12-2011

Regulation of morphogenesis by ammonium transporters in Ustilgo maydis.

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REGULATION OF MORPHOGENESIS BY AMMONIUM TRANSPORTERS IN *USTILGO MAYDIS*

By

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M. Sc. Zoology, University of Delhi, India, 2003

Dissertation
Submitted to the Faculty of the
College of Arts and Sciences of the University of Louisville in
Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Biology, Division of Molecular, Cellular and Developmental Biology,
College of Arts and Sciences
University of Louisville
Louisville, Kentucky

December 2011

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A Dissertation Approved on

September 2, 2011

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DEDICATION

To my dearest parents

and

my loving husband

ACKNOWLEDGEMENT

I take this opportunity to express my sincere gratitude and affection to my mentor Dr. Michael H. Perlin. He has been a wonderful "*Guru*" all these years, guiding, supporting, understanding and encouraging me, as I travelled this phase of my life. I am thankful to him for assigning me a project that made me feel intellectually challenged each day as I unraveled my scientific curiosities, and nurtured the scientist in me. It would be befitting to say that this dissertation would not have been what it is without his support.

I would also like to thank my committee members Dr. Susanna Remold, Dr. Thomas Geoghegan, Dr. Awdhesh Kalia, Dr. Mark Running and my previous committee member Dr. Amy Massey for taking time off their busy schedules and going over my progress reports and yearly presentations. Their critical insights and valuable suggestions have been quite instrumental in channeling my research efforts in a positive direction.

I am deeply indebted to my previous and current lab members, Cau Pham, Charles (Ben) Lovely, David Myers, Su san Toh, Kavita Aulakh, Michael Cooper, Margaret Wallen, Janhavi Deshpande and Seth Adams, for the friendship, love and support that they have shown over the years. I also thank my dear friends Charu Agarwal and Kalyani Putty for the constant encouragement and support and most importantly the space that they have given me to let off some steam, when I needed to. I would also like thank the countless undergraduate students in the 'Goat Lab' for their companionship and

love. I am also thankful to the administrative staff, graduate students and faculty members of the Biology family at the University of Louisville who have contributed to this dissertation in various ways.

This is a great opportunity to express my respect and gratefulness to all my teachers from school onwards for being influential in shaping my personality and inspiring me to do well. I am also truly thankful to my friends, both inside and outside the university, who have been with me throughout the ups and downs in my life. I have cherished each and every moment I have spent with them during these years and would always be indebted to them

I am deeply indebted to my family for their sacrifices, unfaltering love, care, support and faith in me throughout my career. My gratitude to my parents A. G. Pothen and Mary P. Chacko, to my siblings Georgy and Jency and to my extended family is beyond words. Most importantly, I have reached here because of the support, encouragement and love of my best friend, my husband, Dr. Alex John. He had faith in me and instilled my confidence when I doubted myself. Thank you for sticking by me, when I was bitter and depressed and still loving me unconditionally.

Above all, I thank the lord almighty for providing me the strength to step strong, when I was weak, for the wisdom and a sound mind for completing my long journey. For, *I can do all things through Christ, who strengthens me* (Philippians 4:13).

ABSTRACT

REGULATION OF MORPHOGENESIS BY AMMONIUM TRANSPORTERS IN *USTILGO MAYDIS*

Jinny A. Paul

September 2, 2011

Many species of fungi undergo a dimorphic transition to switch from a unicellular yeast-like growth form to a filamentous growth form. *Ustilago maydis* undergoes such a transition, in response to successful mating events and subsequent host cues, to form a filamentous pathogenic form. *U. maydis* also undergoes haploid filamentous growth in response to several environmental cues including, but not limited to nutrient limitation, pH, lipids, among others.

Ammonium transporter proteins (Amts) play an essential role in controlling the dimorphic transition in response to nutrient limitation, specifically nitrogen in the form of ammonium. I demonstrate a role for the *U. maydis* Amts in controlling filamentation under nitrogen limiting and nitrogen replete conditions via signal transduction pathways. I show a functional connection between Amts and the signaling protein, Rho1 GTPase, in *U. maydis*. These experiments suggest that both the high and low affinity ammonium transporters physically interact among themselves and also interact with Rho1 under low ammonium conditions. Epistasis experiments suggest that interaction of high affinity Amt, Ump2, with Rho1 is important for the filamentous growth response. Over-expression of *rho1* reduced the filamentation by haploid cells under low ammonium

conditions. Additionally, *ump2* deletion further eliminated the filamentous growth by haploid cells over-expressing *rho1* under low ammonium conditions. Inferring from these data and the known role of Rho1 in affecting filamentation, I propose a model for Ump2 and Rho1 function in *U. maydis*.

Furthermore, I demonstrate the role of Ump2 in affecting filamentation depends on its expression levels. This study reveals that Ump2 as well as Ump1 expression alters the transcription of genes essential in the mating response pathway and in pathogenicity. Transcription of numerous other genes, some shown to be induced during filamentous growth and a few others, during growth of the fungus inside the host, are also affected by changes in the expression of *U. maydis* Amts. Interestingly, *ump1ump2* double deletion strains are attenuated in their ability to infect the host. These results suggest Amts sense the external environment and transmit information by directly or indirectly affecting one or more signaling pathways to control the morphogenetic fate of *U. maydis*.

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CHAPTER I

GENERAL INTRODUCTION

Fungi as Pathogens

Fungi have a major influence on humans and human related activities including vital functions in many ecosystems. For these and many other reasons fungi are considered to be one of the most important groups of organisms in the world (Mueller and Schmit, 2007). Although the fungal kingdom is estimated to contain over 1.5 million species, only about a 100,000 have been described so far (Hawksworth, 1991; Hawksworth, 2001). A majority of these fungal species are saprophytic but a number of them have been shown to be parasitic, requiring a plant or an animal host for completing their biological cycle. A little more than 400 species are known to cause disease in animals while about 15,000 are estimated to cause diseases in plants (Gonzalez-Fernandez et al., 2010; Hawksworth, 1992).

Plant pathogenic fungi have posed a grave threat to agriculture historically, causing devastating plant epidemics, and they continue to do so even today. For example, the Irish Potato Famine of 1845 to 1851, caused by *Phytopthora infestans*, resulted in the death of one million people from a total of 8 million inhabitants of the region and led another 1.5 million to flee the country. At present the total yield loss due to late blight of potato is estimated at US$2.75 billion each year in developing countries

[http://www.cipotato.org/potato/pests_diseases/late_blight]. Another example is *Magnaporthe grisea* which destroys enough rice in a year that could otherwise feed 60 million people (Dean et al., 2005). Many more plant pathogens (*e.g.*, *Ustilago spp.*, *Fusarium spp.*, *Erysipe spp.*, *Agrobacterium tumifecans*, *Aspergillus flavus*) pose serious threats to agriculture worldwide. The economic costs of infections caused by the *Aspergillus* genus is estimated at ~$45 billion in the US alone (May and Adams, 1997). An estimate of the annual crop losses due to pre- and post- fungal diseases alone stands at 200 billion euros, and an unparalled $600 million is spent annually in US alone on fungicides (Gonzalez-Fernandez et al., 2010). Besides these plant pathogenic fungi, the human pathogenic fungi, *e.g.*, *Cryptococcus neoformans*, *Candida albicans*, *Aspergillus fumigatus*, *Histoplasma capsulatum* and *Pneumocystis jirovecii,* pose a direct threat to human health.

It has been suggested that pathogenic fungi will become virulent as a result of lateral gene transfer, as evidenced in the case of gene transfer between *Stagonospora nodorum* and *Pyrenophora tritici-repentis* for *ToxA* (Friesen et al., 2006). The ability of pathogens, including fungi, bacteria, viruses, to adapt to existing and new conditions, overcoming disease resistance of hosts, and becoming resistant to newly applied chemical treatment is very high. Hence, understanding the biology of diseases, host-pathogen interaction and the expression and physiology of the pathogen are crucial for designing new control strategies.

Besides pathogenic fungi, non-pathogenic fungi like *Agaricus bisporus*, *Pleurotus ostreatus* and truffles, such as *Tuber melanosporum* are important agricultural products in and of themselves (May and Adams, 1997). Non-pathogenic members of the genus

Aspergillus are used in large scale production of citric acid and industrial enzymes like amylases, proteases, and lipases (May and Adams, 1997). Other uses of fungi include antibiotic production using *Penicillium chrysogenum*, the production of bread, beer and wine using *Saccharomyces cerevisiae*, bioremediation using white rot fungi, to name a few (Pointing, 2001). More importantly, fungi have fueled basic scientific research for deducing fundamental biological questions, as exemplified by studies conducted on *S. cerevisiae* and *Schizosaccharomyces pombe* which have contributed to the understanding of biological processes like cell cycle, homologous recombination, *etc.*

Ustilago maydis

The causative agent of corn smut, *U. maydis*, a basidiomycete, naturally infects only maize (*Zea mays*) and its close relative teosonite (*Zea mexicana*), suggesting a very narrow host range. Although the first reports of the smut fungus appeared in the 1730's, *U. maydis* was first described in 1815 by de Candolle [http://www.kew.org/plants-fungi/*Ustilago-maydis*.html] and has been studied by phtyo-pathologists for only over 100 years now.

