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**THE ROLE OF HSL7 IN MORPHOLOGY AND PATHOGENICITY AND ITS
INTERACTION WITH OTHER SIGNALING COMPONENTS IN THE PLANT
PATHOGEN *USTILAGO MAYDIS***

By

**Charles Ben Lovely
B. S. Biology, B. A. Chemistry, University of Louisville, 2002**

**A Dissertation
Submitted to the Faculty of the
College of Arts and Sciences of the University of Louisville
In Partial Fulfillment of the Requirements
For the Degree of**

Doctor of Philosophy

**Department of Biology
University of Louisville
Louisville, Kentucky**

August 2010

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A Dissertation Approved on

May 18, 2010

by the following Dissertation Committee:

Dissertation Director

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ABSTRACT

**THE ROLE OF HSL7 IN MORPHOLOGY AND PATHOGENICITY AND ITS
INTERACTION WITH OTHER SIGNALING COMPONENTS IN THE PLANT
PATHOGEN *USTILAGO MAYDIS*.**

C. Ben Lovely

May 18, 2010

Dimorphism is a highly conserved process in fungi in which a transition between a unicellular, yeast-like growth form and either a unicellular or multicellular, filamentous growth form occurs in response to several different environmental cues. The phytopathogenic fungus *Ustilago maydis* undergoes the dimorphic transition from a yeast-like saprobic growth form to a unicellular, hyphal growth form in response to successful mating and subsequent host cues. In addition, *U. maydis* can also undergo haploid filament formation in response to several environmental cues including low ammonium conditions and growth in lipids or acid pH. On solid media deficient in ammonium (SLAD), *U. maydis* produces a filamentous colony morphology, while in liquid low ammonium media the cells do not form filaments.

The p21-activated protein kinases (PAKs) play a substantial role in regulating the dimorphic transition in fungi. Many activities of the PAKs are relayed through the mitogen-activated protein kinase (MAPK) pathway. The PAK-like Ste20 homologue, Smu1, is required for a normal response to pheromone via up-regulation of pheromone

expression, potentially through the MAPK pathway. Disrupting *smu1* reduced this up-regulation, with the effect more pronounced in the *a2* mating background. Our experiments suggest that Smu1 also plays a role regulating cell length and the filamentous response on solid SLAD media. Yeast two hybrid analysis identified the conserved protein-arginine methyltransferase, Hsl7, as a potential interactor of Smu1. Hsl7 regulates cell length and the filamentous response to solid SLAD, yet in an opposite fashion to Smu1.

Interestingly, simultaneous disruption of *hsl7* and overexpression of *smu1* leads to a hyper-filamentous response on solid SLAD. In addition, the double mutant strain also forms filaments in liquid SLAD, while neither single mutant nor wild type strains display this phenotype. A similar filamentous response in both solid and liquid SLAD was also observed in strains lacking another PAK-like protein kinase involved in cytokinesis and polar growth, Cla4. This was not observed in a strain deleted for the activator of Cla4, the Rho-like GTPase Rac1. My data suggest that Hsl7 may regulate cell cycle progression, while both Smu1 and Cla4 appear to be necessary for the filamentous response in *U. maydis*.

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CHAPTER I

HISTORY OF FUNGI IN DISEASE AND AGRICULTURE

Fungi have played and continue to play an important role in life on this planet. Belonging to a kingdom separate from bacteria, plants, and animals, these eukaryotic organisms are comprised of single-cell species as well as multicellular species. Approximately 70,000 fungal species have been identified; however, estimates on the true number are much greater (Hawksworth, 1991). Found all over the world, these organisms fill about every niche available in the environment going mostly unnoticed as decomposers found in soil. In addition, many serve as symbionts with plants and animals, or even other fungi. However, several species act as opportunistic pathogens of plants and animals, as well. Historically, several pathogenic fungi have had impact on humans worldwide either directly through disease or indirectly by destroying agriculture. *Aspergillus fumigatus*, *Candida albicans*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, and *Pneumocystis carinii* all can cause serious infections in humans and can lead to death. Treatments for many forms of cancer and the current AIDS epidemic are resulting in increases in fungal infections due to systemic immune suppression. Many of these species produce several compounds called secondary metabolites (mycotoxins) that are toxic to animals and are important in the virulence process. *Aspergillus fumigatus* produces several of these mycotoxins, including gliotoxin and sterigmatocystin that are important in virulence of

this fungus (Bok and Keller, 2003). These toxins target cells in the immune system and suppress many of their functions or lead to outright apoptosis of several cell lines.

Several species that do not directly infect humans produce mycotoxins that, when ingested, are highly toxic. *Amanita* species produce amatoxins and are found throughout the world and have been important for many cultures for the religious ceremonies. The species *Amanita muscaria* was featured in the story *Alice in Wonderland* due to its purported hallucinogenic effects. Ergotism (Saint Anthony's fire) is another fungal-based affliction caused by ingesting rye contaminated with *Claviceps purpurea* and has led to several outbreaks in human history. One of the first recorded outbreaks was in the Rhine Valley in Germany in 857 A.D. It has been proposed that the Salem witch trials in the late 17th century were due to an ergotism outbreak (Shelton, 2001 [<http://www.plant.uga.edu/labrat/ergot.htm>]). Many of these same groups of fungi produce other secondary metabolites that are of extreme importance to humans, especially antibiotics. *Penicillium chrysogenum* produces the first identified and widely used antibiotic, penicillin, while *Cephalosporium acremonium* produces cephalosporins. *Cylindrocarpum lucidum* and *Tolypocladium inflatum* both produce cyclosporin.

Several groups of fungi target many plant species important to agriculture or are of agricultural importance themselves. The great Irish potato famine in the mid 19th century that resulted in the deaths of more than a million Irish was triggered by a potato blight (*Phytophthora infestans*) outbreak. Current losses (2009) due to potato blight are in the billions of dollars (International Potato Center [<http://cipotato.org>]). Several other fungal species (e.g., *Ustilago* spp., *Magnaporthe grisea*, *Fusarium* spp., *Erysiphe* spp., *Aspergillus* spp., *C. purpurea*) destroy billions of dollars of crops of several important

crops (corn, wheat, barley, and rice), and can lead to mass starvations or other severe outcomes. Several fungal species are prized agricultural products. The common button mushroom, *Agaricus bisporus*, shiitake mushrooms, *Lentinula edodes*, oyster mushrooms, *Pleurotus ostreatus*, and truffles, *Tuber melanosporum*, are but a few edible mushroom species. Moreover, other species are used in the production of several different food stuffs. Blue cheeses are created by the addition of several different *Penicillium* species, while species of *Saccharomyces*, specifically *Saccharomyces cerevisiae*, are important for making many types of bread and alcoholic beverages. *Aspergillus oryzae* is necessary for the production of soy sauce and saki. Newer uses for fungi have been in the field of bioremediation where it was discovered that several species are able to degrade environmentally toxic compounds, *i.e.*, herbicides/insecticides and fossil fuel byproducts, as well as removing heavy metals. Fungi are highly important in scientific research and studies in various species have lead to the understanding of several significant biological process like cell cycle regulation, genetic maintenance and expression, and pathogenesis. Fungal research continues to expand understanding of biology and its impact on humans.

DIMORPHISM AND DISEASE

Many fungal species display a phenomenon called dimorphism. Dimorphism is a highly conserved process in fungi in which a transition between a unicellular, yeast-like growth form and filamentous, sometimes multicellular, growth form occurs. In the unicellular growth form, fungi divide their nuclei mitotically then undergo cytokinesis resulting in complete, independent cells. The hallmark of filamentous growth is a tube-like structure which may be divided by septa into connected compartments. Albeit

similar to filamentous growth, pseudohyphal growth differs in the form of the filament. In hyphal formation, the septated compartments are uniform and usually remain connected, open to one another through pores in the septal walls (Alexopoulos, *et al.*, 1996). However, in pseudohyphal growth the connected cells undergo normal mitotic division but fail to separate and are reminiscent of individual cells joined end to end with no pores connecting the cells. In addition, while hyphae are a continuous tube in shape, pseudohyphae appear more similar to connected, independent cells. Moreover, both types of filaments can be invasive, allowing the fungus the ability to penetrate substrates on which they grow (Madhani and Fink, 1998).

C. albicans and *S. cerevisiae*, both of the same fungal family, Ascomycota, undergo different dimorphic transitions, where *C. albicans* produces true hyphae and *S. cerevisiae* produces pseudohyphae. Overall the regulation of this transition is similar between the two organisms; however the cues that trigger it can be dramatically different. *C. albicans* forms filaments in response to blood serum and a temperature change, while *S. cerevisiae* transitions from yeast-like cells to pseudohyphae under conditions of low ammonium. The process of dimorphism has been defined as a reversible process in which some fungal species can make a transition from yeast-like to hyphal growth and back (Nadal, *et al.*, 2008). *C. albicans* can readily switch its growth between the yeast-like and hyphal forms and this ability is essential for virulence. However, in other fungi the switch is not reversible and once the switch occurs the organism has to complete its lifecycle. The plant pathogen *Ustilago maydis* must undergo mating and completes its lifecycle in the host plant, ultimately returning to its yeast-like phase upon germination of spores released from the plant (Nadal, *et al.*, 2008).

Fungi switch growth forms in response to various cues, including mating pheromone, environmental conditions, and host cues (in the case of pathogenic species). Depending on the species, several environmental cues can trigger this transition. It is posited that in response to environmental conditions *S. cerevisiae* undergoes the transition to pseudohyphal growth to search for nutrient rich, optimal substrates. *Mucor* species undergo a morphogenic transition in response to type of carbon source present and atmospheric oxygen levels. Yeast-like growth occurs only when a fermentable hexose is available and under anaerobic conditions (Orlowski, 1991). The human pathogens, *Blastomyces dermatitidis*, *H. capsulatum*, and *C. albicans*, all undergo a dimorphic transition when moving from 25⁰C to 37⁰C, though both *B. dermatitidis* and *H. capsulatum* convert from a benign hyphal form to parasitic yeast-like form, whereas *C. albicans* switches from a yeast-like form to a pathogenic hyphal form (Sánchez-Martínez and Pérez-Martín, 2001). Additionally, *C. albicans* can undergo the yeast-like to hyphal transition in response to basic pH, nutrient starvation, and the addition of serum. *U. maydis* undergoes the switch from yeast-like to hyphal growth in response to successful mating and subsequent host cues. The development of stable hyphal filaments occurs only *in planta* and appears to be dependent on signals from the host plant. In pathogenic dimorphic fungi, the ability to infect is linked to the dimorphic transition. Failure of any of the dimorphic pathogenic fungi previously mentioned to undergo a morphological transition attenuates their ability to infect their host species. In addition, *U. maydis* can also undergo haploid filament formation in response to several cues including low ammonium conditions and growth in lipids or acid pH, and this is independent of mating and virulence.

Ustilago maydis

Smut fungi, so named for the darkened spores they produce, were first identified in 1730. They were initially described by Jethro Tull as “black stinking powder,” though the causal agent was not recognized until the 1750’s as coming from other infected, smut containing plants. Subsequent studies identified that spores produced in infected plants were able to cause smut disease in other plants (Money, 2006). *Ustilago maydis* (corn smut) is a basidiomycete fungus that infects maize (*Zea mays*) and teosinte (*Euchlena mexicana*) and was first described in 1815 by de Candolle. *U. maydis* is a plant pathogen but for centuries has been a delicacy (Banuett, 1995). Corn smut disease, or huitlacoche, originated in what is now Mexico and was believed to have mystic powers and be the food of the gods to the Aztecs (Specialty Produce [<http://www.SpecialtyProduce.com>]). Currently, it is a greatly desired commodity in Mexico, valued for its high nutritional value. Huitlacoche is a common staple of Mexican foods and has been cultivated as a cash crop, being potentially more valuable than maize itself (Plant and Fungi, *Ustilago maydis* [<http://www.kew.org/plants-fungi/Ustilago-maydis.htm>]). Though a delicacy in Mesoamerica from prehistoric times until now, it is a potent pathogen and can cost billions of dollars in preventative measures, i.e. fungicides and/or plant genetic modifications, and lost crop yields. Maize is the number one cultivated crop in the U.S. and *U. maydis* is considered an agricultural threat to these maize crops (USDA, 2009 [<http://www.ers.usda.gov/briefing/corn/>]). Breeding programs have lead to almost all corn in the U.S. being genetically identical, making the national crop of corn easily susceptible to a highly virulent disease. *U. maydis* as well as other corn diseases are of great concern to the U.S. both economically and agriculturally. However, this attitude

has not stopped a small community of huitlacoche enthusiasts from trying to open American palates to this Mexican delicacy. They have even renamed it the Mexican truffle.

The life cycle of *U. maydis* (Fig. 1) is divided into three separate stages: a

Figure 1: Representation of the life cycle of *Ustilago maydis*. Haploid sporidia can reproduce asexually by budding. In response to mating pheromone from a compatible partner they form conjugation (mating) filaments and fuse producing an infectious dikaryon which must penetrate the host plant to finish the life cycle. In the plant, hyphal filaments expand and then differentiate by nuclear fusion and produce diploid teliospores that mature and are released. The teliospores germinate, undergo meiosis, then mitosis and give rise to the next generation of haploid sporidia.

unicellular, uninucleate haploid form which is saprophytic, growing on non-living matter; a parasitic, dikaryotic hyphal form; and a diploid form (teliospore), which forms only in tumor-like galls produced *in planta* (for review see Banuett, 1995). Living freely in the environment as cigar-shaped, budding haploid sporidia, dimorphic transition to the dikaryotic filaments, and subsequent teliospore formation, is governed by two mating

type loci, *a* and *b*. Heterothallic in nature, mating requires cells with different alleles at both the *a* locus and *b* locus in order to complete the mating response. The *a* locus, of which only two alleles are known (*a1* and *a2*), consists of at least two open reading frames (ORFs), mating pheromone *a* (*mfa*), which encodes a lipoprotein pheromone, and pheromone receptor *a* (*pra*), a seven-transmembrane-domain pheromone receptor (Banuett, 1995). Furthermore, the *a2* allele contains two additional ORFs, *lga2* and *rga2*, which encode small mitochondrial proteins. These proteins direct mitochondrial inheritance by selectively degrading *a1*-associated mtDNA, promoting *a2* mtDNA inheritance (Fedler, *et al.*, 2009). The pheromone and pheromone receptor constitute the cell recognition system. The *a2* strain responds to basal levels of pheromone from a compatible *a1* strain causing an increase in *a2* pheromone expression. This induction leads to an increase in *a1* pheromone expression promoting the growth of mating filaments (conjugation tubes) from both strains toward one another, ultimately resulting in cell fusion (Smith, *et al.*, 2004).

Cell fusion of haploids gives rise to an infectious dikaryotic filament, where the cells fuse, but the nuclei do not. Cytoplasm in the dikaryotic filament is compressed to the apical tip by retraction septae, dividing the hyphal filament into compartments (Steinberg, *et al.*, 1998). The resulting structure is then bound to completing its lifecycle through maize infection. However, the formation and stability of the dikaryon is dependent on an active heterologous *b* locus that is responsible for self / non-self recognition. The *b* locus encodes two divergently transcribed homeodomain proteins, *bE* and *bW*, which act as a single genetic unit and constitute a heterodimeric transcription factor. The *bE* / *bW* heterodimer likely functions as both a transcriptional activator and

repressor, regulating the expression of genes necessary for pathogenesis in maize. Yet, only dimerization of a *bE* and a *bW* protein from different backgrounds will yield an active transcription factor and pathogenicity. Unlike the *a* locus for which only two alleles have been identified, the current estimate on the number of *b* alleles is over 25, leading to hundreds of possible combinations of *b* alleles and raising several questions about combinations and differences in virulence (Banuett, 1995; Bölker, 2001; Klosterman, *et al.*, 2007). The dikaryon produced from mating of compatible partners then generates an appressorium to penetrate the plant. Unlike *M. grisea* (rice blast fungus) and *Collectotrichum graminicola* (pathogen of corn and wheat), which use turgor pressure to breach the surface of a leaf and gain entry to the plant, *U. maydis* secretes enzymes to cause local destruction of surface tissue in order to enter the plant (Kahmann and Kämper, 2004).

The dikaryon grows filamentously through the plant tissues, seemingly under the radar of the plant, not eliciting an immune response, and leads to the production of galls. Dikaryotic growth is difficult to replicate *in vitro*, indicating that plant cues may play a role in stabilizing the dikaryotic state of growth. The galls produced *in planta* result from plant cell proliferation promoted by the fungus. In the galls, the hyphal filaments undergo karyogamy and differentiate, giving rise to rounded diploid teliospores. These teliospores, which develop only in the plant, mature by producing melanin and a thick, rugged cell wall. The plant tissue making up the galls weakens, becoming fragile and ruptures releasing the darkened, soot-like teliospores into the wind. The tough cell wall of the teliospores protects the spores allowing them to remain dormant over an extended time frame of years. Nonetheless, in favorable conditions the teliospores germinate and

undergo meiosis to form a promycelium. The promycelium consists of four haploid cells that can then undergo subsequent rounds of mitosis and give rise to the next generation of haploid sporidia. Mating between compatible haploid sporidia can occur in a laboratory setting, specifically on axenic, activated charcoal media. Mating two cells in this manner does not give rise to diploid teliospores, but provides a window into studying the regulation of cell morphogenesis and the mating response. Moreover, vegetative diploids, heterozygous at both mating loci, can be produced *in vitro*, allowing studies of post cell fusion events and processes (Banuett, 1995).

REGULATING DIMORPHISM

The dimorphic transition in fungi is a highly regulated process that incorporates several cell signaling pathways that are conserved among dimorphic fungi. Fungal cells need to coordinate the cell cycle, cytoskeletal elements, cell wall enzymes, and other cell components to complete the dimorphic transition. In addition, these various cell elements need to be localized to areas of active remodeling during the dimorphic transition. In nutrient rich conditions, *S. cerevisiae* normally displays isotropic growth producing round yeast-like cells that reproduce asexually by budding. These cells only briefly undergo a polar growth pattern during bud emergence, then the bud resorts to isotropic growth within the bud only (Madden and Snyder, 1998). However, in response to various signaling cues, *S. cerevisiae* can undergo a morphological transition producing polarized growth. Under conditions of low ammonium, yeast cells begin to elongate and, depending on the ploidy of the cells, promote invasive growth on solid media (haploid cells) or pseudohyphal growth (diploid cells). Additionally, pheromones can trigger polar growth as cells of opposite mating background respond to mating pheromone and

arrest in G1, subsequently forming elongated mating projections or shmoo's (Madden and Snyder, 1998). *U. maydis* undergoes similar morphological transitions. Cells are polar in appearance and divide asexually by budding, but the buds never transition from a polar style of growth to isotropic growth as observed in *S. cerevisiae*. Additionally, both nutrient availability and mating pheromone promote filamentous growth in *U. maydis*. Under low ammonium conditions and when sensing mating pheromone from a compatible mating partner, cells can produce filaments (Banuett, 1995; Smith, *et al.*, 2003). In addition, heterozygosity at the *b* locus in haploids can lead to filamentation as well, though this is normally dependent on cell fusion on a suitable host (Brachmann, *et al.*, 2001).

THE cAMP DEPENDENT PROTEIN KINASE A PATHWAY

At least two well studied and conserved signaling pathways regulate the morphogenic transition in *S. cerevisiae*. The first pathway, the cyclic AMP (cAMP) dependent Protein Kinase A (PKA) pathway, is composed of a series of enzymes that regulate cell responses to nutrient conditions by manipulating cAMP levels (Fig. 2, for review see Lengeler, *et al.*, 2000). The PKA pathway comprises two core components, adenylate cyclase (AC) and the heterodimeric module, PKA. The PKA dimer consists of a regulatory subunit and a catalytic subunit and when the heterodimer is constituted, the regulatory subunit inhibits the catalytic subunit function. Activity of the heterodimer is influenced by intracellular cAMP levels, where binding of cAMP to the regulatory subunit causes its dissociation regulatory subunit from the catalytic subunit, freeing the catalytic subunit and allowing it to phosphorylate downstream targets. In *S. cerevisiae*, the activity of PKA promotes agar invasion and pseudohyphal differentiation. Three

Figure 2: General diagram of the cAMP dependent PKA pathway in *S. cerevisiae*. This signaling pathway is important in nutrient sensing in fungi. In *U. maydis* it also plays a central role in pheromone-response and pathogenic development.

genes have been identified that encode catalytic subunits (*tpk1*, 2, and 3) and one gene encodes the regulatory subunit (*bcy1*) (for review see Lengeler, *et al.*, 2000).

In yeast, nutrient signals, specifically fermentable sugars, are perceived by a GPCR system. The GPCR, comprised of Gpr1, a seven-transmembrane domain receptor and Gpa2, the associated $G\alpha$ subunit of the heterotrimeric G-protein, respond to glucose and ammonium levels to trigger a dramatic increase in cAMP levels (Lengeler, *et al.*, 2000; Gagiano, *et al.*, 2002). In addition, the ammonium transceptor, Mep2, triggers an increase in cAMP, though the mechanism is yet to be elucidated.

Adenylate cyclase and phosphodiesterases regulate the intracellular levels of cAMP. Adenylate cyclase converts ATP to cAMP when activated by Gpa2, causing cAMP levels to increase. Subsequently,

cAMP binds to the regulatory subunit of PKA, Bcy1, freeing the catalytic subunits, Tpk1-3. One group of downstream targets of Tpk1-3 is the phosphodiesterases, Pde1 and Pde2. Phosphodiesterases act by converting cAMP to various other phosphate forms. Pde1 is a low affinity phosphodiesterase, while Pde2 is a high affinity phosphodiesterase. In addition, Pde1 acts as a phosphoprotein and has a single PKA phosphorylation site

indicating that it may act in a negative feedback loop serving to limit cAMP levels.

Downstream of PKA, Tpk1-3 phosphorylate and activate the transcriptional activator Flo8, which activates several genes promoting pseudohyphal development, including *muc1* and *flo11*. Additionally, Tpk2 associates with the transcriptional repressor Sfl1 and prevents its function (Lengeler, *et al.*, 2000; Gagliano, *et al.*, 2002).

In *U. maydis*, mating and hyphal development are tightly linked and the cAMP dependent PKA pathway plays a major role in response to mating pheromone (for review see Klosterman, *et al.*, 2007). Four Ga subunits have been identified in *U. maydis*, however, only Gpa3 is important in the mating response (Klosterman, *et al.*, 2007). Similar to *S. cerevisiae*, the *U. maydis* genome contains only one gene that encodes the regulatory subunit of PKA (*ubc1*), but, different from yeast, only two genes encoding catalytic subunits of PKA are present (*adr1* and *uka1*), though *uka1* does not appear to play a role in mating, morphogenesis, and virulence (Gold, *et al.*, 1994; Dürrenberger, *et al.*, 1998). Activation of the adenylate cyclase Uac1 by Gpa3 leads to increased cAMP levels. cAMP binding to Ubc1 frees the catalytic subunits, Adr1 and Uka1, which leads to activation of the Pheromone-Responsive transcription Factor, Prf1 (Kaffarnik, *et al.*, 2003). The activation of Prf1 is necessary for the up-regulation of the *a* and *b* loci in the mating response. Adr1 also targets Crk1, an Ime2-like kinase that is required for proper mating and development of *U. maydis*. Through Adr1, the cAMP-PKA pathway negatively regulates *crk1* expression modulating mating and pathogenic development (Klosterman, *et al.*, 2007).

As observed in *S. cerevisiae*, the cAMP-PKA pathway in *U. maydis* regulates the filamentous response to low ammonium conditions. Whereas increased cAMP levels

lead to pseudohyphal development in *S. cerevisiae*, in *U. maydis* the opposite is true. Increased cAMP levels leads to an active catalytic subunit of PKA, but here it promotes budding instead of the filamentous response. The Ump2 transceptor, the Mep2 homologue in *U. maydis*, promotes the filamentous response by decreasing intracellular cAMP levels. However, the mechanism of cAMP reductions by Ump2 is presently unknown. Interestingly, though intracellular levels of cAMP are opposite between *S. cerevisiae* and *U. maydis* under nitrogen starvation conditions, the overall outcome of this condition is the same. Both *S. cerevisiae* and *U. maydis* transition to filamentous growth through the cAMP-PKA pathway when starved for nitrogen (Smith, *et al.*, 2003).

THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) PATHWAY

While the cAMP-PKA pathway regulates the filamentous response in both *S. cerevisiae* and *U. maydis*, it is one of the two highly conserved and studied pathways involved in the morphogenic transition in both fungi. The cAMP-PKA pathway is primarily involved in nutrient sensing, while the Mitogen-Activated Protein Kinase (MAPK) pathway plays a role in both mating and filamentous growth. Here I will focus on the mating pheromone response pathway (for review see Bardwell, 2005). The MAPK pathway, also known as the Extracellular signal-Regulated Kinase (ERK) pathway, consists of a core module of three phosphoprotein kinases that relay an extracellular signal to the nucleus (Fig. 3). The pathway is a serial cascade of phosphorylation events triggered by, and targeting three protein kinases, a MAPK kinase kinase (MAPKKK), also known as a MAPK / ERK kinase kinase (MEKK), a MAPK kinase (MAPKK / MEKK), and a MAP kinase (MAPK or ERK), which in turn

Figure 3: Diagram of the *S. cerevisiae* MAPK pathway. This pathway plays a role in both pheromone response and pseudohyphal differentiation. The signal is transmitted via activation of a heterotrimeric G-protein which activates a series of phosphorylation events that proceed through the three component module that comprises the MAPK pathway. This ultimately leads to transcriptional regulation of genes involved in either mating or

phosphorylates one or more targeted substrates, some of which are transcription factors. In *S. cerevisiae*, Ste11 is the MAPKKK / MEKK, while Ste7 is the MAPKK / MEK and both Fus3 and Kss1 are MAPKs / ERKs. However, the pathway is more complex, consisting of several more components both upstream and downstream of the core module.

Mating pathway signaling begins with a mating pheromone binding to the seven-transmembrane domain receptor, Ste2 (Ste3 for opposite mating pheromone). Upon the pheromone binding to the receptor, the G $\beta\gamma$ heterodimer complex of Ste18 and Ste4, respectively, binds both p21-activated kinase (PAK) Ste20 and the scaffold and adaptor Ste5. The Ste18 / Ste4 complex is anchored to the plasma membrane by covalent linkage of Ste18 to the lipid bi-layer. Ste5 binds to the Ste18 /

Ste4 complex and to Ste11 bringing Ste11 to the lipid bi-layer. The Ste18 / Ste4 complex also binds Ste20 bringing it to the plasma membrane in the vicinity of Ste5 / Ste11, though some studies suggest that a cryptic membrane binding motif rich with basic residues (BR) localizes Ste20 to the plasma membrane (Takahashi and Pryciak, 2007). In

addition, Ste20 may be brought to the plasma membrane by its association to the monomeric Rho-like GTPase, Cdc42. Cdc42 interacts with Ste20 through the specific CRIB (Cdc42 / Rac Interactive Binding) domain and this interaction activates Ste20 function. Cdc42 binding alleviates the auto-inhibition of the CRIB domain and allows Ste18 / Ste4-mediated signaling (Lamson, *et al.*, 2002). The proximity of Ste20 and Ste11 allows Ste20 to phosphorylate Ste11 and activate it. Additional targets of the Ste18 / Ste4 complex are Far1 and Cdc24. Cdc24 is the Guanine nucleotide Exchange Factor (GEF) for Cdc42 and functions by catalyzing the exchange of GDP with GTP, subsequently activating Cdc42, allowing it to bind to Ste20 and other targets. Far1 acts as an adaptor for Cdc24 and Cdc42. Also, Far1 acts as a downstream target of the MAPK pathway, promoting cell cycle arrest in G1.

Once activated, Ste11 begins the serial cascade of phosphorylation events that pass through Ste7 (the MAPKK / MEK) and then Fus3 and Kss1 (the MAPK / ERK), all organized and isolated by Ste5. Both Fus3 and Kss1 are known to mediate the output of the pheromone response pathway. They regulate the activity of the transcription factor Ste12 by directly phosphorylating Ste12 as well as the Ste12 repressors Dig1 and Dig2. These phosphorylation events lead to Ste12 activity and expression of the mating genes. In addition, Fus3 also targets and activates Far1, promoting a second role for Far1 beyond tethering Cdc24 and Cdc42. Upon activation, Far1 targets the cyclin dependent kinase, Cdc28, and inhibits its function leading to cell cycle arrest at G1 (Bardwell, 2005).

The MAPK pathway also plays a role in the filamentous response to environmental conditions in addition to the mating response (for review see Chen and Thorner, 2007). Though several different components constitute the filamentous

response pathway, the core components of Cdc42, Ste20, Ste11, Ste7, Kss1, and Ste12 are still utilized. Several different transmembrane receptors are necessary for the filamentous response though the MAPK pathway, including Mep2. Signal transduction from membrane to Cdc42 is not clearly understood, but it does require the small monomeric G-protein, Ras2, and the 14-3-3 homologues, Bmh1 and Bmh2. The signal follows an identical path as observed in the mating response pathway, with the exception that only Kss1 is activated, not Fus3 (mating response pathway only). Ste12 up-regulates mating gene expression, but in the filamentous response pathway, Ste12 associates with another transcription factor Tec1p to promote expression of genes involved in the filamentous response.

