. nidulans* (top row in each pair) and *U. maydis* (bottom row in each pair) based upon output from the SMART database (Letunic *et al*. 2004). Indicated domains: RGS (regulator of G-protein signaling); DEP (found in dishevelled, EGL-10, pleckstrin and other signaling proteins, of unknown function); PX (phosphoinositide binding); PXA (PX-associated, of unknown function); black vertical bars (trans-membrane segments). B. Orthologous and functional relationships among selected RGS proteins and Gα subunits in *A. nidulans* and *U. maydis*. Double wavy lines mark putative orthologs. FlbA, FadA, RgsA, GanB and Gpa3 are all known to regulate sporulation (Han *et al*. 2004; Regenfelder *et al*. 1997; Yu *et al.* 1996).



B

A. nidulans		*U. maydis*
FlbA	≃	Rgs1
⊣		
FadA	≃	Gpa1
RgsA	≃	None
⊣		
GanB	≃	Gpa3

Figure 3.2 Phenotypes of selected *U. maydis* strains on various solid media. A. After 2 days of growth on potato dextrose agar supplemented with activated charcoal. B. After 8 days of growth on *U. maydis* minimal medium. C. After 2 days of growth on water agar.

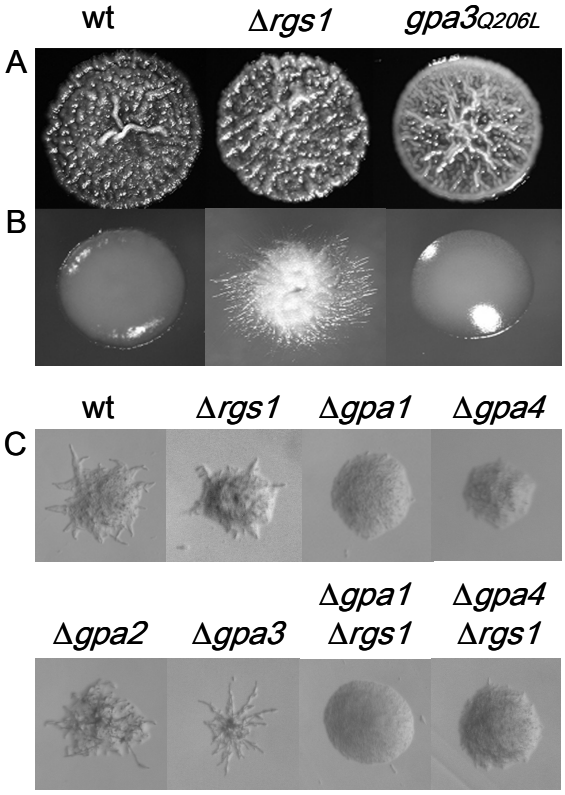

wt
Δrgs1
gpa3Q206L
A
B
wt
Δrgs1
Δgpa1
Δgpa4
C
Δgpa2
Δgpa3
Δgpa1
Δrgs1
Δgpa4
Δrgs1

Figure 3.3 Effect of *rgs1* deletion on mating. Haploid strains were inoculated either individually or coordinately at each spot on the plate. Bright white colonies are filamentous and thus indicate the successful formation of dikaryotic cells. Strain mating types (*a1 b1* and *a2 b2*) are indicated.

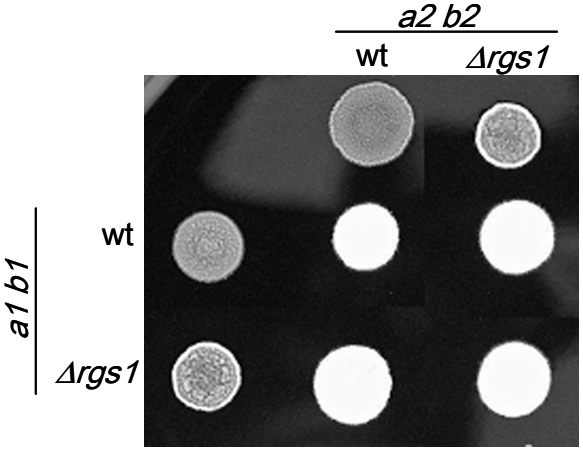

a2 b2
wt
Δrgs1
a1 b1
wt
Δrgs1

Figure 3.4 Effect of *rgs1* deletion on teliosporgenesis. Horizontal sections of basal galls 17 days post inoculation. A. wild-type x wild-type cross; dark patches are masses of mature teliospores. B. *Δrgs1* x *Δrgs1* cross; arrows mark small clusters of immature teliospores. Each gall is representative of more than 20 individual galls.