In the US, yield loss due to *U. maydis* infection is below 2% and the infection rate is low because of the availability of disease resistant maize. However, even a loss of 1% itself is equal to $189 million per year as the value for maize is very high (Martínez-Espinoza et al., 2002). Moreover, depending on climate conditions the loss can increase to about 50%, and thereby pose a potential threat to humans; *U. maydis* is consequently listed as a potential bioweapon by the Ad Hoc Group of the Biological Weapons Convention of the USA (Madden and Wheelis, 2003). The fungus induced tumors can develop on any/or all the green parts of the plant, including stems, leaves, tassels and ears

(Bölker, 2001). The tumors develop as a result of proliferation of the fungal mycelia inside them. Minor infection by *U. maydis* results in the appearance of warts and/or chlorosis or discoloration of the leaves and corn stem, causing little or no harm to the plant. However, severe infection of the corn ear and/or stem leads to sterility or even death. Infection of the corn ear results in the enlargement of the corn kernels, leading to its sterility while tumor formation on the stem, leads to plant death. The enlarged corn kernel (galls) of sweet corn is considered a delicacy in Mexico, known as "huitlacoche" and appears in restaurants outside Mexico as "noveau cuisine". It is believed that the corn galls were used by the Aztecs in their diet as supplements, consistent with the fact that domesticated corn originated in Mesoamerica (Ruiz-Herrera and Martinez-Espinoza, 1998).

U. maydis maintains a biotrophic relation with its host thereby using the living tissue of the host for its proliferation and development (Mendgen and Hahn, 2002). It belongs to a group of fungi that specialize to undergo a dimorphic transition. The characteristic trait of dimorphic fungi is that they alternate between a saprophytic yeast-like form and a dikaryotic filamentous form (or vice versa). The saprophytic yeast-like cells are non-pathogenic, unicellular, cigar shaped cells, approximately 18 μm long and 5 μm wide (Pérez-Martín et al., 2006). These cells, until they come in contact with or sense the pheromones from opposite mating type, grow isotropically and reproduce mitotically by budding. After mating they undergo cytoplasmic fusion with the opposite mating type cell and switch to grow filamentously.

The ease of maintaining and culturing *U. maydis* cells on artificial plates under laboratory conditions, the availability of a sequenced genome of a highly compact,

relatively small size (20.5 million base pairs) containing approximately 6900 protein encoding genes, the accessibility of a well-established gene disruption and transformation system and a very efficient homologous recombination system makes *U. maydis* susceptible to genetic manipulations and a very attractive model to investigate host pathogen interactions (Holliday, 2004; Kämper et al., 2006). Furthermore, understanding of the cell biological processes in *U. maydis* reveal that in many respects it is more closely related to metazoans than to the ascomycetes. Moreover, *U. maydis* shares 20% identity over the entire proteins to humans. Therefore, *U. maydis* could serve as a good model system for investigating complex cellular processes (Steinberg and Perez-Martin, 2008).

Life Cycle of *Ustilago maydis*

In *U. maydis*, sexual development is interconnected to pathogenicity (see Fig. 1). Haploid cells which are saprophytic, multiply by polar budding and are unable to infect the host plant (Bölker, 2001). For a successful infection, haploid cells of compatible mating type have to fuse to form a filamentous dikaryon. The mating between the compatible/opposite mating types is triggered by detection of pheromones [described in detail in a later section]. The dikaryotic hyphae, formed as a result of the fusion of the conjugation tube from the opposite mating types, shows directed tip growth, until they are on the plant surface. Under such conditions the polar tip growth stops and the tip swells to form poorly differentiated, non-melanized appressoria (Brefort et al., 2009; Kahmann and Kämper, 2004). It is speculated that the formation of the infectious structures requires signals from the plant, however, a recent study determined that hydrophobic surface along with the presence of hydroxy-fatty acid can cause

filamentation and can initiate appressoria differentiation (Kahmann and Kämper, 2004; Mendoza-Mendoza et al., 2009). Although the appressorium is generated as a result of compression of cytoplasmic contents towards the apical region, it does not provide the mechanical force, as a result of turgor pressure build up, to penetrate the host as seen in the case of other pathogens like *Magnaporthe grisea* (rice blast fungus) and *Colletotrichum graminicola* (pathogen of corn and wheat) (Kahmann and Kämper, 2004). The filamentous hyphae are presumed to gain entry into the host plant with the aid of lytic enzymes secreted by the fungal cells.

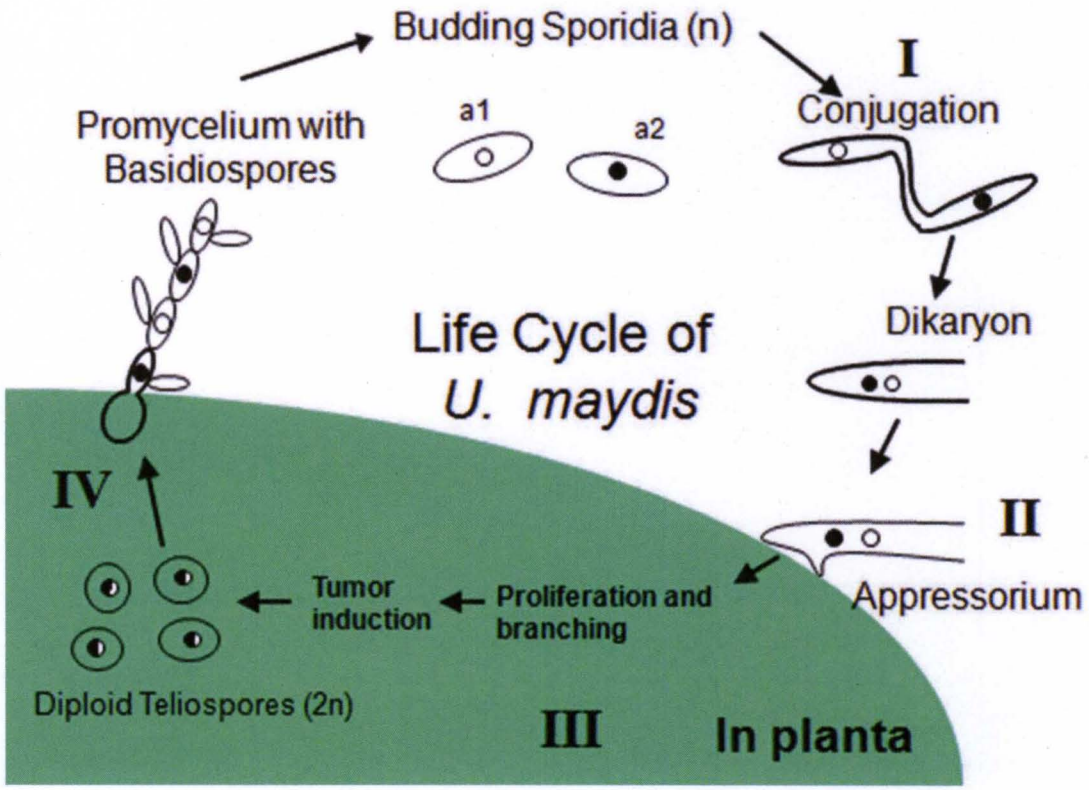

Figure 1: Schematic representation of the *Ustilago maydis* life cycle. Haploid sporidia can reproduce asexually by budding. Conjugation (mating) filaments form in response to pheromone perception, which fuse to form an infectious dikaryon which must penetrate the host plant to finish the life cycle. This is the beginning of sexual reproduction of *U. maydis* and requires the host plant for its commencement and completion. Diploid teliospores only form inside the host plant and once matured get released. Mature teliospores germinate and undergo meiosis and then mitosis to generate new haploid teliospores [reviewed in Banuett, 1995].

During penetration the infected host cell invaginates around the fungal hyphae and this continues until colonization of the epidermal layer (Snetselaar, 1993). This mode of growth continues until the formation of early tumors allowing fungal hyphae to spread into the mesophyll and vascular bundles (Doehlemann et al., 2008). This interaction zone between the plant and the fungus is thought to be involved in exchange of signaling molecule and uptake of nutrients by the fungus. At later stages of infection the hypha grows both intra- and intercellularly (Banuett and Herskowitz, 1996).

Although the fungal hyphae traverse the plant tissue, the plant tissue remains alive and no apparent host defense response is reported. Tumor development is associated with both plant cell enlargement and enhanced cell divisions. In the tumors the cells undergo karyogamy to form diploid hyphae, which proliferate and undergo sporogenesis (hyphal fragmentation, rounding up and differentiation to form melanized diploid teliospores) (Brefort et al., 2009). As the tumors mature, they dry up and rupture to release billions of diploid teliospores. These teliospores, when they land on unsuitable host and under unfavorable environmental conditions lie dormant. But under appropriate growth condition the diploid spores germinate, undergo meiosis to form a stalk known as the promycelium, from which the haploid cells bud off.

It is possible for haploid cells of opposite mating type to fuse and form dikaryotic hyphae in axenic cultures. The formation of the dikaryotic hyphae can be visualized on an agar plate containing activated charcoal. The mating event is evidenced by the presence of colonies that have a white fuzzy appearance resulting from the dikaryotic filament extending into the air (Banuett and Herskowitz, 1989). However, it is not possible to propagate the filaments further than the dikaryotic stage as the fungus needs

to establish and maintain a biotrophic relation with the host for the completion of its life cycle (Brefort et al., 2009).