A similar MAPK pathway exists in *U. maydis* and is responsible for the pheromone response (for review see Klosterman, *et al.*, 2007). Unlike *S. cerevisiae*, several players in this pathway are either not known or poorly understood. Signaling begins with pheromone (Mfa1/2) binding to the pheromone receptor (Pra1/2). However, the connection to the next member in the pathway, Ras2, remains unclear. Through domain examination, Ras2 potentially interacts with the MAPKKK / MEKK Ubc4 / Kpp4 and the potential Ste50-like scaffold, Ubc2. Once activated Ubc4 / Kpp4 phosphorylates Ubc5 / Fuz7 (MAPKK / MEK), which then targets Ubc3 / Kpp2 (MAPK / ERK). Downstream of Ubc3 / Kpp2 are the two known MAPK targets Prf1 and Crk1, both of which lead to induction of the mating loci. In addition, a second MAPK / ERK, Kpp6 leads to plant penetration. Other proteins known to play a role in the mating response are the PAK-like kinases, Smu1 and Cla4. Both kinases display differential mating-type dependent effects, with deleting *smu1* attenuating pheromone expression in

the *a2* mating-type background, whereas deleting *cla4* reduces mating efficiency in the *a1* mating-type background (Smith, *et al.*, 2004; Leveleki, *et al.*, 2004). Beyond the mating response, the MAPK pathway has also been implicated in regulating filamentous growth, though its involvement is not clear.

CROSSTALK BETWEEN THE MAPK AND cAMP-PKA PATHWAY

Studies exploring both the cAMP-PKA and MAPK pathways have revealed several points of cross talk between the two pathways. Lengeler *et al.* (2000) posited three points of intersection between the cAMP-PKA and MAPK pathways in *S. cerevisiae*. Upstream of both pathways, the G-protein Ras2 activates both pathways, though the exact mechanism and points of control have yet to be elucidated. Downstream, activity of the two pathways converge on the expression of *flo11*, a cell surface flocculin that promotes cell to cell adhesion, necessary for pseudohyphal development and agar invasion (Palecek, *et al.*, 2000). Last, high levels of cAMP inhibit MAPK activity, leading to the idea that the cAMP-PKA pathway antagonizes the MAPK pathway at certain points, while at other points the two pathways may work synergistically promoting pseudohyphal development and agar invasion (Lengeler, *et al.*, 2000).

A similar form of crosstalk has also been observed in *U. maydis*, in which several genes act to suppress the constitutively filamentous phenotype of strains lacking the adenylate cyclase, *uac1* (Fig. 4). The suppressor genes are known as *Ustilago* Bypass of Cyclase (Ubc) and rescue the filamentous phenotype observed in the *uac1* deletion strain (Klosterman, *et al.*, 2007). Five genes were isolated that were able to bypass the $\Delta uac1$ phenotype, three of which (Ubc3-5) were the components of the MAPK module. In

Figure 4: Representation of cAMP-PKA and MAPK pathway from *U. maydis*. Both pathways converge on two different targets, Prf1 and Crk1 to modulate mating, morphogenesis, and pathogenicity. Solid lines represent an established interaction, either genetic or physical, Dashed lines are hypothetical / potential interactions. See text for additional information.

In addition, Ubc1 is the regulatory subunit of PKA, and Ubc2 is the Ste5-like adaptor protein (Klosterman, *et al.*, 2007). Moreover, the transcription factor, Prf1, is differentially phosphorylated by both Adr1 (catalytic subunit of PKA) and Ubc3 / Kpp2 (MAPK / ERK) leading to expression of either the *a* mating-type genes (Adr1 dependent)

or expression of the *b* mating-type genes (Adr1 and Ubc3 / Kpp2 dependent). Further, both the cAMP-PKA and MAPK pathway differentially converge on Crk1. cAMP-PKA pathway negatively regulates *crk1* expression, while the MAPK pathway activates Crk1 activity (Klosterman, *et al.*, 2007).

BEYOND THE MAPK PATHWAY

Several additional proteins are involved in the modulation of mating signaling through the MAPK pathway. Many of these proteins also play roles in processes outside of the MAPK pathway. Bem1p is one such protein. It acts as an adaptor and scaffold bringing the Cdc24 / Cdc42 complex in close proximity to Ste5 and the MAPK module, as well as Ste20. Cdc24 localizes to the plasma membrane through pleckstrin homology (PH) and interaction with the adaptor, Far1. Through direct binding, Bem1p also interacts with Ste5 and Ste20, and this association brings Ste20 and the MAPK module together with the Cdc24-Cdc42 complex, allowing Cdc42 to activate Ste20 function on Ste11 (Lyons, *et al.*, 1996; Winters and Pryciak, 2005; Chen and Thorner, 2007).

The activation of Ste11 is just one role Ste20 plays in yeast. As previously mentioned, the PAK-like kinase Ste20 plays an extremely important role in both the MAPK pathway, regulating both the mating response and the filamentous response to environmental conditions, and cell morphogenesis and cytokinesis. The PAKs comprise a large family of highly conserved serine/threonine protein kinases. Ste20 is a downstream target of Cdc42 in both the mating-responsive MAPK pathway and in actin organization in polarized cell growth (Eby, *et al.*, 1998; Holly and Blumer, 1999; Lengeler, *et al.*, 2000). Ste20 also plays a role in the filamentous-responsive MAPK pathway, and mitotic exit (Lengeler, *et al.*, 2000; Höfken and Schiebel, 2002; Chen and

Thorner, 2007). Evidence suggests that Ste20 is required throughout the cell cycle regulating actin and cell polarity (Holly and Blumer, 1999). In addition, Ste20 appears to be regulated by the cell cycle machinery. Cdc28 targets Ste20, altering its downstream outcome. When activated, Ste20 targets the MAPK pathway and the mating response. However, under the activity of pheromone, Far1 triggers a G1 arrest by targeting Cdc28, thereby allowing Ste20 function to proceed through the mating response pathway. In the absence of pheromone, Far1 does not inactivate Cdc28, allowing Cdc28 to target Ste20; this change shifts its function from the mating response pathway to the filamentous response pathway (Oehlen and Cross, 1998; Wu, *et al.*, 1998).

Overall it appears that the key to the differences between the mating-responsive and filamentous-responsive MAPK pathway is the activation of Far1 by presence of mating pheromone. Furthermore, Ste20 is not the only downstream target of the Cdc24 / Cdc42 complex. Cla4, a second PAK-like kinase, is a downstream target of Cdc42 involved in septin formation and cell polarity (Versele and Thorner, 2004). Normal septin formation and Cla4 activity promotes the G2-M transition through the protein kinase Swe1, though early in the cell cycle Cla4 is required for normal actin polarity (Holly and Blumer, 1999; Versele and Thorner, 2005). Cla4 also may play a role in the pheromone response in yeast, potentially through interacting with Cdc42 allowing Ste20 function to promote the mating response. Failure of Cdc42 and Cla4 to interact may reduce MAPK activity by attenuating Ste20 activity and preventing the G1 arrest required for mating, leading to Cla4 and Ste20 antagonism in the mating response (Benton, *et al.*, 1997; Heinrich, *et al.*, 2007).

Several points of overlap in function between Ste20 and Cla4 have been identified. Neither gene product, by itself, is essential, though absence of one gene or the other creates cell defects in mating, pseudohyphal development and agar invasion, proper bud formation, or cytokinesis (Cvrčková, *et al.*, 1995; Benton, *et al.*, 1997; Eby, *et al.*, 1998; Holly and Blumer, 1999; Lengeler *et al.*, 2000; Heinrich *et al.*, 2007). Although deletion of one gene leads to specific associated non-lethal phenotypes, deletion of both *ste20* and *cla4* is lethal and the cells arrest after forming one to two elongated buds (Cvrčková, *et al.*, 1995). Both Ste20 and Cla4 are targets of GTP-bound Cdc42 and play roles in actin polarization, pheromone response, vacuole inheritance, and cytokinesis (Cvrčková, *et al.*, 1995; Benton, *et al.*, 1997; Eby, *et al.*, 1998; Holly and Blumer, 1999; Lengeler *et al.*, 2000; Heinrich *et al.*, 2007). The shared activities of Ste20 and Cla4 may be due to similarity in their structure, differing by only one domain. Cla4 contains a PH domain which facilitates the interaction between Cla4 and the plasma membrane. Ste20 does not contain a PH domain, but is still able to interact with the plasma membrane through the BR region in the N terminus (Takahashi and Pryciak, 2007). Work by Keniry and Sprague (2003) approached the question of overlap between Ste20 and Cla4 by creating chimeras of the two proteins. Their work identified a single amino acid substitution that imparted many Ste20-specific functions to Cla4 without ablating any Cla4-specific functions. In addition, they recognized that several functions of both Ste20 and Cla4 require more than one region of the protein, thereby complicating the ability to fully separate the functions of either.

A similar type of overlap between Ste20 and Cla4 homologues has been observed in *Schizosaccharomyces pombe*, *C. neoformans*, and *U. maydis* (Yang, *et al.*, 1998;

Wang, *et al.*, 2002; Leveleki, *et al.*, 2004; Nichols, *et al.*, 2004; Smith, *et al.*, 2004). In these three cases, the extent of overlapping functions was observed to be distinct between the Ste20 and Cla4 homologues. *S. pombe* provides an interesting picture where in the Shk1 (Ste20 homologue) is essential for viability, mating, cytoskeletal regulation, cell cycle regulation, and response to hyperosmotic conditions, while Shk2 (Cla4 homologue) is dispensable for growth and appears to be redundant to Shk1 (Marcus, *et al.*, 1995; Gilbreth, *et al.*, 1998; Yang, *et al.*, 1998; Bao, *et al.*, 2001; Qyang, *et al.*, 2002). This differs from *S. cerevisiae* in that both Ste20 and Cla4 overlap in function, yet they are separable in many of these functions. Similar to the yeast model, Ste20a/ α (Cla4 homologue) and Pak1 (Ste20 homologue) of *C. neoformans* display overlapping functions in mating, differentiation, cytokinesis and virulence; however, the roles of the two PAKs can be delineated both functionally and temporally (Wang, *et al.*, 2002; Nichols, *et al.*, 2004). Pak1 plays a role in the cell fusion, while Ste20a/ α is essential for maintaining polarity during dikaryotic growth. *U. maydis* displays a unique mating-type specific overlap between Smu1 (Ste20 homologue) and Cla4 in mating and virulence, where both Smu1 and Cla4 have roles in mating and pathogenic development (Leveleki, *et al.*, 2004; Smith, *et al.*, 2004). More specifically, Smu1 is necessary for up-regulation of pheromone expression but the defect is more severe when *smu1* is deleted in the *a2* mating-type background. On the other hand, Cla4 is dispensable for pheromone expression, but necessary for cytokinesis and proper filament formation, yet when lacking *cla4* the defect is greater in the *a1* mating-type background.

Both Ste20 and Cla4 homologues are involved in a myriad of functions that need to be modulated to respond to a given set of conditions. Several proteins modulate Ste20

function in *S. cerevisiae*, the Ste18 / Ste4 complex, Cdc42, Bem1, Ste5, Bmh1, Bmh2, and Cdc28 activate and modulate Ste20 activity between the mating responsive and filamentous-responsive MAPK pathway (Roberts, *et al.*, 1997; Cabib, *et al.*, 1998; Bardwell, 2005). Another protein demonstrated to modulate Ste20 activity is the putative protein-arginine methyltransferase (PRMT), Hsl7 (Fujita, *et al.*, 1999). PRMTs facilitate methylation on arginine residues and play roles in signal transduction, RNA processing, and transcriptional regulation (Ma, 2000). Hsl7 has been shown to have methylation activity *in vitro*, though no methylation activity has been observed *in vivo* (Lee, *et al.*, 2000; Miranda, *et al.*, 2006). Hsl7 was initially identified through a synthetic lethal screen examining second site mutations that were sensitive to the absence of the N-terminus of histone H3 (Ma, *et al.*, 1996). Several studies have demonstrated that Hsl7 regulates cell cycle by acting as an adaptor between Hsl1 and Swe1, targeting Swe1 for degradation and thus promoting G2-M transition (Ma, *et al.*, 1996; Shulewitz, *et al.*, 1999; Cid, *et al.*, 2001; La Valle and Wittenberg, 2001; Asano, *et al.*, 2005). Fujita, *et al.* (1999) demonstrated that Hsl7 also interacts both *in vitro* and *in vivo* with Ste20, competing with Cdc42, and negatively regulates the filamentous response to low ammonium conditions. However, evidence from Shulewitz, *et al.* (1999) indicates that Hsl7 influence on the filamentous response may be independent of Ste20, instead promoting cell elongation through a Swe1-specific G2-M delay. Hsl7 homologues in both *S. pombe* and *Xenopus laevis* also regulate cell cycle in a Wee1 (Swe1 homologue) dependent manner (Gilbreth, *et al.*, 1996 and 1998; Yamada, *et al.*, 2004). Skb1 (the Hsl7 homologue in *S. pombe*) interacts with the *S. pombe* Ste20 homologue, Shk1, and regulates cell cycle and the response to hyperosmotic conditions (Gilbreth, *et al.*, 1998;

Bao, *et al.*, 2001). Under these hyperosmotic conditions, Skb1 displays an increase in its methyltransferase activity (Bao, *et al.*, 2001). Also known as Jbp1 and PRMT5, the human Hsl7 homologue interacts with the non-receptor tyrosine kinase, Jak2, and exhibits methyltransferase activity *in vivo* (Pollack, *et al.*, 1999; Rho, *et al.*, 2001). The methyltransferase activity has been linked to histone H3 and regulates both cell cycle progression at G1-S and tumor suppression by targeting the tumor suppressors, ST7 and p53, through the translation initiation factor eIF4E (Pal, *et al.*, 2004; Scoumanne, *et al.*, 2009).

INITIAL RESEARCH INTEREST OF THIS DISSERTATION

The Perlin lab has been and continues to explore several members of the MAPK and cAMP-PKA signaling pathways in *U. maydis*. These pathways, as described above, are critical in regulating the mating response, morphogenesis, and virulence. Both Hsl7 and Ste20 homologues are important components in regulating the activity of the MAPK pathway. In *S. cerevisiae*, Ste20 plays a role in both the mating and filamentous response MAPK pathways and several different proteins differentiate and direct Ste20 activity towards one of the two possible outcomes of the MAPK pathways (Lengeler *et al.*, 2000; Bardwell, 2005). Hsl7 is one of these proteins, acting as a negative regulator of Ste20 in the filamentous response pathway (Fujita, *et al.*, 1999).

My interest in Hsl7 is based on the identification and characterization of Smu1, a Ste20 homologue in *U. maydis* (Smith, *et al.*, 2004). Smu1 is required for proper mating and pathogenicity. Specifically, Smu1 plays a role in the up-regulation of pheromone (*mfa*) expression. The presence of a putative Hsl7 in *U. maydis* encouraged me to question whether the product of *hsl7* interacts with Smu1, as observed in *S. cerevisiae*,

and what role it might play in the mating response and pathogenicity. My initial observations led me to explore the role of Hsl7 in the filamentous response to low ammonium conditions. In addition, it triggered questions of the regulation of filament formation through the Rho-like G protein, Rac1, and by Cla4. In the remainder of this dissertation, I present data that suggest that both Smu1, with Hsl7, and Cla4 play a role in the cell polarity filament formation and cell wall localization under conditions of low ammonium.

CHAPTER II
THE METHYLTRANSFERASE HSL7 AND THE PAK KINASE SMU1
INTERACTIONS AFFECT CELL SIZE, THE FILAMENTOUS RESPONSE, AND
PATHOGENICITY
INTRODUCTION

p21-activated protein kinases (PAKs) comprise a large, highly conserved family of serine/threonine protein kinases, involved in a myriad of cellular functions including cell cycle regulation, cytoskeletal organization, mating responses, cell polarity, cell morphogenesis, cell separation, and response to environmental conditions (Roberts and Fink, 1994; Cvrčková, *et al.*, 1995; Marcus, *et al.*, 1995; Gilbreth, *et al.*, 1996 and 1998; Leberer, *et al.*, 1997; Eby, *et al.*, 1998; Holly and Blumer, 1999; Fujita, *et al.*, 1999; Bao, *et al.*, 2001; Höfken and Schiebel, 2002; Qyang, *et al.*, 2002; Wiley *et al.*, 2003; Leveleki, *et al.*, 2004; Smith, *et al.*, 2004; Heinrich, *et al.*, 2007; Böhmer, *et al.*, 2008). Homologues of the PAK family are known to activate the conserved, three component module, mitogen activated protein kinase (MAPK) pathway (Lengeler *et al.*, 2000; García-Pedrajas, *et al.*, 2008). These pathways are conserved from fungi to humans and play a role in several fungal processes, including mating, cell morphology, growth under conditions of high osmolarity, and filamentous growth. This pathway consists of a serial cascade of phosphorylation events triggered by, and targeting three protein kinases, a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAP kinase

(MAPK), which in turn phosphorylates one or more targeted substrates, some of which are transcription factors. In *Saccharomyces cerevisiae*, both mating and invasive growth / pseudohyphal differentiation depend on multiple elements of a single MAPK cascade (Roberts and Fink, 1994). In *Schizosaccharomyces pombe*, the MAPK pathway is involved in the mating response and subsequent meiosis and sporulation. Several components are shared between the two subsets of the pathways including the PAK, MAPKKK and the MAPKK (Neiman *et al.*, 1993; Marcus, *et al.*, 1995; García-Pedrajas, *et al.*, 2008). Discriminating between various inputs and the eventual desired outputs is dependent on a series of scaffolding and adaptor proteins. In addition, upstream activators and co-activators dissect eventual outputs through spatial regulation of components of this pathway. Upstream of the PAK's, and subsequently the MAPK pathway, is a series of conserved Rho/Rac-like GTP-binding proteins (GTPases) which bind and regulate the localization and activity of the PAK's (Marcus, *et al.*, 1995; García-Pedrajas, *et al.*, 2008).

One member of the Rho/Rac family of GTPases is Cdc42. Cdc42 plays a role in regulating cell proliferation, polarity, and differentiation in many eukaryotes. In *S. cerevisiae*, Cdc42 is required for cell polarity, cytokinesis, regulating invasive and pseudohyphal development, and mating of haploid cells (Etienne-Manneville, 2004; Lengeler *et al.*, 2000). Cdc42 specificity and activity is regulated by cycling between GTP (active) and GDP (inactive) states via two additional groups of proteins, guanine exchange factors (GEFs) and GTPase activating proteins (GAPs). One such GEF is Cdc24, which localizes Cdc42 to the bud neck allowing for proper formation of bud, cytoskeletal reorganization and cytokinesis. A multiprotein complex comprised of

several proteins including, Cdc42 and Scd1 (Cdc24 homologue), plays a similar roles in *S. pombe* (Chang *et al.*, 1999).

Of potential downstream effectors of Cdc42 and Cdc24, the PAKs have been extensively studied (see above). Ste20 (Shk1) and Cla4 (Shk2) are two PAK's first identified and well characterized in *S. cerevisiae* and *S. pombe*, and functions of the two are required in several key aspects of cell development. In yeast simultaneous deletion of both *ste20* and *cla4* is lethal, indicating the two PAK homologues share at least one essential function (Cvrčková, *et al.*, 1995). In addition, Ste20 and Cla4 play roles in many distinct processes. Ste20 is involved in pheromone response pathway, haploid invasive growth pathway, and osmosensing high-osmolarity glycerol (HOG) pathway, while Cla4 regulates septin function and polarized growth as well as cytokinesis. Data have linked Ste20 to cell polarity and cytokinesis and Cla4 to the mating response and both to actin organization (Cvrčková, *et al.*, 1995; Benton, *et al.*, 1997; Eby, *et al.*, 1998; Holly and Blumer, 1999; Lengeler *et al.*, 2000; Heinrich *et al.*, 2007). In *S. pombe*, Shk1 is essential for viability and is required for several cell functions, including mating, cytoskeletal regulation, cell cycle regulation, and response to hyperosmotic conditions, while Shk2, dispensable for growth, appears to be redundant to Shk1 in many functions (Marcus, *et al.*, 1995; Gilbreth, *et al.*, 1998; Yang, *et al.*, 1998; Bao, *et al.*, 2001; Qyang, *et al.*, 2002).

In *U. maydis*, several Rho-like GTPases have been identified, Cdc42, Rac1, Rho1, and Rho3, of which Cdc42, Rac1, and Rho1 regulate cell separation, hyphal development, and cell polarity, respectively (Weinzierl, *et al.*, 2002; Mahlert, *et al.*, 2006; Böhmer, *et al.*, 2008; Pham *et al.*, 2009). In addition, Cdc42, Rac1p, and Rho1 have

been demonstrated to interact *in vitro* with two known PAK's from *U. maydis*, Cla4 and Smu1 (Leveleki, et al., 2004; Pham, *et al.*, 2009). *In vivo* analysis has identified Cla4 to be an effector of Rac1, regulating cell polarity (Leveleki, *et al.*, 2004). A Cdc24 homologue was identified in *U. maydis*, however Cdc24 is the preferred GEF of Rac1, not Cdc42 (Castillo-Lluva, *et al.*, 2007; Alvarez-Tabarés and Pérez-Martín, 2008). A second GEF, Don1, so named due to the mutant colony morphology resembling donuts with a hollow, empty center, was identified as the activator of Cdc42, regulating formation of the second septum between mother and daughter cells. Moreover, a third kinase, Don3, belongs to the germinal centre kinase subfamily of PAK kinases, and is involved in the formation of the second septum between mother and daughter cells in cell separation (Weinzierl *et al.*, 2002). Initially, Don3 was thought to be the effector of Cdc42, though further analysis indicated that the two act independently in the formation of the second septum (Böhmer, *et al.*, 2008). $\Delta don1$ and $\Delta don3$ mutants are defective in cell separation. In addition, $\Delta cdc42$ mutant strains exhibit the same phenotype (Weinzierl *et al.*, 2002).

Hsl7 homologues are known interactors of the PAKs and are conserved from fungi to humans (Ma, *et al.*, 1996; Gilbreth, *et al.*, 1996; Pollack, *et al.*, 1999; Fujita *et al.*, 1999). Hsl7 homologues are putative protein-arginine methyltransferases and are identified by a single methyltransferase domain (Ma, 2000). Hsl7 homologues are involved in a myriad of cellular functions ranging from cell cycle regulation, cell morphogenesis, and response to several environmental conditions (Ma, *et al.*, 1996; Gilbreth, *et al.*, 1996 and 1998; Pollack, *et al.*, 1999; Fujita, *et al.*, 1999; Bao, *et al.*, 2001; Yamada, *et al.*, 2004). Hsl7 from yeast acts as a mitotic inducer by promoting

targeted Swe1 degradation and as a negative regulator of Ste20 in the filamentous response pathway, by acting as a competitive inhibitor of the Ste20 activator Cdc42 (Fujita *et al.*, 1999; La Valle and Wittenberg, 2001; Asano, *et al.*, 2005). Conversely Skb1, the Hsl7 homologue present in *S. pombe*, acts, in concert with Shk1, as a mitotic inhibitor in a Wee1 dependent fashion (Gilbreth, *et al.*, 1998). The study presented here describes the isolation and characterization of an Hsl7 homologue from *U. maydis* and its negative interaction, and subsequent roles, with Smu1. I hypothesized that Hsl7 would act as a negative regulator of Smu1 in the control of the filamentous response to low ammonium conditions, and potentially the mating response and virulence. Here I show that disruption of *hsl7* leads to increases in the filamentous response to low ammonium conditions, while no defects in the mating response and pathogenicity were observed. Concomitant overexpression of *smu1* leads to an exacerbation of the filamentous response, as well as cell separation defects.

MATERIALS AND METHODS

Strains and Growth Conditions. *Ustilago maydis* strains utilized in this study are listed in Table 1. *Saccharomyces cerevisiae* strains AH109 (genotype: *MATa*, *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200 gal4Δ*, *gal80Δ*, *LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3*, *GAL2_{UAS}-GAL2_{TATA}-ADE2*, *URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ*) and Y187 (genotype: *MATa*, *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200 gal4Δ*, *met⁻*, *gal80Δ*, *URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*) were obtained from Clontech (Mountain View, CA) and Dr. S. Ellis (University of Louisville) and used for yeast two-hybrid experiments. *Saccharomyces cerevisiae* strains used for complementation, MJY102 (*MATa*, *ADE2*, *can1-100*, *his3-11,15*, *leu2-3,112*, *LYS2*, *trp1-1*, *ura3-1*, *hsl7-Δ20::HIS3*) and MJY110 (*MATa*, *ADE2*,

TABLE 1. *U. maydis* strains and plasmids used in this study.

Strain / Plasmid	Genotype	Reference
Fungal Strains^A		
FB1 WT	<i>a1b1</i>	<i>Banuett, et al, 1989</i>
$\Delta hsl7$ <i>a1</i>	<i>a1b1 hsl7::cbx^R</i>	This Study
$\Delta_{810-2832}hsl7$ <i>a1</i>	<i>a1b1 hsl7::hyg^R</i>	This Study
<i>hsl7^{Otef}</i> <i>a1</i>	<i>a1b1 P_{otef}-hsl7, cbx^R</i>	This Study
$\Delta smu1$ <i>a1</i>	<i>a1b1 smu1::cbx^R</i>	This Study
<i>smu1^{Otef}</i> <i>a1</i>	<i>a1b1 P_{otef}-smu1, cbx^R</i>	This Study
$\Delta_{810-2832}hsl7 \Delta smu1$ <i>a1</i>	<i>a1b1 hsl7::hyg^R, smu1::cbx^R</i>	This Study
$\Delta_{810-2832}hsl7 smu1Otef a1$	<i>a1b1 hsl7::hyg^R, P_{otef}-smu1, cbx^R</i>	This Study
FB2 WT	<i>a2b2</i>	<i>Banuett, et al, 1989</i>
$\Delta hsl7$ <i>a2</i>	<i>a2b2 hsl7::cbx^R</i>	This Study
$\Delta_{810-2832}hsl7$ <i>a2</i>	<i>a2b2 hsl7::hyg^R</i>	This Study
<i>hsl7^{Otef}</i> <i>a2</i>	<i>a2b2 P_{otef}-hsl7, cbx^R</i>	This Study
$\Delta smu1$ <i>a2</i>	<i>a2b2 smu1::cbx^R</i>	This Study
<i>smu1^{Otef}</i> <i>a2</i>	<i>a2b2 P_{otef}-smu1, cbx^R</i>	This Study
$\Delta_{810-2832}hsl7 \Delta smu1$ <i>a2</i>	<i>a2b2 hsl7::hyg^R, smu1::cbx^R</i>	This Study
$\Delta_{810-2832}hsl7 smu1Otef a2$	<i>a2b2 hsl7::hyg^R, P_{otef}-smu1, cbx^R</i>	This Study
Plasmids		
pOtef- <i>hsl7</i>	<i>P_{otef}-hsl7, cbx^R</i>	This Study
pOtef- <i>smu1</i>	<i>P_{otef}-smu1, cbx^R</i>	This Study
pYES- <i>hsl7</i>	<i>P_{GALI}-hsl7-URA3</i>	This Study
pGADT7- <i>hsl7</i>	<i>P_{GALF}-hsl7-LEU</i>	This Study
pGBKT7- <i>smu1</i>	<i>P_{GALF}-smu1-TRP</i>	This Study

^AAll mutant strains were made in the wild type background strains listed.

can1-100, his3-11,15, leu2-3,112, lys2D::hisG, trp1-1, ura3-1, hsl7-Δ20::HIS3) were a gift of Dr. J. Thorner (University of California, Berkeley), and MOSY 0150 (*MATa, Δste20::TRP*), and MOSY 0151 (*MATa, Δste20::TRP*) were donated by Dr. D. Lew (Duke University). *Escherichia coli* strains, DH5α (Bethesda Research Laboratories,

Bethesda, MD) and TOP10 (Invitrogen, Carlsbad, CA) were utilized for all cloning and subcloning needs.

Ustilago maydis strains were grown at 25°C in YEP (1% yeast extract, 2% peptone), supplemented with sucrose or dextrose, SLAD (0.17% yeast nitrogen base without ammonium sulfate or amino acids (YNB), 2% dextrose, 50 µM ammonium sulfate), and Minimal Media (0.17% YNB, 2% dextrose, 0.3% ammonium nitrate, 6.25% Holliday Salt Solution (Holliday, 1974). All liquid cultures were grown with shaking (260 rpm). Mating media and solid media were made with 1% activated charcoal and/or 2% agar (Holliday, 1974). Stressing media was made with calcofluor white (CFW, Fluorescent Brightener 28, Sigma, St. Louis, MO, 50 µM), congo red (CR, Fisher Scientific Company, Fairlawn, NJ, 15 µg / mL), or sodium chloride (1 M).

Saccharomyces cerevisiae strains were grown at 30°C in YEPD or SD (0.17% YNB, 1X amino acid drop out solution, the BD Matchmaker™ Library Construction & Screening Kits user manual [BD Biosciences, Palo Alto, CA]) supplemented with dextrose or galactose. For complementation assays, yeast strains were grown in SD with either glucose (repressor of P_{GALI} promoter) or galactose (inducer of P_{GALI} promoter).

