GENETIC INVESTIGATIONS ON THE DIMORPHISM AND PATHOGENICITY OF THE CORN SMUT FUNGUS, *USTILAGO MAYDIS*

by

KAREN ELIZABETH SNYDER

Under the Direction of Scott E. Gold

ABSTRACT

Ustilago maydis is the dimorphic fungal pathogen that causes corn smut disease. The purpose of this research was to identify novel genes important for disease development by analyzing a collection of filamentation suppressor mutants, characterizing the morphologically regulated gene termed *uhf1* (upregulated in hyphal form), and amplifying *cap1* (cyclase associated protein) from *U. maydis* for future functional studies. Using molecular complementation, a total of 29 filamentation suppressor mutants were evaluated for filamentous growth. While a few strains were recalcitrant to standard methodologies, 20 strains were complemented either by *ubc1*, *ubc2*, *ubc3*, *ubc4*, *ubc5*, or *hgl1*. Gene disruption was used to investigate the role of *uhf1* on morphology, mating, and pathogenicity. Studies indicated that *uhf1* is necessary for maintaining a wild-type morphology, pigmentation, and growth rate, but is not essential for mating, gall production, or teliospore development. Polymerase Chain Reaction (PCR) was employed to amplify *cap1* from *U. maydis*.

INDEX WORDS: *Ustilago maydis*, Dimorphism, cAMP, MAP kinase cascade, Uhf1, Cap1, WD repeat proteins, Cpc2, RACK1, snoRNA

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B.A., Hood College, 1997

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2003

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May 2003

DEDICATION

This work is dedicated to my family in God's love.

ACKNOWLEDGMENTS

Foremost I want to acknowledge Dr. David Andrews, Dr. Mariola Garcia-Pedrajas, Lori Bennett, Shelly Miller, John Duick Egan, and Dr. Scott Gold for their significant contributions to the work presented in this thesis. Dr. Andrews was responsible for the identification of *uhf1* as well as the sequencing of this gene and *cap1*. Dr. Garcia-Pedrajas conducted all *uhf1* expression experiments. Lori Bennett complemented some of the *ubc* filamentation suppressor strains. Shelly Miller identified the cosmid clone containing *uhf1*. John Duick Egan provided protocols, expertise, guidance, and support. He also provided the *uac1* digoxigenin probe. I would also like to thank Dr. Gold for allowing me to work in his lab and explore molecular genetics in the United States as well as in Mexico. As my advisor, Dr. Gold was critical to the design of these experiments. He was also responsible for crossing strains 2/27 and 7B1 *in planta* and preparing a few confrontational assays of *uhf1*$^{-}$ mutant strains (data not shown). He also obtained the draft *cap1* sequence from Exelisis Inc.

Next, I want to recognize others who have provided assistance with my project. In addition to the lab members listed above, a special thanks goes to Dr. Alfredo Martinez-Espinoza for answering my questions, providing support, and teaching me about REMI mutagenisis. I want to thank Christopher Topp for showing me how to inoculate corn seedlings with *Ustilago maydis*. I also want to thank my committee members Drs. Sarah Covert and Harald Scherm for their help developing my project. Dr. Scherm also provided assistance using statistical software. Next, I want to thank all the participants of the Momany, Covert, Gold, and Bacon Joint Lab Meeting as well as the

Mycology Discussion Members for their helpful insight on my project. I would also like to thank Dr. Timothy Denny for his help with the growth curve experiments. I am also grateful to Ken Davis, Charles Laster, and Mike Andrews for watering and fertilizing my corn plants.

Finally, I want to thank all the remarkable people who have influenced my life and my decision to attend and finish graduate school. Most importantly I want to thank my parents, my sisters, and my husband, Aaron Sechler, who have helped me through some very difficult times. I also want to thank my fellow graduate students as well as Tara Marsh, Joy Taluyo, Rachael Miskill, Maryann Steele, and Dr. Lisa Castlebury for their support and friendship. Additionally, I want to thank everyone at USDA, ARS, NAA, FDWRSU including Dr. Reid Frederick, Marie Carras, and Pete Kujawski for their love, guidance, and much more. These people along with my professors at Hood College including Dr. Kathy Falkenstein and Dr. Craig Laufer provided me with a strong foundation in scientific research.

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CHAPTER 1

INTRODUCTION

Smut fungi comprise a significant group of plant pathogens with approximately 1,200 known members (Agrios, 1997). Occurring worldwide, these basidiomycetes cause extensive yield and quality damage on many agronomically important crops including wheat, corn, and sugarcane. Effective management practices are necessary to minimize these direct and indirect losses caused by smut fungi. Currently, resistant varieties and fungicides are used for large-scale smut management; however, complete control of these pathogens is difficult. Furthermore, application cost, resistance development, and environmental impact are of major concern with regard to fungicide use. Therefore, a better understanding of pathogenicity is needed to improve management practices.

Ustilago maydis is one plant pathogen that is used to investigate the ability of smut fungi to cause disease. Infecting *Zea maydis* (maize) and *Zea mexicana* (teosinte), *U. maydis* is the causal agent of the economically important disease known as corn smut. Typically, losses due to infection are below 2% because disease resistant varieties are planted (Pataky, 1999). However, the potential for significant loss does exist. According to the 2000 U.S. corn production data from the National Corn Grower's Association, a 2% disease loss would have resulted in approximately $370 million of damage. Aside from its economic significance, *U. maydis* is also studied because of the numerous genetic tools available, the ease with which it can be manipulated, and its alternating growth forms.

Similar to human pathogens such as *Histoplasma capsulatum*, *Ustilago maydis* alternates between budding and filamentous growth. Specific morphologies of these and other pathogenic fungi are closely associated with the ability to cause disease (Orlowski, 1994). In *H. capsulatum*, the budding phase is pathogenic, whereas in *U. maydis* the filamentous from is responsible for disease. Hence, studies on this transition from saprophytic to parasitic growth in *U. maydis* are valuable to identify factors important for pathogenicity.

Although haploid cells of *U. maydis* display filamentous growth in response to environmental factors such as pH (Ruiz-Herrera *et al*., 1995), only the filamentous dikaryon causes disease. This infectious hypha is formed when two compatible haploid cells fuse on the plant surface. After mating, the fungus penetrates the host tissue and grows intracellularly and later intercellularly (Snetselaar and Mims, 1994). Continued colonization of the young plant tissues results in symptoms ranging from a small amount of chlorosis to plant death. The most spectacular symptom observed is the production of galls on any above-ground plant part. Inside these hyperplastic and hypertrophic tissues, teliospores are produced. As the primary source of inoculum for following years, resistant teliospores germinate and undergo meiosis to complete the life cycle when they encounter a suitable environment such as the host surface.

Research on fungal dimorphism and pathogenicity strongly supports the role of adenylate cyclase as an important protein involved in these processes. Adenylate cyclase is responsible for producing the second messenger cyclic adenosine monophosphate (cAMP). In the plant pathogens *U. maydis*, *Ceratocystis ulmi*, and *Magnaporthe grisea* the involvement of cAMP in cellular differentiation has been well documented (Gold *et*

al., 1994; Brunton and Gadd, 1989; Adachi and Hamer, 1998). In *U. maydis*, cAMP regulates morphology through a complex signaling pathway together with a parallel MAP kinase cascade (Gold *et al*., 1994; Mayorga and Gold, 1998; Andrews *et al*., 2000; Mayorga and Gold, 2001). Mutation of the *Ustilago* adenylate cyclase gene, *uac1*, results in a constitutively filamentous phenotype, which is non-pathogenic (Barrett *et al*., 1993; Gold *et al*., 1994). Restoration of the budding morphology is accomplished through the addition of cAMP or by the generation of extragenic suppressors. Using molecular complementation, six different genes, mutations in which suppress filamentation of a *uac1*$^-$ strain, have been identified and are termed *ubc1* (*Ustilago* bypass of cyclase), *ubc2*, *ubc3*, *ubc4*, *ubc5*, (Gold *et al*., 1994; Mayorga and Gold, 1998, 1999; Andrews *et al*., 2000) and *hgl1* (hyphal growth locus) (Dürrenberger *et al.*, 2001). Each of these genes is necessary for dimorphism and wild-type pathogenicity.

The first objective of the research presented in this thesis evaluates additional suppressor mutants previously generated from a *uac1*$^-$ strain in order to determine the likelihood of finding new genes important for dimorphism and pathogenicity. Using methods similar to those used to identify the *ubc* class of genes, protoplasts from a total of 21 mutant strains were transformed with *ubc1*, *ubc2*, *ubc3*, *ubc4*, *ubc5* and *hgl1* to determine which gene, if any, complemented their defect and restored filamentous growth. One strain did not show complementation with any of the known genes and was transformed with the genomic library to identify which clone contained the sequence that restored filamentous growth.

Besides utilizing the filamentous *uac1*$^-$ strain in suppressor mutant screens, *uac1*$^-$ has been useful in a subtractive hybridization approach comparing gene regulation in

filamentous and budding growth. Using this technique, differentially expressed genes have been identified. One gene identified as upregulated in filaments is *uhf1* (upregulated in hyphal form). *uhf1* has been sequenced and used to search GenBank's database for descriptions of possible function (Gold *et al*., 2001). A BLAST search (Altshul *et al*., 1997) indicated that this gene is extremely homologous to the single copy, recessive, non-lethal *cpc2* of *Neurospora crassa* (Müller *et al*., 1995). In fact, there is 76% identity at the protein level.

Cpc2 in *N. crassa* is a Gβ-like or WD repeat protein with a role in amino acid utilization (Krüger *et al*., 1990; Müller *et al*., 1995). Present in all eukaryotes, WD proteins are composed of a highly conserved and repetitious amino acid sequence: variable region-GH-variable region-WD (Neer *et al.*, 1994). With conserved WD repeated motifs, these proteins, including Cpc2, may form a stable β-propeller structure or platform for protein-protein interactions (Smith *et al.*, 1999). Despite an expected similar structure, WD proteins are functionally diverse with roles in signal transduction, cytoskeleton assembly, RNA processing, etc. (Smith *et al.*, 1999). Unfortunately, specific protein-protein associations of Cpc2 and all but one of its homologs, RACK1, remain unknown. RACK1 is a protein found in *Rattus norvegicus* (rat) and is referred to as RACK for receptor of activated C kinase (Ron *et al*., 1994). In mammalian systems, this protein binds activated protein kinase C (PKC) (Ron *et al*., 1994), interacts with cAMP phosphodiesterase (Yarwood *et al*., 1999; Steele *et al*., 2001), and regulates Src tyrosine kinases (Chang *et al.*, 1998).

In terms of function in *N. crassa*, *cpc2* interacts with or influences *cpc1*. Cpc1 is a transcriptional activator that binds to promoter consensus regions and regulates

transcription of numerous genes encoding amino acid biosynthetic enzymes (Paluh *et al*., 1988). Although the relationship between these gene products is unclear, studies using amino acid analogs to induce starvation indicate that *cpc2* acts as a weak repressor of *cpc1* transcription (Müller *et al*., 1995). Under conditions in which nutrients are sufficient, *cpc2* is expressed at high levels while *cpc1* expression is low. As a result, amino acid biosynthetic enzymes are not produced. During starvation or when a single amino acid becomes limiting, there is a down regulation of *cpc2* and a derepression of *cpc1* to allow for increased biosynthetic enzyme production and ultimately an increase in amino acid concentration (Müller *et al*., 1995). This amino acid regulation is referred to as "cross pathway control" (Carsiotis and Jones, 1974), hence *cpc*, or "general amino acid control" (Delforge *et al*., 1975) and is also present in other organisms. Orthologs of both *cpc1* and *cpc2* have been identified in the ascomycetes *Saccharomyces cerevisiae* and *Aspergillus nidulans* (Hoffman *et al*., 1999; Hoffman *et al*., 2000; Chantrel *et al*., 1998) and are functionally interchangeable among these fungi (Paluh and Yanofsky, 1991; Wanke *et al*., 1997). Complementation of *cpc2* has also been observed across eukaryotic kingdoms. RACK1 of rat complements mutations in *cpc2* and *cpcB* in *S. cerevisiae* and *A. nidulans*, respectively (Hoffman *et al*., 1999; Hoffman *et al*., 2000). This ability, along with extremely high protein sequence similarity, suggests a highly conserved structure as well as function. RACK1 is a known receptor for activated protein kinase C and is thought to form a β-propeller structure similar to Gβ proteins (Smith *et al*., 1999), suggesting that related proteins are involved in kinase signal transduction (Hoffman *et al*., 1999). Perhaps the function of RACK1 has a role similar to the heterotrimeric Gβ protein in the cAMP signaling pathway.

In addition to having a role in amino acid biosynthesis, *cpc2* and its orthologs are also involved in fertility and growth. In *N. crassa*, *cpc2*$^{-}$ strains are unable to form protoperithecia (Müller *et al*., 1995). Similarly, a null mutant in *A. nidulans* is also unable to form sexual structures. Apparently there is a defect in the events prior to or slightly after karyogamy because only microcleistothecia filled with hyphae are formed; ascospores are not produced (Hoffman *et al*., 2000). In contrast, ascospores are formed by a diploid *S. cerevisiae* deletion strain (Chantrel *et al.*, 1998). Tetrad analysis of the spores indicates that the two mutant spores have delayed germination (Chantrel *et al.*, 1998). The growth rate of *S. cerevisiae* and *N. crassa* is also reduced by a mutation in the *cpc2* homologous genes (Hoffman *et al*., 1999; Müller *et al*., 1995). Furthermore, the mutation of the *cpc2* ortholog also results in an increased cell size for *S. cerevisiae* (Hoffman *et al*., 1999). This interesting fungal biology associated with *cpc2* suggests that the homologous *U. maydis* gene, *uhf1*, which exhibits greater expression during filamentous growth, may also be important for spore production and disease.

The second objective of this research investigates the role of *uhf1* in *U. maydis* in terms of morphology, mating, and pathogenicity. The hypothesis was that this gene has a role in dimorphism and pathogenicity. Specifically, it was predicted that cells would have an altered appearance and/or growth rate, a reduced mating ability, and a defect in teliospore production. To test this prediction, a mutation in the wild-type copy of the gene was constructed by inserting the gene conferring carboxin resistance, *cbxR*. Microscopic observations, growth and gene expression experiments, mating assays, and pathogenicity studies were conducted to examine the role of this gene. Overall, these studies suggest that *uhf1* does have a role in morphogenesis and pathogenicity.

Another protein shown to influence dimorphism in other organisms is Cap, cyclase associated protein. Conserved in many eukaryotes, this protein is important for both adenylate cyclase activation and actin organization. The N-terminal portion of Cap is responsible for adenylate cyclase binding, and the C-terminus is important for actin binding (Freeman *et al*, 1996). As previously discussed, in *U. maydis* adenylate cyclase is necessary for the bud-hypha transition, a process that requires a dynamic cytoskeleton. Composed of free actin monomers and polymerized microfilaments, the cytoskeleton responds to signals from growth factors and other stimuli to rearrange the cell for current and future events. In *S. cerevisiae*, Cap1 is responsible for cytoskeleton assembly and mutations of this protein result in an abnormal morphology (Freeman and Field, 2000). Recently, a protein similar to the yeast Cap1 was identified in the human pathogen *Candida albicans* and found to be important for disease development and wild-type germ tube formation (Bahn and Sundstrom, 2001). Since *C. albicans* is a dimorphic fungus that alternates between budding and filamentous morphologies, it is likely that the gene product in *U. maydis* may also be necessary for pathogenicity. The third objective of this research was to clone and begin characterization of the *U. maydis cap1* gene to determine its importance in the cAMP signaling pathway.

The research presented in this thesis investigated genes important for dimorphism and disease development of the corn smut pathogen, *Ustilago maydis*. The first objective was to analyze a collection of second-site suppressor mutants termed *ubc* (*Ustilago* bypass of cyclase). Results indicate that there was a low probability of identifying new genes from this collection since 20 out of 21 strains, which produced protoplasts according to standard methods, were complemented by either *ubc1*, *ubc2*, *ubc3*, *ubc4*, *ubc5* or *hgl1*.

Strain 1/46 was the only transformable strain identified that was not complemented by any of the known genes or those in the genomic library. Further investigations on this strain as well as the five strains that were recalcitrant to standard techniques may identify additional genes involved in dimorphic growth. The second objective was to investigate the role of *uhf1*, a gene identified by subtractive hybridization comparing gene expression during filamentous versus budding growth. Similar to its orthologs in *N. crassa* and *S. cerevisiae*, *uhf1* is necessary for the wild-type morphology and growth rate. Slow-growing disruption mutants are irregularly shaped and produce a dark pigment after a few days in liquid broth. Compatible mutants are able to mate and cause galls *in planta* indicating that this gene is not required for pathogenicity. However, statistical analysis suggests that mutation of *uhf1* negatively impacted fungal virulence. Objective three was to begin characterization of *cap1*, which encodes the cyclase associated protein (CAP). This gene was amplified using PCR and cloned. The gene was partially sequenced.

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CHAPTER 2

SYSTEMATIC ANALYSIS OF *UBC* FILAMENTATION SUPPRESSOR MUTANTS

The dimorphic phytopathogen *Ustilago maydis* alternates between budding and filamentous growth, but only the filamentous form of the fungus causes disease. Previously, a constitutively filamentous *uac1*$^{-}$ strain was obtained and mutagenized to produce suppressor mutations termed *ubc* (*Ustilago* bypass of cyclase). In attempt to identify a gene that restored filamentous growth to these budding mutant strains, each strain was independently transformed with the six known suppressor genes *ubc1*, *ubc2*, *ubc3*, *ubc4*, *ubc5*, or *hgl1*. Complementation with one of the known genes was observed for 20 out of the 29 strains analyzed. Strain 1/46 was not complemented with any of the six known genes and was transformed with a genomic library. A genomic cosmid clone was not identified that restored filamentous growth to this strain. Protoplasts were not obtained from strains 12/51, 12/53, 12/57, 12/70, and 12/75 using standard conditions. Further investigations on these five strains as well as strain 1/46 may identify additional *ubc* genes. However, based on the repeated isolation of mutants affected in each *ubc* gene, it is not likely that additional informative mutants can be generated in a cost effective or time efficient manner.

INTRODUCTION

Ustilago maydis is an agronomically important plant pathogen that infects both *Zea maydis* (maize) and its wild relative *Zea mexicana* (teosinte). In maize, this fungal

pathogen reduces grain quality and quantity by colonizing tissues and altering the morphology of the plant. Symptoms of an infected plant include chlorosis, galls, and plant death. Galls, which can be found on any above-ground plant part, are the result of increased plant cell division and cell enlargement. Inside these irregular and thickened tissues the pathogen completes its life cycle by producing hundreds of diploid teliospores (Christensen, 1963). Once these spores germinate, haploid basidiospores are produced. These cells are saprophytic and may undergo asexual reproduction by budding. Fusion of compatible haploid cells having differences at both the *a* and *b* mating type loci results in a filamentous dikaryon. This form of the fungus is pathogenic and penetrates host tissue to cause disease.

Similar to *U. maydis*, *Candida albicans*, a major fungal pathogen of humans, also displays dimorphic growth. In response to changes in pH, temperature, and other environmental factors, *C. albicans* switches from budding to filamentous growth. While both forms of the fungus are present in host tissue, the impact of each morphological form is unknown (Bahn and Sundstrom, 2001). However, there is reduced virulence when filamentous growth is not observed (Bahn and Sundstrom, 2001; Ghannoum *et al*., 1995, Lo *et al*., 1997; Sobel *et al*., 1984). Genetic investigations on *U. maydis* and *C. albicans* as well as other dimorphic fungi have revealed a tight correlation between dimorphism and pathogenicity (Gold *et al*., 2001; Sánchez-Martínez and Pérez-Martín, 2001). Interestingly, many of the genes important for both of these processes have been found to be members of signaling pathways.

In *U. maydis* the pheromone response pathway influences dimorphism and pathogenicity. Initiated by the interaction of a pheromone encoded by *mfa* and a receptor

encoded by *pra*, the pheromone response pathway shows similarity to the pathway observed in *Saccharomyces cerevisiae* (Banuett, 1998). Once the pheromone is bound to the receptor, the signal is thought to be transmitted by a MAP kinase cascade. Genes that encode the MAP kinase cascade have been determined in *U. maydis* and are *ubc4* (*kpp4*) (Andrews *et al*., 2000; Müller *et al*., 2001, abstract), *ubc5* (*fuz7*)(Andrews *et al.*, 2000; Banuett and Herskowitz, 1994), and *ubc3* (*kpp2*) (Mayorga and Gold, 1998; Müller *et al*., 1999). The putative adaptor protein encoded by *ubc2* may also function in this pathway because it shows a genetic interaction with *ubc4* (Mayorga and Gold, 1998). Additionally, *ubc2* mutants are unresponsive to mating pheromone (Mayorga and Gold, 2001). As shown in pathogenicity studies, each of these genes is necessary for wild-type virulence. Deletions in *ubc4*, *ubc5*, or *ubc2* eliminate gall production (Muller *et al.*, 2001, abstract; Mayorga and Gold, 2001), whereas a deletion of *ubc3* results in reduced disease levels (Mayorga and Gold, 1999).