Mating in *U. maydis*

Sexual reproduction in fungi has evolved in a way to develop genetic barriers to prevent selfing. The population is divided into two different mating types based on the genes that determine incompatibility between two cells. Ascomycetes, one of the two major groups of fungi, have only two mating types while the Basidiomycetes may have several thousands (Souza et al., 2003). These two major groups of fungi differ greatly in the number of genes and alleles present at the mating loci. In Ascomycetes, *e.g.*, *S. cerevisiae*, mating is bipolar, *i.e.*, there is one type of mating locus with two different alleles; the Basidiomycetes have a more complex system with both bipolar and tetrapolar mating systems (Casselton, 2002).

U. maydis is heterothallic in nature and must undergo mating to switch from a haploid budding cell to a filamentous dikayron. This switch is an essential strategy that the fungus undertakes to infect the host plant. Therefore mating is an essential part of pathogenicity as haploid cells are normally non-pathogenic. A tetrapolar mating system (illustrated in Fig. 2) is present in *U. maydis*, wherein the two different unlinked loci, govern self/non-self recognition, cellular fusion and post fusion cellular events (Banuett and Herskowitz, 1989). The two loci that determine the success of the mating event are the *a*- and the *b*- mating loci.

The *a*- type mating locus has two alleles *a1* and *a2*, which have been identified as idiomorphs. *a1* and *a2* each contain a large sequence of DNA, 4.5 kbp and 8 kbp respectively, with no DNA similarity to each other and small parts of the non-

homologous regions near the boundaries to the flanking sequence containing regions that are required for mating activity (Bölker et al., 1992; Froeliger and Leong, 1991). Analysis of these two idiomorphs revealed that they control cell mating and fusion of the haploid cell and that each encodes at least two open reading frames (ORFs) (Banuett, 1995; Ruiz-Herrera and Martinez-Espinoza, 1998). The two ORFs code for: 1) a precursor of lipoprotein mating pheromone a (mfa), displaying a characteristic sequence of prenylated proteins at the C-terminus and, 2) the G protein coupled pheromone receptor a (pra) (Bölker et al., 1992; Spellig et al., 1994).

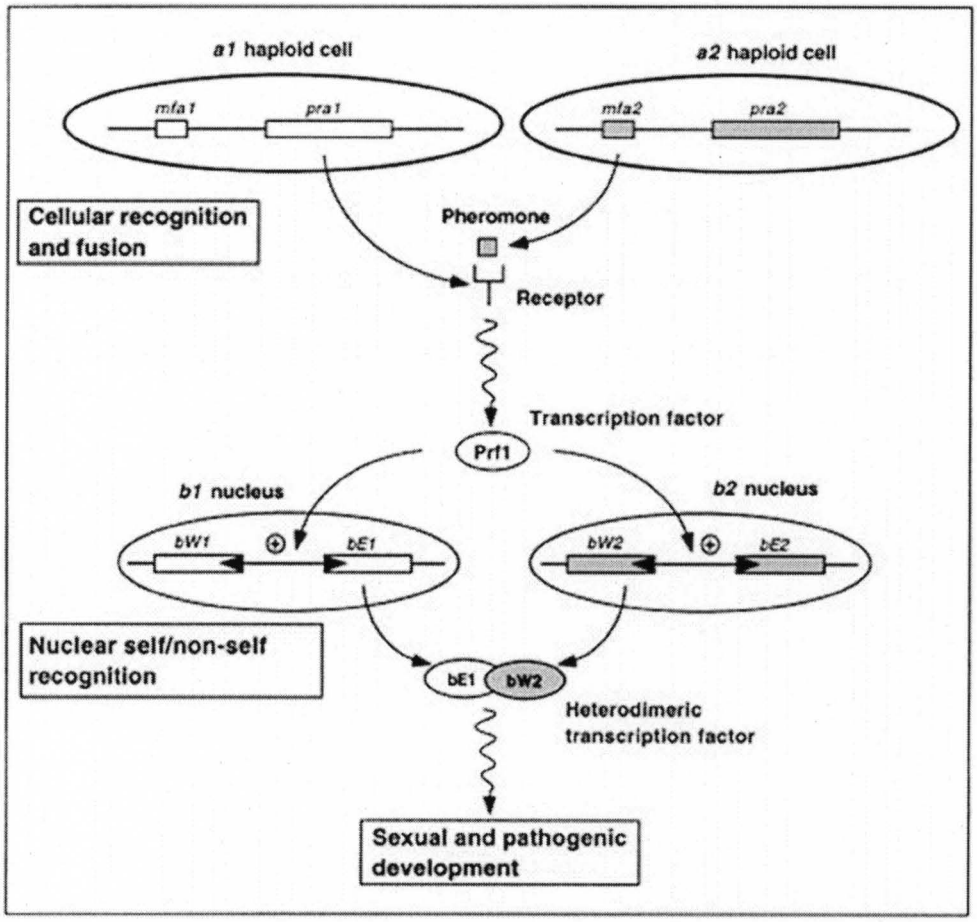

Figure 2: Interaction of the two mating loci in *U. maydis* (Bölker, 2001).

During mating, haploid cells carrying the *a1* idiomorphs synthesize a1 specific pheromone encoded by *mfa1* and the receptor for the a2 pheromone from *pra1*. Analogously, cells containing the *a2* alleles synthesize their pheromone from *mfa2* and the pheromone receptor from *pra2*. As a result of pheromone perception from the opposite mating type, the cells arrest in G2 phase and this leads to increased expression of pheromone and receptor. They subsequently grow filamentously by developing a conjugation tube which they extend to fuse with the opposite mating type, culminating in the formation of a dikaryon (Garcia-Muse et al., 2003). The dikaryon would be a dead end cell type due to cell-cycle arrest, but being present on the host, the plant-host signal releases the cell-cycle arrest (Nadal et al., 2008). This is unlike the case seen in *S. cerevisiae*, where the pheromone and pheromone receptor gene expression ceases after cellular fusion. In *U. maydis* a-mating locus is also essential for post fusion events. Sequence analysis of the *a2* idiomorph, identified two more ORFs *lga2* and *rga2*, (Urban et al., 1996). These proteins are shown to encode small mitochondrial proteins, important for correct mitochondrial inheritance (Fedler et al., 2009). The study by Fedler *et al* revealed that *lga2* and *rga2* of *a2* determine which parental mitochondria would be inherited by the daughter cells. This is achieved by selectively degrading the mtDNA of *a1*, the process being mediated by *lga2*, thereby encouraging uniparental mtDNA inheritance. This study affirms the role of the *a* locus in mtDNA inheritance. However, *lga2* is not essential for pathogenic development despite being a direct target for *b* transcription factor (Romeis et al., 2000).

Although the dikaryon is formed as a result of cellular fusion between cells of opposite mating types, its survival and growth depends on the product of the *b*-mating

locus gene products. In nature *b* loci are estimated to have as many as 25 alleles (Puhalla, 1970). The *b* locus encodes two different genes that are divergently transcribed to encode bE and bW homeodomain proteins, which form a heterodimeric transcription factor to act as a single genetic unit (Gillissen et al., 1992). The bE and bW proteins contain 410 and 626 amino acid proteins, respectively, and are involved in recognition of self/non-self during mating. The functional heterodimer between bE and bW would form only if they are derived from different alleles, *i.e.*, bE1 would interact with bW2, bW3 *etc* but not with bW1 (Kämper et al., 1995). Although the organization of the bE and bW are similar, consisting of a variable region in the amino terminal region and a conserved region in the remaining region of the protein, they do not exhibit any kind of sequence similarity (Banuett, 1995). Kämper et al (1995) suggest that the highly variable stretch of about 100 amino acids at the N-terminus is responsible for the allele specific recognition system. Although the b transcription factor can form as a result of heterodimerization between, for example bE1 and bW1, cells where only this occurs cannot infect the host plant as the transcription factor is not active and the strain is not pathogenic to the maize plant. Thus, the bE/bW heterodimer is the master regulator that controls the expression of genes that are required for subsequent pathogenic development. This is confirmed by the fact that a haploid cell constructed to carry a chimeric *b* locus consisting of bE and bW from different mating types, is able to infect the host and complete the sexual life cycle (Bölker, 1995). DNA microarray analysis suggest that the bE/bW heterodimer differentially regulates the expression of more than 246 genes, most of which are indirect targets of the transcription factor and many of these genes are shown to be essential for pathogenesis (Feldbrügge et al., 2004; Kahmann and Kämper, 2004).

Factors Affecting Morphogenesis and its Regulation

Morphogenesis is a dynamic feature associated with the growth and development of fungi. Fungal cells display varying growth forms, either yeast-like or filamentous, while others have the ability to switch from yeast-like to filamentous form. In contrast to the yeast-like cells which reproduce by budding and undertake isotropic growth, the filamentous growth is associated with apical growth extension. Although the crosslinking between the fungal cell wall components (β-1,3-glucan, mannoproteins and chitin) make the cell wall very rigid and restricts cell growth, cells secrete lytic enzymes such as chitinase and glucanase, which help soften the cell wall and thereby aid in growth at defined regions of the cell (Adams, 2004; Smits et al., 2001). Therefore, reprogramming of the cellular machinery needs to be accurately coordinated during the morphogenetic switch. Understanding the mechanism and regulation of the switch between different morphogenetic stages is important as it is a virulence determinant. Plant pathogen *U. maydis* and animal pathogens such as *C. neoformans* and *C. albicans* must switch from budding to filamentous form in order to cause disease (Sánchez-Martínez and Pérez-Martín, 2001; Wickes et al., 1996).