E. coli strains were grown at 37°C in LB (Luria-Bertani [Bertani, 1951]) and / or Circle Grow media (MP Biomedicals, LLC, Solon, OH).

Primer Design. Primers were designed with the Primer3 program (S. Rosen and H. J. Skaletsky, 1997 [<http://frodo.wi.mit.edu/primer3/>]). Primers were obtained from Eurofins MWG Operon (Huntsville, AL) and are listed in Table 2.

PCR. PCR and Gradient PCR was run on a PTC100 thermal controller (MJ Research Inc., San Francisco, CA) and a DNA Engine thermal cycler (Bio Rad

TABLE 2. Primers used for PCR and Sequencing.

Primers	Sequences (5' → 3')
Hsl 5'2	TGGCTTCACGCTCGTTCACT
Hsl 3'2	AGGATCGGGGGCGATATTGT
cHsl Left	TTCACCATGCCGTCTTCC
cHsl Right	GATCGGGGGCGATATTGTA
Hsl Left	TCTGTCCGATCTCCAATTCC
Hsl Right	CCTCGTTGTCCTTCAATGGT
Hsl Sfi Up	TTCGGCCATCTAGGCCATGCCAAGGTAGGATGCGTAG
Hsl Sfi Down	TGAGGCCTGAGTGGCCCGAATATGGAACGGGAAATG
5Flank Left	GCATTCTCTTCGTCTGCTACCT
5Flank Right Sfi	TTCGGCCATCTAGGCCAGCCTTTCGTGTTTGCTAC
NSmu5	TTCTAGACTTCTCCTCCTCCTCCT
NSmu3	GATTGTTCGTGGTGCGAGTT
Smu1000F	ACGCTCCATACCATCTCGTC
Smu1000R	GAACACTTTGAGGCAGCACA

Laboratories, Hercules, CA), respectively. PCR reactions were run with an initial denaturing temperature of 94°C for 4 minutes, followed by 34 cycles of a second denaturing temperature of 94°C for 30 seconds, annealing temperature ranging from 56°C to 62°C for 30 seconds, and extension temperature of 72°C for 1 minute per 1000 base pairs. A final extension temperature of 72°C for 2 minutes longer than the cycle extension was used to complete the reaction.

Comparison of *hsl7* / *skb1* homologues. Amino acid sequences from five Hsl7 homologues found in Entrez Gene Database (<http://www.ncbi.nlm.nih.gov/gene>) were analyzed using the BLASTP tool from the *ncbi* website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences used were from *Homo sapiens* (PRMT5; Isoform A, accession no. NP_001034708.1 and Isoform B, accession no. NP_006100.2), *Xenopus laevis* (Hsl7; accession no. NP_001084480),

Schizosaccharomyces pombe (Skb1; accession no. CAA17909), and *Saccharomyces cerevisiae* (Hsl7; accession no. NP_009691).

Genetic Manipulation and Vector Construction. PCR products were separated by gel electrophoresis on 0.6% agarose gel (Agarose LE, USB Corp, Cleveland, OH) and purified using the GeneClean III kit (MP Biomedicals, Solon, OH). PCR products were cloned into pCR2.1 TOPO vector (Invitrogen). Plasmid DNA was purified using the Wizard miniprep kit (Promega, Madison, WI). DNA samples were sequenced by the Nucleic Acids Core Facility (Center for Genetics and Molecular Medicine, University of Louisville, Louisville, KY), Eurofins MWG Operon (Huntsville, AL), or AGCT Inc. (Wheeling, IL). Restriction enzymes were purchased from New England Biolabs, Inc. (Bevelery, MA) or American Allied Biochemical, Inc. (Aurora, CO).

Deletion and overexpression of *hsl7* and *smu1* in *U. maydis* were obtained by homologous recombination as described previously (Brachmann *et al.*, 2004). The *hsl7* deletion constructs were created using the *SfiI* technique (Brachmann *et al.*, 2004; Kämper, 2004). Upstream (up-flank) and downstream (down-flank) fragments, approximately 1 kb in length each, of *hsl7* were amplified and used as flanking regions. Both carboxin and hygromycin resistance cassettes were used as markers and were cloned between the up and down flanks in the *SfiI* sites. The Δ *smu1* construct was created using a 4.4 kb PCR product from strain 10/18 (Δ *smu1* a1b1 in the 521 background, Smith *et al.*, 2004) generated with primers smu1000F and smu1000R. The *smu1* expression construct was produced by amplifying the *smu1* ORF with Nsmu5 and Nsmu3 primers and cloning the product into pCR2.1 TOPO (Invitrogen). To provide constitutive expression, the *smu1* ORF was then excised and cloned after the *P_{otef}*

promoter into the *Bam*HI and *Not*I sites of the p123 vector (Weber, *et al.*, 2006). The *hsl7* expression vector was constructed by excising the *hsl7* ORF from pCR2.1-*hsl7* and cloning it after the *P_{otef}* promoter into the *Eco*RI site. *Otef* expression vectors were linearized using *Ssp*I before transforming *U. maydis*, with selection for carboxin resistance (Brachmann *et al.*, 2001). Recombination at the *ip* locus would yield the carboxin resistant transformants. Disruption and over-expression constructs were either digested by restriction endonucleases, or a PCR product was amplified, purified, and used to transform *U. maydis* protoplasts. Potential transformants were verified by PCR.

Yeast two-hybrid analysis. Direct yeast two-hybrid assays were conducted following the BD Matchmaker™ Library Construction & Screening Kits user manual (BD Biosciences). Two vectors, pGBKT7 (bait vector) and pGADT7 (prey vector), contain a series of restriction sites in frame with the 3' end of the GAL4 DNA-BD and the GAL4 DNA-AD, respectively, creating fusion proteins with proteins of interest. Directed assays were performed by introducing the bait and prey vectors into *S. cerevisiae* strain AH109 by co-transformation. A positive interaction was assessed after 3 days growth on –trp/-leu/-his/-ade synthetic drop out media (QDO). The prey fusion protein vector pGA-*hsl7* was constructed by excising the *hsl7* ORF and cloning after the P_{GAL4} promoter into the *Eco*RI site of pGADT7. The *smu1* ORF was excised and cloned after the P_{GAL4} promoter into the *Bam*HI and *Not*I sites of pGBKT7.

Cell length, growth rate, cell viability on stressing media, mating assays, and plant pathogenesis. Cell density was measured spectrophotometrically in liquid culture. Exponential phase of growth (OD₆₀₀ of 0.5 to 0.7) was obtained by measuring the optical densities of cells growing in liquid media for 24 hours. Then cultures were diluted to an

optical density of 0.1 in fresh YEPS and allowed to grow for an additional 3.5 hrs. Cell length was measured in exponential growth (OD₆₀₀ of 0.5 to 0.7) from cells grown in YEPS and analyzed using MetaMorph imaging software (MDS Analytical Technologies Inc., Mississauga, Ontario, Canada). Growth rate was determined by growing cell cultures in YEPS broth for 24 hours, then diluting to an OD₆₀₀ of 0.023 in 50 mL of fresh YEPS. Cultures were allowed to stabilize for 4.5 hours, then optical densities were taken every hour for 11 hours. Stressor viability assays were performed by spotting 5 µL of serial diluted cultures of exponentially growing cells, starting at a concentration of 10⁷ cells / mL and decreasing to 10⁴ cells / mL. The cultures were spotted on media containing CFW (50 µM), CR (15 µg / mL), or sodium chloride (1 M). Mating assays were performed using a concentration of 10⁷ cells / mL and spotting 10 µL on charcoal plates as previously described (Gold *et al.*, 1997). Plant infection using 8 day old Golden Bantam corn seedlings (Bunton Seed Co., Louisville, KY and W. Atlee Burpee & Co., Warminster, PA) was performed with a cell density of 1 x 10⁵ cells / mL for haploid strains as previously described (Gold *et al.*, 1997). Virulence was rated by a disease index on a scale of 0 to 5 with 0 = no symptoms, 1 = chlorosis and / or anthocyanin biosynthesis, 2 = small leaf galls, 3 = small leaf and stem galls, 4 = large galls, and 5 = plant death. A disease rating was assigned to each plant 7, 10, 14, 17, and 21 days post inoculation (+8 days post planting) and the indices for each strain were averaged to give a measure per strain. Three independent trials of approximately 20 plants were performed, averaged, and analyzed.

Statistical Analysis. Cell length measures were analyzed using one way ANOVA with a Dunnett's Multiple Comparison Test in Graphpad Prism 5.02 (Graphpad

Software Inc., La Jolla, CA), while mating type specific comparisons were made using a Student's t-test in Microsoft Excel 2007 (Microsoft Corp., Redmond, WA). Growth rate data were analyzed using one way ANOVA with a Tukey's post hoc analysis in Graphpad Prism 5.02. Statistical analysis of the disease index measures were performed using a Kruskal-Wallis ANOVA with a Dunn's Multiple Comparison Test in Graphpad Prism 5.02.

Staining, micro-colonies, and microscopy. Staining of *U. maydis* cells in exponential growth (OD_{600} of 0.5 to 0.7) was obtained by treating 5 μ L of cells with 1 μ L of 10 μ g / mL of CFW (2 μ g / mL; specific for β -glucan of cell wall) or 100 μ g / mL wheat germ agglutinin (WGA, 17 μ g / mL; Tetramethylrhodamine conjugate, [Invitrogen], specific for chitin of cell wall,). Nucleic acid staining used 10 μ M Syto 11 (5 nM green fluorescent nucleic acid stain) or 1 μ g / mL DAPI (1 mg / mL 4',6-diamidino-2-phenylindole, blue cyan nucleic acid stain[Sigma]). Syto 11-stained cells were visualized using a FITC filter system. For visualization of CFW and DAPI stained cells, a UV-filter set was used. WGA stained cells were visualized using a TRITC filter set, and all stained images were taken on a Nikon Eclipse TE 200 Microscope (Nikon Inc., Melvin, NY) using MetaMorph imaging software. Non stained cell images were taken of cells in exponential growth (OD_{600} of 0.5 to 0.7) on a Nikon Eclipse E800 microscope using Spot imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI) and analyzed using MetaMorph. To help visualize cell morphologies early in colony development, a micro-colony system was designed. Micro-colonies were prepared by inoculating 5 μ L of cell culture in exponential growth (OD_{600} of 0.5 to 0.7) onto a 60-80 μ L media spot on a sterile microscope slide and then covering with a sterile cover slip.

The inoculated slides were placed, elevated, in a sterile glass petri dish with a small volume of sterile water (to prevent desiccation of media). After 48 hr incubation (YEPS) or 72 hr incubation (SLAD), slides were viewed under 10x, 20x, and / or 60x magnification on a Nikon Eclipse E800 microscope using Spot imaging software. Colony morphology images were taken on a Nikon Eclipse SMZ-U microscope using Spot imaging software. All photos were processed with Photoshop Elements 6 (Adobe, San Jose, CA).

RESULTS

Smu1 plays a role in cell length and filamentation as a response to low ammonium conditions. Previous work (Smith *et al.*, 2004) determined that Smu1 was involved in the mating response pathway, differentially influencing pheromone expression, and therefore is necessary for pathogenicity. It was also observed that Smu1 played a subtle role in the filamentous response (Smith *et al.*, 2004). In addition, the phenotypes observed in the *Δsmu1* mutant strains were mating-type specific, primarily affecting the a2b2 mating-type background. These mating-type specific phenotypes have also been observed for the *U. maydis* gene products Cla4 (Leveleki, *et al.*, 2004) and Rho1 (Pham, *et al.*, 2009). However, the filamentous response to low ammonium conditions and cell morphology of *smu1* mutant strains had not been examined in detail. To explore these areas, *smu1* disruption strains were generated by amplifying and purifying a 4.4 kb PCR fragment from strain 10/18 (*Δsmu1 a1b1* [background strain 521], Smith, *et al.*, 2004) and transforming *U. maydis* cells to disrupt the target gene. Disruptions were made in both FB1 *a1b1* and FB2 *a2b2* haploid background strains and confirmation of the positive gene integrations was made by PCR (data not shown).

Overexpression of the *smu1* ORF was obtained by amplifying and cloning the *smu1* ORF after the *P_{Otef}* promoter; the linearized plasmid was then integrated into the *ip* locus in *U. maydis*. The *smu1^{Otef}* construct and its integration in the FB1 *a1b1* and FB2 *a2b2* strains was also confirmed by PCR (data not shown).

Δsmu1 strains generated in the FB background exhibited a reduction in the mating response primarily in the *a2b2* mating type background demonstrated previously in another genetic background (Fig. 5 and Smith *et al.*, 2004). Building upon these results, I found that the *Δsmu1* strains showed a decrease in cell length, though not statistically significant in *a1b1*, while those with overexpression of the *smu1* ORF displayed elongated cell morphology in comparison to the wild type progenitor cells (Fig. 6A and Table 3). It was observed that the decrease in cell length showed statistical significance in the *a2b2* background only, while the *a1b1* strain was decreased cell length, but this was not statistically significant. The increase of cell length in the *smu1^{Otef}* was only observed and statistically significant in the *a1b1* background. Comparisons between the *a1b1* and *a2b2 smu1* mutant strains indicated that the differences between the mating specific strains were significant in both the disruption and overexpression strains (Table 4). These results are consistent with the mating-type specific phenotypes previously observed in the *smu1* mutant backgrounds.

The Smu1 homolog from *S. pombe*, Shk1p, plays a role in cell cycle regulation displaying a decrease in cell length when *shk1* expression is repressed, while increasing in cell length when *shk1* is overexpressed (Gilbreth, *et al.*, 1998). This is similar to what has been observed in *smu1* mutants. To explore additional roles Smu1 might play in the cell cycle, the growth rates of *Δsmu1* and *smu1^{Otef}* strains were examined. *Δsmu1*, *smu1^{Otef}*,

Figure 5: Plate mating assays for $\Delta_{810-2832}$ *hsl7*, *hsl7*^{O_{lef}}, Δ *smu1*, *smu1*^{O_{lef}}, $\Delta_{810-2832}$ *hsl7* Δ *smu1*, $\Delta_{810-2832}$ *hsl7* *smu1*^{O_{lef}} strains. Equal mixtures of haploid strains of opposite mating-type background were plated onto YPD plates containing activated charcoal. A positive mating reaction produced a white “fuz” phenotype of aerial hyphae production. See text for description of results.

TABLE 3. Measures of cell length across all strains.

Strain	n	Length ^A (in μm)	Comparison	p^B
<i>WT a1b1</i>	91	19.27 +/- .43	<i>WT a1b1</i> v. <i>WT a2b2</i>	N.S.
$\Delta_{810-2832}$ <i>hsl7 a1</i>	167	25.22 +/- .45	<i>WT a1b1</i> v. $\Delta_{810-2832}$ <i>hsl7 a1</i>	> .001
<i>hsl7^{Otef} a1</i>	227	18.66 +/- .27	<i>WT a1b1</i> v. <i>hsl7^{Otef} a1</i>	N.S.
Δ <i>smu1 a1</i>	380	18.64 +/- .16	<i>WT a1b1</i> v. Δ <i>smu1 a1</i>	N.S.
<i>smu1^{Otef} a1</i>	308	21.75 +/- .28	<i>WT a1b1</i> v. <i>smu1^{Otef} a1</i>	> .001
$\Delta_{810-2832}$ <i>hsl7 Δsmu1 a1</i>	221	25.25 +/- .33	<i>WT a1b1</i> v. $\Delta_{810-2832}$ <i>hsl7 Δsmu1 a1</i>	> .001
$\Delta_{810-2832}$ <i>hsl7 smu1^{Otef} a1</i>	143	27.86 +/- .45	<i>WT a1b1</i> v. $\Delta_{810-2832}$ <i>hsl7 smu1^{Otef} a1</i>	> .001
<i>WT a2b2</i>	213	19.14 +/- .31		
$\Delta_{810-2832}$ <i>hsl7 a2</i>	406	24.78 +/- .32	<i>WT a2b2</i> v. $\Delta_{810-2832}$ <i>hsl7 a2</i>	> .001
<i>hsl7^{Otef} a2</i>	173	17.31 +/- .29	<i>WT a2b2</i> v. <i>hsl7^{Otef} a2</i>	> .01
Δ <i>smu1 a2</i>	281	17.64 +/- .18	<i>WT a2b2</i> v. Δ <i>smu1 a2</i>	> .05
<i>smu1^{Otef} a2</i>	224	19.66 +/- .30	<i>WT a2b2</i> v. <i>smu1^{Otef} a2</i>	N.S.
$\Delta_{810-2832}$ <i>hsl7 Δsmu1 a2</i>	137	24.25 +/- .45	<i>WT a2b2</i> v. $\Delta_{810-2832}$ <i>hsl7 Δsmu1 a2</i>	> .001
$\Delta_{810-2832}$ <i>hsl7 smu1^{Otef} a2</i>	89	27.33 +/- .86	<i>WT a2b2</i> v. $\Delta_{810-2832}$ <i>hsl7 smu1^{Otef} a2</i>	> .001

^A Cell length values are averages, +/- S.E.

^B Statistical analysis was performed using a one way ANOVA with a Dunnett's Multiple Comparison Test.

N.S. = Not Significant

TABLE 4. Mating-type specific cell length differences.

Strain	n	Length ^A (in μm)	Comparison	p^B
Δ <i>smu1 a1</i>	380	18.64 +/- .16		
Δ <i>smu1 a2</i>	281	17.64 +/- .18	Δ <i>smu1 a1</i> v. Δ <i>smu1 a2</i>	> .001
<i>smu1^{Otef} a1</i>	308	21.75 +/- .28		
<i>smu1^{Otef} a2</i>	224	19.66 +/- .30	<i>smu1^{Otef} a1</i> v. <i>smu1^{Otef} a2</i>	> .001
<i>hsl7^{Otef} a1</i>	227	18.66 +/- .27		
<i>hsl7^{Otef} a2</i>	173	17.31 +/- .29	<i>hsl7^{Otef} a1</i> v. <i>hsl7^{Otef} a2</i>	> .001

^A Cell length values are averages, +/- S.E.

^B Statistical analysis was performed using a Student's t-test.

N.S. = Not Significant

and wild type cells were grown in YEPS media and optical densities were taken every hour for eleven hours. The optical densities were plotted in Excel and doubling times were determined and analyzed (described in Materials and methods). Examination of rates of growth of the *smu1* mutants indicated no differences from those of the wild type progenitor strains (Table 5).

TABLE 5. Growth rate analysis of mutant strains.^A

Strain	Double Time ^B	<i>p</i> ^C
<i>WT a1b1</i>	2.76 +/- .18	
<i>Δsmu1 a1</i>	2.79 +/- .16	0.82
<i>smu1^{Otef} a1</i>	2.81 +/- .10	0.56
<i>Δ₈₁₀₋₂₈₃₂hsl7 a1</i>	2.68 +/- .15	0.43
<i>hsl7^{Otef} a1</i>	2.71 +/- .13	0.56
<i>Δ₈₁₀₋₂₈₃₂hsl7 Δsmu1 a1</i>	2.86 +/- .19	0.38
<i>Δ₈₁₀₋₂₈₃₂hsl7 smu1^{Otef} a1</i>	2.65 +/- .12	0.22
<i>WT a2b2</i>	2.77 +/- .20	0.95
<i>Δsmu1 a2</i>	2.74 +/- .12	0.75
<i>smu1^{Otef} a2</i>	2.61 +/- .15	0.14
<i>Δ₈₁₀₋₂₈₃₂hsl7 a2</i>	2.74 +/- .16	0.79
<i>hsl7^{Otef} a2</i>	2.81 +/- .13	0.69
<i>Δ₈₁₀₋₂₈₃₂hsl7 Δsmu1 a2</i>	2.83 +/- .15	0.55
<i>Δ₈₁₀₋₂₈₃₂hsl7 smu1^{Otef} a2</i>	2.94 +/- .14	0.11

^A Table shows growth rate analysis of six identical experiments per strain.

^B Growth rate expressed as doubling time in hours, +/- S.E.

^C Statistical analysis was performed using a one way ANOVA with a Tukey's Multiple Comparison Test.

Another possible cause of the cell elongation could be misappropriation of cell wall components. Staining of both *Δsmu1* and *smu1^{Otef}* strains with calcofluor white (CFW, a beta-glucan specific stain and cell wall stressor) and wheat germ agglutinin (WGA, chitin specific stain) did not show any cell wall aberrations, indicating that cell

wall material was able to localize and deposit correctly to the growing tip and in the mother daughter septa (Fig. 6B). Thus, *Smu1* does not appear to play a role in cell cycle regulation or cell wall localization and deposition, however work still needs to be done to explore these effects on cell length.

Previous work indicated that *Smu1* plays a role in the filamentous response to low ammonium conditions (Smith *et al.*, 2004). Colony morphology of *smu1* mutant strains compared to their wild type progenitors was examined, via plate colonies and micro-colonies. Strains were grown on either YEPS or low ammonium (SLAD; 50 μ M ammonium) media. Colony morphology of Δ *smu1* and *smu1*^{O₁ef} mutant strains on YEPS displayed no differences when compared to their wild type progenitor strains (Fig. 7A). To further explore the differences in colony morphology, examination of micro-colonies was utilized and enabled a closer view of the responses of the strains on the two types of media. Micro-colonies were generated by inoculating a media spot on a sterile microscope slide, covered with a sterile cover slip, incubating for 2-3 days at 25°C, and then examining under magnification. On YEPS, both at 20x and 60x magnification, neither Δ *smu1* nor *smu1*^{O₁ef} mutant strains displayed any phenotypic difference from that of the wild type progenitor strains (Fig. 7B). Δ *smu1* mutant strains displayed a subtle decrease in the filamentous response when grown on SLAD and this decrease appears to be more substantial in the *a2b2* background. Overexpression strains, on the other hand, showed an increase in the production of filaments on SLAD and this appeared to be greater in the *alb1* background (Fig. 8A and Smith *et al.*, 2004). When grown on SLAD micro-colonies and viewed at 20x magnification, Δ *smu1* strains displayed a decrease in filament formation, which is more severe in the *a2b2* background (Fig. 8B, Inset).

At 60x magnification, the mating-type specific decrease is more evident. *smu1^{Otef}* mutant strain filaments were longer than those observed in the wild type progenitor cells and the effect is again mating-type specific with the *a1b1* mutant background strain producing filaments above what is seen in the *a2b2* mutant background strain (Fig. 8B).

Examination of sensitivity of the *smu1* mutants to the cell wall antagonists CFW and CR indicated no difference to the wild type progenitor strains (data not shown). Acid pH also had no affect on *smu1* mutants (Smith, *et al.*, 2004). Overall, these results indicate that

Figure 8: Filamentous response to low ammonium conditions of *smu1* mutants. Strains were grown on SLAD media and examined for filament formation. *Δsmu1* colonies display a decrease in the ability to form filaments primarily in the *a2b2* mating-type background while *smu1^{Olef}* colonies exhibit an increase in filament formation but only in the *a1b1* mating-type background (A). Micro-colonies were utilized to examine filament formation at the cellular level. *Δsmu1* strains exhibited decreased filament length and number of filaments with a more severe reduction in the *a2b2* mating-type background. The *a1b1 smu1^{Olef}* strain displayed an increase in both filament length and number of filaments (B). Scale bars, 50 μ m – 20x

Smu1 plays a role in cell length and the filamentous response pathway to low ammonium conditions, in addition to the mating response in *U. maydis*.

Identification of an *Ustilago maydis* Hsl7 homolog and construction of *hsl7* specific mutants. As described in the introduction, a putative protein-arginine N-methyltransferase homolog (um15057) was identified in *U. maydis* through a search of the newly available *U. maydis* genome databases (first through an agreement with Exelixis, Inc.; now available publicly through the Whitehead Institute's genome

initiatives and the MumDB Website [<http://mips.helmholtz-muenchen.de/genre/proj/Ustilago>]). Primers were designed to amplify a 3 kb region of DNA from genomic DNA. A BLASTX comparison of the predicted protein showed similarity to various *hsl7* / *skb1* homologues. Annotation on the MumDB website identified a predicted ORF from which primers were designed to amplify a 2.9 kb ORF from cDNA. BLASTP comparison to other *hsl7* / *skb1* homologues showed greatest similarity to the human protein PRMT5 [Isoform A, accession no. NP_001034708.1 and Isoform B, accession no. NP_006100.2; Identities = 207/473 (43%), Positives = 277/473 (58%), Gaps = 41/473 (8%)], *Schizosaccharomyces pombe* protein Skb1 [accession no. CAA17909; Identities = 215/507 (42%), Positives = 290/507 (57%), Gaps = 33/507 (6%)], and *Xenopus laevis* protein Hsl7 [accession no. NP_001084480; Identities = 201/471 (42%), Positives = 273/471 (57%), Gaps = 41/471 (8%)]. Similarity was also seen to the *Saccharomyces cerevisiae* protein Hsl7 [accession no. NP_009691; Identities = 205/660 (31%), Positives = 303/660 (45%), Gaps = 130/660 (19%)]. A search of

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Prmt5A|H.sap      PLOPLMDNLESQTYEVFEKDPKIKYSQYQQAIIYKCLLDVPEEEKDTNVQVIMVLGAGRGP 370
Prmt5B|H.sap      PLOPLMDNLESQTYEVFEKDPKIKYSQYQQAIIYKCLLDVPEEEKDTNVQVIMVLGAGRGP 353
Hsl7|X.lae        PLOPLMDNLESQTYEVFEKDPVKYSQYQQAIIYKCLLDVPEEEKETNIQIIMVLGAGRGP 366
Hsl7|U.may        PLOPLMDNLEGTTYEVFERDPVKYALYEEAVYKALLDRPVTAN-----TLIIVCGAGRGP 387
Skb1|S.pom        PLOPLSYNLENITYEIERDPVKYAEQAIIFSALMDRD-----ESSVTRIIVVAGAGRGP 357
Hsl7|S.cer        PLKPHSDNLLNSTYLTFEKDLVKYDLYESAILEALQDLAPRAS-AKRPLVILVAGAGRGP 390
                  **: *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
                  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

Prmt5A|H.sap      LVNASLRAAQ--ADRRIKLYAVEKNPNAVVTLENWQFEWGSQVTVVSSDMREWVAP-- 426
Prmt5B|H.sap      LVNASLRAAQ--ADRRIKLYAVEKNPNAVVTLENWQFEWGSQVTVVSSDMREWVAP-- 409
Hsl7|X.lae        LVNASLRAAQ--AERKIKVYAVEKNPNAVITLEGWRYEEWGSQVTVVSGDMREWKAP-- 422
Hsl7|U.may        LVDRCLNAADR--AGRAVRLVALEKNPNALVTLQERQALEWGDQVKVQYGDMRKYPVPSS 445
Skb1|S.pom        LVDCALRAAIS--SSRTVDMIALEKNPNAFSMLLMRNQDWAGKVTLVFGDMRTWNPD-- 413
Hsl7|S.cer        LVDRTFKIISMLFMDSKVSIIAIEKNPQAYLYLQKRNFDCWDRVLIKEDMTKWQINEP 450
                  *:  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
                  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

Prmt5A|H.sap      ----EKADIIVSELLGSFADNELSPECLDGAQHFLKDD-GVSIIPGEYTSFLAPISSSKLY 481
Prmt5B|H.sap      ----EKADIIVSELLGSFADNELSPECLDGAQHFLKDD-GVSIIPGEYTSFLAPISSSKLY 464
Hsl7|X.lae        ----EKADIIVSELLGSFGDNELSPECLDGAQHFLKDD-GVSIIPGEYTSYLAPISSSKLY 477
Hsl7|U.may        MA--ERPDIIVSELLGSFGDNELSPECLDGAMRFLKPN-GISIPSSYTAFLSPLSFFKLH 502
Skb1|S.pom        ----YKIDILVSELLGSMGDNELSPECLDGVQHVLDDEETGICIPSSYISYVTPIMSPKLW 469
Hsl7|S.cer        SEKRIQIDLCISELLGSFGCNELSPECLWSIEKYHSHNDTITIPRSYSSYIAPISPLFY 510
                  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

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Figure 9: Hsl7 is a conserved protein arginine methyltransferase. Protein sequence alignment of the single methyltransferase domain (highlighted) indicates that Hsl7 aligns with other known members of the methyltransferase family.

potential domains in the sequence from *U. maydis* yielded only one hit, the Methyltransf_12 domain (Fig. 9). This region showed the highest similarity amongst the homologues with PRMT5 isoforms A and B and Hsl7 from *Xenopus*, (Identities = 64/105 (60%), Positives = 77/105 (73%), Gaps = 4/105 (3%) and Identities = 63/105 (60%), Positives = 79/105 (75%), Gaps = 4/105 (3%), respectively). Skb1 was next in similarity, (Identities = 53/99 (53%), Positives = 64/99 (64%), Gaps = 4/99 (4%), followed by Hsl7 from *S. cerevisiae*, Identities = 47/106 (44%), Positives = 62/106 (58%), Gaps = 4/106 (3%).