The cAMP signaling pathway is also essential for filamentous growth and disease development (Sánchez-Martínez and Pérez-Martín, 2001, review). Produced from the conversion of ATP by adenylate cyclase, cAMP is a second messenger that has been shown to be important for a variety of cellular processes. In the plant pathogens *U. maydis*, *Ceratocystis ulmi*, and *Magnaporthe grisea* the involvement of cAMP in cellular differentiation has been well-documented (Gold *et al*., 1994; Brunton and Gadd, 1989; Adachi and Hamer, 1998). For example, in the rice blast fungus, *M. grisea*, cAMP is important for both growth and virulence (Choi and Dean, 1997). Besides reducing vegetative growth, deletion of adenylate cyclase also prevents appressorium formation. Hence, penetration into the host does not occur because of the loss of the infection

structure morphogenesis. Similarly in *U. maydis*, cAMP is necessary for infection (Barrett *et al*., 1993; Gold *et al.*, 1994). cAMP binds to the regulatory subunit of PKA, Ubc1, and releases the catalytic subunit, Adr1. It is this catalytic subunit that phosphorylates target proteins responsible for budding growth. One of these putative targets is Hgl1, a likely regulatory protein (Dürrenberger *et al.,* 2001). Without the production of the second messenger, PKA is inactive and the fungus grows as filamentous cells, which are non-pathogenic even in compatible mutant crosses, indicating that active PKA is essential for disease (Barrett *et al*., 1993; Gold *et al*., 1994). Similarly, without Adr1 filamentous growth is observed (Dürrenberger *et al.,* 2001).

Many of the genes identified in the pheromone and cAMP pathways have been found by studying second mutations in the filamentous *uac1*$^{-}$ or *adr1*$^{-}$ strains that cause a budding phenotype. Briefly, the process by which these filamentation suppressor strains are analyzed consists of generating protoplasts from budding cells and transforming them with a genomic library. Then, genes that restore filamentous growth are determined. The goal of this research was to analyze additional *ubc* filamentation suppressor mutants in attempt to discover other genes in the complex signaling pathway that would also be important for disease development.

METHODS AND MATERIALS

Strains and culture conditions

The *ubc* filamentation suppressor mutants used in this study were previously derived from the constitutively filamentous *uac1*$^{-}$ strain as spontaneous or UV induced mutants (Mayorga and Gold, 1998). Many of these mutant strains are listed in Table 2.1. Other

strains examined in this study that were not transformed are 12/49, 12/51, 12/53, 12/57, 12/70, and 12/75. Each strain was maintained on potato dextrose agar (PDA, Difco) supplemented to 2% agar and grown at 30°C. *U. maydis* transformants were grown on double complete medium with 1M sorbitol (DCM-S) and amended with hygromycin B (300 μg/ml; Calbiochem) (Barrett *et al*., 1993). Complemented transformants were maintained on PDA containing hygromycin B (150 μg/ml). Both potato dextrose broth (PDB, Difco) and YEPS (1% yeast extract, 2% peptone, 2% sucrose) were used for liquid cultures. For protoplast production, broth cultures were started in 5 ml PDB. Then, 100 μl, 250 μl, or 500 μl of the overnight culture was used to inoculate 100 ml of YEPS. Additional inoculum (up to 1.5 ml) was required for strains 12/53 and 12/57. All broth cultures were grown at 30°C with shaking at 250 rpm. Most YEPS cultures were grown between 16 and 23 h until the $O.D._{600}$ was approximately 0.8.

Protoplast formation and transformation

Protoplasts of most mutant strains were generated according to Tsukuda *et al*. (1988) using 20 mg lysing enzyme (Sigma) suspended in 200 μl lysing enzyme buffer (25 mM Tris-HCl pH 7.5, 100 mM $CaCl_2$, 1 M sorbitol). Protoplasts were stored in a final concentration of 7% DMSO at –80ºC, and remained competent for several months.

While most mutants formed protoplasts readily, some strains did not; these were 12/51, 12/53, 12/57, 12/70, and 12/75. Few protoplasts were obtained from the strains after the standard 10 min incubation period with lysing enzyme. Strains 12/51, 12/53, and 12/57 required at least 25 min to produce a few protoplasts. Glucanex (Novo Nordisk Ferment Ltd.), was used as a second lysing enzyme to attempt to increase protoplast yields. Studies on strain 12/57 began using 200 μl of a 10% Glucanex solution in the

lysing enzyme buffer described above and slowly increasing the volume of the enzyme solution. Glucanex was also tested at 5%, 10%, 15%, and 20% in OM Buffer (1.2 M $MgSO_4$, 10 mM Na_2HPO_4) (Garcia-Pedrajas, pers comm.). Similar methods as in Tsukuda *et al*. (1988) were followed for protoplast formation, except OM buffer was used instead of SCS. Cells were incubated with the enzyme solution for at least 20 min.

Besides testing the effect of a second enzyme on protoplast formation, the effect of media was also tested on small samples to determine if the osmotic sensitivity of the 12/70 and 12/75 cells could be altered to increase protoplast yield. Prior to protoplast production, strains were grown in half and whole-strength YEPS with the addition of 0 M, 0.25 M, 0.5 M, and 1 M sorbitol. Then, using 5 ml of culture and proportional amounts of buffer and lysing enzymes, protoplasts were generated following Tsukuda *et al*. (1988). The effect of salt on the morphology of the cells was also tested on strain 12/75. $MgCl_2$ and NaCl were added to YEPS at 0 M, 0.25 M, 0.5 M, and 1 M. Either one colony from a plate or 10 µl of an overnight culture was used to inoculate 5 ml broth cultures in these experiments.

Transformations were prepared on ice as described by Tsukuda *et al.* (1988), with some modification. First, 500 µl STC was added after a 15 min incubation with a solution of 40% (wt/vol) polyethylene glycol 3350 (Sigma) in STC. Second, cells were spun at 2500 rpm to pellet the cells and remove the supernatant. Finally, cells were resuspended in 200 µl STC and spread directly onto prepared DCM-S plates containing hygromycin B. Growth was observed after 3 days at 30°C.

Uncharacterized mutants were transformed independently with autonomously replicating plasmids conferring hygromycin resistance as a selectable marker and either

ubc1, *ubc2*, *ubc3*, *ubc4*, *ubc5*, or *hgl1* (Mayorga and Gold, 1998; Dürrenberger *et al*., 2001). As a positive control for transformation rate and negative control for complementation, an additional transformation was carried out using the empty vector plasmid phyg101, which confers hygromycin resistance (Mayorga and Gold, 1998). Mock transformations were also set up without DNA to control for background growth. Transformants were observed initially after 3 days of growth at 30°C for complementation as indicated by the production of white filamentous hyphae.

Since complementation of strain 1/46 was not observed with any of the known genes, this strain was also transformed with a *U. maydis* genomic cosmid library. Representing an approximate 12-fold coverage of the genome, this library was previously constructed with about 40 kb inserts in the cosmid vector pJW42 containing ampicillin resistance and the *U. maydis* derived autonomously replicating sequence (ARS) (Barrett *et al*., 1993). The library was organized into a series of pooled fractions each having approximately 1000 clones.

One transformant of strain 1/46 appeared to have partial complementation. In attempt to isolate the transforming cosmid that allowed this phenotype, genomic DNA was extracted from the transformant and used to electroporate *Escherichia coli* DH10B cells (Gibco BRL, Gaithersburg, MD). Ampicillin resistant colonies were obtained on LB (Luria Bertani) plates containing ampicillin (100 μg/ml). Plasmid DNA of this clone was used to re-transform strain 1/46.

DNA extractions and manipulations

Approximately 1 μg of plasmid DNA was used in each transformation reaction. This DNA was obtained using alkaline lysis followed by a series of purification steps using

CsCl or polyethylene glycol (Sambrook *et al*., 1989). Phenol:Cloroform extractions were omitted. The concentration of the DNA was estimated using gel electrophoresis and *Hind* III digested λ DNA as a loading standard for comparison.

Genomic DNA extractions from *U. maydis* were accomplished following Elder *et al.* (1983) with three modifications. First, cells were vortexed with glass beads, lysis buffer (0.5 M NaCl, 0.01 M EDTA pH 8.0, 0.2 M Tris pH 7.5, 1% SDS), and phenol:chloroform:isoamyl alcohol (PCI) (25:24:1) for 4.5 min. Second, two additional PCI extractions were performed. Finally, cold 95% EtOH was used to precipitate the DNA.

RESULTS

In this study, analysis of 29 *ubc* filamentation suppressor mutants was attempted in order to determine the probability of finding new genes and possibly discovering a new gene important for both dimorphism and pathogenicity. Once efficient protoplasts were obtained from a mutant strain, transformations with known genes were conducted to identify genes that would restore filamentous growth. Twenty of the strains were complemented with one of the six known genes (Table 2.1). The most common gene required for complementation was *ubc3*; this gene complemented eight strains. The genes that least frequently restored filamentous growth were *ubc2* and *hgl1*; these genes only complemented one strain each. *ubc1*, *ubc4*, and *ubc5* complemented three, four, and three strains each, respectively. In general, all of these complemented strains had very similar multiple budding phenotypes prior to complementation. They also easily produced protoplasts. Protoplasts of strains 12/4, 12/35, 12/49, and 12/59 were not

produced since a reversion to filamentous growth was observed when these strains were removed from storage.

Numerous protoplasts were obtained from the spontaneous budding mutant, strain 1/46. Unlike the 20 UV generated strains that were complemented by a known gene, strain 1/46 was not complemented by *ubc1*, *ubc2*, *ubc3*, *ubc4*, *ubc5*, or *hgl1* (Table 2.1). Additional transformations of strain 1/46 with the genomic library pools were therefore conducted. Most transformants were obtained using pools 1, 4, and 9 (Table 2.2). The least number of transformants were obtained using pool 2. All of these transformants had a budding growth phenotype. Transformations with pool 5 identified one transformant that displayed some filamentous growth on both DCM-S and PDA plates. To characterize the plasmid that appeared to partially complement strain 1/46, total genomic DNA was isolated and then used to transform bacterial cells. The isolated plasmid from this transformant was not of the expected 40 kb size and did not confer filamentous growth in subsequent transformations.

Protoplasts of strains 12/51, 12/53, 12/57, 12/70, and 12/75 were not easily obtained. Each of these strains had an intersting phenotype (Figure 2.1) that was unlike the multiple budding phenotype of the other strains examined in this study or in previously described *ubc* mutant strains (Gold *et al*., 1994; Mayorga and Gold, 1998; Andrews *et al*., 2000). While some single cells were observed for each of the strains, many of the cells seemed to form pseudohyphal strands with multiple branching buds. Some of the cells of strains 12/53, 12/70, and 12/75 were curled at the ends. Growth of strains 12/51, 12/53, and 12/57 also seemed to be slow compared to other strains in this study.

In attempt to maximize protoplast formation from these strains two experiments were designed to test the effectiveness of Glucanex and the addition of sorbitol to the growth medium. Overall, the use of Glucanex did not enhance the protoplast yield on the strain on which it was tested, strain 12/57. In the second experiment, protoplasts of strains 12/70 and 12/75 were obtained by growing the cells in 5 ml YEPS containing 0.25 M sorbitol overnight. Higher concentrations of sorbitol appeared to reduce the growth of the mutant strains. Transformations with the phyg101 control plasmid were conducted on those strains in which some protoplasts were obtained. The few transformants of strains 12/51, 12/57, 12/75 were frosty white and could not easily be distinguished from complemented filamentous strains. Transformants were not obtained from strain 12/70.

The effect of adding either $MgCl_2$ or NaCl to the media was also tested to see if additional salt would cause morphological changes that would allow increased protoplast production. Treatments did not significantly alter the morphology of the strains because each culture contained a variety of cell shapes. Broth cultures with higher concentrations of salt did not seem to grow as well as cultures without the addition.

DISCUSSION

Identification of genes important for the dimorphic growth transition of a variety of pathogenic fungi has also identified genes necessary for virulence (Orlowski, 1994). In the host-pathogen relationship of maize and *U. maydis*, several genes have been discovered that are necessary for both dimorphism and pathogenicity (Gold *et al*., 2001). The key to this research has been the identification of constitutively filamentous strains with either a mutation in adenylate cyclase (Barrett *et al*., 1993; Gold *et al*., 1994) or the

catalytic subunit of PKA (Dürrenberger *et al*., 1998). Taking advantage of this filamentous phenotype, six genes that suppress filamentous growth were discovered and characterized. The six identified genes that suppress filamentous growth are termed *ubc1* (*Ustilago* bypass of cyclase), *ubc2*, *ubc3*, *ubc4*, *ubc5*, (Gold *et al*., 1994; Mayorga and Gold, 1998, 1999; Andrews *et al*., 2000) and *hgl1* (hyphal growth locus) (Dürrenberger *et al.*, 2001). Each of these genes is necessary for dimorphism and wild-type pathogenicity. The purpose of this research was to attempt to identify additional genes through the systematic analysis of second-site suppressor mutant strains. Using molecular complementation, the *ubc* filamentation suppressor mutants were tested for complementation with either *ubc1*, *ubc2*, *ubc3*, *ubc4*, *ubc5*, or *hgl1* and in one case a genomic library. By screening each mutant for complementation with known genes, the likelihood of finding new genes in this assay was determined.

The collection of *ubc* filamentation suppressor mutants was previously generated from a *uac1*$^{-}$ strain via spontaneous mutations or UV mutagenesis. Creation of extragenic mutations in these strains resulted in a budding phenotype that was not like that of a wild-type strain. Many of these double mutants display a multiple budding phenotype. Apparently, the second mutations in these strains bypass the need for cAMP, but do not restore the normally regulated pathway necessary for wild-type cellular differentiation.

In wild-type cells, cAMP is required for the activation of the protein kinase PKA. Binding of this second messenger to the regulatory subunit of PKA allows the catalytic subunit to be released from an inhibitory complex with the regulatory subunit. The free catalytic subunit is then able to phosphorylate serine and thereonine residues on target

proteins. When cAMP is not produced, as in the case of the *uac1*$^{-}$ disruption strain (Martinez *et al*., manuscript in preparation), these substrate proteins are not phosphorylated and therefore have an altered function with the downstream morphological result of filamentous growth.

In this study, mutants were individually transformed with each of the known genes on separate cosmids. The presence of the ARS sequence allows for approximately 25 plasmid copies per cell with a presumed consequence of over-production of encoded proteins. If a strain were lacking a particular gene and respective protein, the addition of the corresponding cosmid would make up for this defect and restore the *uac1*$^{-}$ phenotype. In this study, restoration of the budding phenotype was observed in 20 different strains. Interestingly, almost half of these UV generated mutant strains were complemented by *ubc*3. In a similar study with primarily spontaneous mutants, Mayorga and Gold (1998) reported that *ubc1* complemented the greatest number of mutants suggesting that it was the most common hot spot for spontaneous mutation.

The only strain not complemented that produced numerous protoplasts and was easily transformed was strain 1/46 (this chapter; Mayorga and Gold, 1998). This strain was tested with the six known genes as well as a genomic library. Apparent partial complementation was observed with clones in a few of the genomic pools; however, the filamentous phenotype was not always visible on PDA or through additional culturing on DCM-S. One transformant from cosmid pool 5 displayed some filamentous growth on both PDA and DCM-S plates similar to other complemented strains. However, its filamentous growth was not as intense as observed for the other complemented strains. The isolated plasmid from this transformant did not allow for filamentous growth in

subsequent transformations, indicating that the previous weak filamentous growth of the original transformation was likely an artifact.

Three explanations for the inability to complement strain 1/46 are that the wild-type copy of the mutant gene is not in the tested cosmid library, that the strain has more than 1 mutation, or that a dominant mutation is the cause of the suppressor phenotype. Although there is not a definitive way to determine if the complementing gene is in the library, an increase in the number of transformants would provide more evidence. Specifically, at least 1000 transformants should be obtained from each pool since each one contains approximately 1000 clones. Several transformations were prepared using protoplasts of strain 1/46 and the genomic library, but less than 500 transformants were observed with each pool, despite the fact that over a thousand transformants were observed with the control plasmid. It is also possible that complementation was not observed because strain 1/46 has multiple mutations in different independent genes. While the likelihood of transforming with 1 clone is low, the probability is lower with more than 1 clone. A final explanation for the lack of complementation with strain 1/46 is that the strain contains a dominant mutation. If strain 1/46 has a dominant mutation a complementing gene would never be found in this library because the dominant mutant phenotype would be epistatic to the wild-type phenotype conferred by the normal allele. In order to clone the gene responsible for a dominant mutation, a genomic library of strain 1/46 would be needed to transform a *uac1*$^{-}$ strain and restore budding growth.

In contrast to strain 1/46, strains 12/51, 12/53, 12/57, 12/70, and 12/75 had irregular pseudohyphal/multiple budding phenotypes and were unable to form protoplasts using standard conditions. At least two different possibilities exist for the inability to produce

competent protoplasts. First, the cells may require additional solutes to maintain the internal osmotic pressure and prevent autolysis. This condition has been observed in yeast cells with defects in *slg1* (Jacoby *et al*., 1998). To test this hypothesis with *U. maydis* strains, increasing amounts of sorbitol were added to the media, but protoplast production efficiency appeared to be unaffected. Furthermore, the supplementation of $MgCl_2$ and NaCl did not alter the cell shape. Another reason few protoplasts were produced from these second-site suppressor strains could be that the additional mutation altered the cell wall composition. The increased time required to produce a few protoplasts suggests strengthening of the cell wall by an unknown mechanism. Additionally, the use of up to 20% Glucanex, another lysing enzyme that is used on filamentous fungi, also supports this hypothesis.

In conclusion, analysis of 29 mutants was attempted in this study. While a few strains were difficult to study, most strains were complemented with known genes that encoded members of both the cAMP signaling pathway and MAP kinase cascade. Only one strain that could be studied using conventional methods did not complement with any of the known genes. Additionally, although only one strain each was identified here with mutations in *ubc2* and *hgl1*, previous to this study, three additional alleles of *ubc2* and at least one of *hgl1* had been identified. These results suggest a low probability of finding new genes important for dimorphism using this technique and strain collection.

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Table 2.1. Phenotype and number of transformants from complementation assays[a]

Strain	phyg101	*ubc1*	*ubc2*	*ubc3*	*ubc4*	*ubc5*	*hgl1*
1/46	-[b] (>1000)[c]	- (195)	- (32)	- (349)	- (894)	- (474)	- (58)
12/1	- (16)	- (12)	- (75)	+[d] **(5)**	- (10)	NT[e]	- (3)
12/2	- (894)	- (300)	- (216)	+ **(217)**	- (400)	- (73)	- (132)
12/3	- (309)	- (16)	+ **(52)**	- (28)	- (64)	- (20)	- (31)
12/4[f]	- (>500)	- (>500)	- (>500)	- (>500)	- (>250)	NT	- (>100)
12/8	- (>1000)	- (6)	- (1)	+ **(33)**	- (3)	- (5)	- (2)
12/11	- (>1000)	- (>100)	- (100)	- (48)	- (10)	+ **(>540)**	- (5)
12/12	- (>1000)	- (167)	- (141)	+ **(72)**	- (174)	- (77)	- (307)
12/14	- (913)	- (77)	- (153)	- (92)	- (163)	+ **(268)**	- (177)
12/19	- (77)	- (12)	- (24)	- (26)	- (94)	+ **(61)**	- (48)
12/20	- (202)	- (11)	- (91)	+ **(16)**	- (112)	- (214)	- (80)
12/22	- (50)	- (10)	- (26)	- (26)	+ **(5)**	NT	- (7)
12/25	- (>500)	- (7)	- (75)	- (24)	+ **(24)**	NT	- (5)
12/27	- (50)	- (45)	- (65)	+ **(100)**	- (65)	NT	- (20)
12/32	- (>1000)	- (83)	- (6)	+ **(57)**	NT	- (5)	- (1)
12/35[f]	- (11)	- (2)	NT	NT	NT	NT	NT
12/39	- (21)	- (3)	- (22)	+ **(13)**	- (4)	NT	- (12)
12/43	- (>50)	- (>50)	NT	NT	+ **(17)**	NT	NT
12/48	- (>500)	- (>100)	- (>500)	- (>500)	+ **(>100)**	NT	- (>100)
12/59[f]	- (5)	+ **(17)**	- (3)	- (2)	- (2)	NT	- (2)
12/62	- (422)	+ **(472)**	- (1)	- (2)	- (4)	- (10)	NT
12/65	- (>800)	- (571)	- (410)	- (134)	- (472)	- (121)	+ **(694)**
12/67	- (>500)	+ **(>500)**	NT	NT	NT	NT	NT

[a]Table includes data collected by Lori Bennett.

[b](-) = Complementation was not observed.

[c]Total number of complemented colonies.

[d](+) = Complementation was observed.

[e]NT = Not tested.

[f]Further studies on the previously transformed strains 12/4, 12/35, and 12/59 were not possible since a reversion to filamentous growth was observed when these strains were removed from storage.