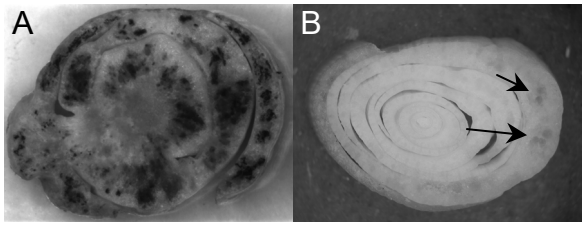
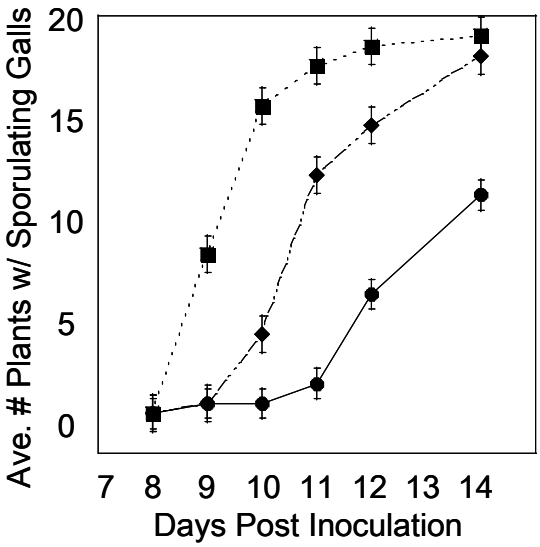

A
B

Figure 3.5 Role of Gpa1 and Gpa4 in temporal patterns of *in planta* sporulation. Eleven day old Golden Bantam corn plants were inoculated with compatible crosses: *wt* x *wt*●, *Δgpa1* x *Δgpa1*■, and *Δgpa4* x *Δgpa4*◆. In two separate experiments, twenty plants per treatment were scored daily for galls producing spores (mean ± SEM). No spores were observed between initial inoculation and eight days post inoculation.


Ave. # Plants w/ Sporulating Galls
20
15
10
5
0
7 8 9 10 11 12 13 14
Days Post Inoculation

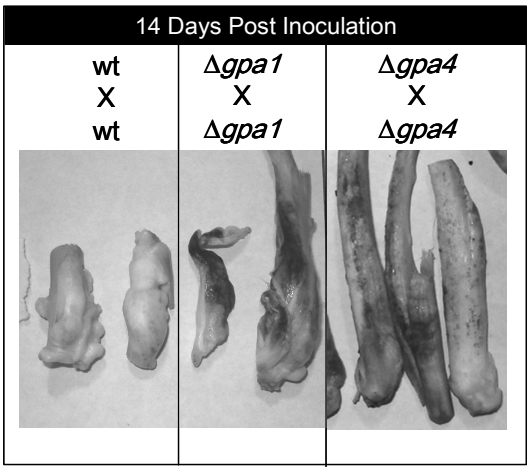

14 Days Post Inoculation
wt X wt
Δgpa1 X Δgpa1
Δgpa4 X Δgpa4

Figure 3.6 Phenotypic effect and transcript abundance in *rgs1* over-expression. A. Phenotypes of selected strains after 24 hours growth in liquid YEPS, or B. 48 hours growth on double complete medium + charcoal. C. Total RNA blots probed with 268 bp *rgs1* fragment: lane 1, wt; lane 2, P*gap*::*rgs1* (*a1 b1*); lane 3, P*gap*::*rgs1* (*a2 b2*); lane 4, $gpa3^{Q206L}$; lane 5, $gpa3^{Q206L}$ p*gap*::*rgs1* (*a1 b1*); lane 6, $gpa3^{Q206L}$ p*gap*::*rgs1* (*a2 b2*). Bottom panels in C show corresponding ethidium bromide-stained gels.