Morphological switch is triggered by a number of signals. For example, non-pathogenic, morphogenetic transitions are undertaken by *S. cerevisiae* in response to different extracellular signals. The isotropically growing haploid cells would switch to form a shmoo (change in shape to become elongate and pear shaped) in response to pheromone from the opposite mating partner (Pruyne and Bretscher, 2000). Furthermore, diploid *S. cerevisiae* cells switch from unicellular yeast form to a pseudohyhal form (cells elongate, fail to abscise following cytokinesis and remain attached to form chains of

cells) under nitrogen limiting conditions; whereas in the case of haploid cells, nitrogen limitation would promote invasive growth on the solid media (Lorenz and Heitman, 1998). Such transitions in growth form might help the cells forage for nutrients under adverse conditions. In a similar way, *U. maydis* undergoes a dimorphic switch in response to mating interactions, or upon sensing environmental conditions like low ammonium, acidic pH, and lipids (Klose et al., 2004; Martinez-Espinoza et al., 2004; Smith et al., 2003). Moreover, *U. maydis* cells, diploid, dikaryotic or haploid, with heterozygous *b* mating type locus, form infectious filaments when they encounter a suitable host, suggesting the importance of host cues in causing the dimorphic transition (Klosterman et al., 2007). Although filamentous growth is seen with respect to different cues, all the filaments are not functionally equal and are not effective in causing the disease state. Involvement of the two important signal transduction pathways, mitogen activated protein kinase (MAPK) pathway and the protein kinase A (PKA) pathway, has been implicated in regulating mating, integrating the different environmental cues, and in controlling morphogenetic transition, thereby causing the disease states.

MAPK Pathway

The MAPK pathway is conserved from yeast to humans and consists of MAP kinases belonging to a family of serine/threonine protein kinases involved in transducing a variety of signals. In fungi, MAPK have been implicated in regulating mating, filamentous growth, cell integrity and stress response; see Fig. 3, (Tudzynski and Sharon, 2003). The pheromone receptors in both *S. cerevisiae* and *U. maydis* are coupled to a trimeric G protein. Mating between opposite mating types produces a pheromone that is perceived by the pheromone receptor of the other mating type. The binding of the

pheromone to the pheromone receptor causes the heterotrimeric G protein to disassociate into Gα and Gβγ subunits. The liberated Gβγ subunit in turn activates the MAP kinase signaling pathway. More than one Gα protein is encoded in the genome of *S. cerevisiae* and *U. maydis*. *S. cerevisiae* has two isoforms of Gα, Gpa1 and Gpa2. Gpa1 has been found to regulate mating/pheromone sensing through the MAPK pathway while Gpa2 regulates nutrient sensing *via* the PKA pathway. *U. maydis* encodes 4 α subunits for the heterotrimeric G protein (Gpa1 through Gpa4); of these, Gpa3 was the only one shown to affect mating and filament formation. Though earlier implicated as being involved in the pheromone response pathway, Gpa3 is closely related to Gpa2 from *S. cerevisiae* and seems to control nutrient sensing via the cAMP-PKA pathway (Lengeler et al., 2000).

The MAPK cascade consists of a series of kinases, MAPKK kinase (MAPKKK/MEPKK), MAPK kinase (MAPKK/ERK kinase [MEK]) and MAP kinase (MAPK or ERK) (Bardwell, 2005). Each kinase in the pathway is activated by phosphorylation by the previous kinase. During mating, as a result of pheromone perception, the Gβγ complex binds to Ste20, a p21-

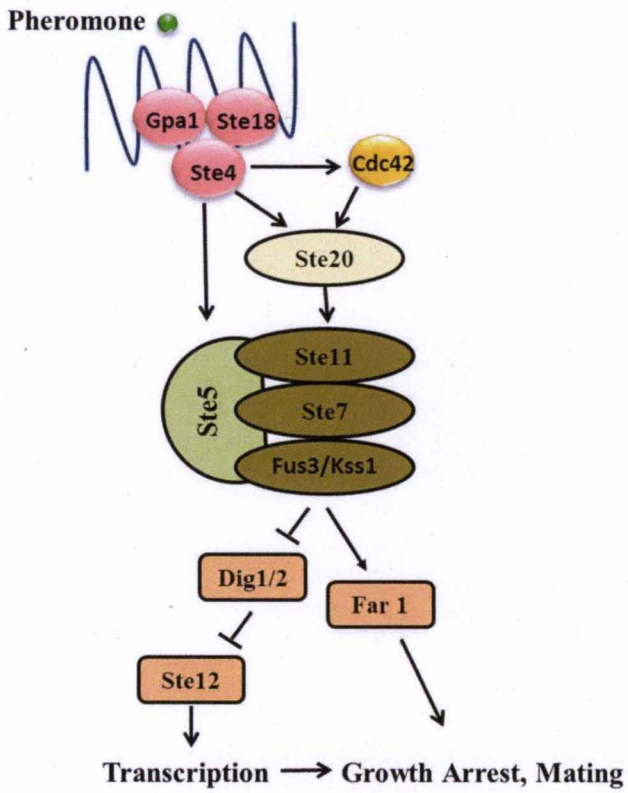

Figure 3: Illustration of the *S. cerevisiae* MAPK signal transduction pathway. Cell growth is arrested and cells undergo mating as a result of pheromone sensing. See text for a detailed description.

activated kinase and to Ste5, the adaptor protein that holds the different modules of the MAPK together. The binding of Gβγ to Ste20 facilitates the interaction between Ste20 and the MAP kinase signaling cascade at the plasma membrane. Ste20 phosphorylates MAPKKK (Ste11) to activate it. This in turn phosphorylates and activates MAPKK (Ste7), which phosphorylates and activates either MAPK (Fus3) or MAPK (Kss1). Both Fus3 and Kss1 repress the activity of the transcription factor Ste12 by being bound to it in their inactive state. Activated Fus3 and Kss1 leads to phosphorylation of Ste12 and Dig1/Dig2 (negative regulator of Ste12), thereby releasing the negative regulation on Ste12, allowing it to transcribe pheromone response genes (Bardwell, 2005). The MAPK pathway is also involved in filamentous growth in response to nutrient limitation. The core components remain identical to the pheromone response pathway with the exception that several different transmembrane receptors are necessary for initiation of the filamentous response. Although the signal follows an identical path, it is only Kss1 that is activated. To activate the expression of genes involved in the filamentous response pathway, Ste12 associates with another transcription factor Tec1p (Chen and Thorner, 2007).

U. maydis shares a MAPK pathway similar to that seen in *S. cerevisiae*. As a result of pheromone stimulation of the pheromone receptor, the signal gets relayed onto the MAPK pathway and this is accomplished by the trimeric G-protein, possibly via interaction with a Ste20 homologue (Smu1; (Smith et al., 2004)). The MAPK pathway in *U. maydis* consists of the MAPKKK (Kpp4 or Ubc4), the MAPKK (Fuz7 or Ubc5) and MAPK (Kpp2 or Ubc3 and Kpp6). Kpp6 shows functional redundancy to Ubc3 for mating but is shown to be essential for pathogenicity (Brachmann et al., 2003). Other

auxiliary proteins are also important for the functioning of the pheromone response pathway.

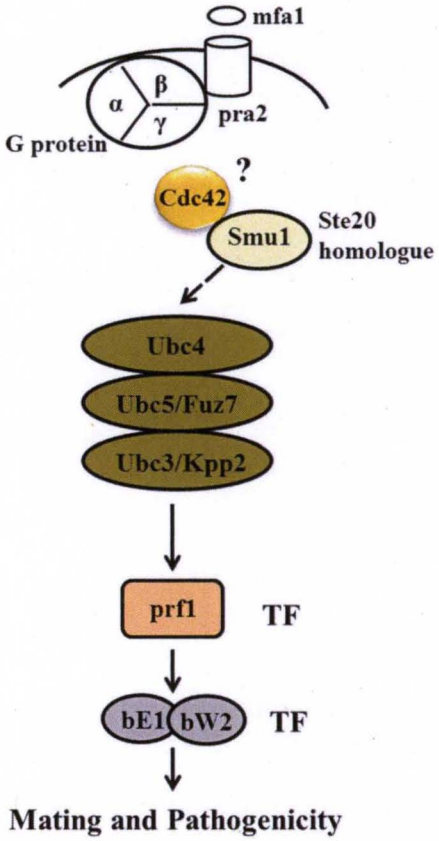

Figure 4: Illustration of MAPK signaling cascade in *U. maydis*.

The MAPK stimulates a downstream transcription factor in *U. maydis*, similar to the observation made in yeast. Transcription factor Prf1 (see Fig. 4) has been identified in *U. maydis* and is activated by the MAPK Ubc3 (Kaffarnik et al., 2003). Prf1 has phosphorylation sites for both MAPK and the protein kinase A (see below). Phosphorylation by either the MAPK or PKA pathways determines which transcriptional program is affected. Induction of *a*- genes requires intact phosphorylation sites on Prf1 for PKA, while induction of *b* genes requires intact PKA and MAPK sites on Prf1 (Kaffarnik et al., 2003).

cAMP-PKA pathway

The cyclic AMP-dependent Protein Kinase A pathway (PKA) works in parallel to the MAPK pathway and is important in controlling morphogenetic transitions resulting from nutrient limiting conditions. The core components of the PKA signaling pathway include the adenylate cyclase (AC) and the heterodimeric PKA module. The heterodimeric PKA module consists of a catalytic subunit and a regulatory subunit (a repressor of the catalytic subunit). In response to appropriate environmental cues, the G protein coupled membrane receptor activates adenylyl cyclase to make cAMP from ATP.

cAMP affects the dimerization of the PKA module. It binds to the regulatory subunit, causing the regulatory subunit to dissociate from the catalytic subunit. The catalytic subunit in its unbound state is active and can phosphorylate downstream transcription factors; see Fig. 5.