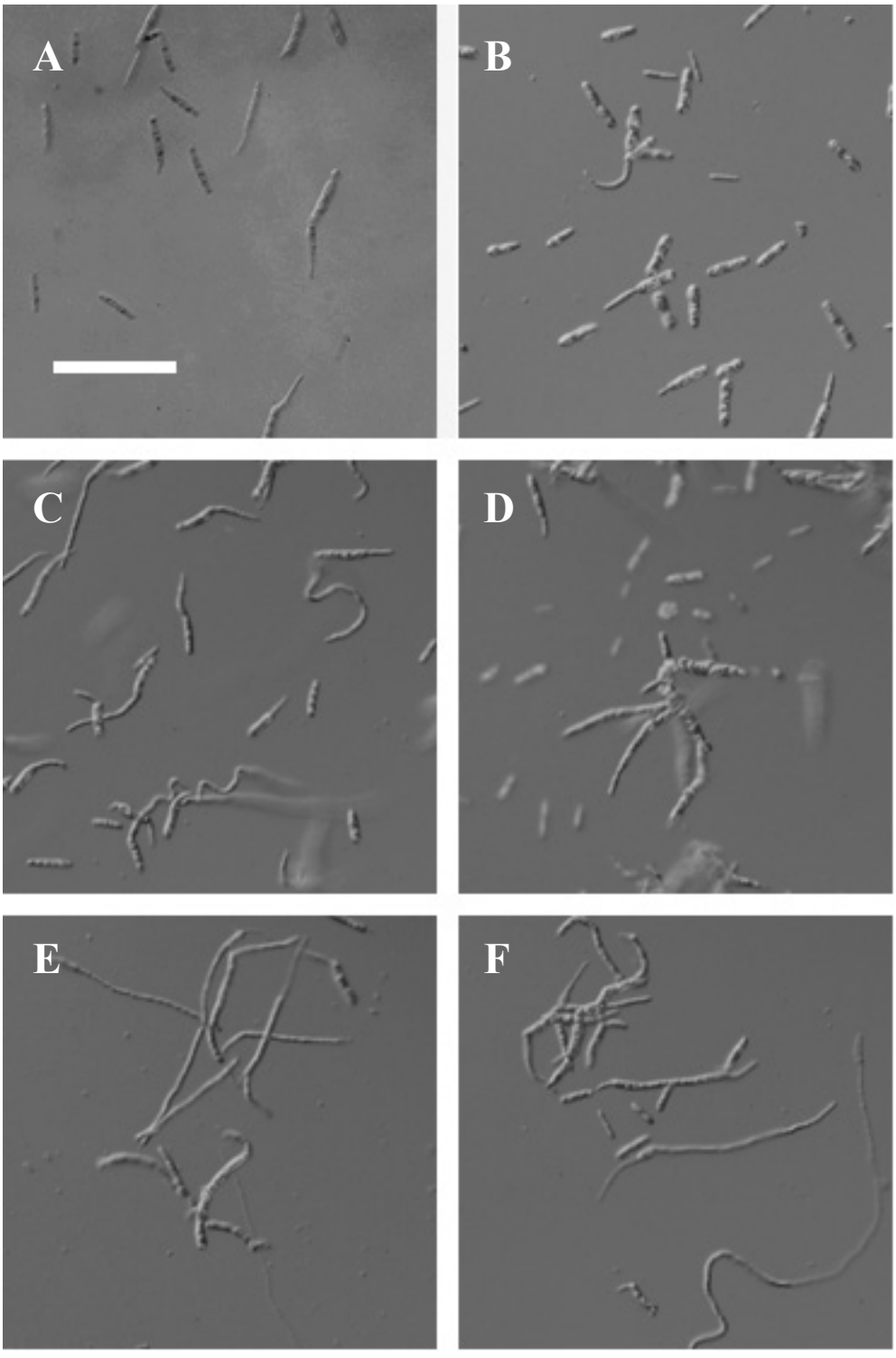
Table 2.2. Phenotype and number of transformants obtained using strain 1/46 and the genomic library pools

	Genomic Library Pools							
Strain	1	2	4	5	6	7	8	9
1/46	-[a] (482)[b]	- (48)	- (413)	- (111)	- (183)	- (138)	- (274)	- (405)

[a](-) = Complementation was not observed.

[b]Total number of transformants obtained.

Figure 2.1. Cellular morphology of the *ubc* mutants recalcitrant to protoplast production. Strains were grown overnight in PDB at 30°C. Panels are: (A) wild-type strain 1/2, (B) 12/51, (C) 12/53, (D) 12/57, (E) 12/70, and (F) 12/75. Photographs were taken using the Digital Camera Fine Pix S1 Pro (Fujifilm) and compiled using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA). Bar shown in A is 50 µm.


A
B
C
D
E
F

CHAPTER 3

CHARACTERIZATION OF *UHF1*, A *CPC2* HOMOLOG UPREGULATED DURING FILAMENTOUS GROWTH

Ustilago maydis, the causal agent of corn smut, is a dimorphic fungus that displays both budding and filamentous morphologies. While budding haploid cells are saprophytic, filamentous dikaryotic cells are pathogenic. The elucidation of genetic factors responsible for this morphological transition has also revealed genes important for disease development. To discover other factors important for dimorphism, subtractive hybridization was previously employed to identify genes upregulated during filamentous growth. One of the genes discovered using this technique was *uhf1* (upregulated in hyphal form). Molecular characterization of this gene has revealed an open reading frame of 1842 bp interrupted by two introns. One intron appears to contain a Box C/D small nucleolar RNA (snoRNA). The protein encoded by *uhf1* is composed of 314 amino acids that form 7 WD repeats. Based on amino acid sequence identities, Uhf1 has been placed within the RACK1 subclass of WD proteins. Insertional disruption of *uhf1* demonstrated that this gene is necessary for maintaining a wild-type morphology, pigmentation, growth rate, and virulence, but was not essential for mating, gall production, or teliospore development. Analysis of over 200 progeny suggests that *uhf1* is linked to the *a* mating type locus.

INTRODUCTION

Dimorphism and pathogenicity are intimately associated in numerous fungi (Orlowski, 1994) including the human pathogens *Candida albicans* and *Histoplasma capsulatum*. Both of these fungi display budding and filamentous morphologies in response to specific environmental conditions. The two forms of *C. albicans* are present in mammalian tissues and are necessary for wild-type virulence. When filamentous growth is not observed, there is a reduction in pathogenicity (Bahn and Sundstrom, 2001; Ghannoum *et al*., 1995; Lo *et al*., 1997; Sobel *et al*., 1984). The budding form of *H. capsulatum* is the only form of the fungus found inside mammalian tissues; filamentous growth has only been observed in soil. Thus, the budding form of this pathogen is responsible for disease. A similar correlation between dimorphism and pathogenicity also exists for several dimorphic phytopathogens. *Ceratocyctis ulmi*, *Taphrina deformans*, and *Ustilago maydis*, are a few examples of plant pathogens that have alternate growth forms (Orlowski, 1994; Mix, 1935).

U. maydis, the causal agent of corn smut, is a basidiomycete that displays both budding and filamentous growth. Once a teliospore germinates and undergoes meiosis, four haploid basidiospores are produced. These cells are saprophytic and exhibit asexual reproduction. The filamentous form of the fungus is observed when two compatible basidiospores or their mitotic progeny interact and fuse. The formation of this infectious cell type is determined by a tetrapolar mating system, which requires differences at both the *a* and *b* loci. The genes at the *a* locus are necessary for cell fusion and encode a pheromone and a receptor. The *b* locus encodes two homeodomain products that

combine in interallelic combinations and are responsible for the formation of the filamentous dikaryon.

The pheromone response pathway also has a role in fungal dimorphism and disease development. Expression of the pheromone and receptor encoded by genes at the *a* locus are controlled by the pheromone response factor, Prf1 (Hartmann *et al*., 1999). When this transcriptional regulator is mutated, production of the pheromone and receptor is severely limited, rendering the mutant sterile and unable to form the filamentous dikaryon (Hartmann *et al*., 1996). Containing several potential phosphorylation sites for MAP kinases (Hartmann *et al*., 1996), Prf1 is thought to function analogously to the pheromone responsive transcriptional regulator in yeast, Ste12 (Song *et al*., 1991; Oehlen and Cross, 1997; Pi *et al*., 1997), and downstream of the pheromone-responsive MAP kinase cascade (Hartmann *et al*., 1999). The three members of the MAP kinase cascade are encoded by *ubc4* (*kpp4*) (Andrews *et al*., 2000; Muller *et al*., 2001, abstract), *ubc5* (*fuz7*) (Andrews *et al.*, 2000; Banuett and Herskowitz, 1994), and *ubc3* (*kpp2*) (Mayorga and Gold, 1998; Müller, *et al*., 1999). Deletions in any of these genes reduce infection (Müller *et al.*, 2001, abstract; Mayorga and Gold, 1999). Additionally, the putative adaptor protein encoded by *ubc2* is also necessary for full virulence of the pathogen (Mayorga and Gold, 2001). *ubc2* is predicted to be in the pheromone response pathway because of its genetic interaction with *ubc4*, its morphogenetic unresponsiveness to mating pheromone, and its phenotype in a $uac1^-$ background, which mimics mutations in *ubc4* as well as the other MAP kinase cascade members (Mayorga and Gold, 2001, 1998).

In contrast to the pheromone response pathway, the activated cAMP signaling pathway represses filamentation. Produced by adenylate cyclase, cAMP binds to the regulatory subunit of PKA, Ubc1, to release its catalytic subunit, Adr1 (Dürrenberger *et al.,* 1998). Then, Adr1 phosphorylates target proteins responsible for budding growth. One of these targets is Hgl1, a putative regulatory protein (Dürrenberger *et al.,* 2001). Without the production of the second messenger molecule, PKA is inactive and the haploid fungus grows as filamentous cells, which are non-pathogenic (Barret *et al*., 1993; Gold *et al*., 1994). Another target of Adr1 seems to be Prf1 because it is activated by a mutation in *ubc1* and has several potential PKA phosphorylation sites (Hartmann *et al*., 1996). To date, Prf1 is the only protein in *U. maydis* that has been identified in which the cAMP and pheromone response pathways converge.

The discovery of constitutively filamentous mutants deficient in either adenylate cyclase, *uac1*, or the catalytic subunit of PKA, *adr1*, has had a significant impact on elucidating members of the cAMP signaling pathway in *U. maydis*. Besides using these filamentous strains in forward genetic studies to identify genes that suppress filamentous growth, they can also be used in subtractive hybridization assays to identify genes upregulated during filamentous growth. Gold *et al*. (2001) reported the isolation of approximately 40 differentially expressed genes that may be important for disease development using Suppression Subtractive Hybridization PCR (Diatchenko *et al*., 1996). The research described in this chapter characterizes one of these morphologically regulated genes, *uhf1* (upregulated in hyphal form).

Orthologous to a variety of eukaryotic genes, *uhf1* is most identical to *cpc2* of *Neurospora crassa* and *cpcB* of *Aspergillus nidulans*. Both of these genes encode

products referred to as WD proteins because they are composed of a repetitious amino acid sequence that typically begins with glycine (G) and histidine (H) and ends with tryptophan (W) and aspartic acid (D) (Smith *et al*., 1999; Neer *et al*., 1994). Despite an expected similar structure, WD proteins are functionally diverse with roles in signal transduction, cytoskeleton assembly, RNA processing, etc. (Neer *et al*., 1994; Smith *et al.*, 1999). In *N. crassa* and *A. nidulans*, mutational analysis of *cpc*2 and *cpcB*, respectively, demonstrates that these genes are essential for fertility because neither ascospores nor sexual structures are produced (Müller *et al*., 1995; Hoffmann *et al*., 1999). Additionally, *N. crassa cpc2* mutants have a reduced growth rate.

The purpose of this research was to determine the role of *uhf1* in *U. maydis* with an emphasis on morphology, mating, and pathogenicity. Using molecular transformation, mutant strains were created by homologous recombination with a disruption construct. Mutant cells were found to have an altered morphological phenotype and a reduced growth rate. *uhf1* was determined to be non-essential for mating, gall production, and teliospore development. Two mutant copies of the gene reduced virulence. Analysis of over 200 progeny suggested that this morphologically regulated gene is linked to the *a* mating type locus.

MATERIALS AND METHODS

Fungal and bacterial strains and culture conditions

U. maydis strains used in this chapter are listed in Table 3.1. Cultures were maintained on potato dextrose agar (PDA, Difco) supplemented to 2% agar and grown at 30°C. Transformants were grown on double complete medium with 1 M sorbitol (DCM-

S) and 4.3 μM carboxin (Vitavax 34, Gustafson) (Barrett *et al*., 1993). Resistant progeny strains were selected on PDA amended with either 4.3 μM or 14.4 μM carboxin. Both potato dextrose broth (PDB, Difco) and YEPS (1% yeast extract, 2% peptone, 2% sucrose) were used for liquid cultures. For protoplast production, broth cultures were started in 5 ml PDB. Then, 50 μl, 100 μl, or 500 μl of an overnight culture was used to inoculate 100 ml of YEPS. All broth cultures were grown at 30°C with shaking at 250 rpm. Most YEPS cultures were grown between 16 and 22 h until the $O.D._{600}$ was approximately 0.8.

Bacterial cultures were grown using liquid and solid LB (Luria Bertani) medium amended with ampicillin (100 μg/ml) at 37°C.

Gene identification

uhf1 was identified using Clonetech's PCR Select cDNA Subtraction kit to identify genes upregulated during filamentous growth. Briefly, cDNA fragments were produced from mRNA isolated from the wild-type budding strain 1/2 and subtracted from cDNA fragments derived from the constitutively filamentous strain 1/9 (Gold *et al*., 1997; Gold *et al*., 2001). Approximately 2 μg of $polyA^+$ RNA isolated from total RNA using the PolyATtract mRNA Isolation System 3 (Promega) was used for the subtraction process. Total RNA was extracted using the method by Stiekema *et al*. (1988). After differentially expressed sequences were obtained, they were cloned into TA cloning vectors (Invitrogen). Next, dot blots were prepared by spotting approximately 250 ng plasmid DNA onto Zeta-Probe membrane (BioRad). These blots were probed with radioactive first-strand cDNAs from budding cells and filamentous cells to identify genes upregulated during filamentous growth. Then, select differential clones were partially

sequenced and used as probes to identify similar fragments and limit redundancy of further analysis. Radioactive probes were made using random hexamers and ^{32}P-dCTP. The radioactive *uhf1* probe used for the dot blots was made using the Prime a Gene Labeling System (Promega) and the initial cloned cDNA element. Probes were added to a hybridization solution composed of 50% formamide, 5X SSC, 50 mM Na_2HPO_4, and 3% SDS according to the Zeta-Probe membrane instruction manual (BioRad). Blots were pre-hybridized for 4 h and hybridized overnight at 42°C. The first set of washes was conducted at 42°C in 40 mM Na_2HPO_4, 1 mM EDTA, and 5% SDS. The second set of washes was performed at 65°C in 40 mM Na_2HPO_4, 1 mM EDTA, and 1% SDS.

Once a partial sequence of the cDNA clone was obtained, PCR primers were developed to amplify the gene from a *U. maydis* genomic cosmid library using sib selection. Representing an approximate 12-fold coverage of the genome, this library was previously constructed with about 40 kb inserts in the cosmid vector pJW42 (Barrett *et al.*, 1993).

DNA extractions

Plasmid DNA was obtained using alkaline lysis followed by a series of purification steps using polyethylene glycol (Sambrook *et al*., 1989). Phenol:cloroform extractions were omitted. DNA for sequencing was obtained using the Qiaprep miniprep kit (Qiagen).

Genomic DNA extractions of *U. maydis* were accomplished following Elder *et al.* (1983) with three modifications. First, cells were vortexed with glass beads, lysis buffer (0.5 M NaCl, 0.01 M EDTA, pH 8, 0.2 M Tris, pH 7.5, 1% SDS), and phenol:chloroform:isoamyl alcohol (PCI) (25:24:1) for 4.5 min. Second, two additional

PCI extractions were performed. Finally, cold 95% EtOH was used to precipitate the DNA. DNA concentrations were estimated using gel electrophoresis and *Hind* III digested λ phage DNA as a loading standard for comparison.

Sequencing

Sequencing was performed on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems) using an ABI Prism Big Dye Terminator Cycle Sequencing Reaction kit (PE Applied Biosystems) according to the manufacturer's directions. The coding region along with approximately 1260 bases prior to the predicted start codon and 500 bases after the tentative stop codon were sequenced. The putative full-length cDNA sequence was also determined by sequencing the cDNA clone obtained using Clonetech's PCR Select cDNA Subtraction kit. The sequenced cDNA fragment included only the transcribed region that corresponds to position 1264-3106 (Figure 3.1) of the genomic sequence.

MacDNASIS Pro v3.5 was used to align sequence contigs. BLAST searches (Altshul *et al*., 1997) were used to identify *uhf1* orthologs and compare protein sequences pairwise (Tatusova and Madden, 1999).

Gene expression

Differential *uhf1* expression was examined in strains 1/2 and 1/9. Cells of each strain were scraped from PDA plates, homogenized, and used to inoculate 5 ml PDB. The next day, 200 ml PDB was inoculated with cells from the overnight 5-ml culture. Seventeen hours later, cells were harvested by centrifugation and immediately frozen in liquid nitrogen for total RNA extraction.

Transcription of *uhf1* was determined for strain 1/52 (*a1 b1 uac1::ble ubc2-2*) during temperature shift assays. All time point cell samples taken for RNA extraction as described below were harvested by centrifugation, immediately frozen in liquid nitrogen, and stored at -80°C until used. Cultures were initiated in tubes with 5 ml PDB inoculated with a colony of strain 1/52 which had been grown at 30°C on PDA. After overnight growth in these tubes at 30°C and 250 rpm, 4 ml were used to inoculate two flasks containing 250 ml PDB. These flasks were incubated at either 30°C or 18°C for 1 or 2 days, respectively. At the end of this period, a 100 ml sample was removed from each flask and referred to as the time 0 sample. A second 100 ml sample was added to 200 ml of fresh PDB already at the desired shift temperature. Each flask was then incubated at the desired shift temperature to initiate the morphological transition, from either budding to filamentous (shift from 30°C to 18°C) or from filamentous to budding (shift from 18°C to 30°C). In each shift experiment, 100 ml samples were taken every 2 h for the first 24 h. Then, a final 48 h sample was also taken. Immediately following the removal of a sample, 100 ml fresh PDB at the appropriate temperature was added to compensate and encourage continuous growth.

Expression of the *uhf1* transcript was examined for strains 1/2C10 and 1/2C12. To produce tissue for total RNA extraction, 5 ml PDB were inoculated with cells from PDA plates and incubated overnight. Cells from this culture were used to inoculate 100 ml PDB and incubated overnight. Tissue was harvested by centrifugation and immediately frozen in liquid nitrogen.

Total RNA was extracted using 5 ml TRIzol Reagent® (Invitrogen Life Technologies) for approximately 1 g of cells according to the manufacturer's directions.

RNA was precipitated by adding 1.5 ml isopropanol and 1.5 ml salt solution (1.2 M NaCl, 0.8 M sodium citrate) to approximately 3 ml of the RNA sample. Water treated with diethyl pyrocarbonate was used to resuspend the RNA. Approximately 15 μg total RNA was run on formaldehyde gels using MOPS buffer (0.2 M MOPS, pH 7.0, 60 mM NaOAc, 10 mM EDTA). Northern blots were prepared using positively charged nylon membranes (Hybond-XL, Amersham Pharmacia Biotech) and 10X SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7) according to Ausubel *et al*. (2002). After baking the membrane at 80°C for 2 h, blots were pre-hybridized for 4 h in Church buffer (5 mM EDTA, 0.25 M Na_2HPO_4, 1% casein hydrolysate, 7% SDS, 0.17% H_3PO_4) at 68°C. Hybridizations were performed overnight at 68°C in Church buffer containing a radioactive probe.

Similar to the probe used in the dot blots, the radioactive *uhf1* probe was generated from the initial cDNA clone obtained from the subtraction procedure, but using PCR and the M13 forward and reverse primers. The Qiaquick PCR Purification kit (Qiagen) was used to purify the fragment. Then, 50 ng was labeled with ^{32}P-dCTP using the RadPrime DNA Labeling System (Invitrogen Life Technologies). This probe was initially used for hybridization with the mRNA transcripts (data not shown). In order to determine if the entire *uhf1* gene was transcribed, a second radioactive probe was generated that corresponds to position 1795-2294 (Figure 3.1), a location downstream of the carboxin insertion.

As a loading control, membranes were stripped and hybridized with a *cbxR* probe generated using the 2262 bp *Sac* I fragment from pGR3 (Richard *et al*., 1992).

Deletion construct design

Polymerase chain reaction was used to amplify the entire gene using the following forward and reverse primers: UmUhf- ACAGCGTTCTCGTCAGTC and UmUhfHind – GGGAAGCTTTGAGTCTCTATCGACAAG (Figure 3.1). The reverse primer UmUhfHind contains a *Hind* III restriction site. Amplification reactions contained 50 mM KCl, 10 mM Tris pH8.3, 2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 200 μM of each dNTP, 1 μM each primer, 1 unit Taq polymerase (Engelke *et al*., 1990; Pluthero, 1993), and approximately 250 ng cosmid DNA in a final volume of 50 μl. Reactions were performed in an Amplitron II Thermocycler (Barnstead/Thermolyne). Samples were heated at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension of 8 min at 72°C was also included.