Hsl7 interacts with Smu1 *in vitro*. Hsl7 homologues have been shown to interact with Ste20-like p21-activated kinases (PAKs) in both *S. cerevisiae* and *S. pombe* and to play a role in the filamentous response to low ammonium conditions as well as cell cycle regulation and response to hyperosmotic conditions (Ma, *et al.*, 1996; Gilbreth, *et al.*, 1996 and 1998; Fujita, *et al.*, 1999; Bao, *et al.*, 2001). To determine if Hsl7 from *U. maydis* interacts with Smu1, the Ste20 PAK homolog in *U. maydis*, yeast two-hybrid analysis was utilized. The ORF's of both *hsl7* and *smu1* were cloned after the Gal4 promoter of yeast based vectors. The *hsl7* ORF was cloned into pGADT7 “prey” vector and fused to the activation domain of the Gal4 transcription factor, while the *smu1* ORF was cloned into the pGBKT7 “bait” vector and fused to the DNA binding domain of the Gal4 transcription factor. During a positive reaction the two proteins of interest interact, reconstituting the transcription factor and activating reporter genes, allowing for growth on auxotrophic media. Both pGA-*hsl7* and pGB-*smu1* vectors were co-transformed into the AH109 yeast background strain and plated onto double dropout media (DDO), with growth confirming successful transformation of both plasmids. Several colonies were

subsequently restreaked on to quadruple dropout media (QDO), a more stringent selective media and a positive interaction was determined by colony growth. Through this process it was observed that Hsl7 and Smu1 interacted *in vitro*, growing on QDO. Two other proteins known not to interact with Hsl7, Rho1 and Pdc1 (Pham, *et al.*, 2009), served as negative controls; when these were used in yeast two-hybrid experiments against Hsl7, the transformants failed to grow on QDO. Smu1 and Rho1, which were previously shown to interact *in vitro* (Pham, *et al.*, 2009), did show a positive interaction, growing on QDO (Fig. 10). This is a strong indication that Hsl7 and Smu1 potentially interact *in vivo*.

Figure 10: Hsl7 interacts with Smu1 *in vitro*. Yeast two-hybrid analysis of Hsl7 and Smu1 interaction. Hsl7, cloned in pGADT7, and Smu1, cloned into pGBKT7, were co-transformed into AH109; a positive interaction is indicated by growth on restrictive media. Hsl7, cloned into pGBKT7 was tested against both Rho1 and Pdc1, cloned into pGADT7 as negative controls. pGB-Smu1 was tested against pGA-Rho1 as a positive control.

Disruption of *hsl7* increases cell length and the filamentous response in low ammonium conditions. From the sequence identified as *hsl7*, two disruption constructs were created. These two disruption constructs were produced using an *Sfi*-based approach by inserting either a carboxin or hygromycin resistance cassette between an upstream and downstream flank of genomic DNA. The constructs were excised by restriction endonucleases, purified and used to transform *U. maydis*. Disruptions of *hsl7* were made in both an FB1 *a1b1* haploid strain and an FB2 *a2b2* haploid strain and confirmation of the gene disruptions were confirmed by PCR (data not shown). The

initial construct was created before the MumDB website annotated the *hsl7* ORF. This construct retains the first 810 base pairs of the ORF. After annotation of the *hsl7* ORF was completed on the MumDB website and confirmed in our lab by sequencing of cDNA (data not shown), a complete deletion of the *hsl7* ORF was generated. All further examination did not reveal any phenotypic differences between strains with complete deletion and partial disruption constructs. Thus, the remainder of the experiments will focus on the partial disruption strains ($\Delta_{810-2832}hsl7$). Overexpression of the *hsl7* ORF was obtained by first amplifying and cloning the *hsl7* ORF after the P_{Otef} promoter; the linearized plasmid was integrated via recombination into the *ip* locus in both the *alb1* and *a2b2* FB strains of *U. maydis*. Generation of the appropriate construct and transformants was confirmed by PCR (data not shown). The fact that deleting *hsl7* in haploid strains still allowed cell growth and reproduction indicates that Hsl7 is not essential for cell viability.

Disruption strains ($\Delta_{810-2832}hsl7$) displayed elongated cell morphology, while overexpression of the *hsl7* ORF showed a decrease in cell length compared to the wild types (Fig. 11A and Table 3). Statistical evidence indicates that the elongated cell length of the disruption strains is significantly different from the wild type progenitor strains, independent of mating-type background. In addition, there was no significant difference between the $\Delta_{810-2832}hsl7 alb1$ and $\Delta_{810-2832}hsl7 a2b2$ strains. The cell length decrease was significant in the *a2b2 hsl7^{Otef}* strain only. The comparison between the *alb1* and *a2b2 hsl7^{Otef}* strains indicated that the difference in cell length between the mating-type specific mutant strains was significant (Table 4). This is an interesting result, potentially

Figure 11: Hsl7 affects cell length but not cell wall localization. $\Delta hsl7$ cells display an increase in cell length while *hsl7^{Otef}* cells exhibit a decrease in cell length but only in the *a1b1* mating-type strain (A). Neither $\Delta hsl7$ nor *hsl7^{Otef}* cells display any defects in cell wall localization (B). Scale bars, 10 μ m.

adding Hsl7 to a growing list of proteins (*e.g.* Smu1, Cla4, and Rho1) whose expression differently affects haploids of one mating-type over the other.

The effect of Hsl7 on cell length is opposite to a phenotype observed in *skb1* mutant strains of *S. pombe*. $\Delta skb1$ strains were decreased in cell length, where as *skb1* overexpression strains were increased in cell length (Gilbreth, *et al.*, 1996). The cell length outcomes observed in *skb1* mutant strains were determined to be due to a

perturbation in cell cycle regulation (Gilbreth, *et al.*, 1998). On the other hand, deletion of *hsl7* in *S. cerevisiae* leads to a slightly larger cell morphology and an elongated bud morphology, indicating that the absence of Hsl7 causes a delay in cell cycle progression resulting in an elongated bud formation (Shulewitz, *et al.*, 1999). To explore this, the growth rates of $\Delta_{810-2832}hsl7$ and *hsl7*^{O_{tef}} strains were determined. Compared to the wild type progenitor strains, no difference in the rates of growth was observed in *hsl7* mutant strains (Table 5). In addition, localization of cell wall components was explored. Staining of both $\Delta_{810-2832}hsl7$ and *hsl7*^{O_{tef}} strains with CFW and WGA did not show any aberrations in cell wall material localization and deposition (Fig. 11B). This indicates that Hsl7 does not appear to regulate growth rate, nor have any effect on localization and deposition of cell wall material in the cell.

Hsl7 of *S. cerevisiae* and Skb1 of *S. pombe* are involved in the response to environmental conditions (Fujita, *et al.*, 1999; Bao, *et al.*, 2001). This led me to investigate of the role Hsl7 of *U. maydis* might play in the response to environmental stimuli. Examination of $\Delta_{810-2832}hsl7$ colonies on YEPS consistently showed small spikes protruding from the colonies, whereas the wild type progenitor strains showed smooth edges (Fig. 12A, see arrows). While different from the wild type progenitor strains, this phenotype was subtle and not indicative of a filamentous response. Strains over expressing *hsl7* displayed no differences in colony morphology compared to that of the wild type progenitor strains when grown on YEPS (Fig. 12A). Examination of YEPS micro-colonies at 20x magnification, $\Delta_{810-2832}hsl7$ strains displayed a phenotype that could be described as “directional growth,” where groups of cells parallel to one another run in a similar direction, different to that of another group of parallel cells (Fig. 12B,

Figure 12: Disruption of *hsl7* leads to a directional growth phenotype, while overexpression of *hsl7* did not affect colony morphology on rich media. Grown on rich media, $\Delta_{810-2832}hsl7$ colonies exhibit small protrusions of cells extending away from the colony edge. The colony morphology of *hsl7*^{Otef} mutant strains did not differ from that of the wild type strains, producing smooth colonies (A, see arrows). Examination of micro-colonies of $\Delta_{810-2832}hsl7$ mutant strain indicated “directional growth” where groups of parallel cells grow in a specific direction different from another group of cells. *hsl7*^{Otef} mutant strains did not differ from the wild type strains (B). Scale bars, 50 μ m – 20x inset image, 40 μ m – 60x image.

Inset). At 60x magnification, $\Delta_{810-2832}hsl7$ strains displayed elongated cellular morphology similar to the increase in length observed in individual $\Delta_{810-2832}hsl7$ cells (Fig. 12B). Also observed was a polarized growth pattern with several cells within a group growing in specific directions, different from group to group. Wild type progenitor cells did not display this directional growth phenotype, displaying unorganized distribution and arrangement of cells. This observation explains the small spikes seen in the colonies of the $\Delta_{810-2832}hsl7$ strains as groups of parallel cells growing in a specific

direction independent of other groups of cells. Overexpression of *hsl7* did not result in any observable phenotypic difference when the strains were grown on YEPS (Fig. 12B).

$\Delta_{810-2832}hsl7$ strains grown on SLAD displayed an increase in the filamentous response, with longer filaments and a colony morphology that can be described as “spiney” (Fig. 13A). Overexpression of *hsl7* did display a subtle decrease, but not

elimination, of the filamentous response to low ammonium conditions primarily in the *a2b2* mating background, indicating the Hsl7 plays a role in the filamentous response to

low ammonium conditions in *U. maydis* (Fig. 13A). These results are similar to the phenotypes of *hsl7* mutants in response to low ammonium conditions in *S. cerevisiae* (Fujita, *et al.*, 1999). $\Delta_{810-2832}hsl7$ strains grown on SLAD micro-colonies produced the same directional growth phenotype observed for these strains on YEPS media when viewed at 20x magnification (Fig. 13B, Inset). At 60x magnification elongated highly branched filaments were evident. The filaments were longer and more highly branched than those observed in the wild type progenitor cells. The directional highly elongated filaments produced by $\Delta_{810-2832}hsl7$ explain the observed “spiney” phenotype in colony morphology on SLAD media (Fig. 13B). A dramatic reduction in filament formation was observed for *hsl7^{Otef}* strains on SLAD when viewed at both 20x and 60x magnification (Fig. 13B). It appears that Smu1 and Hsl7 both play a role in cell elongation and the filamentous response to low ammonium conditions, but their effects tended to be opposite to one another. An increase in cell length and the filamentous response was observed in the $\Delta_{810-2832}hsl7$ and *smu1^{Otef}* strains, while a decrease in cell length and a reduction the filamentous response on low ammonium media were seen in the $\Delta smu1$ and *hsl7^{Otef}* strains, primarily in the *a2b2* strains (in cell length only for *a2b2 hsl7^{Otef}* strains). In addition, the *smu1^{Otef}* colonies grown on SLAD did not display the directional growth phenotype observed in the $\Delta_{810-2832}hsl7$ mutant background.

To determine if Hsl7 is involved in the production of filaments as a response to pheromone, plate mating assays were utilized. Assessment of mating efficiency relies on the production of white aerial hyphae on the black background of YPD-activated charcoal media when strains of opposite mating-type are mixed and spotted. Wild type and the $\Delta_{810-2832}hsl7$, *hsl7^{Otef}* mutant strains were mixed and spotted onto YPD-activated charcoal

media. No differences in mating efficiencies were observed among these strains (Fig. 5). The mating assay indicates that Hsl7 does not appear to play a significant role in the mating response, ultimately having no effect on filament formation due to pheromone. Exploration of the cell wall stressors, CFW and CR, also yielded no discernable differences compared to the wild type progenitor strains (data not shown). Taken together, these results indicate that Hsl7 plays a role in the filamentous response pathway to low ammonium conditions, but not responses to pheromone or cell wall stressors.

Disruption of *smu1* in a $\Delta_{810-2832}$ *hsl7* background does not rescue any of the phenotypes associated with the $\Delta_{810-2832}$ *hsl7* background. In order to determine if there is a genetic interaction between Hsl7 and Smu1 *in vivo*, double disruption mutants were created. In both the *a1b1* and the *a2b2* $\Delta_{810-2832}$ *hsl7* backgrounds, a *smu1* disruption construct was introduced by the same method previously described and this was confirmed by PCR (data not shown). The mutants were viable, demonstrating that the absence of both Hsl7 and Smu1 is not lethal. The disruption of *smu1* in the $\Delta_{810-2832}$ *hsl7* background did not attenuate the elongated phenotype of the $\Delta_{810-2832}$ *hsl7* strains (Fig. 14A and Table 3), nor did it have any effect on the rate of growth (Table 5). Staining of the cell walls of the double disruption strains with CFW and WGA did not reveal any defect in the localization and deposition of cell wall material (Fig. 14B). Examination of colony formation on YEPS indicated that disruption of *smu1* displayed a similar morphology of spikes protruding from the colonies, as observed in the $\Delta_{810-2832}$ *hsl7* strains colony (Fig. 15A) pointing to the directional growth phenotype observed in the $\Delta_{810-2832}$ *hsl7* strains. Further examination of colony morphology on micro-colonies indicated that disruption of *smu1* in the $\Delta_{810-2832}$ *hsl7* background did not alleviate the

Figure 14: Disrupting *smu1* in a $\Delta_{810-2832}hsl7$ background does not rescue cell length increases observed in the $\Delta_{810-2832}hsl7$ background, nor does it affect cell wall localization. $\Delta_{810-2832}hsl7 \Delta smu1$ cells display an increase in cell length comparable to $\Delta_{810-2832}hsl7$ mutant strains (A). $\Delta_{810-2832}hsl7 \Delta smu1$ cells do not display any defects in cell wall localization (B). Scale bars, 10 μ m.

directional growth phenotype observed previously (Fig. 15B). On SLAD, $\Delta_{810-2832}hsl7 \Delta smu1$ strains exhibited the same increase in the filamentous response to SLAD media (Fig. 16A) resulting in the “spiney” morphology seen in the $\Delta_{810-2832}hsl7$ strains presented earlier. The increase in the filamentous response to low ammonium conditions was also observed on SLAD micro-colonies (Fig. 16B). In sum, disruption of *smu1* did not rescue the colony morphology aberrations seen in the $\Delta_{810-2832}hsl7$ strains. In addition, CFW or CR did not adversely affect the $\Delta_{810-2832}hsl7 \Delta smu1$ strains relative to the wild type strains. Moreover, disruption of *smu1* in the $\Delta_{810-2832}hsl7$ background did not alleviate the mating type specific defects in the mating reaction observed in the *smu1* disruption

Figure 15: $\Delta_{810-2832}hsl7 \Delta smu1$ strains produce the directional growth phenotype on rich media. Small protrusions were observed in the $\Delta_{810-2832}hsl7 \Delta smu1$ colonies (A, see arrows). Micro-colonies of $\Delta_{810-2832}hsl7 \Delta smu1$ displayed the “directional growth” phenotype of parallel groups of cells growing in a specific direction (B). Scale bars, 50 μm – 20x inset image, 40 μm – 60x image.

background (Fig. 5).

Overexpression of *smu1* in a $\Delta_{810-2832}hsl7$ background exacerbates phenotypes associated with the $\Delta_{810-2832}hsl7$ background on rich media. To continue the examination of a possible genetic interaction between *hsl7* and *smu1*, $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strains were created by introducing the *smu1^{Otef}* construct into the $\Delta_{810-2832}hsl7 alb1$ and $a2b2$ background strains as previously described. Examination of cell

Figure 16: $\Delta_{810-2832}hsl7 \Delta smu1$ mutants are hyper-filamentous on SLAD. On SLAD media, $\Delta_{810-2832}hsl7 \Delta smu1$ colonies display an increase in the ability to form filaments comparable to that of $\Delta_{810-2832}hsl7$ colonies (A). Micro-colonies were utilized to examine filament formation at the cellular level. $\Delta_{810-2832}hsl7 \Delta smu1$ strains were increased in filament length and branching, as well as displaying “directional growth.” Thus, disruption of *smu1* does not alleviate $\Delta_{810-2832}hsl7$ associated phenotypes (B). Scale bars, 50 μm – 20x inset image; 40 μm – 60x image.

morphology indicated that $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strains were greatly increased in cell length when compared to the wild type progenitor strains (Fig. 17A and Table 3).

Also when compared to $\Delta_{810-2832}hsl7$ or $smu1^{Otef}$ single mutant strains, the $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strains displayed increased cell lengths beyond either single mutant strain

Figure 17: $\Delta_{810-2832}hsl7 smu1^{Otef}$ cells are dramatically increased in cell length and the *a2b2* mating-type strain displays defects in cell separation. $\Delta_{810-2832}hsl7 smu1^{Otef}$ cells display an increase in cell length above and beyond any previously examined strain. The *a2b2* mating-type strain exhibits cell separation defects (A). The $\Delta_{810-2832}hsl7 smu1^{Otef}$ strains did not display any defects in cell wall localization with both mother and daughter cell septa properly formed, though the *a2b2* mating-type background cells did not correctly separate (B, see arrows). A small proportion of $\Delta_{810-2832}hsl7 smu1^{Otef}$ cells displayed lateral budding that occurred at cross wall septa (C, see arrows). Scale bars, 10 μ m.

(Table 6), yet the growth were similar to wild type strains (Table 5). In the *a2b2* background, the $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strain displayed mother daughter cell separation defects (Fig. 17A). Normal mother daughter cell separation in *U. maydis* is completed by the formation of two distinct septa, a primary septum in the mother cell and then, subsequently, a secondary septum in the daughter cell. The two septa outline a fragmentation zone that disintegrates, releasing the daughter cell from the mother cell (Weinzierl, *et al.*, 2002). To explore whether $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strains also

TABLE 6. Cell length differences of the $\Delta_{810-2832}hsl7 smu1^{Otef}$ strains.

Strain	n	Length ^A (in μm)	Comparison	p^B
$\Delta_{810-2832}hsl7 a1$	167	25.22 +/- .45	$\Delta_{810-2832}hsl7 a1$ vs. $\Delta_{810-2832}hsl7 a2$	0.45
$smu1^{Otef} a1$	308	21.75 +/- .28	$\Delta_{810-2832}hsl7 a1$ vs. $\Delta_{810-2832}hsl7 smu1^{Otef} a1$	> .001
$\Delta_{810-2832}hsl7 smu1^{Otef} a1$	143	27.86 +/- .45	$smu1^{Otef} a1$ vs. $\Delta_{810-2832}hsl7 smu1^{Otef} a1$	> .001
$\Delta_{810-2832}hsl7 a2$	406	24.78 +/- .32		
$smu1^{Otef} a2$	224	19.66 +/- .30	$\Delta_{810-2832}hsl7 a2$ vs. $\Delta_{810-2832}hsl7 smu1^{Otef} a2$	> .001
$\Delta_{810-2832}hsl7 smu1^{Otef} a2$	89	27.33 +/- .86	$smu1^{Otef} a2$ vs. $\Delta_{810-2832}hsl7 smu1^{Otef} a2$	> .001

^A Cell length values are averages, +/- S.E.

^B Statistical analysis was performed using a Student's t-test.

N.S. = Not Significant

have deficiencies in septum formation, cell wall localization was examined with CFW and WGA. Neither $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strains showed any sign of cell wall aberrations, indicating that cell wall material is able to localize and deposit correctly to the growing tip and in the formation of the two distinct septa between mother daughter cells (Fig. 17B). In the $a2b2 \Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strain, staining identified both the primary and secondary septa, yet the cells remained connected (Fig. 17B, see arrows). However, a small subset of $a2b2 \Delta_{810-2832}hsl7 smu1^{Otef}$ mutant cells produced cross wall septa, while failing to form the normal mother daughter separation septa (Fig. 17C, see arrows). Two potential explanations could be that the cells are either unable to synthesize or are unable to localize all the vesicular elements and membrane aggregates required for defragmentation between the mother daughter septa. A closer examination of the $a2b2 \Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strain also indicated the formation of lateral buds that appear to branch at a point where a cross wall is formed (Fig. 17C, see arrows).

To further explore the pseudohyphal phenotype observed in the $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strain (primarily in the $a2b2$ background), two cell morphology aspects were examined: cell wall localization and number of nuclei. To this end, the cells were

stained with WGA, CFW, and DAPI (nucleic acid stain – DNA specific). Staining with WGA and CFW again indicated no aberrations of the localization and deposition of cell wall material in the $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strains, yet the *a2b2* mutant strain failed to separate correctly, forming pseudohyphae, with random cross wall septa interspersed in the cells (Fig. 18). The observed cross walls were similar to a central septum,

Figure 18: The *a2b2* mating-type $\Delta_{810-2832}hsl7 smu1^{Otef}$ strain is multinucleic. *a1b1* $\Delta_{810-2832}hsl7 smu1^{Otef}$ cells are elongated but display a single nucleus per cell. The *a2b2* $\Delta_{810-2832}hsl7 smu1^{Otef}$ cells exhibited a single nucleus per septated compartment (see arrows). Scale bars, 10 μ m.

observed in the fission yeast, *S. pombe* (Sipiczki, 2007). Visualization of *a1b1* $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant cells also stained with DAPI exhibited a single bright spot representing the nucleus, comparable to the wild type progenitor strains (Fig. 18). When stained with DAPI, the *a2b2* $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strain displayed several connected cells, each with a single nucleus (Fig. 18, see arrows). This is different from what was observed in the wild type strains, where cells are separate from one another and contain a single nucleus. In addition, the pseudohyphal phenotype was only observed in the *a2b2* mating-type background, not in the *a1b1* $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strain.

To further explore the defect in mother daughter cell separation and lateral bud formation, colony and micro-colony morphologies on YEPS media were examined. The $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains exhibited a filamentous colony morphology; this is drastically different than what is observed for the wild type progenitor strains or any of the other mutant strains previously tested (Fig. 19A). Micro-colony examination of $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains displayed a mating-type specific filamentous growth response at 20x magnification. The *alb1* $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strain displayed directional growth similar to the $\Delta_{810-2832}hsl7$ mutant strains, however, the *a2b2* $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strain exhibited filamentous growth (Fig. 19B, Inset). At 60x magnification, the differences between the *alb1* and the *a2b2* mutant strains can be better observed. The *alb1* $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strain exhibited directional growth and pseudohyphal development of the cells making up the colony, although observations of the *a2b2* $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strain displayed long single filaments and what appeared to be multiple areas of random cytoplasmic evacuation within each filament (Fig. 19B, see arrows). CFW staining of an *a2b2* $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strain micro-colony identified septated hyphae (Fig. 19C, see arrows).

$\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains display a hyper-filamentous response to SLAD media. Observations of the $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strain colonies on SLAD media detected hyper-filamentous growth and again a more pronounced effect was seen in one of the two mating-types (Fig. 20A). The *alb1* $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strain displayed the “spiney” morphology observed in the $\Delta_{810-2832}hsl7$ mutant strains, whereas the *a2b2* $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strain developed extremely long single filaments growing in a single direction from the filamentous edge of the colony. This phenotype,

Figure 19: On rich media, the $\Delta_{810-2832}hsl7 smu1^{Otef}$ strains are filamentous. $\Delta_{810-2832}hsl7 smu1^{Otef}$ strains produce filamentous colonies on rich media unlike wild type strains which are smooth in morphology (A). Micro-colonies of the *a1b1* $\Delta_{810-2832}hsl7 smu1^{Otef}$ strain displayed the "directional growth" phenotype while the cells were dramatically elongated. The *a1b1* $\Delta_{810-2832}hsl7 smu1^{Otef}$ strain exhibited filamentous growth with random areas of cytoplasmic evacuation within the filaments (B, see arrows). Scale bars, 50 μ m – 20x inset image, 40 μ m – 60x image. Staining of micro-colonies of the *a2b2* $\Delta_{810-2832}hsl7 smu1^{Otef}$ strain with calcofluor white indicated that the filaments are septated hyphae (C, see arrows). Scale bars, 20 μ m.

best described as a "starburst" phenotype, was not observed in any other mutant previously examined. In addition, the single long filaments displayed in *a2b2* $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strain appear to have a darkened region at the end of each

Figure 20: On SLAD media, the $\Delta_{810-2832}hsl7 smu1^{Otef}$ strains are hyper-filamentous. $\Delta_{810-2832}hsl7 smu1^{Otef}$ strains produce exaggerated filamentous colonies on SLAD media and are dramatically more filamentous than wild type strains. Also filaments of the *a2b2* $\Delta_{810-2832}hsl7 smu1^{Otef}$ strain exhibited darkened regions at the tips of empty filaments (A, see arrows). Micro-colonies of the $\Delta_{810-2832}hsl7 smu1^{Otef}$ strains displayed extremely elongated filaments and the *a2b2* $\Delta_{810-2832}hsl7 smu1^{Otef}$ strain exhibited random areas of cytoplasmic evacuation within the filaments (B, see arrows). Scale bars, 50 μm – 20x inset image, 40 μm – 60x image. Staining of micro-colonies of the $\Delta_{810-2832}hsl7 smu1^{Otef}$ strains with rhodamine-labeled WGA indicated chitin localization defects (C, see arrows). Scale bars, 20 μm .

filament emerging from a clear empty filament (Fig. 20A see arrows). The cytoplasmic evacuation is similar, yet more extensive, than what is observed when the *a2b2* $\Delta_{810-2832}$ *hsl7 smu1^{Otef}* mutant strain is grown on YEPS media. To get a closer examination of the effects of low ammonium conditions on these specific mutants, micro-colonies were again utilized. At 20x magnification, the expanse of filamentous growth of the $\Delta_{810-2832}$ *hsl7 smu1^{Otef}* mutant strains can be seen (Fig. 20B, Inset). At 60x magnification, the *alb1* $\Delta_{810-2832}$ *hsl7 smu1^{Otef}* mutant strain displayed long branched filaments and random cytoplasmic evacuation; moreover, the *a2b2* $\Delta_{810-2832}$ *hsl7 smu1^{Otef}* mutant strain filaments were exceedingly long and branched compared to those of the *alb1* and exhibited dramatic regions of cytoplasmic evacuation (Fig. 20B, see arrows). Staining of the micro-colonies of the $\Delta_{810-2832}$ *hsl7 smu1^{Otef}* mutant strains indicated chitin delocalization throughout the length of the filaments of both the *alb1* and *a2b2* mutant strains (Fig. 20C).

To explore cell wall deposition in the $\Delta_{810-2832}$ *hsl7 smu1^{Otef}* mutant strains, the strains were grown in liquid SLAD. The strains were grown overnight (due to their slower growth in SLAD media than in YEPS media) and stained with CFW and WGA. Initial observations of wild type progenitor cells indicated that wild type cells do not filament in liquid SLAD (Fig. 21) as opposed to solid SLAD media. In addition, the previously described mutant strains grown in liquid SLAD also did not display filamentous growth, nor any aberrations in cell wall localization and deposition or number of nuclei (Fig. 22). However, the $\Delta_{810-2832}$ *hsl7 smu1^{Otef}* mutant strains exhibited irregular bubbled hyphae morphology observed on solid SLAD media, but upon staining with CFW and WGA, very few cross walls or septa were observed (Fig. 21). Moreover,

Figure 21: $\Delta_{810-2832}hsl7 smu1^{Otef}$ strains produce filaments in liquid SLAD and display defects in cell wall localization. $\Delta_{810-2832}hsl7 smu1^{Otef}$ cells produce filaments in response to liquid SLAD while *WT* cells do not. $\Delta_{810-2832}hsl7 smu1^{Otef}$ cells also exhibit a “bubbled” phenotype similar to pseudohyphae, yet at the narrowing of pseudohyphae, where a septum should form, very few septa appeared in the filaments (See arrows). Scale bars, 10 μ m.

cell wall staining indicated that both chitin and the β -glucan were delocalized and unevenly distributed throughout the elongated cells. The bubbled hyphae appeared similar to pseudohyphae. Yet, where the missing septa should have formed in pseudohyphae, there was a decrease in the localization of cell wall material, while the few cross walls that were present, formed randomly within the bubbled hyphae (Fig. 21, see arrows). There was, however, a corresponding increase in the localization of cell wall material to the widest point of the bubble (Fig. 21). The only major difference observed between the mating-types of the $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strains was greater bubbling appearance of the filaments from the *a2b2* mating-type background, compared to the *a1b1* $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strain (Fig. 21).

To determine if the $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strains are true hyphae or pseudohyphae, they were stained with WGA, CFW, and DAPI. The *a1b1* $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strain displayed an extremely long filamentous cell, containing no more

Figure 22: $\Delta_{810-2832}hsl7$, $hsl7^{Oref}$, $\Delta smu1$, $smu1^{Oref}$, and $\Delta_{810-2832}hsl7 \Delta smu1$ strains were stained with CFW, WGA, and Syto 11. $\Delta_{810-2832}hsl7$, $hsl7^{Oref}$, $\Delta smu1$, and $\Delta_{810-2832}hsl7 \Delta smu1$ strains do not display any defects in cell wall localization and nuclear content when grown in liquid low ammonium. Scale bars, 10 μ m.

than one cross wall and a single brightly stained nucleus per filamentous section of the cell (Fig. 23A, see arrows). However, $a2b2 \Delta_{810-2832}hsl7 smu1^{Oref}$ mutant strain exhibited multiple cells growing in a bi-polar fashion, separated by randomly interspersed cross

Figure 23: The mating-type specific effects of $\Delta_{810-2832}hsl7 smu1^{Otef}$ strains in liquid SLAD. *alb1* $\Delta_{810-2832}hsl7 smu1^{Otef}$ cells exhibit long filaments with a single, centrally located nucleus and septa (see arrows). The *a2b2* $\Delta_{810-2832}hsl7 smu1^{Otef}$ cells exhibited a “bubbled” hyphal phenotype with randomly spaced septa that separate the individual nuclei into compartments (see arrows). Scale bars, 10 μ m.

wall septa. These cross walls do separate the multiple nuclei observed to a single nucleus per compartment. However, the cross walls were irregularly separated, with several forming in the middle of cell-like structures. Other septa were detected between two bubbled regions of the structure, similar to pseudohyphal cells where septa form between cells but the cells do not properly separate (Fig. 23B, see arrows). Overall these data lead to a conclusion that together Hsl7 and Smu1 play a role in the regulation of the filamentous response and cell separation in low ammonium conditions.