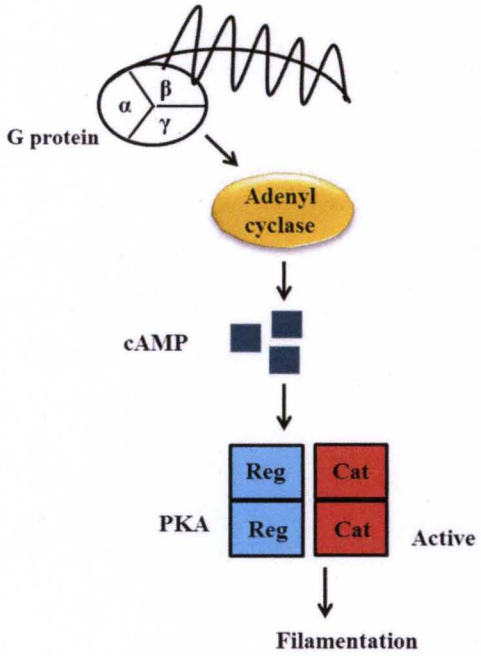

Figure 5: Generalized representation of cAMP signaling cascade.

In *S. cerevisiae*, the PKA regulatory subunit is encoded by a single gene, *BCY1*, while there are three genes encoding the catalytic subunit, TPK1, TPK2, TPK3 (Lengeler et al., 2000). Signals of nutrient availability are sensed by sensors for fermentable carbon source and nitrogen source and transmitted to the nucleus. The signal for fermentable sugar is perceived by GPCR system, involving the Gpr1 (G protein coupled receptor) and the Gα subunit (Gpa2) of the heterotrimeric G protein, which in turn activates the adenylyl cyclase leading to increase in cAMP levels (Lorenz et al., 2000). The cAMP levels are reduced by feedback regulation by Tpk1 and Tpk3 (catalytic subunits) and they have a negative control on the filamentation process (Nikawa J, 1987). cAMP levels are also controlled by phosphodiesterases, which decrease the level of cAMP in the cell. The PKA pathway appears to be important for nitrogen sensing in *S. cerevisiae*. Low ammonium conditions are sensed by Mep2 (ammonium transporter)

transceptor, which is proposed to act upstream of the G protein Gpa2, Ras2 and cAMP to control filamentation (Lorenz and Heitman, 1997; Lorenz and Heitman, 1998).

The *U. maydis* genome also contains genes for the components of the PKA pathway. The *U. maydis* genome contains one gene that codes for the PKA regulatory subunit (Ubc1) and two genes that code for the catalytic subunits (Adr1 and Uka1) (Durrenberger et al., 1998; Gold et al., 1994). However, the role of Uka1 is unclear, since its deletion does not result in phenotypes associated with a non-functional PKA pathway. The binding of cAMP to the regulatory subunit liberates the catalytic subunit to be active, causing the cells to have a budding morphology. A multiple budding phenotype was observed when the regulatory subunit of PKA was deleted, resulting in a constitutively hyperactive catalytic subunit (Gold et al., 1994). Alternatively, when the catalytic subunit is inactive, the haploid cells display filamentous morphology. The adenylyl cyclase is activated by the dissociated Gpa3 subunit of the trimeric G-protein, leading to increased cAMP levels (Lee et al., 2003). Intracellular pools of cAMP which affect the fate of PKA are also influenced by phosphodiesterases in *U. maydis* (Agarwal et al., 2010)

The PKA pathway, along with the MAPK pathway, regulates morphological changes associated with mating in fungi (Kronstad et al., 1998). Activation of the PKA pathway in *U. maydis* is required for the induction of pheromone responsive genes (Lee et al., 2003). The *U. maydis gpa3* deletion mutants (α subunit of G-protein) are sterile and displayed phenotypes similar to *uac1* mutant (adenylyl cyclase) (Krüger et al., 1998). Addition of exogenous cAMP reversed these morphological changes. Mating between strains mutated in the regulatory (*ubc1-1* allele) subunit is also greatly attenuated (Gold et al., 1994). Also, as mentioned earlier, activation of Prf1 by PKA is required for the

induction of *a*- and *b*-mating loci genes. Such evidence suggests that pheromone signal is also transmitted to the nucleus via the PKA pathway (Lee et al., 2003).

As observed in the case of *S. cerevisiae*, the morphological switch in *U. maydis* is also associated with nutrient limitation, specifically low ammonium (see Fig. 6). Under low ammonium conditions, in contrast to what is observed in *S. cerevisiae*, intracellular cAMP levels are low. This causes the regulatory subunit to be bound to the catalytic subunit of PKA, causing it to be inactive. The level of cAMP is hypothesized to be maintained by the ammonium transporter proteins Ump1 and Ump2 in *U. maydis*, Ump1 increasing and Ump2 decreasing the level of cAMP. However the mechanism by which this is achieved is still unclear (Smith et al., 2003). Although the levels of intracellular cAMP are different under low ammonium conditions in *S. cerevisiae* and *U. maydis*, the consequence is the same- filamentous growth. Therefore, it was proposed that pseudohyphal growth due to nitrogen limitation is dependent on the PKA pathway (Smith et al., 2003).

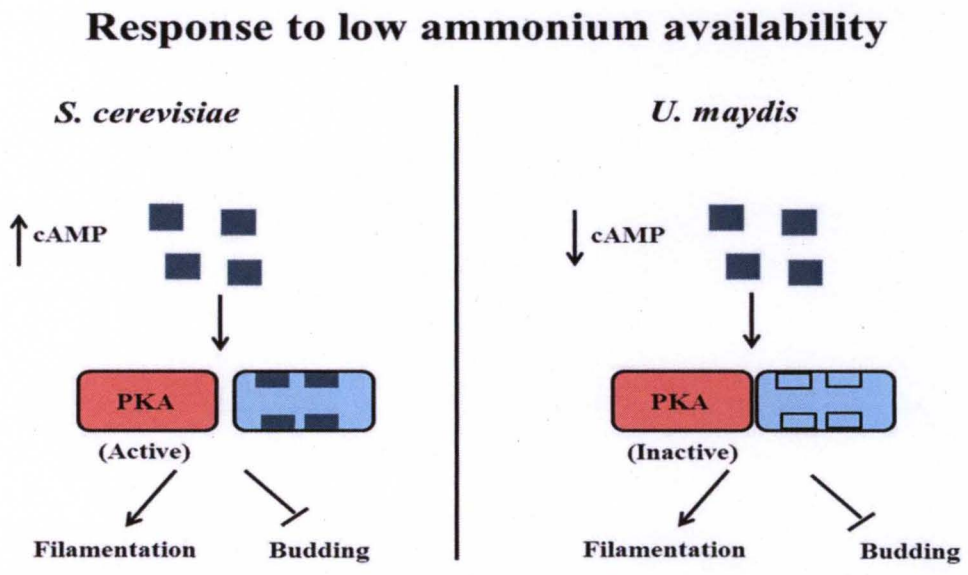

Figure 6: Response of cAMP-PKA pathway to low ammonium condition in *S. cerevisiae* and *U. maydis*.

Ammonium Transporters

Ammonium is an important player in nitrogen metabolism in bacteria, plants and animals. Though it is not the preferred nitrogen source in all organisms, nitrogen from other sources are transformed to ammonium before it gets incorporated into biosynthetic pathways (Wirén and Merrick, 2004). For many decades it was speculated that the flux of ammonia across the membranes, seen in many biological systems, could be attributed to free diffusion of NH_3. Studies conducted in plants to predict the direction and likelihood of NH_3 and NH_4^+ across membranes, concluded that cells would have evolved transporters for both substrates NH_3 and NH_4^+, thereby allowing cellular import and compartmentalization of ammonia (Wirén and Merrick, 2004).

Although the first idea of an active ammonium transporter was obtained from the fungus *Penicillium chrysogenum* in 1970, it was not until 1994 that a *S. cerevisiae* mutant was discovered that grew very slowly in minimal media containing less than 1 mM ammonium as the sole nitrogen source (Marini et al., 1994). Use of genomic and cDNA libraries of *S. cerevisiae* and *Arabadopsis thaliana* to complement the growth defect of the mutant *S. cerevisiae* strain led to the identification, respectively, of *S. cerevisiae* Mep1 and *Arabadopsis thaliana AtAMT1; 1*(Marini et al., 1994; Ninnemann et al., 1994). Since then, additional homologoues (designated Amt, for ammonium transporter, or Mep, methyl ammonium permease) have been identified in the major kingdoms of living organisms, representatives being found in eubacteria, archea, fungi, nematode, worms, insects and fish (Marini et al., 2006; Wirén and Merrick, 2004).