The 2586 bp amplicon was cloned into the vector pCR2.1 TOPO (Invitrogen) and subjected to further DNA manipulations to generate the disruption construct. First, the vector was digested with *Hind* III and the resulting *uhf1* insert was placed into the multiple cloning site of pUC18 (Invitrogen) because this vector did not have an *Eco*R V site. Then, this recombinant plasmid, puhfHP.puc, was electroporated into DH10B cells (Gibco BRL, Gaithersburg, MD) (Ausubel *et al*., 2002). Next, this recombinant was digested with *Eco*R V. Meanwhile, the *cbxR* gene, which confers resistance to carboxin, was isolated from pGR3 (Richard *et al*., 1992) using the restriction enzyme *Eco*ICR1. Then, *cbxR* was inserted into the *Eco*R V site of puhfHP.puc with *cbxR* transcription in the opposite orientation to the transcription of *uhf1*. Finally, the resulting recombinant

plasmid called pCDuhf (plasmid containing a carboxin disrupted *uhf1*) was electroporated into DH10B cells (Figure 3.2).

Southern blot analysis of *uhf1* disruption strains

To determine if the *U. maydis* transformants had been produced by homologous disruption of the *uhf1* gene, Southern blot hybridization was employed. At least 2 µg genomic DNA was digested with *Pvu* II, run on an agarose gel, and blotted onto a positively charged nylon membrane (Boehringer Mannheim) using standard methods (Ausubel *et al*., 2002). Blots were pre-hybridized for at least 3 h at 55°C in standard hybridization solution containing 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, and 1% blocking solution (Boehringer Mannheim). Hybridizations were conducted overnight at 55°C in approximately 5 ml of standard hybridization solution containing a digoxigenin labeled DNA probe. The *uhf1* probe, which binds to the region directly after the carboxin insertion (position 1795-2294, Figure 3.1), was prepared using Dig High Prime (Boehringer Mannheim) according to the manufacturer's specifications. Before labeling the 499 bp PCR fragment, it was gel-purified using a Qiaquick Gel Extraction Kit (Qiagen). Membranes were initially washed at low stringency (two room temperature washes using 2X SSC, 0.1% SDS). Then, two high stringency washes were conducted (68°C wash using 0.2X SSC, 0.1% SDS).

Protoplast production and transformation of *U. maydis*

Protoplasts of wild-type strains 1/2 and 2/9 were generated according to Tsukuda *et al*. (1988) using 20 mg lysing enzyme (Sigma) suspended in 200 µl lysing enzyme buffer (25 mM Tris-HCl pH 7.5, 100 mM $CaCl_2$, 1 M sorbitol). Protoplasts were stored at -80°C after resuspension in STC and the addition of DMSO to a final concentration of

7%. Transformations were performed using approximately 2 μg of digested plasmid DNA. Transformations with no DNA and undigested pGR3 (Richard *et al*., 1992) were also performed as negative and positive controls, respectively.

Morphology and growth

The morphology of select mutant strains was analyzed using light microscopy on an Olympus BH-2 microscope. Color changes were determined by visual comparisons between wild-type and mutant broth cultures started at the same time. Growth rates of strains 1/2, 1/2C10, 2D4, 2D6, 3F1, 3F5, 6C6, 7A4, 7B1, 7B1, 7C1, 7D3, and 8C1 were determined by quantifying the cell concentration of 5-ml cultures over time. Two test tubes for each strain were prepared containing 5 ml PDB and approximately $1x10^5$ cells/ml and placed into a shaking incubator, 250 rpm, 30°C. Cells were counted using a hemacytometer at 4-h intervals. The log of the cell concentration was plotted and a regression line determined for each strain during logarithmic growth using Statistical Analysis Software (SAS Institute Inc., Cary, N.C.). The generation time was calculated during logarithmic growth using the following equations: $(\log N - \log N_o)/t = K$ and $t_g = 0.3/K$ where N = the number of cells, N_o = the initial number of cells, t = time, K = the slope of the growth increase curve, and t_g = generation time (Miller, 1974).

Mating and pathogenicity tests:

Mating type test reactions were prepared on complete medium containing 1% charcoal (Holliday, 1974) by co-spotting 5 μl of an overnight broth culture with an equal volume of one of the tester strains: 2/8, 2/9, 2/11, or 2/14 (Table 3.1). Plates were incubated at 30°C for 24 h. Mating was scored according to the production of white filamentous hyphae typical of the dikaryon.

Pathogenicity studies were conducted using Sweet Corn CN214/F Golden Bantam (Territorial Seed Co., lot #548-28) seed. Plants were grown in Pro-mix soil (Premier) with 5 seeds per 4-inch pot. Plants were fertilized with Peter's Peat Lite Special fertilizer (20:10:20) weekly. Plants were maintained in a growth chamber (model E15; Conviron Inman, SC) with 12-h day/night cycles at 30°C. Seven days after planting, 20 plants were inoculated with a mixture of 10^6 cells/ml of each compatible strain using a 26 gauge 3/8" needle. The following cell mixtures were tested: 1/2 x 2/9; 1/2C10 x 2/9; 1/2C12 x 2/9; 1/2 x 7B1; 1/2 x 122A5; 7B1 x 1/2C10; 122A5 x 1/2C12. Cell concentrations were determined using a hemacytometer. Inoculations with sterile water were performed as a negative control. Cell suspensions or water were injected into the culm just above the soil line until fluid flowed from the whorl indicating that the culm chamber had been filled with inoculum.

The severity of infection was determined on a 0 to 5 scale (Gold *et al.*, 1997) at 7, 10, and 14 days post inoculation. These ratings are 0, no symptoms; 1, anthocyanin and/or chlorosis; 2, leaf galls; 3, small stem galls; 4, large stem galls; 5, plant death. Three independent repeats of the pathogenicity experiments were conducted, and these were considered replications (blocks) for statistical analysis. One-way analysis of variance was performed using Statistical Analysis Software (SAS Institute Inc., Cary, N.C.) for a randomized complete block design. Significantly different means were separated using the least significant difference test at $P = 0.05$.

Teliospore isolation and germination

Galled maize tissues were collected at least 3 weeks after inoculation with compatible *U. maydis* strains. Fresh or dried tissues were briefly rinsed with sterile water and ground

in a mortar containing approximately 25 ml 1.5% $CuSO_4$. The spore suspension was filtered through sterile cheese cloth and incubated overnight. Next, spores were centrifuged for 5 min at 4500 rpm. The supernatant was discarded and the spores were resuspended and washed twice with 50 ml sterile water. Finally, teliospores were resuspended in 1 ml sterile water and plated onto PDA containing ampicillin (100 μg/ml) and streptomycin (100 μg/ml). After a 30-h incubation period at 30°C, microcolonies were removed from the plate using a sterile cotton swab and resuspended in 5 ml PDB. Then, serial dilutions were plated onto PDA containing ampicillin and streptomycin. Single colonies were collected for further analysis.

Creation of *uhf1* disruption mutants in a *uac1*$^-$ background

Strains 7B1 and 122A5 were independently crossed with strain 2/27 *in planta* for the production of teliospores. Three weeks after inoculation, teliospores were isolated and germinated to obtain progeny strains that might contain defects in both *uhf1* and *uac1*. Over 130 progeny were collected from each cross and screened on selective media: PDA containing hygromycin B (150 μg/ml), PDA containing carboxin (14.4 μM), and PDA containing phleomycin (30 μg/ml). DNA from 33 putative double mutants was extracted and digested with *Bam* H1. Southern blots were probed with a 2019 bp fragment that specifically binds to *uac1*. The probe was amplified from pFuz60 (Barrett *et al*., 1993) using the following primers: UAC1F2- GGTCAACATGCTTCCTCGC and UAC1R2- TCGAATTGAATGACACATC. The probe was purified with the PCR Clean-up kit (Qiagen) and randomly labeled using Dig High Prime (Boehringer Mannheim) according to the manufacturer's instructions.

RESULTS

uhf1 isolation and expression

The cloned cDNA fragment containing *uhf1* was one of the largest fragments obtained using the subtractive hybridization kit. On average most fragments were about 400 bp, whereas the *uhf1* fragment was about 1200 bp and contained the entire predicted open reading frame of the gene. Dot blots indicate that this fragment was unique out of 210 cDNA clones randomly picked for further analysis.

Total RNA of strains 1/2 and 1/9 was used to examine *uhf1* expression during budding and filamentous growth, respectively. Northern blot analysis shows that while the gene is expressed in both budding and filamentous cells, it is upregulated during filamentous growth (Figure 3.3). The RNA transcript level in strain 1/9 is at least 2.4 times greater than the level observed in strain 1/2 as calculated using a phosphoimager (Garcia-Pedrajas, pers comm).

High expression of *uhf1* is also observed during filamentous growth of the temperature-sensitive strain 1/52. This strain has the same defect in adenylate cyclase as strain 1/9, but also contains a second temperature-sensitive mutation in *ubc2* that bypasses the need for cAMP for budding growth under non-permissive conditions. Thus, at 30°C budding growth is observed, while at the permissive temperature, 18°C, the fungus shifts to filamentous growth typical of strain 1/9. In a temperature shift assay, high *uhf1* expression was initiated within two hours after shifting from 30°C to 18°C (Figure 3.4). In the reverse experiment cells grown at 18°C were shifted to 30°C to promote budding growth. Expression of *uhf1* increased within 2 h after the temperature

shift. This expression gradually decreased with time as budding growth was restored to the haploid strain (Figure 3.4).

***uhf1* contains a putative snoRNA**

Comparison of sequences derived from the genomic cosmid containing *uhf1* and the putative full-length cDNA identified a 1842 bp ORF interrupted by 2 introns (Figure 3.1). Both introns contain the consensus sequences for 5′ and 3′ splice sites as well as the internal site for lariat formation in filamentous fungi (Balance, 1991). The first intron of 522 bp is located in the middle of the initiation start codon. The second intron is 379 bp and appears to contain a Box C/D snoRNA (small nucleolar RNA) similar to U24 in *S. cerevisiae* (Qu *et al*., 1995).

Box C/D snoRNAs are identified by having two short sequences termed Box C and Box D that allow for the formation of a structure involved in the processing and specific 2′-O-methylation of rRNA (Kiss-Laszlo *et al*., 1996). Box C is located at the 5′ end of the sequence and has the consensus of UGAUGU (Qu *et al*., 1995) or UGAUGA (Narayanan, 2001). Downstream of Box C is Box D with the consensus CUGA (Narayanan, 2001). The second intron in *U. maydis* has 100% homology to Box D, but not to Box C. Instead, the sequence found in *U. maydis* as well as in *N. crassa* conforms to the following sequence: Au/gGAUG. Although the entire Box C consensus is present in *A. nidulans* and *S. cerevisiae* (Hoffmann *et al*., 2000; Hoffmann *et al*., 1999), the partial consensus in *U. maydis* and *N. crassa* may still represent Box C because homology exists in the same region as Box C in the snoRNA of *A. nidulans* when the genomic sequences are aligned. Besides containing a putative Box C/D motif, U24 also has some sequence complementary to 28S rRNA. The second intron in *uhf1* reveals two

regions of 11 bp and 10 bp that are identical to regions of the snoRNA found in the rpL7a gene in *Homo sapiens* that are complementary to 28S rRNA (Qu *et al*., 1995). These regions in the *U. maydis uhf1* gene are AATATTTGCTA and CACCAAGATC, respectively (Figure 3.1).

Uhf1 is composed of 7 WD repeats

uhf1 is predicted to encode a protein of 314 amino acids with 7 WD repeats. Using BLAST (Altshul *et al*., 1997), strong identity was observed with a group of eukaryotic proteins completely composed of WD repeats (Figure 3.5). The protein most identical to Uhf1 is Cpc2 of *N. crassa* (Müller *et al*., 1995). These two proteins are 76% identical. The shared amino acid sequence identities between Uhf1 and other members of this protein family are: 74% for CpcB of *A. nidulans* (Hoffmann *et al*., 2000), 71% for RACK1 of *Rattus norvegicus* (rat) (Ron *et al*., 1994), and 53% for Cpc2/Asc1 of *S. cerevisiae* (Hoffmann *et al*., 1999).

Mutagenesis of *uhf1*

Once the disruption construct was obtained, it was used to transform strains 1/2 and 2/9. Strains 1/2C7, 1/2C10, and 1/2C12 were derived from transformed protoplasts of strain 1/2. Unfortunately, a carboxin-resistant transformant from strain 2/9 was not identified using this technique. Therefore, an alternative method was required to obtain an *uhf1* disruption strain in the *a2 b2* mating type. Strains 1/2C10 and 1/2C12 were separately crossed with strain 2/9 and injected into young corn seedlings. Teliospores were germinated and over 200 progeny from each cross were evaluated to identify carboxin-resistant *uhf1* disruption strains. The progeny strains mentioned in this chapter that were derived from the parental strains 1/2C10 and 2/9 were 2D4, 2D6, 3F1, 3F5,

6C6, 7A4, 7B1, 7B1, 7C1, 7D3, and 8C1. Strain 122A5 was obtained from the cross between 1/2C12 and 2/9. Strains 1/2C7, 1/2C10, 1/2C12, 7B1, and 122A5 were tested for an *uhf1* disruption using Southern blot analysis (Figure 3.6). None of these strains contained the wild-type fragment produced by strain 1/2. Strain 1/2C7, the ectopic insertion transformant, produced both the wild-type and mutant bands. Northern blots were used to determine whether or not the gene was transcribed in the *uhf1* mutant strains (Figure 3.3). Hybridizing transcripts were produced by all strains tested regardless of the *uhf1* disruption. However, the *uhf1* transcript observed in the mutant cells was much larger than transcript obtained from wild-type cells and did not hybridize with the carboxin probe (Figure 3.3).

Analysis of progeny

Initially, progeny from crosses 1/2C10 x 2/9 and 1/2C12 x 2/9 were analyzed to find *uhf1* mutant colonies with the *a2 b2* mating type. However, difficulties finding this mating type required the screening of numerous colonies. While screening hundreds of progeny from these crosses as well as the progeny from cross 122A5 x 1/2, unexpected observations were made. Deviation from the expected 1:1 ratio for carboxin-resistant to sensitive strains was observed in crosses 1/2C10 x 2/9, 1/2C12 x 2/9, and 122A5 x 1/2 (Table 3.2). High Chi-square values indicate that there is a low probability that the observed values fit the 1:1 hypothesis. Mating types were also predicted to segregate in a 1:1:1:1 ratio, but another relationship was observed (Table 3.3). Analysis of a total of 258 progeny that mated normally with a single mating type tester suggested a linkage between *uhf1* and the *a* mating type locus. Carboxin-resistant colonies of the crosses between 1/2C10 or 1/2C12 with 2/9 were most often in the *a1* mating type background,

whereas carboxin-sensitive progeny from the 1/2C12 x 2/9 cross were most often in the *a2* mating type background. The mating type of carboxin-sensitive strains from the cross 1/2C10 x 2/9 were not determined. The opposite correlation was found in the reciprocal cross of 122A5 x 1/2 (Table 3.3). Chi-square values indicate that the hypothesis of independent assortment must be rejected, thus *uhf1* and the *a* mating type locus are linked. These data suggest a map distance of approximately 7 m.u. between *uhf1* and the *a* mating type locus.

While screening progeny for mating type identification, 20 strains that did not display filamentous growth on charcoal plates alone had the ability to mate with two mating type partners. All but one of these strains mated with tester strains that had differences at the *a* mating type locus, but were equal at the *b* mating type locus ($a \neq$, $b =$). Only one of these strains apparently heterozygous at the *a* locus was carboxin-sensitive, consistent with a rare recombination between the *uhf1* and *a* mating type loci.

Phenotypic analysis of *uhf1* mutant strains

After extended incubation, *uhf1* mutant strains were observed to produce a dark pigment. To determine if this phenotype was consistent among the progeny, all colonies examined for mating type identification were also observed for production of a dark-colored pigment when grown for an extended period in PDB at 30°C. Typically after 36 h, broth cultures of *uhf1* mutant strains began producing a brown color that intensified with time. The pigment was observed in the broth as well as inside some of the cells (Figure 3.7, 3.8). Mutant strains with the ability to mate with more than one mating type partner did not exhibit pigmentation. After screening hundreds of *uhf1* mutant progeny, a range of pigment intensities was observed from light brown to an almost black color.

When measures were taken to control the number of cells a more consistent brown color was apparent. Two out of 135 carboxin-resistant strains did not produce the brown pigment. Four out of 123 carboxin-sensitive strains darkened after 3 days. During the course of the growth curve experiments, wild-type broth cultures were found to darken when incubated for a prolonged period, but this did not occur until a few days after carboxin-resistant strains had already changed color (Figure 3.7).

In addition to producing an unknown pigment, *uhf1* mutant cells have an altered morphology (Figure 3.8). Many mutant cells appear to have a thickened cell wall and rounded ends. Some mutant cells display a more drastic appearance similar to a short string of small round spheres. The length of the mutant cells also seems to vary. Typically, the wild-type cells observed in this study did not produce these phenotypes. Using standard growth conditions wild-type cells had a fusiform shape and a uniform length. Only when wild-type cells were kept in broth culture for extended incubation periods did the appearance of these cells change: they became highly vacuolated and sometimes appeared to contain swellings.

The *uhf1* disruption had an influence on culture doubling time. In a controlled growth curve experiment of 10 progeny derived from cross 1/2C10 with 2/9, strains containing the *uhf1* disruption had an average generation time of 3.00 h (Table 3.4). The wild-type strain had a generation time of only 1.81 h. As observed in this experiment (data not shown) and in a second growth curve experiment (Figure 3.9), *uhf1* disruption mutants were also found to reach lower final cell concentrations as compared to wild-type strains. Consistently, *uhf* mutant strains had approximately 50% fewer cells than

wild-type strains at the stationary growth phase. Clearly, strains wild-type for *uhf1* had similar growth patterns that differed greatly from *uhf1* mutant strains.

Mating and pathogenicity of *uhf1* mutant strains

Strains with the *uhf1* insertion mutation were able to mate and cause disease *in planta*. The mating reaction between two compatible *uhf1* mutant strains (1/2C10 with 7B1 and 1/2C12 with 122A5) produced a strong filamentous reaction that was similar to the reaction observed between compatible wild-type strains (Figure 3.10). Pathogenicity studies indicate that wild-type *uhf1* is not absolutely required for disease, the development of galls, or the production of teliospores (Table 3.5, Figure 3.11). However, differences in disease intensity were observed among the 8 crosses performed on young corn seedlings. ANOVA indicates that crosses 1/2C10 x 7B1 and 1/2C12 x 122A5 were significantly different from crosses with at least one wild-type parental strain at a confidence level of $P = 0.05$. These two crosses were also significantly different from each other at $P = 0.05$.

Strains with mutations in both *uhf1* and *uac1* were not identified in the screening of over 100 progeny collected from crosses 2/27 x 7B1 and 2/27 x 122A5. The colony morphology of those progeny obtained in this experiment varied and it was unclear if many of the colonies were homogeneous since after several streaks onto PDA these same filamentous, budding, or intermediate frosty phenotypes were still observed. Selection using hygromycin or carboxin was effective. Phleomycin selection was not effective for identifying strains carrying the *uac1::ble* mutation. If the incubation time was limited to 1 day, very little growth of any of the colonies was observed and upon extended incubation even wild-type strains grew well on medium containing phleomycin at the

concentration used. Therefore, Southern blot analysis was relied on for the identification of a double mutant. The analyzed strains were observed as having both the wild-type and mutant copy of *uac1* or the wild-type copy of the gene alone (data not shown).

DISCUSSION

While *U. maydis* displays both budding and filamentous morphologies during its life cycle, only the budding stage of the fungus can be maintained in culture since wild-type filamentous growth requires host tissues. Thus, the obligate nature of this fungus has hindered the identification of genes necessary for filamentous growth and subsequent disease development. The isolation of constitutively filamentous and non-pathogenic haploid strains with defects in either adenylate cyclase, *uac1*, or the catalytic subunit of PKA, *adr1*, have had a profound impact on the elucidation of key genes responsible for filamentous growth and disease (Barrett *et al*., 1993; Gold *et al*., 1994; Dürrenberger *et al*., 1998). Using forward genetic approaches to identify genes that restore filamentous growth to second-site suppressors of *uac1*$^-$ or *adr1*$^-$ strains, several members of the cAMP and MAP kinase pathways were identified (Gold *et al*., 1994; Mayorga and Gold, 1998, 1999, 2001; Andrews *et al*., 2000; Dürrenberger *et al*., 2001). Subtractive hybridization has also been a useful tool to detect numerous differentially expressed genes from filamentous cells of an *uac1*$^-$ strain in comparison to wild-type budding cells (Gold *et al*., 2001). The research reported here focuses on *uhf1*, a gene upregulated during filamentous growth. Disruption of this gene indicates that it is not essential for growth, but is required to maintain a wild-type cellular morphology and growth rate. Despite growth defects, two *uhf1* mutant strains are able to mate and produce galls *in planta*, but

with reduced virulence. Analysis of over 200 progeny also suggest that this gene is linked to the *a* mating type locus with an estimated map distance of 7 m.u.