$\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strains are attenuated for virulence. To assess the roles of Hsl7 and Smu1 in virulence in *U. maydis*, identical compatible mutant strains of opposite mating-type were mixed and injected into maize seedlings (8 days post planting). Measures of virulence were taken at 7, 10, 14, 17, and 21 days post

inoculation and three independent trials were performed. The cross of an *alb1* and an *a2b2* of a single mutant background were compared to a wild type progenitor cross. The mutant backgrounds examined were $\Delta_{810-2832}hsl7$, $hsl7^{Otef}$, $\Delta smu1$, $smu1^{Otef}$, $\Delta_{810-2832}hsl7 \Delta smu1$, and $\Delta_{810-2832}hsl7 smu1^{Otef}$. The virulence for each infection was measured by a disease index, ranking the severity of the disease symptoms on a scale from 0 to 5. The results of a Kruskal-Wallis ANOVA with a Dunn's Multiple Comparisons Test, shown in Table 7, indicate that although slightly decreased for virulence compared to the wild type progenitor cross, $\Delta_{810-2832}hsl7$, $hsl7^{Otef}$, and $smu1^{Otef}$ did not show a statistical difference compared to the wild type controls. The results are also presented in a percent of symptom formation graph (Doehlemann, *et al.*, 2009; see Fig. 24). The $\Delta_{810-2832}hsl7$, $hsl7^{Otef}$, and $smu1^{Otef}$ mutant strains all displayed a similar reduction in plant death (black shade on graph, Fig. 24) as well as an increase in symptom-free plants (Green shade on graph, Fig. 24). The $\Delta smu1$, $\Delta_{810-2832}hsl7 \Delta smu1$, and $\Delta_{810-2832}hsl7 smu1^{Otef}$ crosses all exhibited various decreases in virulence and they were all statistically different to the wild type controls in Kruskal-Wallis ANOVA with a Dunn's Multiple Comparisons Test ($p < .001$). In addition, the $\Delta smu1$, $\Delta_{810-2832}hsl7 \Delta smu1$, and $\Delta_{810-2832}hsl7 smu1^{Otef}$ crosses displayed a decrease in plant death and an increase in plants with a 0 or a 1 on the disease index scale (Fig. 24). The Dunn's Multiple Comparisons Test indicated that there was no significant difference in the reduction of virulence between the $\Delta smu1$ and $\Delta_{810-2832}hsl7 \Delta smu1$ mutant strains. The Dunn's Multiple Comparisons Test also indicated that the final mutant strain, $\Delta_{810-2832}hsl7 smu1^{Otef}$, was significantly less virulent than all other compared strains (Table 7).

In addition to the measures of virulence taken at 21 dpi, virulence measures were

TABLE 7. Pathogenicity of mutant strains^A.

Treatment ^B	Strains	Plants Tested	Disease rating ^C by number of affected plants						Disease Index ^D
			0 Points	1 Point	2 Points	3 Points	4 Points	5 Points	
1	<i>WT</i>	58	1	5	3	1	12	36	4.17
2	$\Delta_{810-2832}hsl7$	59	3	8	4	4	12	28	3.66
3	<i>hsl7^{Otef}</i>	59	4	9	7	0	14	25	3.46
4	$\Delta smu1$	59	4	8	17	5	14	11	2.85***
5	<i>smu1^{Otef}</i>	59	4	6	3	4	15	27	3.71
6	$\Delta_{810-2832}hsl7 \Delta smu1$	59	10	13	12	4	11	9	2.34***
7	$\Delta_{810-2832}hsl7 smu1^{Otef}$	59	12	29	10	0	4	4	1.44***

^A Table shows combined data from three identical experiments of 19 to 20 plants each reported for 21 days postinoculation.

^B Treatment consisted of the inoculation of 10^5 cells ml^{-1} for each of the strains.

^C The disease rating is measured on a scale of 0 to 5 points based on the severity of symptoms as follows: 0 points, no disease symptoms; 1 point, chlorosis / anthocyanin production; 2 points, small leaf galls; 3 points, small galls on stems; 4 points, large stem galls; 5 points, plant death.

^D The disease index is calculated as the sum of the disease ratings divided by the number of plants tested and statistical analysis was performed using a Kruskal-Wallis ANOVA with a Dunn's Multiple Comparison Test from three independent experiments.

*** p value less than .001.

taken at 7, 10, 14, and 17 dpi and disease indices for each time measure were calculated.

The change in pathogenicity was calculated as the slope of a linear regression and was compared to the slope of the wild type strains. The $\Delta_{810-2832}hsl7$, *hsl7^{Otef}*, and *smu1^{Otef}* strains did not show a statistical difference compared to the wild type controls. The $\Delta smu1$, $\Delta_{810-2832}hsl7 \Delta smu1$, and $\Delta_{810-2832}hsl7 smu1^{Otef}$ crosses all exhibited significant decreases compared to the wild type controls (Table 8). In addition, there was no significant difference between $\Delta smu1$ and $\Delta_{810-2832}hsl7 \Delta smu1$. The decrease slope of the $\Delta_{810-2832}hsl7 smu1^{Otef}$ was significant compared to both the $\Delta smu1$ and $\Delta_{810-2832}hsl7 \Delta smu1$

Figure 24: Disease symptom formation in various *U. maydis* mutant strains. All strains were rated at 21 dpi and represent three independent experiments. Plants were inoculated with one paired background, wild type, $\Delta_{810-2832}hsl7$, $hsl7^{Otef}$, $\Delta smu1$, $smu1^{Otef}$, $\Delta_{810-2832}hsl7 \Delta smu1$, $\Delta_{810-2832}hsl7 smu1^{Otef}$. The graph displays the percentage of plants with a specific symptom of infections. Both $\Delta smu1$ and $\Delta_{810-2832}hsl7 \Delta smu1$ exhibited a decrease in the percentage of dead plants, while the $\Delta_{810-2832}hsl7 smu1^{Otef}$ displayed a decrease of disease severity across all plants.

strains. These results point to an interesting interaction between Hsl7 and Smu1 in the ability to cause disease.

DISCUSSION

Smu1 affects cell length and the filamentous response to low ammonium

conditions. Previous work identified the Ste20 homologue Smu1 and its roles in mating and pathogenicity (Smith, *et al.*, 2004). In addition, disruption of *smu1* decreases cell length, though only significantly in the *a2b2*, and the filamentous response to low ammonium conditions. Conversely, ectopic overexpression of *smu1* under the constitutively active Otef promoter displayed a significant increase in cell length in the

TABLE 8. Changes in the rate of infection.^A

Strains ^B	Slope ^C		<i>p</i> ^D
<i>WT</i>	0.20 ± 0.023		
<i>Δsmu1</i>	0.18 ± 0.022	<i>WT</i> v. <i>Δ₈₁₀₋₂₈₃₂hsl7</i>	N.S.
<i>smu1^{Otef}</i>	0.17 ± 0.017	<i>WT</i> v. <i>hsl7^{Otef}</i>	N.S.
<i>Δ₈₁₀₋₂₈₃₂hsl7</i>	0.12 ± 0.008	<i>WT</i> v. <i>Δsmu1</i>	> .05
<i>hsl7^{Otef}</i>	0.18 ± 0.012	<i>WT</i> v. <i>smu1^{Otef}</i>	N.S.
<i>Δ₈₁₀₋₂₈₃₂hsl7 Δsmu1</i>	0.09 ± 0.006	<i>WT</i> v. <i>Δ₈₁₀₋₂₈₃₂hsl7 Δsmu1</i>	> .05
<i>Δ₈₁₀₋₂₈₃₂hsl7 smu1^{Otef}</i>	0.06 ± 0.009	<i>WT</i> v. <i>Δ₈₁₀₋₂₈₃₂hsl7 smu1^{Otef}</i>	> .05
		<i>Δsmu1</i> v. <i>Δ₈₁₀₋₂₈₃₂hsl7 Δsmu1</i>	> .05
		<i>Δsmu1</i> v. <i>Δ₈₁₀₋₂₈₃₂hsl7 smu1^{Otef}</i>	> .05
		<i>Δ₈₁₀₋₂₈₃₂hsl7 Δsmu1</i> v. <i>Δ₈₁₀₋₂₈₃₂hsl7 smu1^{Otef}</i>	> .05

^A Table shows combined data from three identical experiments of 19 to 20 plants each reported for 7, 10, 14, 17, and 21 days postinoculation.

^B Strains were mixtures of opposite mating background and co-inoculated.

^C Disease index was calculated for each time point and used as a measure of pathogenicity. A slope for each mutant strain was calculated from a linear regression and compared with the slope of the wild type strain.

^D Slopes of regression were compared using Graphpad Prism 5.02.

N.S. = Not Significant

alb1 mating-type background only. Growth rate analysis did not indicate any differences in either of the *smu1* mutant strains; however, growth analysis may have not picked up subtle variations in the cell cycle of the mutant strains. Another possibility is that the cell length perturbations may not be due to cell cycle, but due to cytoskeletal defects potentially in actin or microtubule dynamics and polarization (Holly and Blumer, 1999; Qyang, *et al.*, 2002). It is interesting to think that Smu1 may play a role in cytoskeletal organization, as well as regulating mating pheromone expression. Work in both *S. cerevisiae* and *S. pombe* suggests that Ste20 homologues are involved in both the MAPK pathway and cell polarity through actin organization (Roberts and Fink, 1994; Marcus, *et*

al., 1995; Gilbreth, *et al.*, 1996 and 1998; Leberer, *et al.*, 1997; Eby, *et al.*, 1998; Holly and Blumer, 1999; Fujita, *et al.*, 1999; Bao, *et al.*, 2001; Qyang, *et al.*, 2002). Evidence also suggests that Ste20 also plays a role in mitotic exit (Höfken and Schiebel, 2002). In addition, staining of the cell wall components, β -glucan and chitin, with CFW and fluorescently labeled WGA, respectively, did not indicate any defects in cell wall localization and deposition. Smu1 also displayed a subtle role in the filamentous response pathway to low ammonium conditions indicating Smu1 plays a role in the filamentous response pathway. And, this decrease was mating-type specific, occurring primarily in the *a2b2* mating-type background. Overexpression of *smu1* resulted in an increase in the filamentous response to SLAD media, which was confined to the *a1b1* mating-type specific background. Ste20-like kinases have been demonstrated to be involved in responses to environmental conditions regulating the MAPK pathway (Lengeler, *et al.*, 2000; Bao, *et al.*, 2001). Smu1 could potentially regulate the filamentous response to low ammonium conditions via activation of the MAPK pathway; however this has yet to be established.

Hsl7 negatively affects cell length and the filamentous response to SLAD.

Studies of the Ste20 homologues from *S. cerevisiae* and *S. pombe* indicated that the respective Hsl7 homologues interact with the PAK kinases both *in vitro* and *in vivo* (Fujita, *et al.*, 1999; Gilbreth, *et al.*, 1996 and 1998; Bao, *et al.*, 2001). Here I identified Hsl7, a sole protein-arginine methyltransferase homolog in *U. maydis*. Haploid *U. maydis* cells containing either a disruption or an ectopic overexpression of the *hsl7* gene exhibited cell length effects. The $\Delta_{810-2832}hsl7$ mutant strains exhibit an increase in cell length, independent of mating-type background, while the *hsl7*^{O_{tef}} mutant strains

displayed a decrease in cell length, only significant in the *a2b2* mating-type background. The results were similar yet opposite to those in *smu1* mutant strains. Deletion of *hsl7* in *S. cerevisiae* caused a slight increase in cell size and an increase in bud length, while deletion of *skb1* in *S. pombe* created a decrease in cell length (Gilbreth, *et al.*, 1996 and 1998; Shulewitz, *et al.*, 1999). Conversely, overexpression of *skb1* caused an increase in cell length. Both Hsl7 and Skb1 have been shown to play a role in cell cycle regulation in *S. cerevisiae* and *S. pombe*, respectively, although resulting in opposite effects in the G₂-M transition (Gilbreth, *et al.*, 1996 and 1998; Ma, *et al.*, 1996; Shulewitz, *et al.*, 1999). Growth rate analysis and cell wall staining indicated no differences between the *hsl7* mutant strains and the wild type strains. Additionally, the $\Delta_{810-2832}hsl7$ mutant strains did display small protrusions from colonies grown on YEPS media. Analysis of the micro-colonies of the $\Delta_{810-2832}hsl7$ mutant strains indicated a “directional growth” phenotype, where groups of cells grew parallel to one another, yet independent from other groups of parallel cells giving rise to small protrusions from the general outline of the colony. An increase in cell to cell adhesion may explain the “directional growth” phenotype. Flo11p, a cell surface flocculin involved in cell-cell adhesion, is required for haploid invasive growth as well as diploid pseudohyphal development in *S. cerevisiae* (Palecek, *et al.*, 2000). Mutating *hsl7* in *S. cerevisiae* enhances agar invasion as well as filamentous growth independent of *flo11* expression. In *U. maydis*, Hsl7 could play a similar role repressing cell to cell adhesion that may be required for filament formation.

The Hsl7 homologues of *S. cerevisiae* and *S. pombe* have been shown to play roles in the filamentous response to low ammonium conditions and hyperosmotic conditions, respectively (Fujita, *et al.*, 1999 and Bao, *et al.*, 2001). While the Δ_{810-}

$\Delta_{2832}hsl7$ and $hsl7^{Oref}$ mutant strains were not sensitive to hyperosmotic conditions, nor to cell wall stressing agents or acid pH conditions, they did display perturbations in the filamentous response to low ammonium. The $\Delta_{810-2832}hsl7$ and $hsl7^{Oref}$ mutant strains displayed an increase and a decrease in the ability to form filaments on SLAD, respectively. Additionally, the $\Delta_{810-2832}hsl7$ mutant strains also exhibited the “directional growth” previously observed on rich media. In *S. cerevisiae*, Hsl7 plays a role in the filamentous response to low ammonium conditions, initially by acting as a negative regulator of Ste20 in the filamentous response pathway (Fujita, *et al.*, 1999). However, additional studies point to Hsl7 regulating the filamentous response to low ammonium conditions via regulating Swe1 and the G₂-M transition (La Valle and Wittenberg, 2001). Deletion of *hsl7* leads to accumulation and stabilization of Swe1, with a freeze in the entry into mitosis, promoting cell elongation. Overall, this indicates that *hsl7*, in addition, and potentially independent to *smu1*, plays a role in cell length and the filamentous response to low ammonium conditions.

Disrupting *smu1* in a $\Delta_{810-2832}hsl7$ background does not rescue any $\Delta_{810-2832}hsl7$ associated phenotypes. Studies of the Ste20 homologues from *S. cerevisiae* and *S. pombe* indicated that the respective Hsl7 homologues interact with the PAK kinases both *in vitro* and *in vivo* (Fujita, *et al.*, 1999; Gilbreth, *et al.*, 1996 and 1998; Bao, *et al.*, 2001). Yeast two-hybrid analysis indicated that the protein product of the predicted Hsl7 ORF of *U. maydis* interacted with Smu1 *in vitro*. Based on these results, a disruption of *smu1* was introduced in a $\Delta_{810-2832}hsl7$ background to examine a possible genetic interaction between the two gene products. In both the *S. cerevisiae* and the *S. pombe*, the Hsl7 homologue is upstream of the Ste20 homologue, modulating its function (Fujita,

et al., 1999 and Bao, *et al.*, 2001). The disruption of *smu1* in the $\Delta_{810-2832}hsl7$ background did not rescue any of the $\Delta_{810-2832}hsl7$ associated phenotypes. Cell length increases, directional growth, and increases in the filamentous response to low ammonium conditions were unaffected in the double disruption strains compared with the $\Delta_{810-2832}hsl7$ mutant strains. In addition, no new phenotypes were identified in the double disruption strains. These results stand at odds with models in both *S. cerevisiae* and *S. pombe* predicting the interaction of the Hsl7 homologues and PAK kinase (Ste20 and Shk1) homologues (Fujita, *et al.*, 1999; Gilbreth, *et al.*, 1996 and 1998; Bao, *et al.*, 2001). Both Ste20 and Shk1 have been shown to be downstream of the Hsl7 homologue, whose function modulates the subsequent PAK kinase functions.

Overexpressing *smu1* in a $\Delta_{810-2832}hsl7$ background exacerbates $\Delta_{810-2832}hsl7$ associated phenotypes. To further explore a potential genetic interaction, an ectopic copy of *smu1* was overexpressed from the P_{Oief} promoter in the $\Delta_{810-2832}hsl7$ background. Such double mutant cells were extremely elongated, above and beyond any previously examined strain in this work. Cell separation defects were observed only in the *a2b2* mating-type background. In addition, a filamentous colony morphology with septated hyphal cells was also observed. Overall this double mutant background exacerbates the phenotypes observed in the $\Delta_{810-2832}hsl7$ background. Under conditions of low ammonium, the $\Delta_{810-2832}hsl7 smu1^{Oief}$ mutant strain colonies displayed a hyper-filamentous response with extremely elongated filaments with massive chitin delocalization. And again mating-type specific phenotypes were observed, with the *a2b2* mating-type background exhibiting cytoplasmic evacuation. To better explore the observed cell wall defects in the $\Delta_{810-2832}hsl7 smu1^{Oief}$ mutant strains, the cells were

grown in liquid SLAD (low ammonium) media. Interestingly, only the $\Delta_{810-2832}hsl7$ *smu1^{Otef}* mutant strains were able to form pseudohyphal filaments when grown in liquid SLAD, whereas all other strains examined did not produce filaments. Unlike solid media, where growing cells quickly exhaust the local supply of ammonium, cells grown in liquid media have constant availability to nutrients, even those in low amounts, due to the continuous circulation that occurs in the liquid medium. Another requirement for filament formation may be contact with a solid surface as in solid media. Additionally, the filamentous response to SLAD conditions in solid media may be driven by a form of quorum sensing, where cell density plays a role in triggering the filamentous response. Quorum sensing has been observed in *C. albicans* (Hogan, 2006). Also, *A. niger* exhibited spatial regulation of genes involved in nitrate metabolism, independent of carbon source and nitrate concentration (Levin *et al.*, 2007). Staining of the $\Delta_{810-2832}hsl7$ *smu1^{Otef}* mutant strains again revealed chitin delocalization while β -glucan localization was unaffected. Chitin, which normally localizes to the growing apical tip of *U. maydis*, as well as to the two septa formed during cytokinesis, was distributed throughout the cell walls of the mutant strains. The chitin localization was not ubiquitous though, as regions of the pseudohyphal filaments did display more chitin localization than areas of the cell wall. Staining of the filaments with DAPI indicated an interesting dichotomy between the mating-type specific mutant strains. The *alb1* mutant strain exhibited a single long filament containing a single, centrally located, nucleus, whereas the *a2b2* exhibited true pseudohyphae that grew in a bi-polar fashion with each compartment containing a single nucleus. Taken as a whole, it appears that Smu1, in concert with Hsl7, is involved in localizing chitin to the proper mother daughter septa in response to low ammonium

conditions, and the response to this concerted action is mating-type dependent. In *U. maydis*, the activity of Hsl7 does not appear to regulate the activity of Smu1, but instead the genetic manipulations introduced in the $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains promotes a synergism creating the hyper-filamentous response to low ammonium conditions.

The disruption of *hsl7* does not alleviate the reduction in virulence associated with disruption of *smu1*. Smith *et al.* (2004) demonstrated that disruption of *smu1* decreased virulence of *U. maydis* in a dosage dependent manner. This decrease was significant when both haploid strains were disrupted for *smu1*. Kruskal-Wallis ANOVA analysis of the previously described mutant strains indicated no significant effects on virulence from $\Delta_{810-2832}hsl7$, $hsl7^{Otef}$, or $smu1^{Otef}$ mutant strains. The significant decrease in virulence of $\Delta smu1$ mutant ($p < .001$) was consistent with previous observations (Smith, *et al.*, 2004), the $\Delta_{810-2832}hsl7\ \Delta smu1$ mutant strain was significantly decreased for virulence compared to the wild type strains as well ($p < .001$), yet no significant difference was observed between the $\Delta_{810-2832}hsl7\ \Delta smu1$ and $\Delta smu1$ mutant strains. However, the $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains exhibited a dramatic decrease in virulence, lower than all other strains examined. The decrease in virulence was significant in comparison to all previously examined strains, indicating that the role of Hsl7, through Smu1, on pathogenicity is difficult to determine. The $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains did retain the ability to mate on YPD-charcoal media, however, the *a2b2* mating-type specific mutant strain exhibited the white aerial hyphae “fuz” of a positive mating reaction in the absence of the opposite mating partner. The *a1b1* $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strain, when mixed with the opposite mating partner, did display an increase in the mating response compared to wild type strains (Supplemental Fig. 5). It

appears that the reduction in virulence in the $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains is not due to inability to activate the *a* locus controlling cell recognition and cell fusion, but could be an aspect of the *b* locus that regulates sexual and pathogenic development.

Conclusions. I have demonstrated the cloning and characterization of the Hsl7 homologue from *U. maydis* and its genetic interaction with Smu1, the Ste20 homologue. The homologue described in this report, Hsl7, has greater amino acid similarity to Skb1 from *S. pombe*, but shares many roles identified in both Hsl7 and Skb1 from *S. cerevisiae* and *S. pombe*, respectively. In yeast, Ste20 potentially regulates both the mating response and morphogenesis subsets of the MAPK pathway (Roberts and Fink, 1994; Leberer, *et al.*, 1997). Hsl7 interacts with Ste20 in a negative manner regulating morphogenesis through the MAPK under low ammonium conditions, but is independent of the mating reaction responsibilities of the MAPK (Fujita *et al.*, 1999). In addition, Ste20 may play an indirect role in cell cycle regulation through actin polarization and septin formation (Cvrčková, *et al.*, 1995; Holly and Blumer, 1999; Höfken and Schiebel, 2002). In contrast, in fission yeast, Shk1 acts parallel to the MAPK pathway in morphological responses but is predicted to act through the MAPK module in regard to the mating response (Marcus, *et al.*, 1995). Here again, Skb1 acts upon Shk1, in a positive manner regulating cell cycle and the response to hyperosmotic conditions (Gilbreth, *et al.*, 1996 and 1998; Bao, *et al.*, 2001).

I demonstrated in *U. maydis* that Hsl7 negatively regulates cell length and the filamentous response to low ammonium conditions, partially through Smu1. In particular, disrupting *hsl7*, while concomitantly overexpressing *smu1*, negatively affects mother daughter cell separation in rich media, but only in the *a2b2* mating-type background.

However, in low ammonium conditions both mating-type mutant strains are affected for cell separation. The *alb1* mutant strain produces extremely long filaments with very few septa and a single nucleus, whereas the *a2b2* mutant strain produces pseudohyphae, each containing a single nucleus separated by mother daughter cell septa or cross wall septa. The defects in cell separation of the $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains were similar to phenotypes observed in *U. maydis* $\Delta cdc42$, $\Delta don1$, and $\Delta don3$ mutant strains. The $\Delta cdc42$, $\Delta don1$, and $\Delta don3$ mutant strains, which do not display any aberrant cell morphology phenotypes, are defective in mother daughter cell separation, specifically, failing to create the second septum in the daughter cell between which vesicular elements and membrane aggregates accumulate (Weinzierl, *et al.*, 2002 and Mahlert, *et al.*, 2006). At closer examination, however, the phenotype associated with the $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains are different than the daughter septum formation defects observed in $\Delta cdc42$, $\Delta don1$, and $\Delta don3$ mutant strains. The $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains are able to form both mother and daughter septa as well as cross wall septa and lateral buds. Also, the loss of the *cdc42*, *don1*, and *don3* gene products contribute to a polar growth pattern observed in the “don” mutant phenotype of *U. maydis*. In wild type haploid cells, budding alternates between the poles of cells rather than unipolar budding that is observed in pseudohyphae (Jacobs, *et al.*, 1994). The failure of the cells to form the daughter septum and separate, in combination with the polar growth pattern, contributes to the “don” phenotype, where cells along the outer edge of the colony grow faster than the inner cells pulling them outward away from the center of the colony (Weinzierl, *et al.*, 2002). The phenotypes exhibited by the $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains also vary from the “don” mutant strains in that the center of the colony has not pulled away

creating a “donut” phenotype with a hollow center. It is possible that the link between mother daughter cells is not strong enough in this specific mutant background to maintain the “don” phenotype. As the newer generations of cells grow outward, the fragmentation zones between the mother and daughter septa are pulled apart preventing the older, more centrally located cells from being torn from the center of the colony. It is also possible that the $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains do not undergo the unipolar growth pattern observed in the $\Delta don1$, $\Delta don3$, and $\Delta cdc42$ mutants, but rather the cells undergo a bipolar growth pattern.

Recent work has delineated Cdc42 and Don3 as independent co-stimulators of cytokinesis with Don3 activity preceding Cdc42 activity in formation of the secondary septin (Böhmer, *et al.*, 2008). Here Don3 is predicted to phosphorylate, subsequently activating, a putative landmark protein allowing for Cdc42 dependent septin and actomyosin ring formation. Rho-type GTPases have been shown to be involved in cytoskeletal organization and formation of the actomyosin ring in yeast (Tapon and Hall, 1997; Eby, *et al.*, 1998; Nelson, 2003). Additionally Ste20 is also involved in actin cytoskeleton organization and is a downstream effector Cdc42 in this process (Eby, *et al.*, 1998). It is possible that Smu1 may be a downstream effector of Cdc42 in *U. maydis*, potentially regulating actin cytoskeleton dynamics and formation of the actomyosin and septin rings. An interaction through yeast two-hybrid analysis indicated that Cdc42 interacts with the CRIB domain of Smu1 *in vitro* (Leveleki, *et al.*, 2004). This, coupled with the positive yeast two-hybrid interaction between Smu1 and Hsl7, could explain the inability of $\Delta_{810-2832}hsl7\ smu1^{Otef}$ mutant strains to undergo mother daughter cell separation under low ammonium conditions, ultimately enhancing the filamentous

response. Here Hsl7 would be acting as a negative regulator of Smu1. However, this idea fails to explain the inability of *smu1* disruption mutants to rescue phenotypes associated with loss of *hsl7*. An alternate hypothesis, *i.e.*, loss of *hsl7* potentially delaying cell cycle progression, while overexpression of *smu1* promotes the filamentous response, possibly via the MAPK pathway, explains the observed phenotypes in both the $\Delta_{810-2832}hsl7 \Delta smu1$ mutant strains and the $\Delta_{810-2832}hsl7 smu1^{Otef}$ mutant strains. Hsl7 may act on cell cycle, promoting entry into mitosis by targeting Wee1 for degradation. Absence of Hsl7 would delay the cell cycle leading to an increase in cell length as is observed in *S. cerevisiae* (Shulewitz, *et al.*, 1999). This cell cycle delay in the absence of Hsl7 may act in concert with the overexpression of *smu1*, leading to up regulation of the filamentous response to low ammonium conditions that is exacerbated under conditions that promote this response.

CHAPTER III

CLA4, BUT NOT RAC1, REGULATES THE FILAMENTOUS RESPONSE TO LOW AMMONIUM CONDITIONS

INTRODUCTION

Many fungal pathogens exhibit a dimorphism in which the cells transition from either a budding morphology to a hyphal morphology or vice versa. The morphological transition of dimorphic fungi is governed by a several different regulatory pathways, which mediate cell cycle regulation, cytoskeletal organization, and differential gene expression. Members of the Rho/Rac family of GTP-binding proteins (GTPases), including Cdc42 and Rac1, have been implicated in many of these pathways, regulating actin organization, cell polarity, and cytokinesis (Nakano, *et al.*, 1997; Cabib, *et al.*, 1998; Madden and Snyder, 1998; Lengeler, *et al.*, 2000; Tolliday, *et al.*, 2002; Nelson, 2003; Etienne-Manneville, 2004; Bassilana and Arkowitz, 2006; Mahlert *et al.*, 2006). The activity of these GTP-binding proteins is dependent upon two additional groups of proteins, guanine exchange factors (GEFs), which activate their respective targets, and GTPase activating proteins (GAPs) that inactivate the GTPases. When activated, Rho/Rac GTPases modulate localization and activity of several downstream effectors including p21-activated protein kinases (PAKs). The PAKs comprise a large family of serine/threonine protein kinases that regulate many cell processes including cell cycle, cytoskeletal organization, and polar growth (Cvrčková, *et al.*, 1995; Marcus, *et al.*, 1995;

Gilbreth, *et al.*, 1996 and 1998; Eby, *et al.*, 1998; Holly and Blumer, 1999; Bao, *et al.*, 2001; Höfken and Schiebel, 2002; Qyang, *et al.*, 2002; Wiley *et al.*, 2003; Leveleki, *et al.*, 2004; Smith, *et al.*, 2004). Evidence suggests that PAKs regulate the conserved mitogen activated protein kinase (MAPK) pathway in several of these processes (Lengeler *et al.*, 2000; García-Pedrajas, *et al.*, 2008). The MAPK pathway is a three-component module consisting of three protein kinases, a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAP kinase (MAPK), which regulate the dimorphic transition in response to mating factors, environmental conditions, or for virulence (Lengeler *et al.*, 2000; García-Pedrajas, *et al.*, 2008). The pathways in *Saccharomyces cerevisiae* regulating mating, growth in hyperosmotic conditions, and pseudohyphal development are some of the best understood (Lengeler *et al.*, 2000; García-Pedrajas, *et al.*, 2008).