The presence of Amt homologues was extended to primates with the discovery of RBC Rhesus proteins (Rh) bearing marginal similarity (20-27%) to the Amt's at the

amino acid level (Cherif-Zahar et al., 1990; Liu et al., 2001; Marini et al., 1997; Marini et al., 2000). Later, non-erythroid Rh proteins were determined to be present in the kidney, liver and skin of human and mouse (Liu et al., 2001; Ludewig et al., 2001). Experiments suggest that the human Rh glycoprotein can function as an ammonium transporter and is important in maintaining the homeostasis of ammonium in the brain and other organs (Tremblay and Hallenbeck, 2009). Correspondingly, studies conducted on sea squirt (the closest invertebrate relative of humans sharing about 80% genes) show that absence of Amt affects brain development (Marino et al., 2007). Furthermore, the Rh proteins, specifically the RhAG (erythroid) and RhGK (non-erythroid from kidney), when expressed in the Mep mutant of *S. cerevisiae* were able to rescue the growth defect under low nitrogen conditions (Marini et al., 2000; Westhoff et al., 2004). Phylogenetic analyses revealed that Rh proteins are a subfamily of Amt proteins. Furthermore, Rh proteins are also present in the slime mold *Dictyostelium discoideum,* marine sponge *Geodia cydonium*, nematode *Caenorhabditis elegans* and fruit fly *Drosophila melanogaster* (Liu et al., 2001; Ludewig et al., 2001). Interestingly, both *C. elegans* and *D. melanogaster* contain both Amt and Rh proteins (Ludewig et al., 2001).

Topology of Ammonium Transporter Proteins

Amt proteins are encoded in most of the known genomes and in many cases multiple copies of the *amt* gene is present. For example, two genes encoding ammonium transporters are found in *U. maydis*, three in *S. cerevisiae* and ten putative *amt* genes are found in rice (Marini et al., 1997; Smith et al., 2003; Suenaga et al., 2003). Current data based on hydropathy profiles suggest that the common membrane topology of Amt/Mep proteins consists of 11 trans-membrane (TM) helices with an extracytoplasmic N-

terminus and a cytoplasmic C-terminus. The C-terminus is predicted to be about 30 residues in size but can comprise up to 150 residues in some cases; see Fig. 7 (Thomas et al., 2000; Wirén and Merrick, 2004). Bioinformatic analyses show that there are two distinct signature sequences present in TM helix 5 and TM helix 10 (Saier et al., 1999). Although, *E. coli* AmtB protein is predicted to have 12 TM helicies with the first TM being a signal peptide that gets cleaved off during assembly of the protein, the final protein monomer has 11TM helices (Khademi et al., 2004; Thornton et al., 2006; Zheng et al., 2004). It is suggested that the differences in the number of TM segments and the kinetics of NH_4^+ uptake in subgroups of the Amt superfamily could be an adaptation to the respective environments in which the organisms are found (Liu et al., 2001).

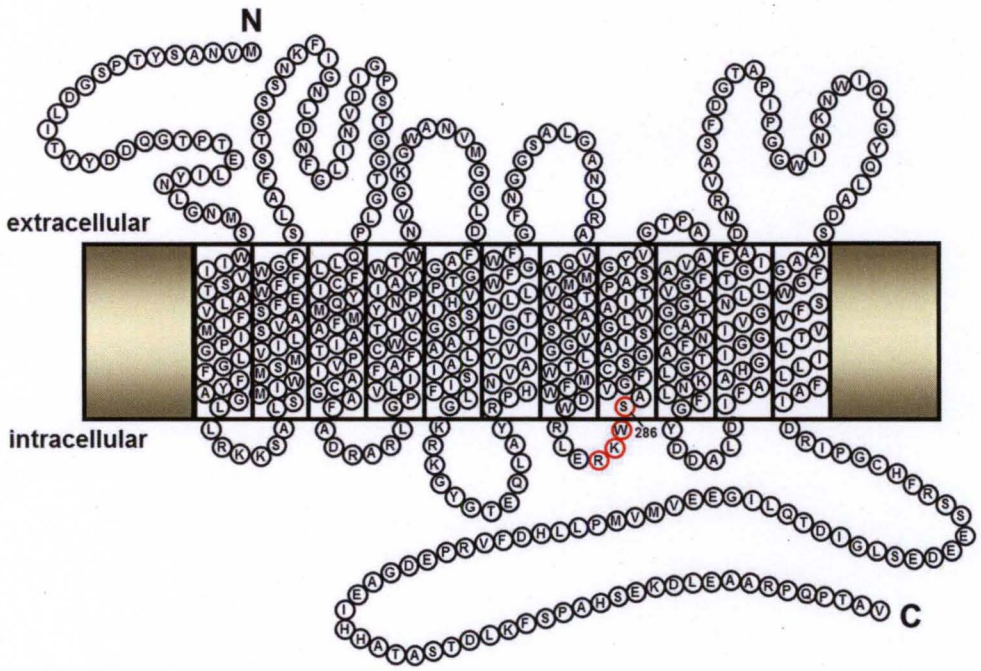

Figure 7: Predicted membrane topology of Ump2. The putative PKA phosphorylation site is highlighted in red. Courtesy: David Smith.

Mode of Action of Amt Proteins

Ammonium exists as a mixed species of NH_3 and NH_4^+, the ratio of which depends on the pH of the local environment. Therefore, which of the molecules is the substrate for the Amt/Mep transporters has been a controversial issue. The 3D structure of the Amt from *E. coli* suggests that the narrow substrate conduction channel is hydrophobic (Khademi et al., 2004; Zheng et al., 2004). The size and properties of the pore suggest that conduction of NH_3 is biologically relevant. Also, the transport of NH_4^+ would be energetically disfavored over NH_3 (Li et al., 2006). At least for bacterial AmtB protein, it is suggested that the NH_4^+ is deprotonated during entry into the pore and then re-protonated on the cytoplasmic side (Khademi et al., 2004; Merrick et al., 2006; Zheng et al., 2004). Studies conducted on the Mep proteins in *S. cerevisiae* suggest that proteins in the same family can display different fundamental mechanisms of transport. This study hypothesized, following the identification of important histidine residues affecting transport function depending on extracellular pH, that ScMep2 transports NH_3 following deprotonation of NH_4^+ as proposed for the bacterial Amt, whereas ScMep1 and ScMep3 could directly transport NH_4^+. Histidine to Glutamate substitution at position 194 and 168 in the *S. cerevisiae* Mep2 and the *E. coli* AmtB, respectively, allowed bypassing of the deprotonation event (Boeckstaens et al., 2008).

Ammonium Transporters in Fungi

Genes predicted to encode Amt proteins have been identified in all the sequenced fungal genomes. The fungal ammonium permeases cluster distinctly from that of animals, plants and bacteria in the phylogenetic tree created using protein sequences (Teichert et al., 2008). The tertiary and possibly the quaternary structure of the fungal/ all Amt

proteins is believed to be similar to the structure of *E. coli* AmtB and *A. fulgidus* Amt-1, as a high level of homology is observed within the Amt subfamily (Li et al., 2006). Fungi encode multiple Amts; there are three each in *S. cerevisiae*, *H. cylindrosporum* and four in *Neurospora crassa*. These multiple Amts can be divided into two groups based on their affinity for ammonium. In *S. cerevisiae*, the high affinity ammonium transporters ScMep1 and ScMep2 have K_ms for NH_4^+ in the range of <1 to 10 μM and the low affinity ammonium transporter ScMep3 has a K_m in the range of 1.4–2.1 mM. Among ScMep1 and ScMep2, Mep2 is the highest affinity ammonium transporter in yeast. Upon phylogenetic analysis of the Amt proteins, the distinct grouping of the high affinity and the low affinity suggests that proteins in the same affinity group may have common features (Wirén and Merrick, 2004).

Regulation of Fungal Amt Proteins

Although many nutrient permeases are regulated at the post-translational and transcriptional levels, no clear evidence of post-translational modification or protein interaction has yet been reported for the fungal Amts that suggests a possible mode of regulation. In *S. cerevisiae* only the high affinity ammonium transporter Mep2 is shown to be N-glycosylated, however the significance of this glycosylation is not evident and does not seem to be important for its function (Marini and Andre, 2000). A trans-dominant mutation of Mep1 is shown to inhibit the transporter function of Mep3 in *S. cerevisiae*, implying a possible interaction or regulatory effect of one ammonium transporter on the other (Marini et al., 2000). Although this interaction is not necessary for transport function of each of these transporters, it is observed that the sum of individual ammonium transporter activities is higher than the transport activity observed

in wild type cells, suggesting the possibility for cross regulation (Marini et al., 2000). Modulation of the ammonium permease, at least in the case of *S. cerevisiae*, by phosphorylation and dephosphorylation is suggested to be important for the sensing function associated with a subgroup of the fungal Amts. Mutation studies and phospho-mimicing experiments on the PKA phosphorylation sites identified in *S. cerevisiae* Mep2 and *U. maydis* Ump2 reveal that the phosphorylation status of this site is necessary yet not sufficient for the sensing function of these two proteins (Smith et al., 2003). The expression of *S. cerevisiae* Meps is also regulated by GATA transcription factors, which mediate nitrogen catabolite repression in this organism (Marini et al., 1997). Ammonium permeases from *A. nidulans* and *F. oxysporum* were also shown to be under nitrogen catabolite repression and the full expression of the Meps requires the GATA transcription factors (Monahan et al., 2006; Teichert et al., 2008). Although GATA binding sequences were identified in the sequence of the *U. maydis ump2* gene, GATA transcription factor/s still needs to be identified in this organism and their effects on the expression of Umps needs to be determined. Another likely regulator of the Mep proteins, in some fungi, is the Npr1 serine/threonine kinase. It is required for optimal uptake of ammonium when *S. cerevisiae* cells are grown under non preferable nitrogen sources (Dubois and Grenson, 1979). In *S. cerevisiae* it is observed that the ability to grow under low ammonium conditions is lost in the absence of this kinase (Boeckstaens et al., 2007). However the role of Npr1 kinase is variable on the two Mep proteins present in *C. albicans*. In this organism, Mep1 activity is unaffected in the absence of the Npr1 kinase while the activity of Mep2 is abolished (Neuhauser et al., 2011).