Sequence analysis of *uhf1* revealed a high degree of identity to a class of eukaryotic genes that encode proteins known as WD repeats. These proteins are referred to as WD proteins because they are composed of a repetitious amino acid sequence that typically begins with glycine (G) and histidine (H) and ends with tryptophan (W) and aspartic acid (D) (Smith *et al*., 1999; Neer *et al*., 1994). Differences among these proteins include the amino acid composition within the WD repeat, the number of WD repeats, and the presence or absence of amino or carboxy terminal extensions. It is this variability that probably provides functional specialization (Neer *et al.*, 1994). From regulating cell division to transmembrane signaling, proteins with WD repeats are involved in a plethora of cellular functions (Neer *et al*., 1994; Smith *et al*., 1999). Probably the most well-studied WD protein is the Gβ subunit of the heterotrimeric G protein. Unlike many of the WD proteins, both the function and structure of this subunit are known. As an important signaling molecule that interacts with Gγ, adenylate cyclase, phospholipase C, etc. (Birnbaumer, 1992), the Gβ subunit forms a β-propeller fold with three potential surfaces for protein interactions (Smith *et al*., 1999).

Similar to Gβ, Uhf1 also has 7 WD repeats and may form a similar scaffold for protein interactions, but it is most identical to proteins contained within the RACK1 subfamily of WD repeats because it lacks an amino terminal extension; Gβ has an amino extension. RACK1 has been identified in *R. norvegicus* and is 100% identical to the ortholog in *Homo sapiens* at the protein level (Ron *et al*., 1994). In mammalian systems this protein binds activated protein kinase C (PKC) (Ron *et al*., 1994), interacts with

cAMP phosphodiesterase (Yarwood *et al*., 1999; Steele *et al*., 2001), and regulates Src tyrosine kinases (Chang *et al.*, 1998). While the protein inhibits Src activity, it does not seem to inhibit PKC activity (Chang *et al*., 1998; Ron *et al*. 1994). Chang *et al*. (1998) suggests that RACK1 is required for localizing cytosolic βPKC, an isoform of PKC in humans, to the plasma membrane. A similar protein has also been shown to directly interact with the Ran1 kinase for nuclear localization in *Schizosaccharomyces pombe* (McLeod *et al*., 2000). Ran1 is an essential protein in the fission yeast where it regulates meiotic development (Nurse, 1985). Similar to defects in *cpc2* of *N. crassa* and *cpcB* of *A. nidulans*, loss of *cpc2* function in *S. pombe* also reduces sexual differentiation (Müller *et al*., 1995; Hoffman *et al*., 2000; McLeod *et al*., 2000). Furthermore, in the fission yeast *cpc2* is epistatic to adenylate cyclase in terms of sexual differentiation, but does not downregulate cAMP production (McLeod *et al*., 2000).

Consistent with the high degree of identity within this collection of proteins having WD repeats, there is also functional complementation. Mutations in *cpc*2 in *S. pombe* and its orthologs in *S. cerevisiae* and *A. nidulans* are all complemented by the addition of RACK1 (McLeod *et al*., 2000; Hoffman *et al*., 1999; Hoffman *et al*., 2000). Additionally, Cpc2 of *N. crassa* also complements a *S. cerevisiae cpc2* mutant (Hoffman *et al*., 1999). Taken together, these data provide strong suggestive evidence that Uhf1 is likely to have a similar structure and function to homologs in other organisms.

Another common feature among members in the RACK1 subfamily is that several genes contain an intron at a conserved location. At this site the U24 snoRNA has been identified in both *cpcB* of *A. nidulans* and *cpc2* of *S. cerevisiae* (Hoffman *et al*., 1999; Hoffman *et al*., 2000). Functioning independently of its corresponding gene, deletion of

the intronic U24 snoRNA is not associated with phenotypes described for fungal strains with deletions of either *cpcB* or *cpc2* (Hoffman *et al*., 1999; Hoffman *et al*., 2000). The research in this chapter identifies putative snoRNAs in *uhf1* and *cpc2* of *U. maydis* and *N. crassa,* respectively. Although complete identity to the Box C sequence is not observed in either organism, the central core of the sequence element is conserved. Additionally, both the Box D and 28S complementary segments are present. Structural analysis should confirm the identification of these intronic snoRNAs.

The goal of the research presented in this chapter was to determine the function of Uhf1 by disrupting gene function by insertional mutagenesis. Southern and northern blot analyses indicate that while *uhf1* was successfully disrupted, its transcription was not prevented. Hybridization with a probe homologous to a region located 3′ to the carboxin insertion indicated the production of a large transcript that did not hybridize to the *cbxR* probe in subsequent northern blots. The inability of the *cbxR* probe to hybridize to the mutant transcript, suggests that *cbxR* was spliced from the mutant transcript. Alternate splicing and continued *uhf1* transcription may explain the production of the large mutant transcripts of strains 1/2C10 and 1/2C12 that were clearly different than the one produced by the wild-type strain. Future sequencing of the mutant transcript will provide insight on this mutation and its effect on the protein. It is unkown whether or not the putative snoRNA was correctly spliced.

Functional analysis of Uhf1 was accomplished by inserting 2262 bp within the first intron immediately after the initiation codon. The phenotype associated with *uhf1* mutant strains indicates a dysfunctional protein, however, the exact mutation is unclear. Perhaps the mutation had an affect on protein folding. It has been suggested that proteins that

encode β-propeller structures, such as the one predicted for RACK1 and its orthologs, maintain their ring structures through the interaction of the β strand of the amino terminus with the β strands located at the carboxy terminus of the protein (Smith *et al*., 1999; Garcia Higuera *et al*., 1996). Removal of the initial 30 amino acids in RACK1 causes changes in the ability of the protein to fold correctly (Garcia-Higuera *et al*., 1996).

In both *S. cerevisiae* and *S. pombe*, deletions in the *cpc2* ortholog result in an abnormal cellular morphology (Hoffmann *et al*., 1999; McLeod *et al*., 2000). Specifically, the mutant cells of these fungi are larger than wild-type cells. Morphological differences are also observed in *U. maydis*. Many *uhf1* mutant cells have rounded ends and produce an unknown dark pigment when incubated in liquid culture for a few days. This phenotype is somewhat similar to *U. maydis* cells with mutations in both *adr1* (catalytic subunit of PKA) and *hgl1* (a regulatory protein) (Dürrenberger, 2001). However, *uhf1* mutant cells appear to be most like *U. maydis* haploid cells that are in the process of forming chlamydospores. Kush and Schauz (1989) reported the induction of wild-type budding cells to differentiate and form chains of four rounded spores when grown in a rich medium and shifted to a poor medium: minimal medium containing a low amount of carbon and no nitrogen. Under these starvation conditions, a progression of cellular shapes were observed as the cells enlarged, swelled at certain locations, and formed chlamydospores in either a synchronous or successive manner. *uhf1* mutant cells exhibit many of these cellular states observed during chlamydospore development.

Originally *uhf1* was identified in an assay to find genes upregulated during filamentous growth. Subsequent experiments using the temperature-sensitive strain 1/52,

which has defects in both *uac1* and *ubc2*, also provided similar results. Increased *uhf1* transcription occurred shortly after budding cells of strain 1/52 were shifted to a permissive temperature and began growing as filaments. In contrast, expression of *uhf1* from cells changing from filaments to buds decreased, but this effect was not immediate. Transcription decreased after 10 h.

In addition to morphology, *uhf1* may also be influenced by nutrient availability. The *uhf1* orthologs of *N. crassa*, *A. nidulans*, and *S. cerevisiae* (Müller *et al*., 1995; Hoffmann *et al*., 1999; Hoffmann *et al*., 2000) have been shown to be influenced by the "general amino acid control" (Delforge *et al*., 1975) or "cross pathway control" (Carsiotis *et al*., 1974) system. In these fungi, starvation for a specific amino acid leads to a down regulation of the *uhf1* ortholog and the production of a specific group of amino acids. For example, in *N. crassa*, histidine starvation causes a decrease of *cpc2* transcription (Müller *et al*., 1995) and activates the synthesis of not only histidine, but also tryptophan, and arginine (Carsiotis *et al*., 1974). Consequently, the gene responsible for regulating transcription of the amino acid biosynthetic enzymes in *N. crassa, cpc1*, is upregulated. Similar results have also been observed in *A. nidulans* and *S. cerevisiae* (Hoffmann *et al*., 1999; Hoffmann *et al*., 2000).

The dark pigment produced by a*dr1*$^{-}$ *hgl1*$^{-}$ cells, by wild-type cells shifted to nutrient deprived broth, and by *uhf1* mutant cells may be some type of melanin or melanin precursor. Similar to many other fungal spores, the teliospores of *U. maydis* are darkly pigmented. Besides being important for virulence in some pathogens, such as *Magnaporthe grisea* (Howard and Ferrari, 1989; Howard and Valent, 1996), the melanin polymer is also important for spore survival (Bell and Wheeler, 1986). The production of

pigment and rounding of chlamydospore-like cells in *uhf1* mutants suggest that Uhf1 functions to inhibit pathways leading to production of thick walled pigmented cells such as teliospores or chlamydospores. Since *hgl1*$^-$ strains also produce a dark pigment, perhaps *hgl1* is also an inhibitor of these survival pathways.

Color variations were observed among the broth cultures of progeny strains derived from crosses of wild-type and *uhf1* mutant cells. While some broth cultures were light brown, others were almost black. The variation of the amount of pigment produced by mutant cells is consistent with Kush and Schauz (1989) who report that specific requirements must be met in order for the complete production of chlamydospores. When screening numerous colonies for mating type determination and pigment production, neither the age nor the number of cells was controlled. Only during growth curve experiments was the cell age and initial cell concentration controlled. Continued observation of the cultures used in the growth curve experiments revealed relatively minor color differences among the mutant strains, indicating that both culture age and cell number may have been contributing factors to the observed color differences in the progeny screen.

Within this study a few progeny strains were observed to display unexpected pigmentation phenotypes. Carboxin-resistant strains with the ability to mate with more than one mating type partner and with the inability to mate with themselves displayed wild-type pigmentation. This observation may be explained by the presence of a wild-type copy of *uhf1* in the mutant strains. Since *uhf1* is linked to the *a* mating-type locus the only way to get two mutant copies of *uhf1* in these dual mating type strains (*a1a2*) is by recombination between *a* and *uhf1*. Another rare atypical group of strains was

composed of normally mating non-pigmented carboxin-resistant strains (2/135) and pigmented carboxin-sensitive strains (4/123). These results are more difficult to explain, but are likely due to spontaneous mutations. In broth culture, cells of *U. maydis* are susceptible to spontaneous mutations that affect carboxin sensitivity and color (Keon *et al*., 1991; Dürrenberger *et al*., 2001).

Reduced growth is a common characteristic observed in fungi with mutations in the *uhf1* ortholog. Mutations of *cpc2* in *N. crassa* and *S. cerevisiae* result in a reduced growth rate by at least 25% (Krüger *et al*., 1990; Hoffmann *et al*., 1999), whereas a mutation in the orthologous gene in *S. pombe* causes a reduction in the final cell concentration (McLeod *et al*., 2000). The *uhf1* mutant cells of *U. maydis* exhibit both of these characteristics. First, *uhf1* mutant cells require about an hour longer to double as compared to wild-type cells. Second, *uhf1* mutant cells do not reach the same cell density as wild-type cells. Since cell size varies in *uhf1* mutant strains, perhaps the biomass of the mutant and wild-type cultures are identical, but this was not tested in this study. It is unknown whether these reduced growth phenotypes are associated with continuous stress or if there is a delay in the cell cycle. Deletion of *cpc2* in *S. pombe* delays cellular division (McLeod *et al*., 2000).

Disruption of *uhf1* suggests that this gene is not important for mating or pathogenicity since compatible *uhf1* mutant strains are able to mate, cause galls *in planta*, and produce teliospores. However, this gene is important for virulence. Pathogenicity data show that the inoculation of two compatible mutant strains into corn seedlings results in significantly less disease than plants inoculated with compatible wild-type strains. This

result indicates that at least one functional copy of the gene is necessary for full virulence.

Unfortunately, it is unknown whether or not teliospores of these crosses are able to germinate. This would be a future topic to explore since fertility defects are associated with the loss of *uhf1* orthologs in *N. crassa*, *A. nidulans*, and *S. pombe* (Müller *et al*., 1995; Hoffmann *et al*., 2000; McLeod *et al*., 2000). Hoffmann *et al*. (2000) suggested that defects slightly prior to or after karyogamy were a result of a loss of *cpcB* in *A. nidulans*. If a similar correlation exists in *U. maydis*, then it may be likely that the teliospores would not germinate since it is inside these developing spores that karyogamy takes place.

According to Mendelian genetics, the disruption of a single gene should segregate from its corresponding wild-type allele in a 1:1 ratio. Therefore, it was hypothesized that an equal number of carboxin-resistant and sensitive strains would be collected from the crosses between *uhf1* mutant strains and wild-type strains. Since this ratio was not observed by Chi-square analysis, it was suggested that another factor was likely responsible for the greatly reduced numbers of carboxin-resistant progeny. This bias may be explained by the slow growth phenotype discovered in *uhf1* mutant strains. The longer generation times of these mutant strains may have allowed for the preferential selection of faster-growing carboxin-sensitive strains on medium lacking carboxin. Then, by using dilution platings to collect single colonies for analysis, the bias may have been enhanced by diluting out the slow-growing resistant mutants.

Mating type determination of both carboxin-resistant and sensitive strains was predicted to reveal an equal proportion of each mating type: *a1 b1*, *a1 b2*, *a2 b1*, and *a2*

b2. However, a 1:1:1:1 ratio was not found in the analysis of over 200 progeny that had one mating type partner. Instead, *uhf1* co-segregated with the *a* locus of the parental strain to suggest linkage. Linkage to the *a* locus is not a rare occurrence because several auxotrophic markers that require the addition of specific amino aids, vitamins, or nucleic acid components have previously been shown to be linked to *a* (Holliday, 1961). Furthermore, Holiday's examination of this linkage group, which consists of the markers *me-1*, *ad-1*, *leu-1*, *pan-1* and the *a* mating type locus, suggests that linkage on this chromosome occurs over a region of at least 57.7 m.u. The marker most tightly linked to the *a* locus is *pan-1*, which requires the vitamin supplement pantothenic acid. Interestingly, this marker helped identify the *a2* mating type locus (Froeliger and Leong, 1991). Although it is unclear why the production of methionine (*me-1*), leucine (*leu-1*), adenine/hypoxanthine (*ad-1*), and Uhf1 are linked to the mating type locus, it may be that it is an evolutionary mechanism to keep the genes within the population. Since survival of the pathogen requires the *a* mating type locus for completion of its life cycle, those genes that are tightly linked to the locus are more likely to be maintained.

Identification of a strain with mutations in both *uhf1* and *uac1* was attempted in order to determine the genetic interaction between the genes. Since *uhf1* expression is upregulated in the filamentous strain that contains an *uac1* mutation, it was predicted that the products encoded by these two genes may interact and may be involved in the same signaling pathway. Unfortunately, this analysis could not be conducted because a double mutant was not identified in this study. The most probable explanation for this phenomenon was that few colonies were screened. Varied morphologies and ineffective phleomycin selection made screening difficult. After streaking single colonies obtained

from diluted platings of germinated teliospores onto PDA several times, a consistent phenotype for each progeny strain should have been observed on each plate. However, this was not observed. Instead, many of the streaked plates contained a combination of budding, filamentous, and intermediate fuzzy colonies. The reason for this observation is unknown. Likewise, it is unclear why phleomycin selection was ineffective. Only those strains with the *uac1::ble* disruption should have been able to grow on PDA containing phleomycin, but in this study wild-type strains were observed growing on the medium after 1 day at 30°C.

In conclusion, Uhf1 belongs to highly conserved group of eukaryotic proteins containing 7 WD repeats. It is upregulated during filamentous growth and may be regulated by nutrient availability. Assuming that a mutant allele was created, disruption of the gene indicates that *uhf1* is necessary for maintaining a wild-type morphology and growth rate, but is not essential for mating or disease development. Future isolation of an *uac1 uhf1* double mutant strain is likely to identify genetic relationships between these genes and may lead to a better understanding of the importance of *uhf1* during filamentous growth. Perhaps the protein encoded by *uhf1* is part of a stress regulation pathway that converges with the cAMP signaling pathway.

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Table 3.1. Strains of *U. maydis* used in this study

Strain	Relevant genotype	Reference
1/2	*a1 b1*	Gold *et al.* (1997)
1/2C7 (7/42)[a]	*a1 b1* (ectopic *uhf1::cbx*)	This study
1/2C10 (7/43)	*a1 b1 uhf1::cbx*	This study
1/2C12 (7/44)	*a1 b1 uhf1::cbx*	This study
1/9	*a1 b1 uac1::ble*	Gold *et al.* (1994)
1/52	*a1 b1 uac1::ble ubc2-2*	Mayorga and Gold (1998)
2/8	*a1 b1*	Gold *et al.* (1997)
2/9	*a2 b2*	Gold *et al.* (1997)
2/11	*a2 b1*	This study
2/14	*a1 b2*	This study
2/27	*a1 b1 uac1::ble* with pFuz60KBe	This study
2D4 (7/47)[b]	*a1 b1 uhf1::cbx*	This study
2D6 (7/48)[b]	*a2 b2 uhf1::cbx*	This study
3F1 (7/49)[b]	*a1 b2 uhf1::cbx*	This study
3F5 (7/50)[b]	*a1 b1 uhf1::cbx*	This study
6C6 (7/51)[b]	*a1 b2 uhf1::cbx*	This study
7A4 (7/52)[b]	*a1 b2 uhf1::cbx*	This study
7B1 (7/45)[b]	*a2 b2 uhf1::cbx*	This study
7C1 (7/53)[b]	*a2 b1 uhf1::cbx*	This study
7D3 (7/54)[b]	*a2 b2 uhf1::cbx*	This study
8C1 (7/55)[b]	*a1 b2 uhf1::cbx*	This study
122A5 (7/46)[c]	*a2 b2 uhf1::cbx*	This study

[a]Numbers in parentheses indicate future identification numbers.

[b]Progeny strain from the cross between 1/2C10 and 2/9.

[c]Progeny strain from the cross between 1/2C12 and 2/9.

Table 3.2. Carboxin selection of progeny from *uhf1* mutant strains crossed to wild-type

Cross	Total number tested	Number of cbx^R strains	Number of cbx^S strains	X^{2a}	P
1/2C10 x 2/9	254	77	177	39.37	<0.001
1/2C12 x 2/9	274	112	162	10.124	<0.0025
1/2 x 122A5	103	20	83	38.534	<0.001

[a]Chi-sqaure values were calculated on the null hypothesis that the progeny numbers of cbx^R strains to cbx^S strains would form a 1:1 ratio, degrees of freedom = 1.

Table 3.3. Distribution of the *a* mating type locus in progeny from *uhf1* disruption strains crossed to wild-type

	Resistance values[a]				Sensitivity values							
	O	E	O	E	O	E	O	E	Expected				
Cross	*a1*	*a1*	*a2*	*a2*	*a1*	*a1*	*a2*	*a2*	R:P ratio[b]	*df*	X^2	P	m.u.[c]
1/2C10 x 2/9	42	23	4	23	NA	NA	NA	NA	1:1	1	31.4	<0.001	8.7
1/2C12 x 2/9	71	37	3	37	0	21.5	43	21.5	1:1 & 1:1[d]	3	105.5	<0.001	2.6
1/2 x 122A5	3	7.5	12	7.5	72	40	8	40	1:1 & 1:1[d]	3	56.6	<0.001	11.6

[a]Observed (O) and expected (E) values for the number of carboxin resistant and sensitive progeny containing the *a1* or *a2* mating type locus.

[b]The R:P ratio is the number of progeny with the recombinant (R) mating type compared to the number of progeny with the parental (P) mating type.

[c]Shown are map distances calculated per cross. To calculate the average map distance the combined % recombinants was calculated as follows: (total number of recombinants / total number of progeny) x 100%. The average m.u. is 7.

[d]Denoted 1:1 & 1:1 for analysis of the resistant values separate from the sensitive values. This was done to compensate for growth differences in disruption versus non-disruption isolates. Results were combined to generate a single X^2 value.

Table 3.4. Generation time of an *uhf* disruption mutant and its mutant progeny

Strain	Generation time (h)[a]
1/2	1.81
1/2C10	3.01
2D4	2.47
2D6	2.62
3F1	4.08
3F5	2.52
6C6	2.73
7A4	3.43
7B1	3.10
7C1	3.10
7D3	3.42
8C1	2.56

[a]The generation time (t_g) was calculated from 8-16 h using the equation $t_g = 0.3/K$. K is the slope of the growth increase curve.