In the basidiomycete fungus *Ustilago maydis*, unicellular haploid cells reproduce by budding. Haploid cells of opposite mating backgrounds can fuse and form a diploid filamentous dikaryon. This dikaryon can subsequently infect maize (*Zea mays*). Cell fusion and pathogenic development are controlled by two separate loci, *a* and *b*. The *a* locus encodes a pheromone and pheromone receptor involved in cell recognition and cell fusion. Subsequent pathogenic development is dependent upon the *b* locus which encodes two homeodomain proteins, bE and bW (Kronstad and Leong, 1989). After cell fusion, dimerization of the bE and bW proteins from opposite mating-type backgrounds, yields a functional heterodimer that regulates sexual development and pathogenicity (Kämper, *et al.*, 1995). Differentiation into the infectious dikaryon is dependent upon the activity of this *b* heterodimer. However, *U. maydis* cells can also undergo similar

filamentous differentiation in response to environmental conditions. Lipids, acidic pH, and low ammonium conditions can trigger the filamentous response in a b locus-independent manner (Ruiz-Herrera, *et al.*, 1995; Smith *et al.*, 2003; Klose, *et al.*, 2004).

The highly conserved GTPase, Cdc42 of *S. cerevisiae* regulates both bud formation and polarized cell growth, though two well studied PAKs, Ste20 and Cla4. Through the Cdc42 specific GEF, Cdc24, Cdc42 localizes to the bud neck and contributes, in a Cla4 dependent manner, to the septin ring formation (Versele and Thorner, 2004). Septin ring formation is a critical step in the cell cycle. The failure of septin ring formation results in a Swe1-dependent G₂ delay in cell cycle. Cdc42 and Ste20 also contribute to polarizing the actin cytoskeleton and polarized cell growth. This occurs partially through the MAPK pathway in response to mating cues, as well as low ammonium conditions (Eby, *et al.*, 1998; Lengeler, *et al.*, 2000; Lamson, *et al.*, 2002). While *S. cerevisiae* contains only Cdc42, *U. maydis* contains Cdc42 and a second Rho/Rac GTPase, Rac1. In *U. maydis*, Cdc42, through its specific GEF, Don1, regulates septum formation and mother daughter cell separation (Weinzierl, *et al.*, 2002; Mahlert, *et al.*, 2006; Böhmer, *et al.*, 2008). In *U. maydis*, the activity of Rac1, regulated by the Rac1 specific GEF, Cdc24, focuses on bud formation and morphology, as well as polar growth. Rac1, via Cdc24, localizes to the polar tip of the bud and directs localized cell wall deposition (Leveleki, *et al.*, 2004; Mahlert, *et al.*, 2006). The deposition of cell wall material is Cla4 dependent and promotes apical growth of the bud. Deletion mutants of both *rac1* and *cla4* are unable to form bud necks or undergo proper bud formation; in addition, neither mutant has the ability to form b locus-dependent filaments. Thus Rac1 may act as the master regulator of the filamentous response through Cla4.

To date, no work has focused on the role Rac1 or Cla4 may have in the filamentous response to environmental conditions, such as low ammonium conditions. In contrast the Ste20 homologue, Smu1, is known to have a role in the filamentous response to low ammonium conditions (Smith, *et al.*, 2004; Chapter II). Smu1 appears to regulate the filament formation under low ammonium conditions in a positive fashion. In this study, I explore the roles Rac1 and Cla4 in the filamentous response to low ammonium conditions. I demonstrate that deletion of *rac1* leads to a severe delay in filament formation and a dramatic reduction in filament number compared to wild type strains. Conversely, deletion of *cla4* leads to a hyper-filamentous response under low ammonium condition compared to wild type strains.

MATERIALS and METHODS

Strains. *Ustilago maydis* strains used in this study that were not previously listed in Chapter 2 are listed in Table 9.

TABLE 9. *U. maydis* strains and plasmids used in this study.

Strain / Plasmid	Genotype	Reference
Fungal Strains^A		
FB1 WT	<i>a1b1</i>	<i>Banuett, et al, 1989</i>
Δ <i>cla4 a1</i>	<i>a1b1 cla4::hyg^R</i>	<i>Leveleki et al., 2004</i>
<i>cla4^{Otef} a1</i>	<i>a1b1 P_{otef}-cla4, cbx^R</i>	This Study
<i>rac1^{Otef} a1</i>	<i>a1b1 P_{otef}-rac1, cbx^R</i>	<i>Pham, et al., 2009</i>
FB2 WT	<i>a2b2</i>	<i>Banuett, et al, 1989</i>
Δ <i>cla4 a2</i>	<i>a2b2 cla4::hyg^R</i>	<i>Leveleki et al., 2004</i>
<i>cla4^{Otef} a2</i>	<i>a2b2 P_{otef}-cla4, cbx^R</i>	This Study
Δ <i>rac1 a2</i>	<i>a2b4 rac1::nour^R</i>	<i>Leveleki et al., 2004</i>
Plasmid		
pOtef- <i>cla4</i>	<i>P_{otef}-cla4, cbx^R</i>	This Study

^AAll mutant strains were made in the wild type background strains listed.

Primer Design. Primers were designed as previously described (Materials and methods, Ch. 2) and are listed in Table 10.

TABLE 10. Primers used for PCR and Sequencing.

Primers	Sequences (5' → 3')
NCla5	TTCTAGACGCCACCTCTGGACTACAA
Cla43K	CGGTACCTGAAAAGGGAAGGGAGT

Genetic Manipulation and Vector Construction. Overexpression of *cla4* in *U. maydis* was obtained by homologous recombination as described previously (Brachmann *et al.*, 2004). The *cla4* expression construct was produced by amplifying the *cla4* ORF with NCla5 and Cla43K primers and cloning the product into pCR2.1 TOPO (Invitrogen). To provide constitutive expression, the *cla4* ORF was then excised and cloned after the *P_{otef}* promoter into the *Bam*HI and *Not*I sites of the p123 vector (Weber, *et al.*, 2006). The *cla4^{Otef}* expression vector was linearized using *Ssp*I before transforming *U. maydis*, under selection for carboxin resistance (Brachmann *et al.*, 2001). Recombination at the *ip* locus would yield the carboxin resistant transformants. The *cla4^{Otef}* expression vector was digested with restriction endonucleases, purified, and used to transform *U. maydis* protoplasts. Potential transformants were verified by PCR.

PCR, growth conditions, cell length, statistical analysis, staining, micro-colonies, and microscopy have been described previously (Materials and methods, Chapter II).

RESULTS

***Δcla4* mutant strains are affected in cell length, chitin localization, and cell separation.** Previously Cla4 was found to be important for proper budding and cell morphology (Leveleki, *et al.*, 2004). Deletion of the *cla4* ORF affected cell morphology, causing the cells to appear fatter than the wild type strains (Fig. 25). In

Figure 25: Cla4 plays a role in cytokinesis and cell length. $\Delta cla4$ cells display an increase in cell length in the $a2b2$ mating-type strain only, while $cla4^{Oref}$ cells exhibit an increase in cell length only in the $alb1$ mating-type specific strain. $a2b2 \Delta cla4$ cells also exhibited cytokinesis defects. Scale bars, 10 μm .

addition, cytokinesis was affected in the $\Delta cla4$ mutant strains, with the greater defect occurring in the $a2b2$ mating-type background (Fig. 25 and Leveleki, *et al.*, 2004). The $cla4$ mutant strains were affected in cell length, primarily in the $a2b2$ mating-type background, but this phenotype was not explored (Leveleki, *et al.*, 2004). In the current study, examination of the $\Delta cla4$ mutant strains identified a significant increase in cell length that was specific to the $a2b2$ mating-type background. No significant increase in cell length was observed in the $alb1$ mating-type background (Table 11). Comparisons between the $\Delta cla4 alb1$ and $a2b2$ mutant strains demonstrated a significant difference between the mating-type backgrounds (Table 12). This mating-type specific increase in cell length stands in sharp contrast to the decrease in cell lengths observed in $\Delta smu1$ mutant strains. The significant changes in cell length in both the $\Delta cla4$ and $\Delta smu1$ mutant strains compared to wild type strains are confined to the $a2b2$ mating-type background (Table 12). Comparisons of the mating-type specific effects on cell length between the $\Delta cla4$ and $\Delta smu1$ mutant strains indicated that the lengths were significantly

TABLE 11. Measures of cell length across all strains.

Strain	n	Length ^A (in μm)	Comparison	p^B
<i>WT a1b1</i>	91	19.27 +/- .43	<i>WT a1b1</i> v. <i>WT a2b2</i>	N.S.
<i>Δcla4 a1</i>	408	19.48 +/- .30	<i>WT a1b1</i> v. <i>Δcla4 a1</i>	N.S.
<i>Δsmu1 a1</i>	380	18.64 +/- .16	<i>WT a1b1</i> v. <i>Δsmu1 a1</i>	N.S.
<i>cla4^{Otef} a1</i>	367	21.22 +/- .25	<i>WT a1b1</i> v. <i>cla4^{Otef} a1</i>	> .05
<i>smu1^{Otef} a1</i>	308	21.75 +/- .28	<i>WT a1b1</i> v. <i>smu1^{Otef} a1</i>	> .001
<i>WT a2b2</i>	213	19.14 +/- .31		
<i>Δcla4 a2</i>	111	24.05 +/- .83	<i>WT a2b2</i> v. <i>Δcla4 a2</i>	> .001
<i>Δsmu1 a2</i>	281	17.64 +/- .18	<i>WT a2b2</i> v. <i>Δsmu1 a2</i>	> .05
<i>cla4^{Otef} a2</i>	505	19.69 +/- .20	<i>WT a2b2</i> v. <i>cla4^{Otef} a2</i>	N.S.
<i>smu1^{Otef} a2</i>	224	19.66 +/- .30	<i>WT a2b2</i> v. <i>smu1^{Otef} a2</i>	N.S.

^A Cell length values are averages, +/- S.E.

^B Statistical analysis was performed using a one way ANOVA with a Dunnett's Multiple Comparison Test.

N.S. = Not Significant

TABLE 12. Mating-type specific cell length differences.

Strain	n	Length ^A (in μm)	Comparison	p^B
<i>Δcla4 a1</i>	408	19.48 +/- .58		
<i>Δsmu1 a1</i>	380	18.64 +/- .31	<i>Δcla4 a1</i> vs. <i>Δcla4 a2</i>	> .001
<i>cla4^{Otef} a1</i>	367	21.22 +/- .25	<i>Δcla4 a1</i> vs. <i>Δsmu1 a1</i>	> .001
<i>smu1^{Otef} a1</i>	308	21.75 +/- .48	<i>cla4^{Otef} a1</i> vs. <i>smu1^{Otef} a1</i>	N.S.
<i>Δcla4 a2</i>	111	24.05 +/- .85		
<i>Δsmu1 a2</i>	281	17.64 +/- .29	<i>cla4^{Otef} a1</i> vs. <i>cla4^{Otef} a2</i>	> .001
<i>cla4^{Otef} a2</i>	505	19.69 +/- .20	<i>Δcla4 a2</i> vs. <i>Δsmu1 a2</i>	> .001
<i>smu1^{Otef} a2</i>	224	19.66 +/- .44	<i>cla4^{Otef} a2</i> vs. <i>smu1^{Otef} a2</i>	N.S.

^A Cell length values are averages, +/- S.E.

^B Statistical analysis was performed using a Student's t-test.

N.S. = Not Significant

different, independent of mating-type background.

Leveleki, *et al.* also indicated cell wall defects in chitin localization but not in β -glucan localization. Staining of the $\Delta cla4$ mutant strains with WGA and CFW, confirmed this previous work (Fig. 26, see arrows). Cross walls stained readily with

Figure 26: $\Delta cla4$ strains are multinucleic and exhibit chitin delocalization: $\Delta cla4$ cells appear fatter than wild type cells and when visualized with CFW, contain several cross walls (See arrows). The cells exhibit chitin delocalization when stained with WGA though. In addition, both mating-type strains contained several nuclei, separated by cross walls when stained with DAPI (See arrows). Scale bars, 10 μ m.

CFW, but not with WGA indicating that chitin does not localize to the cross wall septa, whereas the β -glucans do localize to the septa. In addition, chitin deposition occurs all along the cell wall in the $\Delta cla4$ mutant strains, not to the polar tip as in the wild type strains (Fig. 26). Also, co-stained with CFW, DAPI indicated that each compartment contained a single nucleus, further supporting that the $\Delta cla4$ mutant strains divide by contraction at a central septum (Fig. 26, see arrows, and Leveleki, *et al.*, 2004).

Overexpression of *cla4* was generated by amplifying the *cla4* ORF and cloning it after the P_{otef} promoter. The linearized plasmid was then integrated into the *ip* locus of the FB1 *a1b1* and FB2 *a2b2* strains of *U. maydis* and confirmed by PCR (data not shown). Examination of the *cla4^{Otef}* mutant strains did not display any aberrant cell morphologies other than cell elongation (Fig. 26). Also the *cla4^{Otef}* mutant strains did not display any cell wall defects when visualized with CFW and WGA (Fig. 27). Cell length

Figure 27: *cla4^{Otef}* strains did not exhibit any defects in cell wall localization when stained with CFW and WGA. When grown in rich media, *cla4^{Otef}* strains did not display any defects in cell wall localization when stained with CFW and WGA. Scale bars, 10 μ m.

measures indicated a significant increase in length, but only in the *a1b1* mating-type background, while the *a2b2* mating-type background did not display any increase in cell length (Table 11). Comparison between the *a1b1 cla4^{Otef}* and *a2b2 cla4^{Otef}* mutant strains indicated a significant difference in cell length between the mating-type backgrounds (Table 12). This result is identical to the cell length phenotype observed in the *smu1^{Otef}* mutant strains, where the *a1b1* mating-type background displayed an increase in cell length, while the *a2b2* did not (Table 11). In addition, no significant difference was observed when the *cla4^{Otef}* and *smu1^{Otef}* mutant strains of the same mating-type

background were compared (Table 12). While these results are in concordance with the mating-type specific phenotypes previously observed in both *Δcla4* and *Δsmu1* strains, the overall comparisons between the similarity of overexpressing either *cla4* or *smu1* and the differences of disrupting either *cla4* or *smu1* indicate a complex relationship between the two kinases.

The colonies of the *Δcla4* mutant strains are altered in the filamentous response to low ammonium conditions. The *Δcla4* mutant strains do not form filaments during the mating reaction and *in planta* (Leveleki *et al.*, 2004). However, filamentation in response to low ammonium conditions *in vitro* was not examined. With the cell morphology phenotypes in mind, both colony and micro-colony morphologies of *Δcla4* and *cla4^{Oief}* mutant strains were compared. When grown on YEPS media, neither the *Δcla4*, nor the *cla4^{Oief}* mutant strains displayed any difference in colony morphology compared to the wild type progenitor strains (Fig. 28A). To explore colony morphology at the cell level, micro-colonies were utilized. On YEPS, at 20x and 60x magnification, the colony morphology of both the *Δcla4* and *cla4^{Oief}* mutant strains did not vary from the wild type strains (Fig. 28B). The *alb1 Δcla4* mutant strain did consistently exhibit filamentous cells that extended out away from the colony center (Fig. 28B), however, only a few filaments formed from each colony. It does appear that the *Δcla4* mutant strains, particularly the *alb1* mating-type strain, have the ability to form filaments on rich media.

To explore if the *cla4* mutants have the ability to form filaments in response to environmental cues, the strains were grown in low ammonium conditions. The *Δcla4* mutant strains did form filaments on low ammonium, and mating-type specific

Figure 28: Cla4 mutant strains are unaffected for colony morphology on rich media. Neither $\Delta cla4$ nor $cla4^{O1ef}$ colonies display any defects in colony morphology on rich media (A). The micro-colonies of both the $\Delta cla4$ and $cla4^{O1ef}$ strains did not differ to that of the wild type strains, though a few cells of the $a1b1 \Delta cla4$ strain did form filaments (B). Scale bars, 50 μm – 20x inset image, 40 μm – 60x image.

differences in response were observed (Fig. 29A). The $a1b1 \Delta cla4$ mutant strain produced single, dramatically elongated and twisted filaments compared to the wild type $a1b1$ strain (Fig. 29A). The filaments exhibited by the $a1b1 \Delta cla4$ mutant strain appeared to have a similar darkened region at the end of each filament advancing from an empty filament, reminiscent of a phenotype observed in the $a2b2 \Delta_{810-2832} hsl7 smu1^{O1ef}$ mutant strain (Fig. 29A, see arrow; compare with Fig. 14A, Chapter II). The $a2b2 \Delta cla4$ mutant strain displayed a smaller colony size with a thicker filament size when compared

Figure 29: On SLAD media, the $\Delta cla4$ strains display aberrant filament formation. The *a1b1* $\Delta cla4$ strain produces exaggerated filamentous colonies, while the *a2b2* $\Delta cla4$ strain is reduced in the ability to filament on SLAD compared with the wild type strains. Also filaments of the *a1b1* $\Delta cla4$ strain exhibited darkened regions at the tips of empty filaments (A, see arrows). Micro-colonies of the *a1b1* $\Delta cla4$ strain displayed extremely elongated, unbranched filaments with the cytoplasm compressed to the tip by retraction septa, while the *a2b2* $\Delta cla4$ strain exhibited elongated, highly branched filaments (B, see arrows). Scale bars, 50 μm – 20x inset image, 40 μm

with the filaments of the wild type *a2b2* strain (Fig. 29A). Micro-colonies were employed to then examine filament formation in more detail. At 20x magnification, the filaments formed by the *a1b1* $\Delta cla4$ mutant strain were much thicker and more elongated compared to the wild type strain, although they appeared to be single, unbranched filaments (Fig. 29B, inset). The *a2b2* $\Delta cla4$ mutant strain, similar to the *a1b1* $\Delta cla4$ mutant strain also formed thick, elongated filaments. However, unlike the filaments

produced in the *a1b1* mutant strain, the filaments produced in the *a2b2* mutant strain were branched (Fig. 29B, inset). At 60x magnification, the filaments seemingly had the cytoplasm confined to the polar tip by retraction septae. The filaments produced by the *a2b2* Δ *cla4* mutant strain were highly branched filaments, not displaying any of the septated hyphal attributes observed in the *a1b1* Δ *cla4* mutant strain (Fig. 29B). Neither cytoplasmic evacuation nor septae were observed in the *a2b2* Δ *cla4* mutant strain.

Figure 30: The *a1b1* *cla4^{Otef}* mutant strain exhibits an increase in filamentation; however the *a2b2* *cla4^{Otef}* mutant strain is reduced in the filamentous response on SLAD. *cla4^{Otef}* colonies exhibit an increase in filament formation in the *a1b1* mating-type background, while a decrease is observed in the *a2b2* mating-type background (A). Micro-colonies of the *cla4^{Otef}* strains exhibit a lack of filament formation at the cellular level, independent of mating-type background (B). Scale bars, 50 μ m – 20x inset image, 40 μ m – 60x image.

The *cla4^{Otef}* mutant strains formed filaments in response to SLAD media, with the *alb1 cla4^{Otef}* mutant strain showing an increase in the production of filaments compared to the wild type *alb1* strain (Fig. 30A). However, the colonies of the *a2b2 cla4^{Otef}* mutant strain were smaller and exhibited a decrease in filament formation in response to low ammonium conditions compared to their wild type progenitor strain (Fig. 30A). To further explore the mating-type differences observed in the colony morphology of the *cla4^{Otef}* mutant strains, micro-colonies were again employed. At 20x magnification, both the *alb1* and the *a2b2 cla4^{Otef}* mutant strains displayed a dramatic reduction in the ability to form filaments on SLAD (Fig. 30B, inset). At 60x magnification, no filamentous cells appeared, in contrast to what was observed in the wild type strains (Fig. 30B). The reduction of the filamentous formation of the *alb1 cla4^{Otef}* mutant strain on SLAD micro-colonies stands at odds with the colony morphology of *alb1 cla4^{Otef}* under low ammonium conditions.

***Δcla4* mutant strains are filamentous and display cell wall abnormalities in liquid SLAD.** The *Δcla4* mutant strains formed filaments when grown overnight in liquid SLAD, similar to the *Δ₈₁₀₋₂₈₃₂hsl7 smu1^{Otef}* mutant strains. The *Δcla4* mutant strains were grown in liquid SLAD and stained with WGA and CFW. The *alb1 Δcla4* mutant strain formed long, single, septated hyphae, whereas the *a2b2 Δcla4* mutant strain exhibited large multi-branched hyphae with few cross wall septa (Fig. 31, see arrows). The *alb1 Δcla4* mutant strain displayed regularly spaced retraction septa when grown in liquid as was observed on the micro-colonies. Closer examination the *a2b2 Δcla4* mutant strain indicated that the branching of the hyphae occurred at cross wall septae. Both strains also displayed the same chitin localization defects observed when grown in rich

Figure 31: *Δcla4* strains produce filaments in liquid SLAD and display defects in cell wall localization. *Δcla4* cells produce filaments in liquid SLAD while *WT* cells do not. The *a1b1 Δcla4* cells exhibit unbranched septated hyphae while the *a2b2 Δcla4* cells were highly branched displaying very few septa, with branch points occurring at cross wall septa (See arrows). Both *Δcla4* mating-type strains display massive chitin delocalization but no defects in β -glucan localization. Scale bars, 10 μ m.

media, where chitin was not localized to the polar tip of the cells or with any septa that are formed, but all along the cell wall. The *cla4^{Otef}* mutant strains, when grown in liquid SLAD, did not differ from the wild type progenitor strains (Fig. 32B).

To explore if the compartments delineated by retraction septa in the *a1b1 Δcla4* mutant strain contained only a single nucleus or separated multiple nuclei, these cell structures were co-stained with DAPI, CFW, and WGA. The long filaments again exhibited massive chitin delocalization, whereas the β -glucans localized to the septa correctly (Fig. 32A, see arrows). Nuclear staining with DAPI, indicated a single nucleus in the leading compartment of the *a1b1* mutant strain (Fig. 32A, see arrows). Examination of the *a2b2 Δcla4* mutant strain indicated a single nucleus contained between randomly interspersed septa (Fig. 32A, see arrows). However, differences between the mating-type backgrounds indicated different responses to liquid low

Figure 32: *Δcla4* strains display mating-type specific effects in liquid SLAD, while *cla4^{Otef}* strains did not exhibit any defects in cell wall localization and nuclei number. The filaments of the *alb1 Δcla4* cells exhibit a single nucleus and septa confined to the tip of the filament by retraction septa. The *a2b2 Δcla4* cells exhibited randomly spaced septa that separate the individual nuclei into compartments (A, see arrows). The *cla4^{Otef}* strains do not display any defects in cell wall localization (stained with CFW and WGA) or nuclear content (stained with Syto11) (B). Scale bars, 10 μ m.

ammonium conditions. The *alb1 Δcla4* mutant strain displayed regularly spaced retraction septa whereas the *a2b2 Δcla4* mutant strain displayed few septa, although the septa in both mating-type backgrounds did separate each nucleus into an independent compartment.

***Δrac1* is able to form filaments in response to low ammonium conditions, while overexpression of *rac1* leads to filamentation in rich media.** Mahlert, *et al.* showed that overexpression of *rac1* promotes filamentous growth, whereas deletion of *rac1* attenuates filamentous growth. The cell morphology of the two *rac1* mutant strains was distinctly different (Fig. 33). The *Δrac1* mutant strain displayed cells that were fatter

Figure 33: Rac1 plays a role in cytokinesis and cell morphology. *Δrac1* cells displayed cell separation defects. *rac1^{Otef}* cells exhibit a dramatic increase in cell length. Scale bars, 10 μm.

and failed to separate normally compared to the wild type strain. This phenotype is similar to the phenotype observed for the *Δcla4* mutant strains. Overexpression of *rac1* was created by introducing the *rac1^{Otef}* construct into the *alb1* background strain as previously described. The *rac1^{Otef}* mutant strain did form filaments as previously described (Fig. 33 and Mahlert, *et al.*, 2006). With the regulation of filament formation in mind, colony and micro-colony morphology was explored. Deletion of *rac1* did not affect the morphology of colonies or micro-colonies on YEPS (Fig. 34A and B). Overexpressing *rac1* did lead to a filamentous colony and micro-colony phenotype on YEPS (Fig. 34A and B). In addition, the *rac1^{Otef}* mutant strain exhibited directional growth seen previously in the *alb1 Δ₈₁₀₋₂₈₃₂hsl7 smu1^{Otef}* mutant strain.

Figure 34: Rac1 mutant strains differ in colony morphology on rich media. The *Δrac1* strain colonies did not display any defects in colony morphology, whereas the *rac1^{Otef}* strain exhibited a filamentous colony morphology on rich media (A). The micro-colonies of the *Δrac1* strain did not differ from those of the wild type strains, however the *rac1^{Otef}* strain exhibited extremely elongated cells and a “directional growth” phenotype (B). Scale bars, 50 μ m – 20x inset image, 40 μ m – 60x

Under low ammonium conditions, the *Δrac1* mutant strain displayed a dramatic reduction in filament formation, whereas the *rac1^{Otef}* mutant strain exhibited a dramatic increase in filament formation for both colonies and micro-colonies (Fig. 35A and B). The colonies of the *rac1^{Otef}* mutant strain produced larger, thicker filaments than the wild type progenitor strain. Examination of the micro-colonies at 20x magnification revealed elongated filaments, in addition to directional growth (Fig. 35B, inset). At 60x magnification, it became clearer that the long filaments were not highly branched (Fig. 35B). The phenotype was comparable to the phenotype observed in YEPS media, with only the length of the cells increased. The *Δrac1* mutant strain again appeared reduced in the filamentous response to low ammonium when viewed at 20x magnification (Fig. 35B, inset). At 60x magnification, an interesting phenotype was observed; many of the cells were bent or curved in appearance (Fig. 35B). In addition, rounded structures were present at the ends of the majority of cells, while a few cells began to elongate to

Figure 35: The filamentous response to SLAD of the *Rac1* mutant strains vary compared to wild type strains. The $\Delta rac1$ strain displays a dramatic reduction in filament formation while the $rac1^{Otef}$ strain exhibits exaggerated filament formation on SLAD media (A). The $rac1^{Otef}$ strain was hyper-filamentous when observed in SLAD micro-colonies. The $\Delta rac1$ strain exhibited bent, shorter, thickened cells that ended with a rounded structure at the apical tip. A few cells were beginning to form filaments (B, see arrows). Scale bars, 50 μ m – 20x inset image, 40 μ m – 60x image.

potentially form filaments (Fig. 35B, see arrows). The rounded structures are reminiscent of a similar structure observed in a constitutively active *rac1* mutant strain (rac^{Q61L}); however, expression of rac^{Q61L} is lethal (Mahlert, *et al.*, 2006).

The ability of the *rac1* mutants to filament in liquid SLAD was examined since I had observed filament formation of the *cla4* mutant strains under these growth conditions. Interestingly, neither the $\Delta rac1$ nor the $rac1^{Otef}$ mutant strain formed filaments when grown in liquid SLAD (Fig. 36). Both the $\Delta rac1$ and the $rac1^{Otef}$ mutant strain displayed a single nucleus when stained with Syto 11 and no cell wall defects were

Figure 36: Rac1 mutant strains are unaffected in cell wall localization and nuclei number in SLAD media. Neither $\Delta rac1$ nor $rac1^{Otef}$ cells display any defects in cell wall localization or nuclei number on SLAD media. The $rac1^{Otef}$ cells were elongated but did not form filaments in SLAD media. Scale bars, 10 μ m.

observed when stained with CFW and WGA, although the $rac1^{Otef}$ mutant strain was elongated compared to the wild type strain (Fig. 36). This result differs from what was observed in the $\Delta cla4$ mutant strains, where the $\Delta cla4$ mutant strains were filamentous and display cell separation defects.