Ammonium Sensors and Signal Transduction

Diploid *S. cerevisiae* cells differentiate to grow as pseudohyphae under conditions of nutrient limitation, specifically for nitrogen. Investigations of the ammonium transporters in this organism revealed that the presence of the high affinity ammonium transporter, Mep2, is essential for the cells to undertake pseudohyphal growth as a response to low ammonium. However, the Mep2 mutant cells were not deficient in their ability to transport or metabolize ammonium. Thus it was proposed that Mep2 serves to transport ammonium and also acts as a nitrogen sensor to determine when conditions are conducive for pseudohyphal growth (Lorenz and Heitman, 1998). This morphological transition in yeast is connected to the two signaling cascades, MAPK pathway and the pathway involving Gα-Gpa2. In Δ*mep2* strains, that lack pseudohyphal differentiation, the Gpa2 was not activated under low ammonium conditions, and constitutively active Gpa2 was able to restore the pseudohyphal growth in *mep2* mutants, consistent with Mep2 being upstream of Gpa2 and thereby linking it to the PKA signaling pathway (Lorenz and Heitman, 1998). This suggested that Mep2 fulfills a sensing function by linking low ammonium availability to a morphological response (Wirén and Merrick, 2004). Interestingly, heterologous expression of high affinity ammonium transporters from other fungi like HcAmt1, HcAmt2 from *H. cylindrosporum*, Ump2 from *U. maydis* and Mep2 from *C. albicans* can rescue the pseudohyphal growth defect of Δ*mep2*. Therefore it was proposed that the sensing function of fungal Amts is a distinct property of the high affinity ammonium permease (Dabas et al., 2009; Javelle et al., 2003; Wirén and Merrick, 2004).

Ustilago maydis Ammonium Transporters

The *U. maydis* genome encodes two genes for ammonium transporters, namely *ump1* and *ump2*. Of these, Ump1 is the low affinity ammonium transporter and has a K_m of 5-10 mM for methylammonium, whereas the K_m of Ump2 for methylammonium is 15-17 μM and is the high affinity ammonium transporter (Smith et al., 2003). Haploid cells lacking Ump1 are still able to transport ammonium and are filamentous under low ammonium conditions; however cells lacking the high affinity ammonium transporter Ump2 are unable to sense the availability of low nitrogen source and therefore lack the ability to grow filamentously under these conditions. Nevertheless, the *ump2* mutant strains are unaffected in their ability to mate or to cause disease. Ump2 is also speculated to lower cAMP levels inside the cells. All the *U. maydis* mutants that lower the cAMP content (*i.e.*, *gpa3* (Gα subunit of the G-protein), *uac1* (adenylyl cyclase) or conditions that reflect this situation like mutation of the catalytic subunit of PKA, Adr1, grow filamentously (Durrenberger et al., 1998; Gold et al., 1994; Regenfelder et al., 1997)). However, filaments induced under such condition (low cAMP) do not render the haploid strains pathogenic, suggesting that the filaments formed under these conditions are functionally different than filaments formed after a successful mating event (Kahmann and Kämper, 2004).

Unlike *S. cerevisiae* cells that lack the ability to grow under conditions of low ammonium in the absence of all the three ammonium transporters, the double *ump* mutants of *U. maydis* were able to grow under ammonium limiting conditions, suggesting another transporter or mechanism that aids in growth (Smith et al., 2003). Morphological analysis of the Δ*ump1*Δ*ump2* colonies revealed that they produced filaments that were

unusual and had a tangled appearance. This atypical morphology was attributed to the aberrant cAMP levels in the absence of both the Ump1 and Ump2, implying that these proteins maintain cAMP concentrations at a certain level in the wild type cells. Furthermore, the connection of the *U. maydis* ammonium transporters to the cAMP pathway is also supported by the observation that the aberrant phenotype of the double *ump* mutant is eliminated upon supply of exogenous cAMP (Smith et al., 2003).

Research Interest and Hypothesis

Among the many aspects of host pathogen interactions, one of the less examined areas is the acquisition of nutrients by the pathogen during growth within the host. The ability of the pathogen to successfully invade and establish an infection would depend on the fitness of the pathogen in the host, which in turn would depend on the availability of nutrients for survival of the pathogen. *U. maydis*, being a biotroph, would have to manipulate the host to feed on its nutrients while maintaining live host tissue. Very little is known regarding the assimilation of nitrogen by the pathogen inside the host. It is speculated that nutritional status of the plant determines the transcription of the *in planta* induced genes of the pathogen. Snoeijers et al (2000) proposed that nitrogen limitation would be a key regulator in expressing the virulence genes in plant pathogens. Previous studies have confirmed that the *in planta* induced gene *mpg1,* required for pathogenicity, in *Magnaporthe oryzae* or the avirulence gene *Avr9* of the tomato pathogen *Cladosporium fulvum* is also up-regulated under conditions of nitrogen limitation (Ackerveken et al., 1994; Talbot et al., 1993). Studies have found that many genes up-regulated under nitrogen starvation were up-regulated during plant infections as well (Coleman et al., 1997; Divon et al., 2005; Donofrio et al., 2006; López-Berges et al.,

2010). Thus nitrogen seems to be an important cue for the expression of infection related genes.

Although a wide variety of nitrogen sources can be utilized by fungi, one of the preferred sources for nitrogen is ammonium. The *U. maydis* genome encodes two membrane bound proteins associated with the transport of ammonium. My interest is to improve the understanding of the ammonium transporters and their connection to signaling pathways and it stems from an earlier study conducted in our lab to identify and characterize the ammonium transporters in *U. maydis* (Smith et al., 2003). This study found that the high affinity ammonium transporter, in addition to transporting ammonium, also plays a role in sensing the availability of low ammonium in the surrounding environment and is required for a filamentous response to low ammonium. As mentioned previously, transition from the yeast-like budding form to the filamentous form is associated with causing disease on the host plant and is under the control of the two main signal transduction pathways. Additionally sequence analysis of the *ump2* gene predicted the presence of a PKA phosphorylation site in the protein; initial experiments conducted in yeast revealed the importance of this site in controlling the transport and sensing function of the high affinity ammonium transporter by the PKA signaling cascade. These observations suggested a connection between this ammonium transporter and at least one of the signaling pathways. In the present study, I have further shown that the ammonium transporter proteins physically interact with the signaling protein Rho1, providing a plausible conduit for their role(s) in controlling filamentation. Moreover, the roles of *U. maydis* ammonium transporters were investigated for their ability to modulate global transcription. In these analyses, I identified as differentially expressed, several

genes previously described to be important in causing pathogenicity on maize. To summarize, this study provides us with a greater understanding of functional aspects of fungal ammonium transporters, including factors that control their transcription, their effects on transcription of other genes involved in mating and pathogenicity and their physical interactions, both among ammonium transporters and with components of signal transduction pathway.

CHAPTER II

FUNCTIONAL HOMOLOGY OF *M. VIOLACEUM* AND *B. CINEREA* MEPS REVEALED THROUGH HETEROLOGOUS EXPRESSION.

SUMMARY

Proteins belonging to the Amt family are functionally conserved, participate in sensing the availability of nitrogen, specifically ammonium, in the environment, and transport this nutrient into the cell. The *Ustilago maydis* ammonium transporters and the *Saccharomyces cerevisiae* Mep homologues have been extensively examined and mutant/expression systems are well established in these model organisms. Using these two systems we were able to characterize functionally the Meps present in two other fungi, *Microbotryum violaceum* and *Botrytis cinerea*. Our experiments show that, while the MepA of *M. violaceum* does not rescue the filamentation defect of *U. maydis,*the second transporter, MepC, rescues the filamentation defect to some extent. Intriguingly, a complementation assay using the *B. cinerea* (ascomycete) Meps in *S. cerevisiae* (ascomycete) shows that, although all the three Meps were able to rescue the growth defect of the triple Mep mutant of *S. cerevisiae*, none of them were able to rescue the filamentation defect. However, all three Meps from *B. cinerea*, Mep1, Mep2 and Mep3 were able to rescue to the filamentation defect of the *U. maydis* (basidiomycete) *ump2* mutant to some extent. Our study suggests that the genes identified as ammonium

transporters in these two organisms undertake the function of ammonium transporters in both heterologous hosts and as sensors of low ammonium in *U. maydis*.

INTRODUCTION

Nitrogen is an important nutrient source in both unicellular and multicellular organisms for making cellular components and metabolites. The ability to detect and transport nitrogen into the cells is not only essential for survival but also for developmental processes. For example, several pathogenic fungi undergo a dimorphic transition from a yeast-like budding form to a filamentous form, the filamentous form being infectious. Pathogenic fungi like *U. maydis* exist in nature as haploid sporidia and in the presence of the opposite mating type cells and signals from the host plant undergo mating to produce infectious dikaryotic filaments, a prerequisite for invading the host plant. In the presence of abundant nitrogen and carbon, both *U. maydis* and *S. cerevisiae* haploid and diploid cells undergo budding (Smith et al., 2003). However, under nitrogen limiting conditions, both organisms undergo an alternative filamentous growth form that is not directly related to mating or the ability to infect host plants (*i.e.*, in the case of *U. maydis*). Interestingly, formation of conjugation tube and true filaments under the control of the mating loci is initiated under nitrogen starvation under laboratory conditions (Banuett and Herskowitz, 1994). Ammonium transporter genes like *MEP1*, *MEP2* and *MEP3* in *S. cerevisiae* and *ump1* and *ump2* in *U. maydis* are important for the uptake of ammonium as a nitrogen source (Marini et al., 1997; Smith et al., 2003). Moreover, Mep2 and Ump2 are capable of sensing low ammonium availability and transmitting this signal as a trigger for the dimorphic switch.