Table 3.5. Pathogenicity of *uhf1* disruption mutants

Strains Inoculated	Dikaryon *uhf1* /*uhf1*	Total # of plants[e]	Disease Index Values[f] 0	1	2	3	4	5	Disease Index[gh]
1/2 x 2/9	+/+	61	0	7	24	8	7	15	2.98a
1/2C10 x 2/9	-/+	60	0	8	23	9	11	9	2.83a
1/2C12 x 2/9	-/+	60	0	7	24	11	8	10	2.83a
1/2 x 7B1	+/-	60	0	13	25	10	5	7	2.47ab
1/2 x 122A5	+/-	60	0	14	24	8	5	9	2.52a
7B1 x 1/2C10	-/-	60	0	14	30	8	5	3	2.22b
122A5 x 1/2C12	-/-	60	0	38	20	1	1	0	1.42c
Water control	NA[i]	59	12	47	0	0	0	0	0.80d

[e]Total number of plants tested in 3 repetitions.

[f]Disease index values 14 d after inoculation: 0 = no symptoms, 1 = anthocyanin/chlorosis, 2 = small leaf galls, 3 = small stem galls, 4 = large stem galls, 5 = plant death.

[g]The disease index is calculated as the sum of the disease ratings divided by the number of plants.

[h]Disease index values followed by the same letter are not significantly different from each other according to the LSD test (P = 0.05).

[i]NA = not applicable

Figure 3.1. DNA sequence of *uhf1*. Approximately 1300 bp upstream of the putative start codon (bold) and 500 bp downstream of the predicted stop codon were sequenced. The deduced amino acids are given above the sequence of the open reading frame. Two introns interrupt the gene at positions 1266 and 2310 as confirmed by the cDNA sequence. The following consensus sites are underlined: 5' splice site; lariat formation; 3' splice site. Box C and Box D consensus sites located in the second intron are outlined with boxes. Two short sequences identical to regions in the 28 S ribosomal DNA are underlined with a broken line. Forward and reverse arrows indicate positions of the oligonucleotides used to amplify *uhf1*. A *Hind* III recognition sequence was added to the 3´ end of the reverse primer. The *cbxR* insertion was made at the *Eco*R V site at position 1599. (Gene and cDNA sequences were provided by D. Andrews, pers comm).

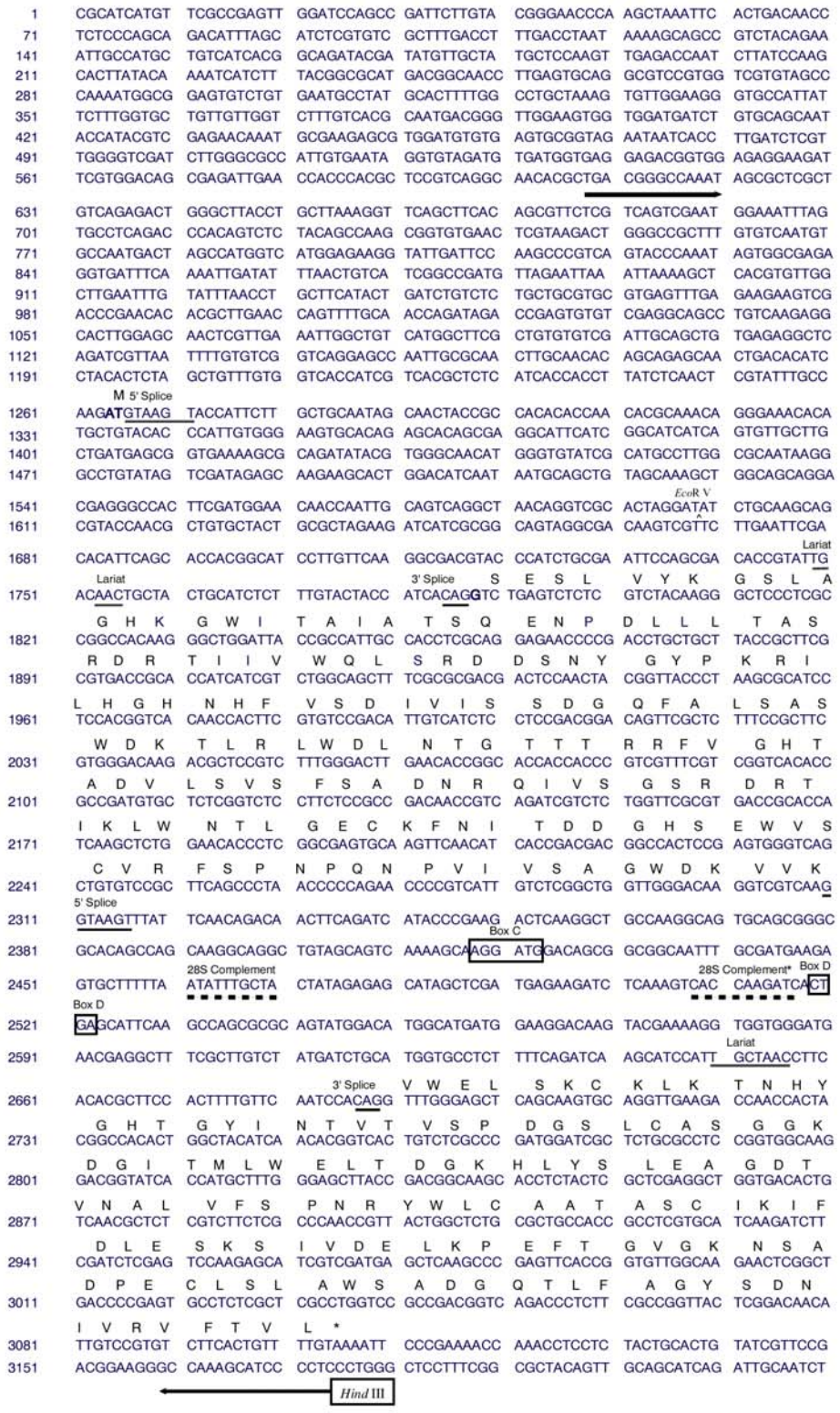

M 5' Splice
Lariat
3' Splice
EcoR V
Box C
28S Complement
28S Complement*
Box D
Hind III

Figure 3.2. Structure of plasmid, pCDuhf. *uhf1* was amplified and cloned into TOPO PCR2.1. Then, this gene was transferred into pUC 18 for digestion with *Eco*R V. The gene conferring carboxin resistance, *cbxR*, was inserted at this location and employed as a selectable marker.

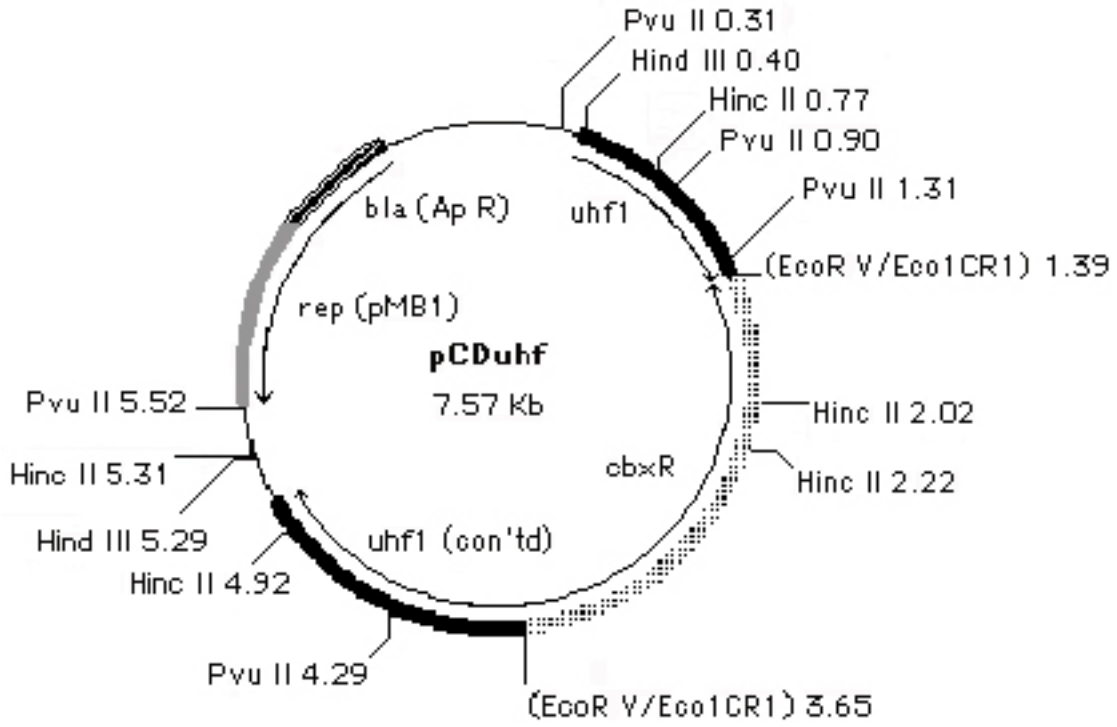

Pvu II 0.31
Hind III 0.40
Hinc II 0.77
Pvu II 0.90
Pvu II 1.31
(EcoR V/Eco1CR1) 1.39
Hinc II 2.02
Hinc II 2.22
cbxR
(EcoR V/Eco1CR1) 3.65
Pvu II 4.29
Hinc II 4.92
Hind III 5.29
Hinc II 5.31
Pvu II 5.52
bla (Ap R)
rep (pMB1)
pCDuhf
7.57 Kb
uhf1
uhf1 (con'td)

Figure 3.3. *uhf1* expression in filamentous and budding cells. A northern blot using total RNA from strains 1/9 (lane 1), 1/2 (lane 2), 1/2C10 (lane 3), and 1/2C12 (lane 4) was prepared and probed with *uhf1* (panel A) and *cbxR* (panel B). Panel C is a control showing the ethidium stained agarose gel prior to transfer. (The northern blot and subsequent hybridizations were performed by M. Garcia-Pedrajas).

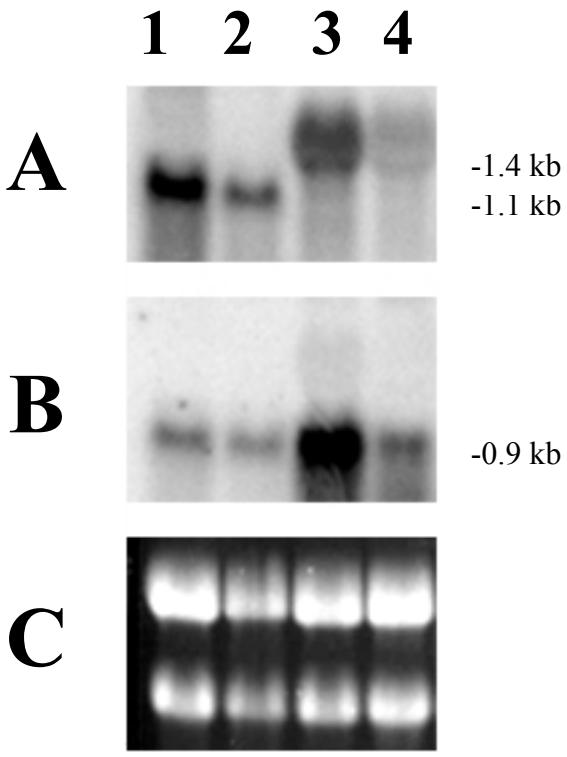
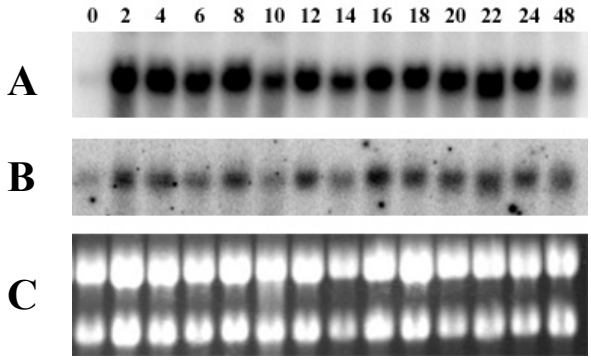

1 2 3 4
A
-1.4 kb
-1.1 kb
B
-0.9 kb
C

Figure 3.4. Temporal expression of *uhf1* using the temperature sensitive *uac1*$^{-}$ *ubc2-2* strain 1/52. The top panels depict *uhf1* expression levels during the transition from budding growth at 30°C to filamentous growth at 18°C. The bottom panels show the opposite transition. Northern blots were prepared using total RNA and probed with *uhf1* (A panels) and *cbxR* (B panels). Numbers on top of the panels indicate sample times in hours after the temperature shift. C panels show the ethidium bromide stained agarose gels prior to transfer. (Northern blots were provided by M. Garcia-Pedrajas, pers comm).

Bud-filament transition



Filament-bud transition

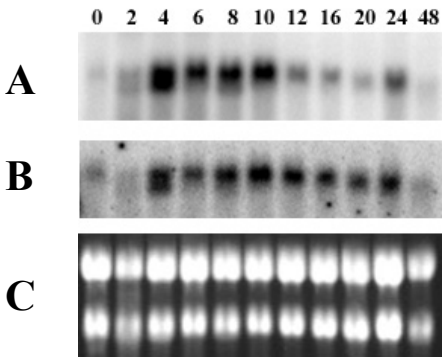


Figure 3.5. Comparison of Uhf1 with orthologous proteins from *N. crassa* (Cpc2, accession number Q01369), *A. nidulans* (CpcB, accession number AAF98065), *S. cerevisiae* (Cpc2*, accession number NP_013834), and *R. norvegicus* (RACK1, accession number NP_570090). Identical amino acids are shaded. Identities are not shown when two pairs of amino acids are present at the same location. The 7 WD repeats are underlined according to Hoffmann *et al*. (1999). Sequences were aligned using the automatic Higgins alignment tool of the MacDNASIS Pro v3.5 program with some modification.

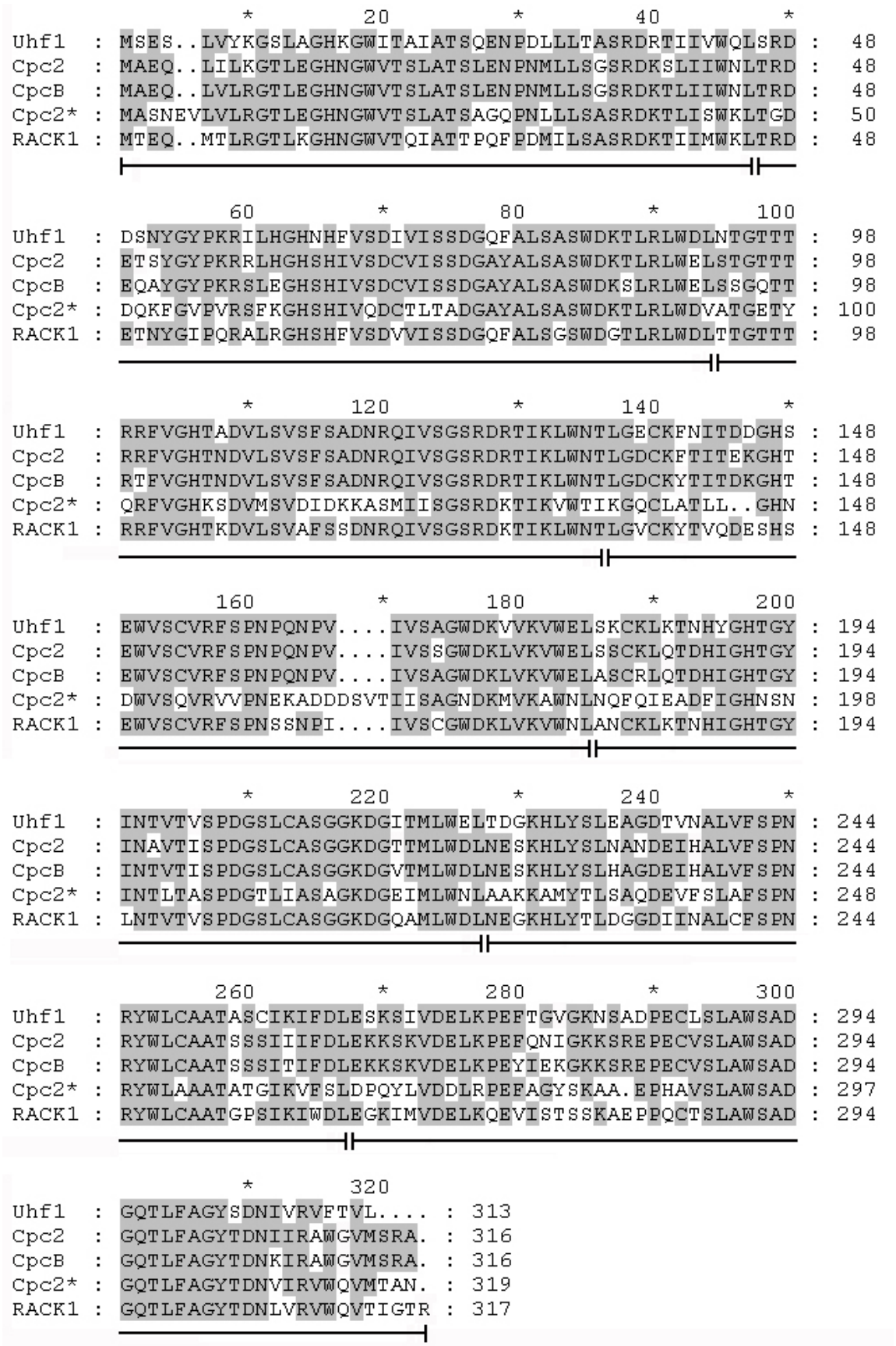

Uhf1 : MSES..LVYKGSLAGHKGWITAIATSQENPDLLLTASRDRTIIVWQLSRD : 48
Cpc2 : MAEQ..LILKGTLEGHNGWVTSLATSLENPNMLLSGSRDKSLIIWNLTRD : 48
CpcB : MAEQ..LVLRGTLEGHNGWVTSLATSLENPNMLLSGSRDKTLIIWNLTRD : 48
Cpc2* : MASNEVLVLRGTLEGHNGWVTSLATSAGQPNLLLSASRDKTLISWKLTGD : 50
RACK1 : MTEQ..MTLRGTLKGHNGWVTQIATTPQFPDMILSASRDKTIIMWKLTRD : 48
Uhf1 : DSNYGYPKRILHGHNHFVSDIVISSDGQFALSASWDKTLRLWDLNTGTTT : 98
Cpc2 : ETSYGYPKRRLHGHSHIVSDCVISSDGAYALSASWDKTLRLWELSTGTTT : 98
CpcB : EQAYGYPKRSLEGHSHIVSDCVISSDGAYALSASWDKSLRLWELSSGQTT : 98
Cpc2* : DQKFGVPVRSFKGHSHIVQDCTLTADGAYALSASWDKTLRLWDVATGETY : 100
RACK1 : ETNYGIPQRALRGHSHFVSDVVISSDGQFALSGSWDGTLRLWDLTTGTTT : 98
Uhf1 : RRFVGHTADVLSVSFSADNRQIVSGSRDRTIKLWNTLGECKFNITDDGHS : 148
Cpc2 : RRFVGHTNDVLSVSFSADNRQIVSGSRDRTIKLWNTLGDCKFTITEKGHT : 148
CpcB : RTFVGHTNDVLSVSFSADNRQIVSGSRDRTIKLWNTLGDCKYTITDKGHT : 148
Cpc2* : QRFVGHKSDVMSVDIDKKASMIISGSRDKTIKVWTIKGQCLATLL..GHN : 148
RACK1 : RRFVGHTKDVLSVAFSSDNRQIVSGSRDKTIKLWNTLGVCKYTVQDESHS : 148
Uhf1 : EWVSCVRFSPNPQNPV....IVSAGWDKVVKVWELSKCKLKTNHYGHTGY : 194
Cpc2 : EWVSCVRFSPNPQNPV....IVSSGWDKLVKVWELSSCKLQTDHIGHTGY : 194
CpcB : EWVSCVRFSPNPQNPV....IVSAGWDKLVKVWELASCRLQTDHIGHTGY : 194
Cpc2* : DWVSQVRVVPNEKADDDSVTIISAGNDKMVKAWNLNQFQIEADFIGHNSN : 198
RACK1 : EWVSCVRFSPNSSNPI....IVSCGWDKLVKVWNLANCKLKTNHIGHTGY : 194
Uhf1 : INTVTVSPDGSLCASGGKDGITMLWELTDGKHLYSLEAGDTVNALVFSPN : 244
Cpc2 : INAVTISPDGSLCASGGKDGTTMLWDLNESKHLYSLNANDEIHALVFSPN : 244
CpcB : INTVTISPDGSLCASGGKDGVTMLWDLNESKHLYSLHAGDEIHALVFSPN : 244
Cpc2* : INTLTASPDGTLIASAGKDGEIMLWNLAAKKAMYTLSAQDEVFSLAFSPN : 248
RACK1 : LNTVTVSPDGSLCASGGKDGQAMLWDLNEGKHLYTLDGGDIINALCFSPN : 244
Uhf1 : RYWLCAATASCIKIFDLESKSIVDELKPEFTGVGKNSADPECLSLAWSAD : 294
Cpc2 : RYWLCAATSSSIIIFDLEKKSKVDELKPEFQNIGKKSREPECVSLAWSAD : 294
CpcB : RYWLCAATSSSITIFDLEKKSKVDELKPEYIEKGKKSREPECVSLAWSAD : 294
Cpc2* : RYWLAAATATGIKVFSLDPQYLVDDLRPEFAGYSKAA.EPHAVSLAWSAD : 297
RACK1 : RYWLCAATGPSIKIWDLEGKIMVDELKQEVISTSSKAEPPQCTSLAWSAD : 294
Uhf1 : GQTLFAGYSDNIVRVFTVL.... : 313
Cpc2 : GQTLFAGYTDNIIRAWGVMSRA. : 316
CpcB : GQTLFAGYTDNKIRAWGVMSRA. : 316
Cpc2* : GQTLFAGYTDNVIRVWQVMTAN. : 319
RACK1 : GQTLFAGYTDNLVRVWQVTIGTR : 317

Figure 3.6. Southern blot analysis of *uhf1* disruption strains. Genomic DNAs from strains 1/2 (lane 1), 2/9 (lane 2), 1/2C7 (lane 3), 1/2C10 (lane 4), 1/2C12 (lane 5), 7B1 (lane 6), and 122A5 (lane 7) were digested with *Pvu* II and run on an agarose gel. Digestion of the *uhf1* cosmid genomic clone, cos75 (lane 8) and the disruption construct, pCDuhf (lane 9) with *Pvu* II were included as controls. The membrane was probed with the *uhf1* digoxigenin labeled probe.