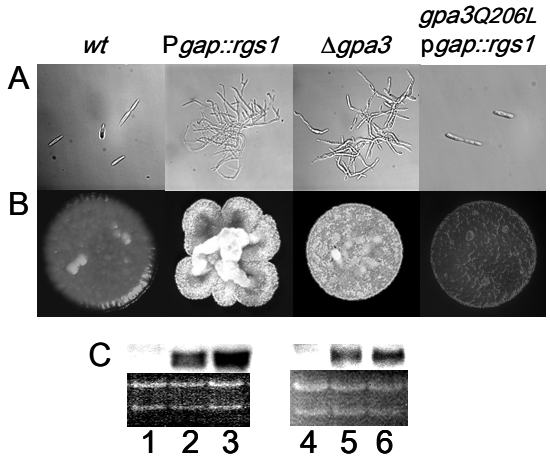
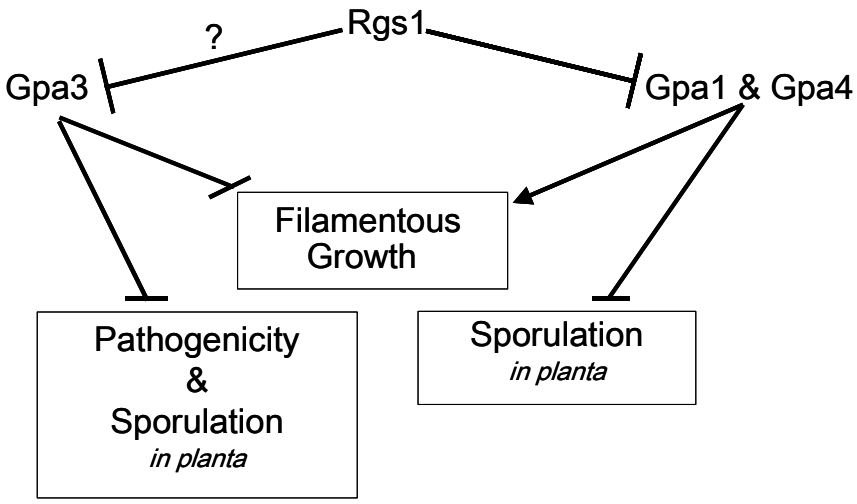

wt
Pgap::rgs1
Δgpa3
gpa3Q206L
pgap::rgs1
A
B
C
1 2 3
4 5 6

Figure 3.7 Model of Rgs1 regulatory networks in *U. maydis*. Rgs1 promotes *in planta* sporulation by deactivating Gpa1, Gpa4, and possibly Gpa3, all of which are negative regulators of this process. Rgs1 suppresses filamentation on minimal medium by deactivating Gpa1 and Gpa4, both of which are essential for filamentous growth under low nutrient conditions. Rgs1 may also stimulate filamentation on rich medium by deactivating Gpa3, which suppresses filamentous growth in this environment. Because the only evidence that Rgs1 interacted with Gpa3 is derived from over-expression data the question mark indicates that the interaction between Rgs1 and Gpa3 should be viewed with some caution as this may not be the case under normal circumstances.


Rgs1
?
Gpa3
Gpa1 & Gpa4
Filamentous
Growth
Pathogenicity
&
Sporulation
in planta
Sporulation
in planta

CHAPTER 4

CONCLUSIONS

For successful infection of a plant by any phytopathogen, the pathogen must first recognize the appropriate host, evade any host defense systems, and in the case of obligate phytopathogenes, must be able to establish growth within the host in a way that does not lead to host death. The obligate phytopathogen, *Cronartium quercuum* f.sp. *fusiforme*, produces basidiospores that recognize, germinate, and establish infection on pine. Here I have described how surface hydrophilicity affects *C. q. fusiforme* basidiospore germination type and how it affects gene expression in the germinating basidiospores. I used a reverse genetics approach to study *in planta* sporulation in *U. maydis*.