Time delay of the filamentous response to low ammonium conditions. The conflicting filamentous phenotypes observed for colonies of the $cla4^{Otef}$ mutant strain and micro-colonies in addition to the dramatic reduction in the filamentous response by the $\Delta rac1$ mutant strain could indicate a potential delay in the filamentous response to low ammonium conditions in these mutant backgrounds. To explore this hypothesis, the $cla4$ and $rac1$ mutant strains were streaked onto SLAD media and incubated at 25° C for 6 days, and images were gathered at both 3 and 6 days post inoculation (dpi). Comparisons between the wild type strains at both 3 and 6 dpi indicated no differences in the

filamentous response between the mating-type backgrounds (Fig. 37A and B). Examination of the *cla4* and *rac1* mutants indicated temporal effects in the filamentous response. At 3 dpi, the Δ *cla4* mutant strains displayed mating-type background differences in colony morphology. The *alb1* Δ *cla4* mutant strain exhibited an increase in filament formation, while the *a2b2* Δ *cla4* mutant strain displayed a decrease in filament formation in comparison to the wild type progenitor strains (Fig. 37A). Though the overall filamentous response was increased for both the *alb1* and the *a2b2* mutant strains, the mating-type specific differences from the wild type strains observed at 3 dpi was maintained at 6 dpi (Fig. 37B). The dramatically elongated filaments exhibited in the *alb1* Δ *cla4* mutant strain at 3dpi, continued to elongate, and by 6 dpi, displayed extremely long, single septated hyphae (Fig. 37B). At 3 dpi, the *cla4*^{Otef} mutant strains displayed similar mating-type background specific phenotypes to those observed in the Δ *cla4* mutant strains (Fig. 37A). The *alb1* mutant strain exhibited an increase, while the *a2b2* mutant strain exhibited a decrease in filament formation compared to the wild type strains. Though the overall colony morphology was different than the wild type strains at 6 dpi, the *a2b2* *cla4*^{Otef} mutant strain still displayed a decrease in the filamentous response, while for the *alb1* *cla4*^{Otef} mutant strains overall filament formation was comparable to the wild type strain (Fig. 37B).

Examination of the Δ *rac1* and *rac1*^{Otef} mutant strains indicated time dependent changes in the filamentous response to SLAD. At, 3 dpi, the Δ *rac1* mutant strain displayed a dramatic decrease in the ability to form filaments on SLAD compared with the wild type strain (Fig. 37A). However, the colony was not smooth as it was when grown on rich media. The subtle filament formation indicated that *rac1* dramatically

Figure 37: Colony morphology of *Cla4* and *Rac1* mutant strains grown on SLAD for 3 and 6 days post inoculation (dpi). Examination of *Cla4* mutants at 3 dpi indicates that the *a1b1* mating-type of both the $\Delta cla4$ and *cla4^{Otef}* mutant strains displayed an increase in the filamentous response compared to wild type strain. The *a2b2* $\Delta cla4$ and *cla4^{Otef}* mutant strains were reduced in filament formation compared to wild type strains. The $\Delta rac1$ strain did not form filaments whereas the *rac1^{Otef}* strain was increased in filamentation on SLAD at 3 dpi (A). At 6 dpi, the *a1b1* $\Delta cla4$ mating-type strain displayed dramatically elongated filaments while the *a1b1* *cla4^{Otef}* mutant strain appeared similar to the wild type strain. The *a2b2* $\Delta cla4$ and *cla4^{Otef}* mutant strains were again reduced in filament formation compared to the wild type strain. The $\Delta rac1$ strain began to form filaments, but they were severely reduced compared to the wild type strain. The *rac1^{Otef}* strain was still increased in filamentation on SLAD at 6 dpi (B).

reduces, but does not eliminate the ability to form filaments under low ammonium

conditions. This idea was borne out when the $\Delta rac1$ mutant strain was examined at 6 dpi.

Although the mutant did not produce filaments that were comparable to those observed in

the wild type strain, the *Arac1* mutant strain did produce filaments in response to SLAD (Fig. 37B). On the other hand, the *rac1*^{Otef} mutant strain displayed an increase in the filamentous response compared to the wild type at 3 dpi (Fig. 37A). At 6 dpi, the *rac1*^{Otef} mutant strain still displayed an increased colony size as well as increased filament formation on SLAD media (Fig. 37B). However, the difference between the *rac1*^{Otef} mutant and the wild type strain at 6 dpi is not as dramatic as the difference that is observed at 3 dpi. Overall these results indicate that of these multiple gene products examined, all the mutant strains were able to filament in response to low ammonium conditions. However, the various mutant strains show a delay in the ability to form filaments under low ammonium conditions.

DISCUSSION

Cla4 plays a role in cell length that is mating-type specific and negatively regulates the filamentous response to low ammonium conditions. Cla4 is a member of the highly conserved p21-activated protein kinase family. Deletion of *cla4* disrupted the ability of *U. maydis* to undergo normal bud formation; subsequently Δ *cla4* cells undergo elongated growth and divide by fission from a central septum. Nuclear staining indicated that each septated compartment contained a single nucleus. In addition, Δ *cla4* mutant strains exhibited defects in chitin localization as well as in the ability to form filaments both in the mating reaction and *in planta*, leading to a decrease in virulence (Leveleki *et al.*, 2004). A significant increase in cell length was observed only in the *a2b2* mating-type background of Δ *cla4* mutant strains. A mating-type specific cell length phenotype was also observed in mutants of the gene *smul*, though Δ *smul* mutants were decreased in cell length compared to wild type strains (Chapter II). Neither of the

mutant strains of the *alb1* mating-type background was significantly different compared to the wild type strains, though the difference between the Δ *cla4* and the Δ *smul alb1* mating-type backgrounds was significant. In *S. cerevisiae*, Cla4, a target of Cdc42, promotes septin collar formation, subsequently allowing targeting of Swe1 to the bud neck for degradation (Versele and Thorner, 2004). In addition, Cla4 is involved in the hyper-phosphorylation of Swe1, targeting Swe1 for degradation and allowing proper and timely transition from G₂-M. Swe1 monitors septin (actin) filament formation and is stabilized when perturbations occur causing a G₂ delay, blocking mitosis (Versele and Thorner, 2004). An effect on cell length was also observed for the strains containing an ectopic gene copy for overexpression of *smul* or *cla4*. Interestingly, both the *cla4*^{Otef} and the *smul*^{Otef} *alb1* mutant strains exhibited a comparable increase in cell length, whereas neither *a2b2* mating-type specific mutant strains were different from the wild type strains or one another. Overall these results present an interesting picture of the relationship between the two PAKs studied in *U. maydis*. In yeast, both Ste20 and Cla4 play a role in cytoskeletal dynamics and septum formation during cell separation, as well as cell cycle regulation (Cvrčková, *et al.*, 1995; Benton *et al.*, 1997; Eby, *et al.*, 1998; Holly and Blumer, 1999; Höfken and Schiebel, 2002; Versele and Thorner, 2004). It is possible that Cla4 in *U. maydis* plays a role in cell cycle regulation by targeting Wee1 for degradation in a Cdc42 / Rac1 dependent manner, thereby regulating a critical step in G₂-M transition and cytokinesis. If so, this would be in addition to the activity of Cla4 in polar growth, though more work needs to be done to clarify this potential role.

Δcla4 mutant strains are unable to form filaments in the mating reaction, though they are capable of cell recognition, cell fusion, and filament formation *in planta*

(Leveleki, *et al.*, 2004). Conversely, the $\Delta cla4$ mutant strains exhibited a filamentous response on low ammonium conditions including liquid SLAD, again with phenotypes that varied according to mating-type. In addition, both $\Delta cla4$ mutant strains displayed defects in chitin but not β -glucan localization. Overexpression of *cla4* resulted in an increase in the filamentous response to SLAD in the *a/b1* mating-type background; however the *a2b2 cla4^{Oref}* mutant strain exhibited a decrease in the filamentous response to SLAD. Interestingly, the *cla4^{Oref}* mutant strain micro-colonies displayed a dramatic decrease in the filamentous response to SLAD independent of mating-type background. Overall the role of Cla4 in the filamentous response to low ammonium conditions is complicated. Cla4 potentially plays a role in polar growth and proper bud formation in addition to possible cell cycle effects. Absence of Cla4 may not directly cause the filamentous response, but rather delay the proper cell cycle timing allowing other mechanisms regulating the filamentous response to act, creating elongated cells.

***rac1* mutant strains are able to form filaments in response to low ammonium conditions.** Cla4 is an effector of the Rho / Rac-like GTPase, Rac1, and regulates polar growth in *U. maydis* (Mahlert, *et al.*, 2006). Deletion of *rac1* leads to attenuation of polar growth, with the cells appearing very similar to those of $\Delta cla4$ mutant strains, being fatter and failing to separate through normal mother daughter septa. This indicates that Rac1 potentially localizes Cla4 to the bud site allowing proper formation of the septin ring, ultimately allowing for proper G₂-M transition. Conversely, overexpression of *rac1* leads to dramatic cell elongation and a polar growth pattern similar to Wee1 overexpression strains (Sgarlata and Pérez-Martín, 2005). Overexpression of Rac1 may sequester Cla4 from the septin ring to the growing polar tip of the bud, delaying Wee1

phosphorylation and subsequent degradation extending G₂, while concomitantly causing polar growth. Equally, the over abundance of Rac1 may simultaneously promote normal cell cycle propagation and polar growth, whereas polar growth is not as dramatic in the wild type strains due to lower stoichiometric levels of Rac1. The *rac1^{Otef}* mutant strain colony and micro-colony results are very similar to the filamentous and directional growth responses observed in the *alb1 Δ₈₁₀₋₂₈₃₂hsl7 smu1^{Otef}* mutant strain indicating a shared role in cell elongation and potentially in cell cycle regulation (Chapter II). Under conditions of low ammonium, the *Δrac1* mutant strain exhibited a marked reduction in the ability to form filaments, though it was observed primarily in micro-colonies that filaments were beginning to form, indicating a potential delay in the filamentous response to low ammonium. The cell morphology of the *Δrac1* mutant strain exhibited two striking features, curved or bent cells and a bulbous structure at the end of the cells. A similar bent phenotype was observed in *Δcla4* mutant strains that form a centrally located septum and separate by contraction, which can result in a bending of daughter cells. Yet the bent phenotype observed in the *Δrac1* mutant strain does not appear to be due to new daughter cells forming from a central septum, but rather the cell itself contorting. Even though the *Δrac1* mutant strain was viable in SLAD media, the rounded structure at the ends of the cells was reminiscent of the constitutively active and lethal form of *rac1*, Rac1^{Q61L} (Leveleki, *et al.*, 2004; Mahlert, *et al.*, 2006). The *rac1^{Otef}* mutant strain produced large thick filaments, similar to the dramatically thickened colony morphology observed in a *Δump1 / Δump2* double mutant (Smith, *et al.*, 2003). In addition, the *rac1^{Otef}* mutant strain exhibited a directional, hyper-filamentous phenotype on micro-colonies. However in liquid SLAD, the *rac1^{Otef}* mutant strain, though elongated

compared to wild type strains, failed to form filaments differing dramatically from phenotypes observed for either the $\Delta_{810-2832}hsl7\ smu1^{Otef}$ or the $\Delta cla4$ mutant strains. These results indicate that the phenotypes observed in low ammonium conditions in the $\Delta_{810-2832}hsl7\ smu1^{Otef}$ or the $\Delta cla4$ mutant strains are independent of Rac1. However, the $\Delta rac1$ mutant strains are able to form filaments, albeit reduced dramatically, indicating that Rac1 plays some role in the filamentous response (see below), but is not absolutely required in all circumstances.

Time effects on the filamentous response to low ammonium conditions.

Responses to nutrient conditions are complex and vary from nutrient to nutrient. *U. maydis* undergoes a morphological transition from a budding phenotype to a filamentous phenotype in response to low ammonium conditions, growth on lipids as the carbon source, or acidic pH (Ruiz-Herrera, *et al.*, 1995; Smith *et al.*, 2003; Klose, *et al.*, 2004). Ammonium is the preferred nitrogen source for many fungi and under low ammonium conditions (< 100 μ m) the colonies of *U. maydis* strains produce a filamentous colony phenotype. The extent of the filamentous response is time dependent, with a greater and greater production of filaments over a greater duration on low ammonium conditions. At 3 dpi, several of the previously examined mutant strains exhibited differences compared to the wild type strains in the ability to form filaments on SLAD media, most dramatically the $\Delta rac1$ mutant strain, which failed to form filaments. At 6 dpi, the increase in the filamentous response of the $cla4^{Otef}$ and $rac1^{Otef}$ in comparison to the wild-type strain had diminished. Conversely, the $\Delta rac1$ mutant strain produced filaments, though they were severely reduced in comparison to those of wild type strains. These results contradict the hypothesis of *rac1* acting as the master regulator of the filamentous

response. The deletion of *rac1* appears to delay the ability of the cells to produce filaments on SLAD media indicating other players are involved in the filamentous response to SLAD media or a parallel pathway regulating the filamentous response. Moreover, the delay in the onset of the filamentous response by *Arac1* mutant strain could be due to changes in the media independent of low ammonium (*i.e.* glucose levels or secondary metabolite production) triggering the filamentous response. Evidence in *Aspergillus niger* indicates that spatial recognition triggers either expression of three genes involved in nitrate reduction at the colony edge or nitrogen metabolic repressor at the colony center (Levin *et al.*, 2007). This spatial gene expression appears to be independent of nitrate concentrations, as well as carbon source. In addition, the expression genes involved in nitrate reduction remained constant upon transfer to fresh media, indicating that accumulation of secondary metabolites does not seem to modulate gene expression. Quorum sensing, a potential explanation for spatial gene expression, has been observed in *C. albicans* where cell density negatively affects biofilm production and pathogenesis (Hogan, 2006). Though not yet demonstrated, a similar process could explain spatial and temporal behaviors of *U. maydis*.

Conclusions. Rho / Rac GTPases have been implicated in regulating cell polarity, cytoskeletal organization, and cytokinesis (Nakano, *et al.*, 1997; Cabib, *et al.*, 1998; Madden and Snyder, 1998; Lengeler, *et al.*, 2000; Tolliday, *et al.*, 2002; Nelson, 2003; Bassilana and Arkowitz, 2006; Mahlert *et al.*, 2006). PAKs have been identified as potential downstream effectors of the Rho / Rac GTPases, contributing to many of the GTPase specific roles (Roberts and Fink, 1994; Cvrčková, *et al.*, 1995; Marcus, *et al.*, 1995; Gilbreth, *et al.*, 1996 and 1998; Leberer, *et al.*, 1997; Eby, *et al.*, 1998; Holly and

Blumer, 1999; Fujita, *et al.*, 1999; Bao, *et al.*, 2001; Höfken and Schiebel, 2002; Qyang, *et al.*, 2002; Wiley *et al.*, 2003; Leveleki, *et al.*, 2004; Smith, *et al.*, 2004; Heinrich, *et al.*, 2007; Böhmer, *et al.*, 2008). In *U. maydis*, Rac1 establishes polar growth, while Cdc42 regulates cell separation, and Rho1 is required for cell polarity and cytokinesis (Mahlert *et al.*, 2006; Pham *et al.*, 2009). Cla4 has been identified as a downstream effector of Rac1, while downstream effectors of Cdc42 and Rho1 have yet to be identified, although Cla4 is a potential prospect (Pham, *et al.*, 2009). Cdc42, through its GEF, Don1, is required for formation of the second septum allowing proper mother daughter cell separation (Mahlert *et al.*, 2006). Cdc42 localization and activity at the second septum requires the activity of the PAK Don3 (Böhmer *et al.*, 2008). Cdc42, which also localizes to the primary septum, is dispensable for polarized growth and filament formation (Mahlert *et al.*, 2006). However, it is interesting to speculate the role of Cdc42 when localized to the primary septum. On the other hand, Rac1 plays a role in polar growth and normal bud formation by its localization to the polar tip, potentially through Cdc24, the Rac1-dependent GEF. Rac1 activity promotes localized cell wall formation in a Cla4 dependent manner (Mahlert *et al.*, 2006). The Δ *cla4* mutant strains display a dramatic disruption in chitin deposition; however the Δ *rac1* mutant strain did not display any defects in chitin deposition. Though both Cla4 and Rac1 are involved in polar growth, the role of Cla4 in chitin deposition is independent of Rac1. Ultimately, Rac1 and Cdc42 have separate roles in *U. maydis*: Rac1, through Cla4, regulates septin apparatus assembly necessary for bud formation, and then promotes polarized cell growth while Cdc42 is required to promote mother daughter cell separation, but is dispensable for bud formation and polar growth (Mahlert, *et al.*, 2006). Thus it appears that Rac1,

through Cla4, acts as the master regulator of filament formation (Mahlert, *et al.*, 2006; Pham, *et al.*, 2009). However, very little work has been performed on Rac1, Cdc42, Rho1, and Cla4 under conditions of low ammonium. In this study, I provide evidence that Cla4 plays a role in the filamentous response to low ammonium conditions. In addition, Rac1 may not be the only upstream activator of Cla4. Moreover, neither Cla4 nor Rac1 are essential in the ability to form filaments under conditions of low ammonium, although the ability to form filaments is greatly reduced in *rac1* deletion strains.

When grown in rich conditions, the Δ *cla4* mutant cells are morphologically different from the cells of the disrupted *U. maydis* PAK-like Ste20 homologue, Smu1. Δ *cla4* cells were septated, containing several independent nuclei having failed to correctly separate, unlike the Δ *smu1* mutant strains which are affected only in cell length (Fig. 5, Chapter II). These results are consistent with the observed roles of Cla4 in cytokinesis and polar growth in *S. cerevisiae* and *C. albicans* (Leberer, *et al.*, 1997; Benton *et al.*, 1997). In addition, the phenotypes observed in the Δ *cla4* were similar to those exhibited in Cdk1, Cdk5, and Wee1 mutants, indicating that Cla4 may play a role in cell cycle similar to Cla4 from *S. cerevisiae* (Asano, *et al.*, 2005; Sgarlata and Pérez-Martín, 2005; Castillo-Lluva *et al.*, 2007). Cell length measures of the Δ *cla4* mutant strains support this idea. An increase in cell length could indicate a delay in the G₂-M transition. Additional support was observed in low ammonium conditions where the Δ *cla4* mutant strains increase filament formation in a mating-type specific manner. This may indicate that though Cla4 is necessary for filament formation in rich conditions, it is dispensable in conditions of low ammonium. Another possibility is that deletion of *cla4*

indirectly promotes filamentous growth by failing to promote cell separation (via binding to Rac1), creating a cell cycle delay, while additional pathways are promoting increased polar growth leading to the filamentous response. The role Rac1 plays in the filamentous response to low ammonium conditions is more complicated. Deletion of *rac1* dramatically delays filament formation, but does not eliminate the ability to form filaments. Conversely, overexpression of *rac1* increased the filamentous response; however the *rac1*^{Otef} mutant strain did not exhibit filament formation when grown in liquid SLAD, though the mutant cells were elongated. The exaggeration of the filamentous response in the Δ *cla4* mutant cells, along with the lack of such a response in either *rac1* mutant strain suggest that the role Cla4 plays in the filamentous response to low ammonium conditions may be independent of Rac1. These results imply that Cla4 may not be the only downstream effector of Rac1, or conversely, Cla4 may have other upstream activators regulating septin assembly and proper cell separation. Potential candidates include Cdc42 and Rho1.

CHAPTER IV

GENERAL DISCUSSION

Dimorphic fungi undergo a process of yeast-like to hyphal transition in response to nutrient, mating, and/or host cues. Studying how these fungi respond to this myriad of cues provides insight into intracellular signaling in response to extracellular signals.

Dimorphic fungi provide an excellent model to study the regulatory pathways associated with cellular morphogenesis due to their ease of genetic manipulation. A complex network of signaling molecules and pathways regulate many essential cellular processes including cell cycle, cytoskeletal organization, cell polarity, cellular morphology, cytokinesis and hyphal development. Exploration of these pathways has identified several proteins that are highly conserved from fungi all the way to humans, as well as the conservation of these pathways as distinct units.

The Rho/Rac family of GTP-binding proteins (GTPases) regulates many of the functions involved in cell growth, development, mating, and differentiation (Nakano, *et al.*, 1997; Cabib, *et al.*, 1998; Madden and Snyder, 1998; Lengeler, *et al.*, 2000; Tolliday, *et al.*, 2002; Nelson, 2003; Leveleki *et al.*, 2004; Mahlert *et al.*, 2006; Pham, *et al.*, 2009). The activity of these proteins is regulated temporally and spatially by an ever growing group of accessory proteins, giving rise to specific landmark effects and subsequent development. Guanine exchange factors (GEFs) are one subset of these accessory proteins and are involved not only in the activation of the GTPases, but in their cellular

localization (Ziman, *et al.*, 1993; Gulli and Peter, 2001; Weinzierl, *et al.*, 2002; Coll, *et al.*, 2003; Mahlert *et al.*, 2006; Schink and Bölker, 2009). In addition, GEFs regulate GTPase specificity for downstream substrates, defining precise processes. In *U. maydis*, three highly conserved Rho/Rac GTPases, Cdc42, Rac1, and Rho1 have been implicated in cell separation, bud formation, cell polarity, and polar growth (Weinzierl, *et al.*, 2002; Leveleki, *et al.*, 2004; Mahlert, *et al.*, 2006; Böhmer, *et al.*, 2008; Pham, *et al.*, 2009). Cdc42 activity requires the specific GEF activity of Don1 while it is proposed that Rac1 requires the GEF activity of Cdc24 (Mahlert, *et al.*, 2006; Castillo-Lluva, *et al.*, 2007; Alvarez-Tabarés and Pérez-Martín, *et al.*, 2008; Hlubek, *et al.*, 2008). Hlubek, *et al.* (2008) indicated that a single amino acid substitution in Rac1 confers Cdc42-specific activities to Rac1. The amino acid substitution promotes these activities by allowing Rac1 to interact with the Cdc42-specific GEF, Don1. A Rho1-specific GEF has yet to be identified, though Cdc24 has been implicated through *in vitro* interaction analyses (Pham *et al.*, 2009).

Many functions of the GTPases depend on the highly conserved family of p-21 activated kinases (PAKs) as downstream effectors (Roberts and Fink, 1994; Cvrčková, *et al.*, 1995; Marcus, *et al.*, 1995; Gilbreth, *et al.*, 1996 and 1998; Leberer, *et al.*, 1997; Eby, *et al.*, 1998; Holly and Blumer, 1999; Fujita, *et al.*, 1999; Bao, *et al.*, 2001; Höfken and Schiebel, 2002; Qyang, *et al.*, 2002; Wiley *et al.*, 2003; Leveleki, *et al.*, 2004; Smith, *et al.*, 2004; Heinrich, *et al.*, 2007; Böhmer, *et al.*, 2008). Two specific PAKs, Cla4 and Ste20, have been identified and studied, and although loss of either PAK usually does not result in a lethal phenotype, loss of both PAKs is lethal (Roberts and Fink, 1994; Cvrčková, *et al.*, 1995; Marcus, *et al.*, 1995; Gilbreth, *et al.*, 1996 and 1998; Leberer, *et*

al., 1997; Eby, *et al.*, 1998; Holly and Blumer, 1999; Fujita, *et al.*, 1999; Bao, *et al.*, 2001; Höfken and Schiebel, 2002; Qyang, *et al.*, 2002; Wiley *et al.*, 2003; Leveleki, *et al.*, 2004; Smith, *et al.*, 2004; Heinrich, *et al.*, 2007; Böhmer, *et al.*, 2008), indicating that although they regulate separate processes, they share at least one essential function (Cvrčková, *et al.*, 1995). Cla4 and Ste20 homologues have been identified and studied in *U. maydis* (Leveleki, *et al.*, 2004; Smith, *et al.*, 2004). Cla4 appears to be an effector of Rac1, regulating cell separation and polarity, while Smu1 is involved in mating pheromone expression and virulence (Leveleki, *et al.*, 2004; Smith, *et al.*, 2004).

PAK activity is also dependent upon several proteins in addition to Rho/Rac GTPases (Lengeler *et al.*, 2000; García-Pedrajas, *et al.*, 2008). Hsl7, a member of the conserved protein-arginine methyltransferase family of proteins, is known to interact with the Ste20 homologues and plays a role in cell cycle regulation, cell morphogenesis, and response to several environmental conditions (Ma, *et al.*, 1996; Gilbreth, *et al.*, 1996 and 1998; Pollack, *et al.*, 1999; Fujita *et al.*, 1999; Bao, *et al.*, 2001). In addition, Hsl7 is known to regulate cell cycle progression in a Wee1 dependent manner (Ma, *et al.*, 1996; Gilbreth, *et al.*, 1998; Shulewitz, *et al.*, 1999; Yamada, *et al.*, 2004; Asano, *et al.*, 2005). In *S. cerevisiae*, Hsl7 targets Swe1 (Wee1 homologue) for degradation by co-localizing Swe1 to the bud neck with Hsl1, allowing for hyper-phosphorylation via Cdc5 and subsequent degradation, promoting G₂–M transition (Cid, *et al.*, 2001; La Valle and Wittenberg, 2001; Asano, *et al.*, 2005).

I was able to demonstrate in *U. maydis* that Smu1, in addition to regulating the expression of mating pheromone and virulence, positively regulates the filamentous response to low ammonium conditions. Moreover, Smu1 affects cell length. Hsl7 plays

a role in cell length and the filamentous response to low ammonium conditions, yet opposite to the role of Smu1. In addition, Hsl7 interacts with Smu1 in yeast two-hybrid analyses, indicating that Hsl7 may play a role in regulating Smu1 activity. Fujita, *et al.* (1999) demonstrated that Hsl7 interacts with Ste20 both *in vitro* and *in vivo*, regulating the filamentous response pathway to low ammonium. Genetic analysis indicates that Hsl7 is upstream of Ste20 and the action of Hsl7 upon Ste20 is negative. Deletion of Hsl7 leads to a filamentous response in nitrogen rich conditions that can be alleviated by concomitant deletion of Ste20, while overexpression of *hsl7* attenuates the filamentous response to low ammonium, a phenotype that can be rescued by co-overexpressing *ste20* (Fujita, *et al.*, 1999). The disruption of *smu1* does not alleviate $\Delta_{810-2832}hsl7$ associated phenotypes, nor does the latter deletion rescue $\Delta smu1$ effects on mating response or virulence. This points to an alternate hypothesis posed in *S. cerevisiae*, in which Hsl7 regulates the filamentous response in a Swe1-dependent manner independent of Ste20 (La Valle and Wittenberg, 2001). Deletion of *hsl7* creates a delay in the G₂-M transition, causing cell elongation and eases the ability of the cells to promote the filamentous response. It is possible that a similar process occurs in *U. maydis*, in which Hsl7 would act on cell cycle regulation, while Smu1 plays a role in the filamentous response pathway. This is supported by data indicating that overexpressing *smu1* while depleting *hsl7* leads to an exacerbation of $\Delta_{810-2832}hsl7$ -associated phenotypes, increasing cell length and the filamentous response to low ammonium. Remarkably, mutant strains lacking *hsl7* while simultaneously overexpressing *smu1* were able to develop filaments in liquid SLAD media. In this study, the only other strains that produced filaments in liquid SLAD were those lacking *cla4*.

The PAK homolog, Cla4, differs from Ste20 homologues in one major regard, Cla4 homologues contains a pleckstrin homology (PH) domain, while Ste20 homologues do not. This domain allows Cla4 to bind to the membrane and localize to polar growth sites as well as regulate cell morphogenesis and mitotic exit (Wild, *et al.*, 2004). In *U. maydis*, Cla4 has been identified as the downstream effector of Rac1, regulating bud formation and morphology and polar growth (Leveleki, *et al.*, 2004; Mahlert, *et al.*, 2006). Moreover, deletion of either *cla4* or *rac1* eliminates b-dependent filamentation. However, on SLAD media, Δ *cla4* strains were still able to produce filaments, being the only other mutant strain to filament in liquid SLAD outside of the Δ ₈₁₀₋₂₈₃₂*hsl7 smu1*^{Oief} strains. Interestingly this filamentous response to SLAD appears to be independent of Rac1 activity. Deletion of *rac1* created a dramatic delay in the filament formation, while overexpression of *rac1* leads to hyphal growth. Yet, neither mutant strain formed filaments in liquid SLAD. It could be that Cla4 limits the filamentous response to low ammonium conditions by either sequestering a component in the filamentous response pathway (potentially Cdc42 or PAK/MAPK activator) or by promoting bud emergence and normal cell cycle progression preventing cell elongation and hyphal growth. *S. cerevisiae* Cla4 binds Cdc42 promoting septin ring formation and proper bud emergence. Subsequently Cla4 is involved in the hyper-phosphorylation of Swe1 promoting G₂-M transition (Versele and Thorner, 2004; Asano, *et al.*, 2005). It is interesting that the two PAKs, Cla4 and Smu1, have opposite roles in the filamentous response pathway, where deletion of *cla4* alone or overexpressing *smu1* in a Δ ₈₁₀₋₂₈₃₂*hsl7* background, leads to a hyper-filamentous response to low ammonium.

In *S. cerevisiae*, deletion of both *ste20* and *cla4* is lethal, indicating that Ste20 and Cla4 share at least one essential function (Cvrčková, *et al.*, 1995). The overlapping role appears to be in actin organization and cell separation (Cvrčková, *et al.*, 1995; Eby, *et al.*, 1998; Höfken and Schiebel, 2002; Kadota, *et al.*, 2004). However, studies by Keniry and Sprague (2003) indicates that different subdomains of Ste20 and Cla4 are functionally separate and governed by different factors. Threonine 818 promotes Ste20 activity in the HOG and pheromone response pathways, whereas two different regions direct invasive growth. Subdomains III to X allow the Cla4-specific function for viability in a $\Delta cln1$, $\Delta cln2$, $\Delta cla4$ strain. It is possible that a similar form of overlap occurs between Cla4 and Smu1 in *U. maydis*. They share similar responsibility in mating and subsequent pathogenic development, though Smu1 is required for proper pheromone based signaling, while Cla4 is dispensable for normal pheromone response but necessary for subsequent b-dependent development. Additionally, they both have a role in the filamentous response to low ammonium, yet they are opposite in how they regulate the response. Cla4 appears to regulate the filamentous response, potentially through promoting bud formation and cell separation. The role of Smu1 is more difficult to understand. Overexpression of *smu1* promotes increased filamentation to SLAD, but only upon simultaneous disruption of *hsl7* do I observe the hyper-filamentous response to low ammonium. This particular set of circumstances points to additional players that one or both, Smu1 and Hsl7, act upon in regulating cell morphogenesis.