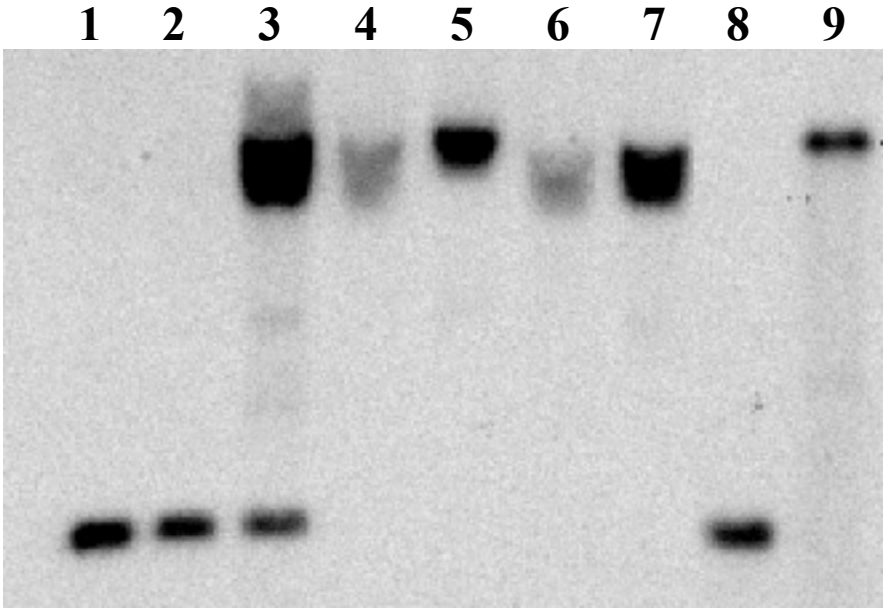

1 2 3 4 5 6 7 8 9

Figure 3.7. Pigment production of *uhf1* mutant cells grown in PDB. Test tubes were inoculated with approximately 1 x 10^5 cells/ml and placed at 30°C with shaking. The following strains are shown: wild-type 1/2 (tube 1), wild-type 2/9 (tube 2), ectopic transformant 1/2C7 (tube 3), 1/2C10 (tube 4), 7B1 (tube 5), 1/2C12 (tube 6), and 122A5 (tube 7). Observations were made at 1 (panel A), 5 (panel B), and 10 (panel C) days. Pictures were taken with the Digital Camera Fine Pix S1 Pro (Fujifilm) and compiled using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA).

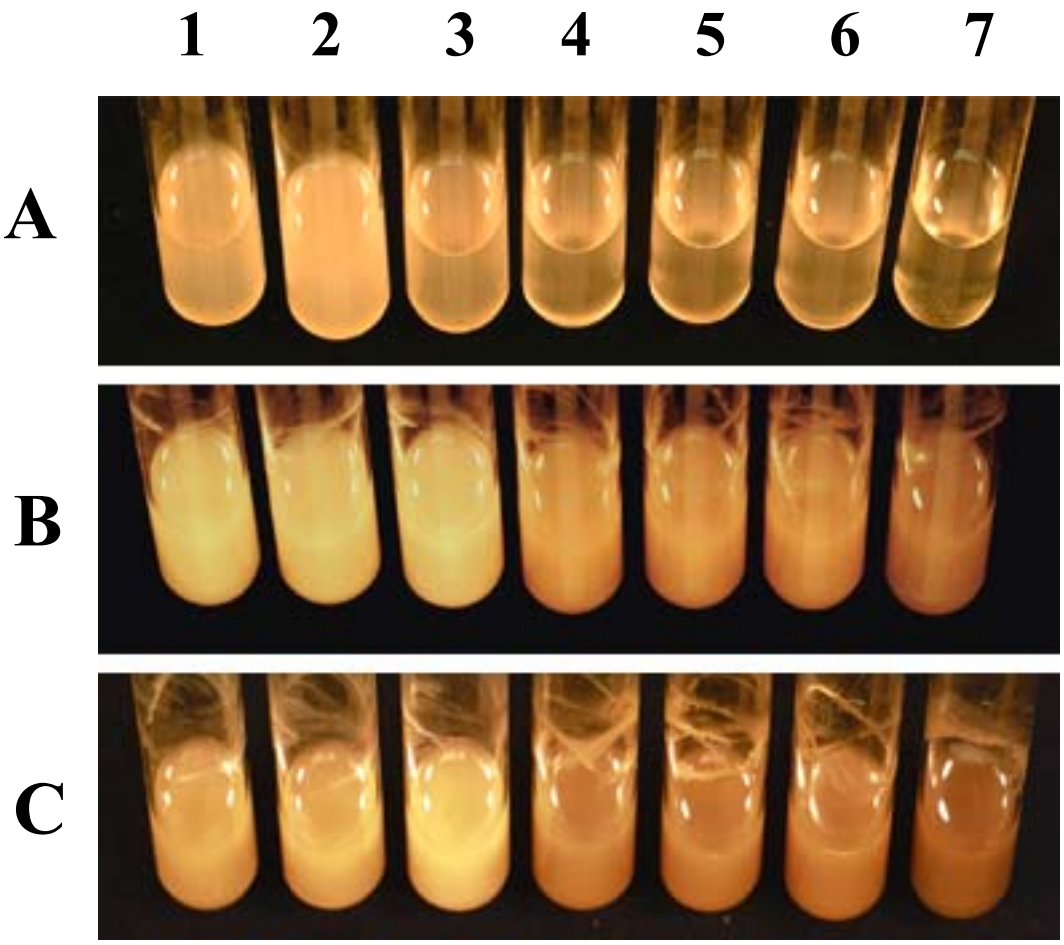

1
2
3
4
5
6
7
A
B
C

Figure 3.8. Cellular morphologies of *uhf1* mutant strains. Panels A-F were taken after 1 day of growth and panels G-M were taken after 10 days of growth in PDB. The panels show the following strains: A and G are of 1/2; B and H are of 1/2C10; C and I are of 7B1; D and J are of 1/2C7; E, K, and L are of 1/2C12; F and M are of 122A5. Pictures were taken with the Digital Camera Fine Pix S1 Pro (Fujifilm) and compiled using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA). All photographs were taken at 200x, except for picture L, which was taken at 400x. Bars shown in A and L are 10 μm and 5 μm, respectively .

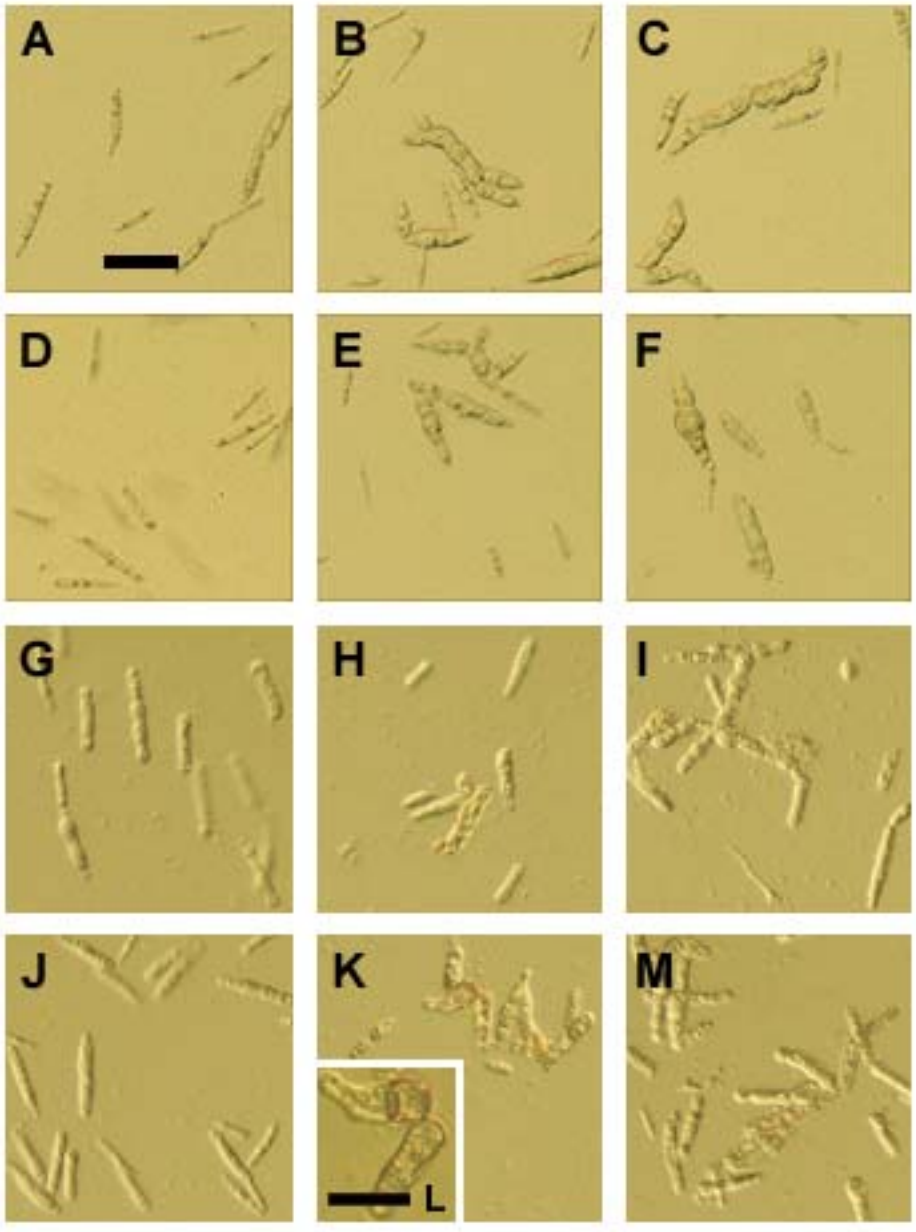

A
B
C
D
E
F
G
H
I
J
K
L
M

Figure 3.9. Growth curve of *uhf1* mutant strains. PDB tubes were inoculated with approximately 1 x 10^5 cells/ml and placed at 30°C with shaking. The cell concentration was determined every 4 h using a hemacytometer. Data shown are the average of two repetitions.

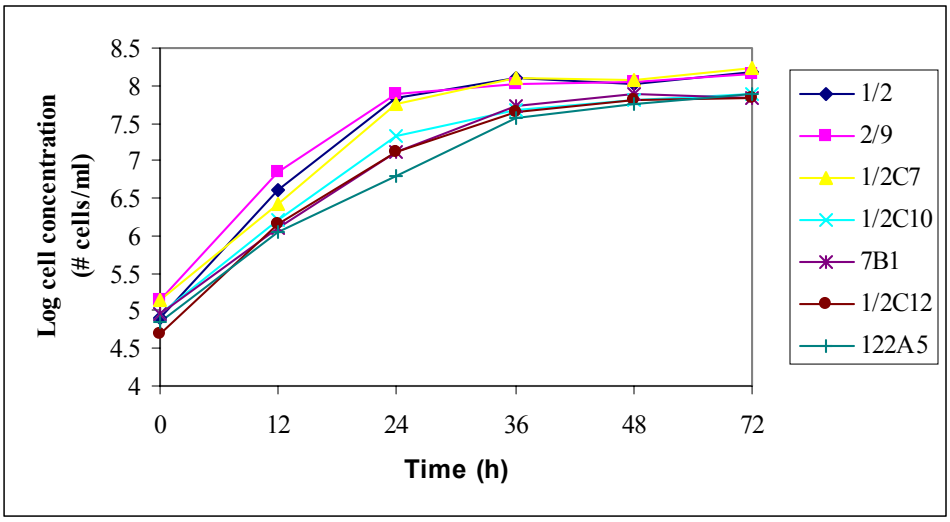

Log cell concentration
(# cells/ml)
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
0
12
24
36
48
72
Time (h)
1/2
2/9
1/2C7
1/2C10
7B1
1/2C12
122A5

Figure 3.10. Mating of *uhf1* mutant strains. Compatible wild-type and mutant cells were co-inoculated onto charcoal medium, incubated overnight, and observed for the production of white filamentous growth typical of the dikaryon. Photograph was taken with a Digital Camera Fine Pix S1 Pro (Fujifilm) and adjusted using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA). Mating of the compatible *uhf1* mutant strains 1/2C12 and 122A5 was identical to that of 1/2C10 and 7B1 (data not shown).

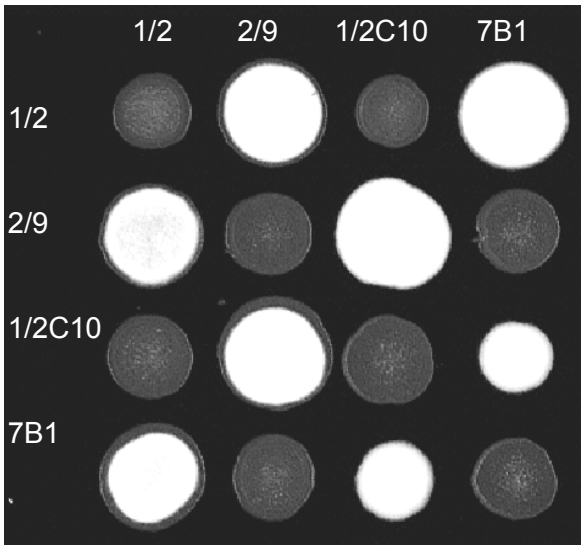
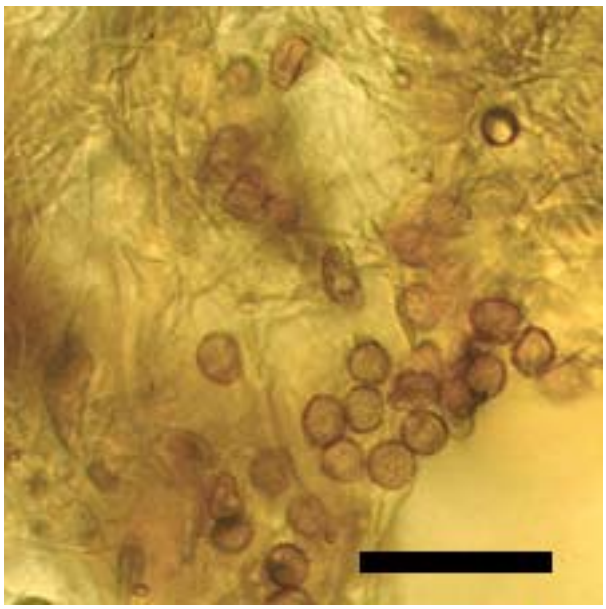

1/2
2/9
1/2C10
7B1
1/2
2/9
1/2C10
7B1

Figure 3.11. Cross section of galled plant tissue containing teliospores produced by the co-inoculation of 1/2C10 and 7B1. Photographs were taken with a Fine Pix S1 Pro (Fujifilm) digital camera and adjusted using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA). Teliospores were also observed when 1/2C12 was co-inoculated with 122A5 (data not shown). Bar shown is 10 μm.



CHAPTER 4

MOLECULAR CHARACTERIZATION OF *CAP1* IN *U. MAYDIS*

Ustilago maydis is a dimorphic fungal pathogen that switches from budding to filamentous growth in order to complete its life cycle. This bud-hypha transition is a highly dynamic process requiring rearrangement of the cytoskeleton and reduced cAMP levels. One protein that is conserved in many eukaryotes that interacts with both the actin cytoskeleton and adenylate cyclase is the cyclase associated protein, CAP. Using primers based on a *cap1*-like sequence identified in a genomic draft sequence provided by Exelisis Inc. (San Francisco, California), a CAP ortholog has been cloned in *U. maydis*.

INTRODUCTION

Fungal dimorphism is characterized by the ability to switch from budding to filamentous growth in response to specific stimuli. Environmental signals that may cause the fungus to change its morphology include changes in pH, temperature, and nutrient availability. Depending on the organism and stimulus, the cAMP signaling pathway may be activated in order for the cell to respond. As a second messenger signaling molecule, cAMP is crucial for dimorphic growth in numerous fungi including the human pathogens *Candida albicans* (Cho *et al.*, 1992; Niimi *et al.*, 1980; Sabie and Gadd, 1992) and *Histoplasma capsulatum* (Medoff *et al*., 1981) as well as the plant pathogens *Ophiostoma ulmi* (Brunton and Gadd, 1989) and *Ustilago maydis* (Gold *et al*., 1994). It has also been shown to control the pseudohyphal growth observed in *Saccharomyces cerevisiae* (Sánchez-Martínez and Pérez-Martín, 2001).

Besides regulating the bud-hypha transition, cAMP is required for the differentiation of filamentous cells into specialized structures. In the rice blast pathogen *Magnaporthe grisea*, cAMP is required for the production of the appressorium (Lee and Dean, 1993). Without this structure, *M. grisea* is unable to penetrate host tissues and subsequently cause disease. Similarly, alterations in the cAMP signaling pathway, which affect the growth transition of dimorphic pathogens, also cause reduced virulence (Orlowski, 1994). Hence, dimorphism and pathogenicity are intimately associated.

In the corn smut pathogen *U. maydis*, the cAMP signaling pathway regulates dimorphism and pathogenicity (Gold *et al*., 2001). Produced from the conversion of ATP by adenylate cyclase, cAMP is bound by the regulatory subunit of PKA. This cAMP binding indirectly activates the catalytic subunit of PKA by triggering its release from the inhibitory regulatory subunit. Once released, the catalytic subunit is activated to phosphorylate target proteins responsible for budding growth. Without the production of the second messenger molecule cAMP, PKA is inactive and the fungus has filamentous growth (Barrett *et al*., 1993; Gold *et al*., 1994), a condition that is normally observed in wild-type dikaryotic cells. In contrast to filamentous dikaryotic cells, filamentous haploid cells are non-pathogenic even in compatible mutant crosses (Barrett *et al*., 1993).

The bud-hypha transition in *U. maydis* as well as in other organisms is a process that requires a dynamic cytoskeleton to adapt to the cells' changing needs. The rearrangement of free actin monomers and polymerized microfilaments is apparently triggered by the cAMP signaling pathway. Conserved in many eukaryotes, CAP (cyclase associated protein) is important for both adenylate cyclase activation and actin organization. The N-terminal portion of the protein is responsible for cyclase binding,

and the C-terminal part of the protein is important for actin binding (Freeman *et al*, 1996). In *S. cerevisiae*, a mutation of *cap1* results in an abnormal cell morphology (Freeman and Field, 2000). Similarly, a mutation in the *cap1* ortholog found in *C. albicans* also causes an abnormal phenotype. Homozygous *cap1*$^{-}$ budding cells of *C. albicans* respond inefficiently to stimuli that cause abundant filamentous growth in wild-type cells. These cells exhibit reduced levels of cAMP and are avirulent (Bahn and Sundstrom, 2001). Interestingly in contrast to *U. maydis*, both *C. albicans* and *S. cerevisiae* require cAMP to stimulate filamentous or pseudohyphal growth, respectively.

The purpose of the research presented in this chapter was to identify *cap1* in *U. maydis* for future functional analysis. Using PCR primers developed from the draft sequence provided by Exelisis Inc. (San Francisco, California), an approximate 3 kb fragment was amplified. Restriction digests and partial sequencing of the cloned product confirmed the identification of the gene. The predicted protein contains 462 amino acids and has a proline rich region.