In chapter II, I determined that surface hydrophilicity affected germination fate and gene expression in *C. q. fusiforme* basidiospores. Surface hydrophilicity affected *C. q. fusiforme* basidiospore germination fate, as the hydrophilicity of the surface was increased from 21% to 54% the percent, the percentage of basidiospores that germinated and germinated directly decreased. Because *C. q. fusiforme* is an obligate phytopathogen, has a complex lifecycle, and has no established transformation system, we cannot perform traditional genetic analysis. Therefore, I took a genomic-based approach to study gene expression in *C. q. fusiforme* germinating basidiospores and exploited the ability of the basidiospores to perform the two different germination types *in vitro* (direct on polystyrene and indirect on glass). I made two suppression subtraction hybridization libraries, one enriched for genes expressed during direct germination and the other enriched for genes expressed during indirect germination. Of the 180

unique gene sequences identified in the combined libraries, seventy-two were unique to the direct library, eighty-seven were unique to the indirect library, and twenty-one were common to both libraries. Interestingly, forty-three of the genes in the combined libraries had significant blast scores (E value < 10-5) to either *U. maydis* or *Cryptococcus neoformans* and sixteen of these could be placed into functional categories. To determine the function of these 43 gene products, a genetic model system, such as *U. maydis*, and reverse genetics approaches could be utilized. If knockouts of the putative orthologs in *U. maydis* have detectable phenotypes, the corresponding *C. q. fusiforme* genes could then be introduced to test their ability to complement functionality.

Macro-arrays were used to test for differential expression between the directly and indirectly germinating basidiospores. Of the previously mentioned 180 cDNAs in the two libraries, I found fourteen to be differentally expressed between the two. To verify the macro-array analysis a semi-quantitative RT-PCR was used on at least four clones that showed high differential expression (data not shown). The *C. q. fusiforme* actin gene was used as an RNA control and loading control in the PCR amplifications.

In Summary, this is the first study to investigate *C. q. fusiforme* basidiospore germination on a molecular scale. Our genetic knowledge base of this process has been increased through the identification of 180 genes that may be involved in host recognition, infection, and basidiospore formation.

In Chapter III, I described how I searched the *U. maydis* genome and found three putative regulator of G-protein signaling (RGS) proteins. Using a reverse genetic approach I determined that *U. maydis* uses at least one RGS, Rgs1, to regulate *in planta* sporulation through the negative regulation of a Gα subunit(s) of a heterotrimeric G-protein(s). At the beginning of this

study only one Gα protein in *U. maydis* , Gpa3, was reported to be involved in *in planta* sporulation (Regenfelder, Spellig *et al*. 1997). I have established that two additional Gα subunits, Gpa1 and Gpa4, are negative regulators of *in planta* sporulation, as well as positive regulators of filamentous growth on low nutrients .

The data presented herein suggests a new model for regulation of *in planta* sporulation in which multiple heterotrimeric G-protein signaling pathways are used instead of one. Establishment of Rgs1 as the negative regulator of the Gα subunits that negatively regulate *in planta* sporulation lays the groundwork for the eventual determination of how the pathogen intercepts signals from the external environment to initiate the switch from vegetative growth to reproductive growth. The ability of an RGS to regulate more than one Gα subunit has not previously been described. The complex phenotype of the *rgs1* and P*gap::rgs1* mutants suggests that Rgs1 can regulate Gpa1, Gpa4, and possibly Gpa3 given the appropriate environment. This model can be tested by destroying the RGS binding sites in the different Gα's in a P*gap::rgs1* genetic background and by investigating the phenotypes of a combination of Gα knockouts. In addition future experiments need to be performed on the two other putative RGS protein sequences that were found. It will be interesting to establish which of the remaining Gα's they regulate and if they, like Rgs1, can regulate different Gαs depending on the conditions.

References

Regenfelder, E., T. Spellig, *et al*. (1997). "G proteins in *Ustilago maydis*: transmission of multiple signals?" The European Molecular Biology Organization Journal **16**(8): 1934-42.