Ultimately, both Smu1 and Cla4 are involved in regulating the filamentous response to low ammonium conditions. Here I have demonstrated that, although absence of Cla4 or overabundance of Smu1, with concomitant Hsl7 absence, is not lethal to *U.*

maydis, either genetic configuration results in a hyper-filamentous response to low ammonium. The interaction between Hsl7 and Smu1 appears to be a convergence of independent roles creating the exacerbated response. The lack of a clear genetic interaction between Hsl7 and Smu1 points to the involvement of the two proteins in separate pathways that culminate in regulating the filamentous response to SLAD. It is possible that Hsl7 plays a role in cell cycle regulation, promoting G₂-M transition as in *S. cerevisiae*, which would explain the increased cell length and increased filamentous response in SLAD. In addition, Smu1 could play a role in the filamentous response to SLAD, potentially through the MAPK pathway. The delay in G₂-M transition created by removing Hsl7 primes the cells for the filamentous response to low ammonium, while simultaneous overexpression of Smu1 potentially leads to hyper-filamentation. This hypothesis allows for the fact that the concurrent disruption of both *hsl7* and *smu1* does not alleviate any phenotypes associated with either single gene disruption strain. Cla4 also contributes to a similar style of response by delaying G₂-M transition through prevention of proper bud formation, potentially through the septin ring formation, allowing the normal filamentation inputs to overdrive the filamentous response, leading to hyper-filamentation.

Studying the PAKs leads to two interesting ideas of signaling pathway overlap and specificity of the various signaling pathways leading to filamentation, and to the input of multiple pathways controlling morphogenesis and development. More work needs to be done to assign cell fate specific activity to both Cla4 and Smu1 under multiple conditions, as well as defining the multitude of potential targets and regulators of both PAKs. Exploring the PAK specific activity of the Rho/Rac family of GTPases

would provide essential insight to the localization and activity of the two families of proteins in dimorphism and in cell fate.

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APPENDIX I

HAPLO-INSUFFICIENCY IN THE HSL7 MUTANT BACKGROUND

INTRODUCTION AND DISCUSSION

Diploid organisms exhibit allelic compensation for most genes. This is defined as a loss of function allele with no discernable phenotype. The one functional allele masks the phenotypic consequences of the loss of the function of the second allele. Ninety seven percent of *S. cerevisiae* genes display allelic compensation (Duetschbaur *et al.*, 2005). However, in up to three percent of genes, loss of function in one allele can lead to harmful effects on the diploid cells and such deviations from wild type phenotypes are termed as haplo-insufficiency (H-I). More commonly observed in multi-cellular organisms, H-I has been shown to cause many problems in humans in chromosome maintenance and gene expression. The transcription factors TWIST and GATA3, as well as the DNA helicase BLM and DNA repair proteins (ATM) all exhibit H-I defects (Duetschbaur *et al.*, 2005). Examples such as these have lead to many diseases such as Marfan syndrome, cancer, and Diamond Blackfan Anemia (DBA) (Baek, *et al.*, 2008; Dr. S Ellis, personal communication).

Though observed in *S. cerevisiae*, *S. pombe*, and *C. albicans*, H-I has not been extensively studied in fungi and only recently has H-I been examined in the maize pathogen *Ustilago maydis* (Dr. C. Pham, PhD dissertation). The two highly conserved genes, the Rho-like GTPase, *rho1*, and the 14-3-3ε homologue, *pdcl*, have demonstrated

H-I defects. Rho1 and Pdc1 are required for maintenance of polarity and cytokinesis in *U. maydis* (Pham, *et al.*, 2009). In addition, diploids lacking one allele of *rho1* (*rho1:Δrho1*) were avirulent as well as unable to produce or detect pheromone, form vacuoles, and produce hyphae. Single copy *pdcl* diploid strains (*pdcl:Δpdcl*) were able to infect plants normally when the method of plant inoculation was stem injection, however when dropped onto the whorl of the plant, a decrease in tumor formation was observed (Dr. C. Pham, personal communication).

In *U. maydis*, mating and pathogenic development are regulated by two highly conserved pathways, the cAMP-dependent PKA pathway and the MAPK pathway (for review, see Klosterman, *et al.*, 2007). Both pathways relay extracellular signals to the nucleus and trigger differential gene expression. One target of the two pathways is the pheromone responsive transcription factor, Prf1, which regulates expression of both the *a* and *b* mating loci. The two pathways also are involved in filamentous response to environmental conditions, i.e., low ammonium (SLAD) and acid pH. The role of the MAPK pathway in regulating both the mating response and filamentous growth may be dependent on the p21-activated protein kinase (PAK) Smu1. Loss of *smu1* attenuated the up-regulation of pheromone expression in the *a2* mating-type background in response to basal levels of pheromone from the *a1* mating-type strain, resulting in a decrease in the overall mating response and, ultimately, virulence (Smith *et al.*, 2004). It was also observed that Smu1 played a role in the filamentous response to low ammonium conditions, though the mechanism for this regulation is not understood (Smith, *et al.*, 2004; Chapter II). In addition, mating-type specific perturbations in cell length were observed in *smu1* mutant strains as previously described (Chapter II).

Similar effects on the cell length and the filamentous response to low ammonium conditions were observed in strains either lacking or overexpressing *hsl7* (Chapter II). Hsl7 is a putative protein-arginine methyltransferase that plays a role in cell cycle regulation in the G2-M transition in *S. cerevisiae*, *S. pombe*, and *X. laevis* (Gilbreth, *et al.*, 1998; La Valle and Wittenberg, 2001; Yamada, *et al.*, 2004; Asano, *et al.*, 2005). In addition, it has been demonstrated that Hsl7 from *S. cerevisiae* regulates the filamentous response to environmental conditions, though the exact mechanism is disputed (Fujita, *et al.*, 1999; La Valle and Wittenberg, 2001). One mechanism of filamentous regulation has Hsl7 interacting with Ste20 in a negative manner (Fujita, *et al.*, 1999). Analogous interactions between Hsl7 homologues and Ste20 homologues have been observed in both *S. pombe* and human (Gilbreth, *et al.*, 1996 and 1998; Bao, *et al.*, 2001; Pollack, *et al.*, 1999). In *U. maydis*, genetic interaction studies previously performed (Chapter II) indicated that the interaction between Smu1 and Hsl7 may not be a direct interaction as observed in *S. cerevisiae*. However, concomitant disruption of *hsl7* and overexpression of *smu1* leads to hyper-filamentation on SLAD and displays mating-type specific defects in cell separation.

The mating-type defects exhibited by *smu1* and *hsl7* mutant strains lead to the exploration of H-I in these strains. To explore H-I in these genes, strains with a single gene replacement with a carboxin drug resistance cassette of either *smu1* or *hsl7* were created in the diploid strain D132. Examination of cell morphology of both the *hsl7::Δhsl7* and *smu1::Δsmu1* diploid cells indicated no difference between either mutant strain and the wild type strain (data not shown). In addition, examination of colony morphology of both the *hsl7::Δhsl7* and the *smu1::Δsmu1* diploid strains on rich (data not

shown) and low ammonium media indicated no difference when compared to the wild type strain (Fig. 38). Next the pathogenicity of both mutant strains was examined. Two

Figure 38: Filamentous response to low ammonium conditions of the diploid mutant strains. The strains were grown on SLAD media for 6 days and examined for filament formation at 3 days (A) and 6 days (B). Neither the *hsl7:Δhsl7* nor the *smul:Δsmul* diploid strains differed from wild type in response to SLAD media.

different methods of inoculation were utilized, the first being injection of an inoculum of cells into the stem of the corn plant; in the second method, cells were dropped into the whorl of the plant. Measures of virulence were taken at 21 days post inoculation (dpi) and three independent trials of approximately 10 plants were performed for both injected (i) and whorl dropped (w) strains. The virulence for each infection was measured by a disease index, ranking the severity of the disease symptoms on a scale from 0 to 5 (as described in Chapter II of this dissertation) and the results of an ANOVA and a Tukey's post-hoc analysis are shown in Table 13. The results are also presented in a percent of symptom formation graph (Doehlemann, *et al.*, 2009; see Fig. 39). The *smul:Δsmul* diploid strain did not display any significant difference from the wild type strain in the ability to infect maize by either method of inoculation. However, the *hsl7:Δhsl7* diploid strain exhibited a statistically significant decrease in virulence compared to the wild type strain when inoculated by injection. However, virulence of the *hsl7:Δhsl7* diploid strain inoculated via whorl drop did not display any difference in pathogenicity compared with the wild type strain. Both the wild type and the *smul:Δsmul* diploid strains were able to

TABLE 13. Pathogenicity of mutant strains^A.

Treatment ^B	Strain	Plants Tested	Disease rating ^C by number of affected plants						Disease Index ^D
			0 Points	1 Point	2 Points	3 Points	4 Points	5 Points	
A	1 <i>D132</i>	30	0	10	12	4	4	0	2.07
	2 <i>D132Δhsl7</i>	30	3	24	3	0	0	0	1.00**
	3 <i>D132Δsmu1</i>	29	2	9	14	2	2	0	1.76
B	1 <i>D132</i>	30	17	12	1	0	0	0	0.47
	2 <i>D132Δhsl7</i>	30	17	13	0	0	0	0	0.43
	3 <i>D132Δsmu1</i>	30	20	10	0	0	0	0	0.33

^A Table shows combined data from three identical experiments of 9 to 10 plants each reported for 21 days postinoculation.

^B Treatment consisted of the inoculation of 10^7 cells ml⁻¹ for each of the strains. Treatment A plants were inoculated by injection. Treatment B plants were inoculated via the fungal strain dropped into the whorl of the plant.

^C The disease rating is measured on a scale of 0 to 5 points based on the severity of symptoms as follows: 0 points, no disease symptoms; 1 point, chlorosis/anthocyanin production; 2 points, small leaf galls; 3 points, small galls on stems; 4 points, large stem galls; 5 points, plant death.

^D The disease index is calculated as the sum of the disease ratings divided by the number of plants tested and statistical analysis was performed using a Kruskal-Wallis ANOVA with a Dunn's Multiple Comparison Test from three independent experiments.

** p value less than .01.

produce many small and several large galls, whereas the *hsl7:Δhsl7* diploid strain only produced a few extremely small galls. A potential explanation for this difference could be that cells inoculated by injection are introduced to plant material that is not as hardy as surface tissue, which is more lignified and harder to penetrate. Thus infections set up by injection may demonstrate defects of the *hsl7:Δhsl7* diploid strain compared to the wild type strain, whereas the difficulty of plant penetration when strains are introduced by whorl drop conceals any difference in virulence between the mutant and wild type strain.

Figure 39: Disease symptom formation in the H-I *U. maydis* mutant strains. All strains were rated at 21 dpi and represent three independent experiments. Plants were inoculated with by one of two methods, injections in the plant stem (i) or dropped in the whorl of the plant (w). The graph displays the percentage of plants with a specific symptom of infections. The *hsl7:Δhsl7* diploid strain failed to produce large galls as observed in both the wild type and *smu1:Δsmu1* diploid strains, but only when inoculated by injection. No difference was observed from either mutant strain when the plants were inoculated by the whorl drop.

H-I in fungi has only been reported in a handful of species, though it is a growing area of research. Work in *S. cerevisiae* has yielded evidence that up to 3% of genes display H-I, while the number of genes in *S. pombe* is 22, and 146 genes in *C. albicans* exhibit H-I (Uhl, *et al.*, 2003; Deutschbauer, *et al.*, 2005; Baek, *et al.*, 2008). Work in *U. maydis* has now implicated 3 genes, *rho1*, *pdcl*, and *hsl7* that show evidence of H-I (This work and Dr. C. Pham, PhD dissertation). H-I phenotypes may be due to one of two defects in expression. In H-I genes, there may be a dosage defect, where the cells require a set minimum amount of protein to promote a specific cell activity and by deleting one of the two alleles of the gene there may be a decrease in overall protein levels. An alternate hypothesis is that a stoichiometric perturbation creates an imbalance in protein

ratios required in regulating a cellular process, thus leading to an H-I defect (Veitia, 2002). The *hsl7:Δhsl7* diploid strain showed a marked reduction in gall formation when inoculated by injection, though no other defects were observed. The lack of an observable phenotype in the *hsl7:Δhsl7* diploid strain when inoculated by whorl drop indicates a confounding phenotype that needs to be further explored.

MATERIALS AND METHODS

Ustilago maydis strains utilized in this study are listed in Table 14.

TABLE 14. *U. maydis* strains and plasmids used in this study.

Fungal Strains ^A		
d132	<i>al1a2b1b2</i>	<i>Kronstad and Leong, 1989</i>
d132 <i>Δhsl7</i>	<i>al1a2b1b2 hsl7, hsl7::cbx^R</i>	This Study
d132 <i>Δsmul</i>	<i>al1a2b1b2 smul, smul::cbx^R</i>	This Study

^AAll mutant strains were made in the wild type background strains listed.

Primer design, PCR, vector construction and deletion construction, growth conditions, and statistical analysis, are described previously (Materials and methods, Chapter II). Plant infection using 8 day old Golden Bantam corn seedlings (Bunton Seed Co., Louisville, KY and W. Atlee Burpee & Co., Warminster, PA) was performed with a cell density of 4×10^7 cells / mL for diploid strains as previously described (Gold *et al.*, 1997). Plant inoculation was performed by either injecting strains into the corn stem or dropping strains into the whorl of the plant and symptoms were recorded for each plant 21 days post inoculation (29 days post planting) and the indices for each strain were averaged to give a measure per strain. Three independent trials of approximately 10 plants were performed, averaged, and analyzed.

APPENDIX II
MATING-TYPE SPECIFIC CELL WALL SENSITIVITY IN A STRAIN
LACKING HSL7

RESULTS AND DISCUSSION

Fungal cells are surrounded by a thick cell wall comprised of three different components, ~60% β -glucans (~50% 1,3- β -glucan; ~10% 1,6- β -glucan), ~40% mannoproteins, and ~1% chitin (Smits, *et al.*, 2001). Cross-linking between the various components of the cell wall creates rigidity in the cell wall and can limit cell growth and morphology. Ultimately, the cell wall needs to be a dynamic environment to allow for developmental morphogenesis. During the cell cycle of *S. cerevisiae*, cell wall plasticity allows for bud development and growth. During both mating filament formation and pseudohyphal development, a highly specific and organized apical form of growth occurs where cell wall material must be localized and deposited at the growing tip, thus promoting cell elongation. In addition, cytokinesis is dependent on deposition and subsequent degradation of a chitin rich septum separating mother and daughter cells (Smits, *et al.*, 2001).

Chitin is a long homopolymer of β -1,4-linked N-acetylglucosamine. As N-acetylglucosamine is excreted and polymerized into long chains, hydrogen bonding between the chains forms microfibrils and crystallizes the chitin (Bowman and Free, 2006). This process occurs at sites of active growth and cell wall remodeling, including

the bud neck during cell separation and the hyphal tip and septa in filamentous fungi, is catalyzed by chitin synthases. In yeast, three chitin synthases (Chs) have been identified and are important, specifically for cell wall repair (Chs1p), primary septum formation (Chs2p), and overall chitin synthesis (Chs3p). Deletion of all three chitin synthases is lethal, indicating chitin is an integral part of the fungal cell wall (Bowman and Free, 2006).

As described previously, a putative protein-arginine N-methyltransferase homolog was identified in *U. maydis* (Chapter II). Hsl7 was demonstrated to interact with the Ste20 homologue, Smu1, in yeast two-hybrid analysis. Subsequent work indicated that Hsl7 plays a role in cell length, potentially by regulating the cell cycle, and the filamentous response to low ammonium conditions. As a means of comparison, two $\Delta hsl7$ mutant strains of opposite mating-type (*MUM11 a1b1* and *MUM12 a2b2*) were obtained from Dr. José Pérez-Martín (Campus de Cantoblanco-UAM, Madrid, Spain). Examination of cellular morphology indicated that both *MUM11* and *MUM12* displayed statistically significant increases in cell length compared to the wild type strains, independent of mating-type background (Fig. 40A and Table 15). In addition, the *MUM* strains and the $\Delta_{810-2832}hsl7$ strains did not differ in cell length, indicating similar effects on cell length from both $\Delta hsl7$ mutant backgrounds. Examination of growth rates in the *MUM* strains did not indicate any perturbations in cell cycle, thus growth was similar to that observed in the $\Delta_{810-2832}hsl7$ background strains (Table 16). However, staining of the cell wall with CFW did indicate aberrations in mother daughter cell separation in both mating-type backgrounds. Both *MUM11* and *MUM12* strains exhibited groups of cells that failed to separate and produced cross walls, though not every cell displayed these

TABLE 15. Measures of cell length of *MUM11* and *MUM12* strains.

Strain	n	Length ^A (in μm)	Comparison	p^B
<i>WT a1b1</i>	91	19.27 +/- .40	<i>WT a1b1</i> v. <i>WT a2b2</i>	0.82
<i>MUM11 a1b1</i>	70	25.11 +/- .62	<i>WT a1b1</i> v. <i>MUM11 a1</i>	> .001
<i>$\Delta_{810-2832}$hsl7 a1</i>	167	25.22 +/- .45	<i>MUM11 a1b1</i> v. <i>$\Delta_{810-2832}$hsl7 a1</i>	N.S.
<i>WT a2b2</i>	213	19.14 +/- .44	<i>MUM11 a1</i> v. <i>MUM12 a2</i>	0.98
<i>MUM12 a2b2</i>	308	25.12 +/- .44	<i>WT a2b2</i> v. <i>MUM12 a2</i>	> .001
<i>$\Delta_{810-2832}$hsl7 a2</i>	406	24.78 +/- .32	<i>MUM12 a2b2</i> v. <i>$\Delta_{810-2832}$hsl7 a2</i>	N.S.

^A Cell length values are averages, +/- S.E.

^B Statistical analysis was performed using a one way ANOVA with a Dunnett's Multiple Comparison Test and a Student's t-test.

TABLE 16. Growth rate analysis of mutant strains^A.

Strains	Double Time ^B	p^C
<i>WT a1b1</i>	2.76 +/- .18	
<i>MUM11 a1b1</i>	2.74 +/- .19	0.82
<i>WT a2b2</i>	2.77 +/- .20	0.95
<i>MUM12 a2b2</i>	2.87 +/- .11	0.31

^A Table shows growth rate analysis of six identical experiments per strain.

^B Growth rate expressed as doubling time in hours, +/- S.E.

^C Statistical analysis was performed using a one way ANOVA with a Tukey's Multiple Comparison Test.

defects (Fig. 40B, see arrows). As a measure, the number of cross walls (cell walls that split a single cell in two, compared to mother daughter septa that divide two different cells) were counted per cluster (or group) of cells (Table 17). In *MUM11*, nearly 48% cross wall formation was observed while no cross walls were observed in the wild type progenitor strain. Whereas in *MUM12*, only 24% cross wall formation was observed, though the wild type *a2b2* mating-type strain produce 12% cross wall formation. Unlike cell morphology and growth rate analysis, cross wall

formation was only observed in the *MUM* strains, not the $\Delta_{810-2832}hsl7$ strains, and the degree of formation was mating-type specific.

Colony and micro-colony morphology was examined to determine if the production of cross walls would affect colony shape and structure. When grown on YEPS media, *MUM11* and *MUM12* displayed the small protrusions observed in the $\Delta_{810-2832}hsl7$ mutant strains (Fig. 41A, see arrows). Investigation of micro-colony morphology

TABLE 17. Cross wall formation.

Strain	Cross walls ^A	Clusters ^B	Percentage
<i>WT a1b1</i>	0	90	0.0%
<i>MUM11 a1b1</i>	76	159	47.8%
<i>WT a2b2</i>	15	123	12.2%
<i>MUM12 a2b2</i>	58	241	24.0%

^A Cross walls were counted as cell walls that split a single cell in two, different than mother / daughter septa cell division.

^B Clusters consist of either single cells or groups of cells that were connected.

revealed the “directional growth” phenotype previously observed (Fig. 41; Chapter II). colonies (Fig. 42A and B). The increase in filament formation on SLAD raised the question of whether loss of *hsl7* affected filament formation during the mating reaction. During a plate mating assay, compatible haploid cells of opposite mating-type background were mixed and spotted on YPD media containing activated charcoal. A positive mating reaction produces aerial hyphae that appear white on the black background. No defects in the mating response were observed in the *MUM* mutant strain when mixed with a compatible wild type or mutant strain (Fig. 43). All the colony morphology and plate mating assay data support the observations made in Chapter II of this dissertation, that Hsl7 plays a role in regulating the filamentous response to SLAD but not the mating response.

All previous data suggest that the independent Δ *hsl7* mutant strains are affected in cell length and the filamentous response to low ammonium conditions, but not in growth rate or the mating response. However, the production of cross walls was only observed in the *MUM* background as the $\Delta_{810-2832}$ *hsl7* mutant strains did not exhibit cross

Figure 41: *MUM* strains exhibit a directional growth phenotype. Grown on rich media, *MUM* colonies exhibit small protrusions of cells extending away from the colony edge (A, see arrows). Examination of micro-colonies of the *MUM* mutant strains indicated “directional growth” where groups of parallel cells grow in a specific direction different from another group of cells (B). Scale bars, 50 μ m – 20x inset image, 40 μ m – 60x image.

wall formation. Specifically, *MUM11* displayed sensitivity to cell wall antagonists. This was seen when the *MUM* strains were spotted on media containing the cell stressors 50 μ M CFW (chitin antagonist), 15 μ g / mL CR (β -glucan antagonist), or the osmotic stressor 1M NaCl. When spotted on media containing CR or CFW, the *MUM11* strain showed a delay or outright lack of growth, respectively, at 48 hours after inoculation (Fig. 44A). However, at 72 hours the growth of *MUM11* on CR had recovered, but no

Figure 42: Filamentous response to low ammonium conditions of *hsl7* mutants. *MUM* colonies display an increase in the ability to form filaments when grown on SLAD (A). Micro-colonies were utilized to examine filament formation at the cellular level. The *MUM* strains were increased in filament length and branching (B). Scale bars, 50 μ m – 20x inset image, 40 μ m – 60x image.

similar recovery was observed on CFW containing media. When spotted on media containing 1M NaCl, again the *MUM11* strain displayed a decrease in growth after 96 hours (Fig. 44A). However, it may be that the sensitivity to 1M NaCl is due a defect in the cell wall structure as demonstrated by CFW and CR sensitivity.

To further explore the lethality of CFW, cells were grown overnight in liquid YEPS media then diluted into fresh YEPS media containing 50 μ M CFW and viewed microscopically after 4 hours. Both wild type strains and the *MUM12* strain were able to

Figure 43: Plate mating assays for the *MUM* strains. Equal mixtures of haploid strains of opposite mating-type background were plated onto YPD plates containing activated charcoal. A positive mating reaction produced a white “fuz” phenotype of aerial hyphae production. See text for description of results.

grow in the stressor media; conversely, the *MUM11* strain was unable to grow.

Examination of cell morphology indicated that the wild type and *MUM12* strains produced cells that were large and highly branched (Fig. 44B). Though CFW stress prevents growth of *MUM11* cells, several were able to be viewed and their morphology appeared to be a group of short, thickened undivided cells with failed buds at the ends of the cell groups (Fig. 44B).

It is possible that CFW is weakening the cell wall of the *MUM11* strain making it extremely susceptible to internal osmotic stresses that wild type cells are easily able to handle. To explore this idea the *MUM* strains were spotted onto CFW stressing media that contained a 1 M concentration of the osmotic stabilizer sorbitol. Sorbitol did partially rescue the growth of the *MUM11* strain (Fig. 45A). The *MUM11* was able to grown in CFW stressor media containing 1M sorbitol and the cell morphology was similar to that observed in the wild type strains, as well as in *MUM12* (Fig 45B). Oddly, this specific media causes bi-polar growth of cells, where cells lose their single polar bud site selection and buds grow from both ends of the cell simultaneously.

Figure 44: Cell wall stressors. Serial dilutions of the *MUM* strains were spotted onto media containing 50 μM calcofluor white (CFW; chitin antagonist), 15 μg / mL congo red (CR; β-glucan antagonist), or the osmotic stressor 1M NaCl. Observations were made at 48hr and 72hr for CFW and CR media, while observations were made at 96hr for 1M NaCl. Growth defects were only observed in *MUM11* both CFW and CR media at 48hr, though growth had rescued by 72hr on CR. At 96hr *MUM11* was sensitive to 1M NaCl (A). Cell morphology was observed when the *MUM* strains were grown in liquid media containing CFW. Again only the *MUM11* strain displayed a different cell morphology from wild type strains and *MUM12*, and fail to grow in liquid CFW media (B). Scale bars, 10 μm.

The sensitivity to CFW of the *MUM11* strain raised the question of how this strain differs from the other two $\Delta hsl7$ mutant strains previously described (Chapter II). As previously described, the $\Delta hsl7$ mutant strains contain a complete deletion of the *hsl7* ORF, while the $\Delta_{810-2832}hsl7$ mutant strains were lacking the nucleotides from 810 to the end of the ORF. In construction of the $\Delta_{810-2832}hsl7$ construct, the first 828 nucleotides that could potentially encode a 275 amino acid truncated protein were left intact.

Figure 45: Rescue of CFW sensitivity by the osmotic stabilizer sorbitol. Serial dilutions of the *MUM* strains were spotted onto media containing 50 μ M CFW and the osmotic stabilizer sorbitol partially rescued the growth defect observed in the *MUM11* strain (A). Cell morphology of cells grown in liquid media containing CFW and 1M sorbitol indicated that though all cell morphology was affected in all strains, the *MUM11* strain grew and was not differently affected in cell morphology compared to wild type strains and *MUM12* (B). Scale bars, 10 μ m.

Exploration of the deletion construct from the *MUM11* strain indicated that *MUM11* is a partial disruption of the *hsl7* ORF. The first 480 nucleotides of the *hsl7* ORF were intact and potentially encode a 159 amino acid truncated protein. It is possible that this truncated protein may be expressed and interferes with either chitin synthesis and / or localization. Eight chitin synthases have been identified in *U. maydis* and appear to work in coordination at the septa in yeast-like cells and hyphae (Weber, *et al.*, 2006). Of the eight chitin synthases, Chs5 and Chs7 are most important in cell morphology and

separation. How the *MUM11* strain is sensitive to CFW stress and if it is affecting chitin synthase localization and / or activity are interesting questions that are currently being explored. Expression, as well as potential localization of the truncated protein is currently being examined.

MATERIALS and METHODS

Strains. *Ustilago maydis* strains utilized in this study are listed in Table 18.

TABLE 18. *U. maydis* strains used in this study.

Strain	Genotype	Reference
Fungal Strains^A		
FB1	<i>a1b1</i>	<i>Banuett, et al, 1989</i>
<i>MUM11</i>	<i>a1b1 hsl7::cbx^R</i>	Dr. José Pérez-Martín
FB2	<i>a2b2</i>	<i>Banuett, et al, 1989</i>
<i>MUM12</i>	<i>a2b2 hsl7::cbx^R</i>	Dr. José Pérez-Martín

^AAll mutant strains were made in the wild type background strains listed.

Growth conditions, cell length, statistical analysis, staining, micro-colonies, and microscopy are described previously (Materials and methods, Chapter II).

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To “P” or not to “P”? The action of methylammonium permeases in fungal dimorphism depends on their ability to be phosphorylated.

Presented at the 22nd Fungal Genetics Conference, Asilomar, CA 2003.

C. Ben Lovely, Gregory E. Shaw, and Michael H. Perlin. Department of Biology, Program on Disease Evolution, University of Louisville, Louisville, Kentucky, USA

Mating Type Specific Signaling in *Ustilago maydis*.

Presented at the 25th Fungal Genetics Conference, Asilomar, CA 2009

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Regulation of Morphogenesis in Response to Low Ammonium in *Ustilago maydis*.

Presented at the Mycology Society of America Conference, Lexington, KY 2010

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Mating Type Specific Signaling in *Ustilago maydis*.

Presented at the Mycology Society of America Conference, Lexington, KY 2010

Publications

C. Ben Lovely and Michael H. Perlin. Department of Biology, Program on Disease Evolution, University of Louisville, Louisville, Kentucky, USA (*Manuscript in Preparation*)

The role of Hsl7 in morphology and pathogenicity in the plant pathogen *Ustilago maydis*.

Cau D. Pham, C. Ben Lovely, Zhanyang Yu, David Myers, Trisha Patel, and Michael H. Perlin. Department of Biology, Program on Disease Evolution, University of Louisville, Louisville, Kentucky, USA (*Manuscript in Preparation*)

Haplo-insufficiency in *Ustilago maydis* reduces pathogenicity.