MATERIALS AND METHODS

Identification of *cap1* in *U. maydis*

The Exelisis database was searched for regions of sequence similarity to previously described *cap1* genes. Then, using the following primers UmCAP (F) - GCGATGCAGGAAGAGTATGA and UmCAP (R) - GATCGAGGAGTGAAGACAAGTG, polymerase chain reaction (PCR) was used to amplify a 3122 bp fragment containing the entire predicted ORF and approximately 400bp on each side. Amplification reactions contained 50 mM KCl, 10 mM Tris pH 8.3,

2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 200 μM of each dNTP, 1 μM each primer, 1 unit Taq polymerase (Engelke *et al*., 1990; Pluthero, 1993), and approximately 40 ng genomic DNA in a final volume of 50 μl. Reactions were performed in an Amplitron II Thermocycler (Barnstead/Thermolyne). Samples were heated at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 4.5 min. A final extension of 8 min at 72°C was also included.

The single band obtained from the PCR reaction was cloned into pCR2.1 TOPO (Invitrogen) and transformed into TOPO 10 F′ bacterial cells according to the manufacturer's directions. The resulting plasmid was called pCAP.

Plasmid DNA was obtained using alkaline lysis on bacterial cells from an overnight culture grown in LB (Luria Bertani) amended with ampicillin (100 μl/ml). Cells were treated with 300 μL TENS (0.1 NaOH, 0.5% SDS, in TE pH8) and 150 μL 3 M NaOAc, pH5.2, briefly vortexed and pelleted. Cellular debris was removed and cold 95% EtOH was used for DNA precipitation. Colonies were screened by digesting plasmid DNA with *Eco*R 1.

Identifying the genomic cosmid clone

A radioactive probe was generated and used to screen the genomic library previously constructed in the cosmid vector pJW42 and stamped onto a series of membranes. The probe was made from the original PCR product that was used in the cloning process. After purifying the PCR product using the Qiaquick PCR Purification kit (Qiagen), approximately 15 ng was labeled with ^{32}P-dCTP using the RadPrime DNA Labeling System (Invitrogen Life Technologies).

Blots were pre-hybridized for 4 h in Church buffer (5 mM EDTA, 0.25 M Na_2HPO_4, 1% casein hydrolysate, 7%SDS, 0.17% H_3PO_4) at 68°C. Hybridizations were performed overnight at 68°C in Church buffer. A second probe that hybridized to an ammonium transporter found in *U. maydis* was also tested in the same blots in conjunction with the *cap1* probe. Using primers UmCAP (F) and UmCAP (R), PCR was used to screen those cosmids that hybridized to the probe solution.

Sequencing

DNA for sequencing was obtained using the Qiaprep miniprep kit (Qiagen). Sequencing was performed on the ABI Prism 310 Genetic Analyzer (PE Applied Biosystems) using the ABI Prism Big Dye Terminator Cycle Sequencing Reaction kit (PE Applied Biosystems) according to the manufacturer's directions.

RESULTS

Translation of the draft Exelisis DNA sequence revealed a protein of 462 amino acids that is homologous to the CAP proteins of *S. cerevisiae* (accession number NP_014261), *Lentinula edodes* (accession number BAA26002), *Dictyostelium discoideum* (accession number P54654), and *C. albicans* (accession number AAD42978) (Figure 4.1). As seen in the protein sequence of these and other CAPs, the predicted *U. maydis* protein has a proline-rich region (amino acids 212 to 220). The predicted protein sequence does not seem to contain the RLE/RLE motif or the common SH3 binding domain (PXXP, P = proline and X = any amino acid) present in the CAP sequence of *C. albicans* (Bahn and Sundstrom, 2001) and other CAP proteins.

Several putative Topo clones containing the *cap1* PCR product were obtained from the TOPO 10 F′ bacterial transformation. Fifteen white colonies were chosen for DNA extraction and subsequent digestion with *Eco*R 1. Two bands of approximately 3 kb and 4 kb were obtained for the *cap1* insert and vector, respectively (Figure 4.2). Plasmid DNAs from two colonies that produced the expected bands were end sequenced. Sequence data agreed with the sequence data obtained from Exelisis indicating that the predicted fragment of *cap1* had been amplified and cloned.

Screening the cosmid library with the probe mixture identified six putative *cap1* clones: 4-2-12A3, 4-4-14F10, 5-4-19E9, 5-5-20B4, 6-5-25D5, and 7-1-26H3. Using the UmCAP primer set, none of the clones were identified as the *cap1* cosmid.

DISCUSSION

The cytoskeleton is an extensive network of filaments that provides support and moves organelles and other cellular components in response to stimuli. Two types of filaments that comprise this dynamic scaffold are microtubules and microfilaments. Consisting of polymerized α- and β-tubulin subunits, microtubules are important for polarized growth and nuclear migration in *U. maydis* (Steinberg *et al*., 2000) as well as in *Schizosaccharomyces pombe* (Drummond and Cross, 2000). Additionally, there is some similarity with the organization of the microtubules with *C. albicans* (Steinberg *et al*., 2000). In *S. cerevisiae*, microtubules also help with nuclear migration but it is the microfilaments, composed of actin, that establish cell polarity. These actin filaments are regulated by Cap1, which binds actin, adenylate cyclase, and the SH3 domains of other proteins. Based on these interactions, Yu *et al*. (1999) suggest that Cap1 links the actin

cytoskeleton and the cAMP signaling pathway to influence morphological changes in response to specific stimuli. The purpose of the research presented in this chapter was to identify *cap1* in *U. maydis* for future functional studies.

Based on the *cap1*-like sequence provided by Exelisis Inc., PCR primers were developed to amplify a target region predicted to contain the entire *cap1* gene. After PCR, the resulting amplicon was cloned and partially sequenced from the fragment ends. Sequence data agreed with the draft sequence from Exlesis Inc. suggesting that the target had been amplified, but subsequent screening of the genomic library with this 3.1 kb fragment failed to identify a cosmid clone. Cosmid clones are neccessary for obtaining the best possible sequences because they contain large pieces of genomic DNA including full-length gene sequences not manipulated by enzymatic reactions such as PCR. It is unclear why a cosmid clone was not successfully identified in this study because hybridization with the dual probe should have identified clones containing either *cap1* or an ammonium transporter. Perhaps there was a problem with the probe, or it may be that a cosmid containing *cap1* is not present in the library. Furture screening attempts should be conducted using a fresh probe independent of other variables.

The predicted translation of the Cap1 protein in *U. maydis* is homologous to the CAPs of *S. cerevisiae*, *L. edodes,* and *C. albicans*. Similar to these proteins and those CAPs found in other organisms, the *U. maydis* protein has a conserved proline-rich region. In contrast to these proteins, the *U. maydis* protein does not appear to contain two regions commonly present in CAP proteins; the RLE/RLE motif that is important for Ras/cAMP dependent signaling (Yu *et al*., 1999) and the SH3 binding domain that is necessary for actin localization. Since the RLE/RLE motif is located near the N terminus

of many CAP proteins, it is possible that the predicted start of the protein is incorrect and the motif is present but is located further upstream. Additionally, absence of this motif as well as the typical SH3 binding consensus may be due to sequencing errors. Potenitially sequence variability is another reason the SH3 binding domain consensus PXXP is not observed in the predicted protein sequence. Cesareni *et al.* (2002) reviewed several exceptions to this consensus and suggested that there is more diversity among SH3 binding domains than previously thought. Therefore, *cap1* in *U. maydis* may bind to SH3 domains. If the RLE/RLE motif and/or SH3 binding domain is not present in *cap1*, then the absence of this region(s) may indicate that it is not necessary for protein function in *U. maydis*. While homology exists among the CAPs of many different organisms, there is functional divergence. For example, deletion of the gene in *S. pombe* causes increased mating and sporulation but does not in *S. cerevisiae* (Kawamukai *et. al*., 1992). In *U. maydis*, this protein may be responsible for another phenotype.

Predicted interactions with cAMP and actin makes Cap1 an interesting protein to explore in the dimorphic plant pathogenic fungus that depends on the cAMP signaling pathway for virulence and filamentous growth. In *C. albicans*, mutation of both CAP1 alleles prevents germ tube formation and filamentous growth under standard conditions and prevents disease. It is unknown what the role of Cap1 has in *U. maydis* especially since two commonly found motifs are not present in the putative protein sequence. Additionally, the role of cAMP in *C. albicans* is different than *U. maydis* because this human pathogen requires cAMP for filamentous growth. Once the cosmid clone is identified and the complete sequence determined, functional studies will reveal the importance of Cap1 in dimorphism and pathogenicity of *U. maydis*.

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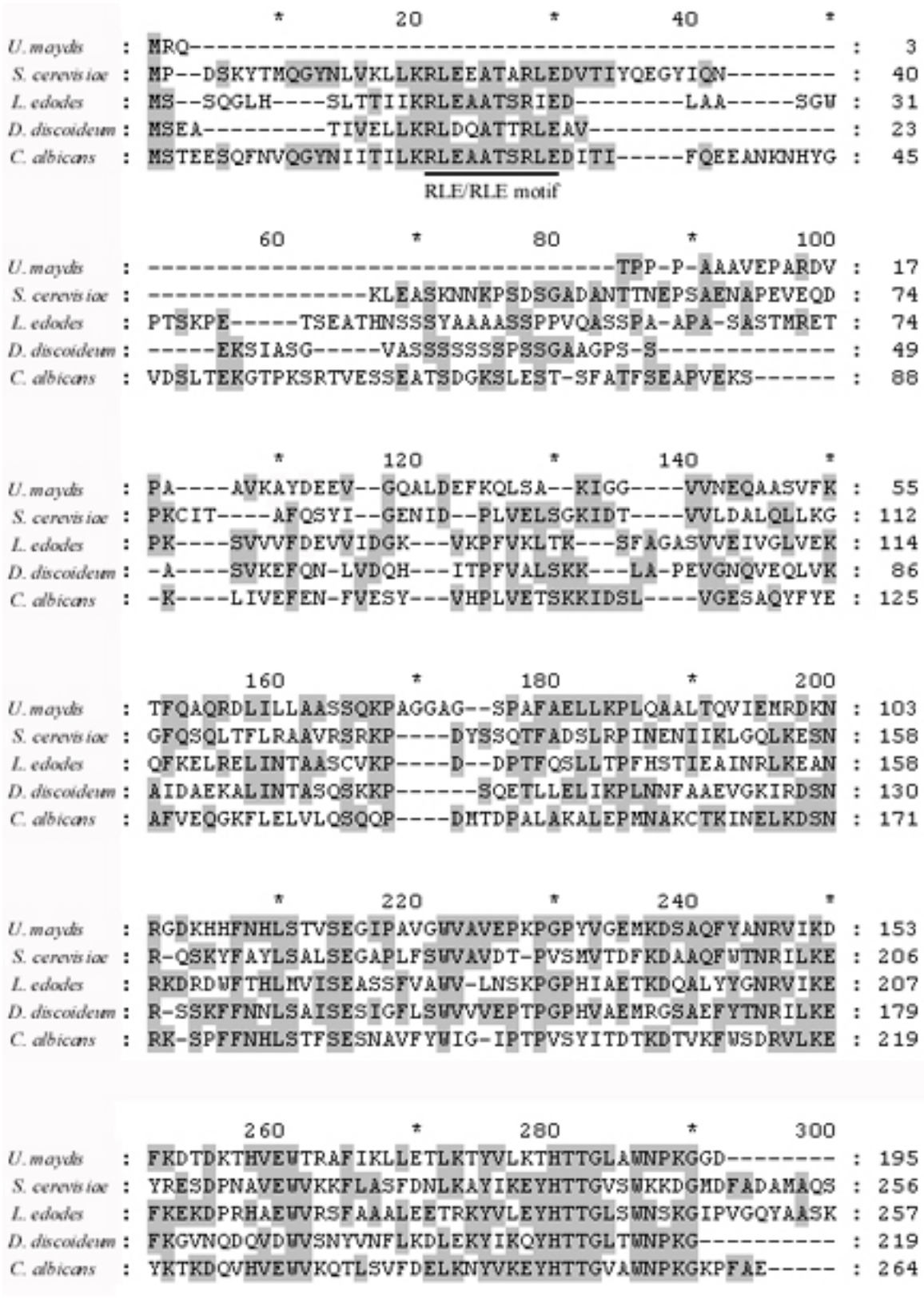
Figure 4.1. Comparison of the putative CAP protein sequence from *U. maydis* to *S. cerevisiae* (accession number NP_014261), *Lentinula edodes* (accession number BAA26002), *Dictyostelium discoideum* (accession number P54654), and *C. albicans* (accession number AAD42978). Identical amino acids are shaded. Identities are not shown when two pairs of amino acids are present at the same location. The RLE/RLE motif, Proline rich region, and SH3 binding domain have been underlined according to the *C. albicans* sequence. Sequences were manually aligned using GeneDoc Multiple Sequence Alignment Editor and Shading Utility version 2.5.000.


RLE/RLE motif
U. maydis
S. cerevisiae
L. edodes
D. discoideum
C. albicans

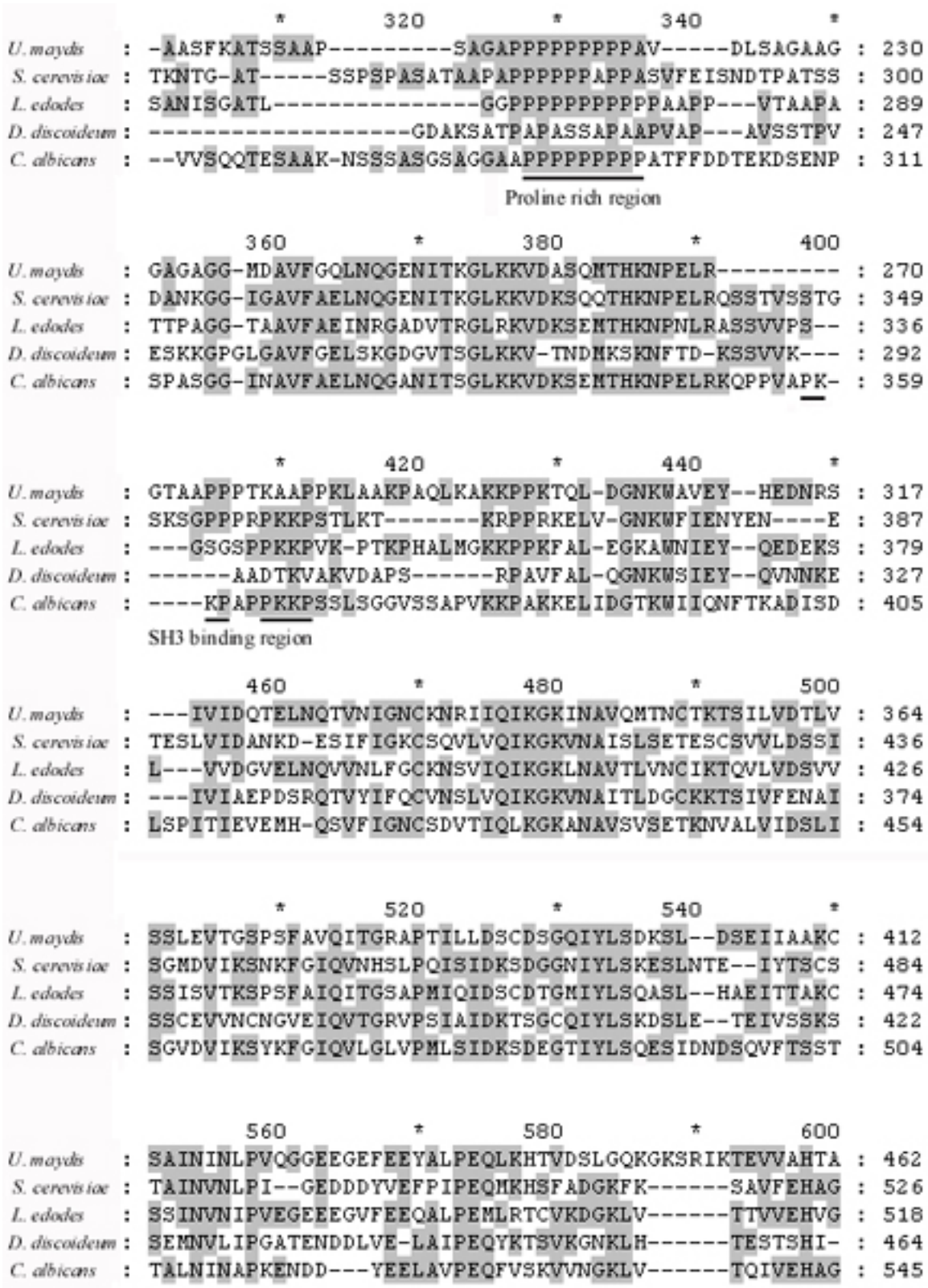
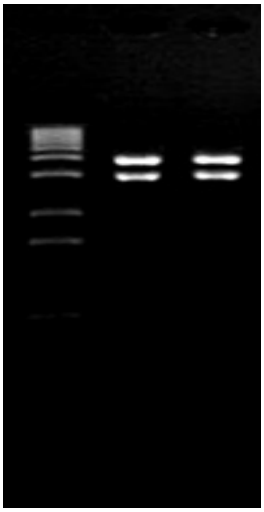

Proline rich region
SH3 binding region

Figure 4.2. Digestion of plasmid DNA from two CAP Topo clones with *Eco*R I. On this agarose gel stained with ethidium bromide, a 1kb ladder (Gibco, BRL) was included as a size comparison (lane 1) for the two clones (lanes 2 and 3). As predicted, digestion with *Eco*R I yields two bands of 3.1 and 3.9 kb representing the inserted fragment and vector, respectively.


1
2
3

CHAPTER 5

CONCLUSION

Genetic investigations on the bud-hypha transition of the phytopathogen, *Ustilago maydis*, have identified genes necessary for dimorphism as well as pathogenicity. Many of these genes have been found to encode members of the cAMP signaling and MAP kinase pathways. A few examples include *ubc3*, *ubc4*, and *ubc5*, which are required to cause wild-type disease symptoms (Mayorga and Gold, 1998; Andrews *et al*., 2000; Müller *et al*., 2001, abstract). The purpose of the research presented in this thesis was to investigate additional genes involved in dimorphism to discover other genes important for virulence. Specifically, the objectives of this study were to analyze a collection of filamentation suppressor mutants, characterize the morphologically regulated gene *uhf1* (upregulated in hyphal form), and begin characterization of *cap1* (cyclase associated protein) in *U. maydis*.

The analysis of 29 filamentation suppressor mutants was conducted to determine the likelihood of finding new genes in a previously made collection of mutant strains termed *ubc* (*Ustilago* bypass of cyclase) (Mayorga and Gold, 1998). These spontaneous or UV-induced mutant strains were derived from a constitutively filamentous adenylate cyclase mutant strain. Each of the suppressor mutant strains were independently transformed with six known suppressor genes, *ubc1*, *ubc2*, *ubc3*, *ubc4*, *ubc5*, and *hgl1*, and evaluated for complementation. Filamentous growth was restored to 20 mutant strains. The remaining strains were either recalcitrant to standard protoplasting methods or had reverted back to filamentous growth. Overall, these data suggest a low probability of

finding new genes important for dimorphism using this technique to evaluate the *ubc* mutant strain collection.

The morphologically regulated gene, *uhf1*, was previously identified in a subtractive hybridization assay conducted to discover genes upregulated during filamentous growth. Using a gene that confers carboxin resistance, *uhf1* was disrupted to determine its function in terms of morphology, mating, and pathogenicity. Functional analyses of *uhf1* suggest that this gene is not necessary for mating, disease development, or teliospore production, but is responsible for maintaining a wild-type morphology, pigmentation, and growth rate. The gene also seems to have a role in virulence since two compatible mutant strains cause significantly less disease than two compatible wild-type strains. After the analysis of over 200 progeny, *uhf1* was found to be linked to the *a* mating type locus. Composed of 7 WD repeats, the protein encoded by *uhf1* is over 70% identical to *cpc2* of *Neurospora crassa,* and *cpcB* of *Aspergillus nidulans*. Expression of the *cpc2* gene in both of these organisms is influenced by amino acid levels (Müller *et al*., 1995; Hoffman *et al*., 2000).

The final objective of this research was to identify *cap1* in *U. maydis*. Conserved in many eukaryotes, the cyclase associated protein is important for adenylate cyclase activation and actin organization. It has also been shown to be involved in dimorphism and pathogenicity of the human pathogen *Candida albicans* (Bahn and Sundstrom, 2001). This protein in *U. maydis* may have a similar role and reduce the amount of disease. Using PCR a 3.1 kb fragment was amplified and partially sequenced to confirm the identity of *cap1*. This cloned product will provide a basis for the future deletion or

disruption of *cap1* to determine its function in terms of morphology, mating, and pathogenicity of the corn smut pathogen.

In conclusion, three different genetic approaches were used in this research to identify and investigate genes likely to be responsible for filamentous and infectious growth of the corn smut pathogen. First, complementation assays identified six *ubc* filamentation suppressor mutants that possibly have defects in undiscovered genes. Second, insertional mutagenesis of *uhf1* revealed that the gene is not essential for filamentous growth in the mating reaction or disease development, despite its upregulation in filamentous cells. Third, PCR identified *cap1* in the phytopathogen. Although further experimentation is necessary, these data help strengthen the knowledge of what genes are required for disease development. Ultimately, this knowledge should help lead to better control methods that apply to not only *U. maydis*, but also related fungal pathogens more difficult to study.

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