Ammonium transporter genes from *S. cerevisiae* have been well studied. The triple *mep* mutant of *S. cerevisiae* is unable to grow under conditions of nitrogen limitation (< 5 mM) (Marini et al., 1997). Deletion of any two of the *MEP* genes does not interfere with the ability to grow under low ammonium conditions; however, disruption of the Mep2 gene alone interferes with ability of the cells to grow filamentously. Similarly, *U. maydis* strains disrupted for *ump2* also fail to filament on low ammonium. This well established system has been used to identify and characterize proteins involved in nitrogen sensing and transport. In this study we utilize the Δ*mep1, 2, 3* mutant of *S. cerevisiae* and the Δ*ump2* mutant of *U. maydis* to explore functionality of the putative ammonium transporters from *Microbotryum violaceum* and *Botrytis cinerea.*

M. violaceum, commonly known as the anther smut fungus, is a pathogen of flowering plants belonging to the Caryophyllaceae family (Garber and Day, 1985). *M. violaceum,* like *U. maydis,* exists as haploid cells which undergo mating in the presence of the opposite mating type, accompanied by host signals leading to the formation of infectious hyphae. However, unlike *U. maydis* which undergoes mating under rich nutrient conditions, mating in *M. violaceum* occurs only under conditions of starvation, in particular, under low nitrogen availability. Two distinct Meps were identified from *M. violaceum* in our lab in an earlier study (Smith et al., 2003). To determine the possible role of these genes in sensing nitrogen levels in the external environment and transport of ammonium into the cell, MepA and MepC were expressed in *S. cerevisiae* triple Mep mutant which shows a defect in both growth and filamentation under low ammonium and the *ump2* mutant of *U. maydis* which shows a defect in filamentation under low

ammonium, in order to test possible functional homology with the respective transporters from these model fungi.

B. cinerea is a necrotrophic pathogen with a broad host range, attacking over 200 plant species (Williamson et al., 2007). It is the causal agent of the gray mold rot disease which affects many economically important flowers, fruits and vegetables (Zheng et al., 2000). Infection by *B. cinerea* is initiated by the conidia, which germinate on the host plant and must sense the availability of nutrients (Van Kan, 2006). One study found that the nutritional requirements for different biological isolates of *B. cinerea* from the same host were different (Cotoras et al., 2009). Typically the nitrogen source available for the pathogen is dependent on the host tissue that is colonized by the pathogen. For example, root pathogens might have access to different nitrogen sources than the leaf pathogens. Pathogens utilize different strategies for infecting their hosts. Some pathogens are specialized for attacking weak plants with slow growth and faster aging due to nitrogen starvation. Excessive nitrogen supply to the plant can increase its susceptibility to diseases. *B. cinerea* is among pathogens that stimulate the disease process due to increased nitrogen supply to the host plant (Snoeijers et al., 2000). Therefore these pathogens would have evolved mechanisms to sense and transport nitrogen in the various available forms. Three putative ammonium transporters, Mep1, Mep2 and Mep3, have been identified in *B. cinerea*. To functionally characterize the *B. cinerea* Meps, again two different heterologous hosts, *S. cerevisiae* and *U. maydis*, were utilized.

MATERIALS and METHODS

Strains and Growth Conditions. The *U. maydis* and *S. cerevisiae* strains used in the study are listed in Table 1. The *U. maydis* haploid strain um2h-2 (*a1b1* Δ*ump2::Hyg*^{R}) (Smith et al., 2003) and the *S. cerevisiae* diploid strain MLY131a/α (Δ*mep1::LEU2/*Δ*mep1::LEU2*Δ*mep2::LEU2/*Δ*mep2::LEU2*Δ*mep3::G418/*Δ*mep3::G418 ura3-52/ura3-52 MAT*a/α) (Lorenz and Heitman, 1998) were each used for complementation assay. *Escherichia coli* strains DH5α [Bethesda Research laboratories] and TOP10 [Invitrogen, Carlsbad, CA] were employed for all cloning purposes.

TABLE 1. Strains used in this study.

Strain	Genotype	Reference
S. cerevisiae		
MLY131a/α	Δ*mep1* Δ*mep2*Δ*mep3MATa/α*	*Lorenz, et al, 1998*
MLY131a/α-pYES-BcMep1	Δ*mep1* Δ*mep2*Δ*mep3MATa/α-Pgal bcMep1*	This Study
MLY131a/α-pYES-BcMep2	Δ*mep1* Δ*mep2*Δ*mep3MATa/α-Pgal bcMep2*	This Study
MLY131a/α-pYES-BcMep3	Δ*mep1* Δ*mep2*Δ*mep3MATa/α-Pgal bcMep3*	This Study
U. maydis		
um2h-2	*a1b1 ump2::hyg*^{R}	*Smith, et al, 2003*
um2h-2-MvMepA	*a1b1 ump2::hyg*^{R}*P*$_{otef}$*-MvmepA, cbxR*	This Study
um2h-2-MvMepB	*a1b1 ump2::hyg*^{R}*P*$_{otef}$*-MvmepC, cbxR*	This Study
um2h-2-BcMep1	*a1b1 ump2::hyg*^{R}*P*$_{otef}$*-Bcmep1, cbxR*	This Study
um2h-2-BcMep2	*a1b1 ump2::hyg*^{R}*P*$_{otef}$*-Bcmep2, cbxR*	This Study
um2h-2-BcMep3	*a1b1 ump2::hyg*^{R}*P*$_{otef}$*-Bcmep3, cbxR*	This Study

U. maydis cells were grown at 28-30° in YEPS (1% yeast extract, 2% each of peptone and sucrose with or without 1.5% agar). For *U. maydis* complementation, synthetic low ammonium media (SLAD) was used as a limiting nitrogen source. SLAD was made with 0.17% yeast nitrogen base (YNB) without amino acids or ammonium sulphate, 50 μM ammonium sulphate, 2% dextrose with or without 2% agar (Smith et

al., 2003). For yeast complementation, cells were plated onto SD medium (0.17% YNB, 1X amino acid drop-out solution and 2% agar) and the putative transformants were transferred to SLADG (0.17% yeast nitrogen base without amino acids or ammonium sulphate, 2% galactose and 0.2% glucose plus 50 μM ammonium sulphate) (Smith et al., 2003) and observed for growth and colony morphology for 5 days at 30°C.

Vector Construction. The *M. violaceum* MepA and MepC and the *B. cinerea* Mep1, 2 and 3 constitutive expression vector for *U. maydis* complementation were constructed by amplifying and cloning the ORFs for the respective genes into pCR2.1 TOPO [Invitrogen]. These ORFs were identified for *M. violaceum* from previous cloning (MepA; (Smith et al., 2003)) or via inspection of the draft sequence of the *M. violaceum* genome (http://www.broadinstitute.org/annotation/genome/Microbotryum_violaceum). For *B. cinerea*, the ORFs were used as these were determined from the cDNAs (R. Martín-Dominquez and E. Benito, personal communication). These ORFs were then excised and cloned after the P_{otef} promoter into the *Sfi1* and *Not1* sites of the p123 vector(Weber et al., 2006). These expression vectors were linearized using *Ssp1* before transforming the um2h-2 *U. maydis* strain, with selection for carboxin resistance via recombination at the *ip* locus (Brachmann et al., 2001; Loubradou et al., 2001). The pYES-MepA, pYES-Mep1, pYES-Mep2, pYES-Mep3 yeast expression vectors for the complementation experiment in *S. cerevisiae* were generated by PCR amplification of the ORF for the respective genes and cloning into pYES2.1/V5-His-TOPO [Invitrogen]. The pYES-MepC expression vector was similarly generated by PCR from *M. violaceum* genomic DNA and cloning into pYES2.1/V5-His-TOPO [Invitrogen, Carlsbad, CA].

RNA Isolation and Expression Analysis. RNA isolation from *U. maydis* and *S. cerevisiae* was performed using the TRIzol reagent protocol from Invitrogen, with modifications [Invitrogen, Carlsbad, CA]. *U. maydis* and *S. cerevisiae* cells were grown on agar plates for 2-5 days. The cells were scraped off the plates and homogenized under liquid nitrogen with a mortar and pestle. Roughly 100 mg of the homogenate was treated with 1 ml of TRIzol and processed further for RNA extraction. The RNA samples were treated with DNaseI [New England Biolabs, Ipswich, MA] before being used for RT-PCR. RETROscript kit from Ambion was used for RT-PCR using 1 μg of RNA as the starting material.

RESULTS

***M. violaceum* MepA is unable to rescue the filamentation defect of the *ump2* knockout mutant while MepC can partially rescue the filamentation defect.** An initial screening using degenerate primers yielded two distinct genes encoding Meps from *M. violaceum*. The entire DNA sequence of one of the putative transporters, MepA, was determined and was found to have a number of introns (Smith et al., 2003). The near complete sequence of the second Mep, MepC, was determined using a DNA walking kit [Seegene Inc., Seoul, South Korea] and the draft assembly of the *M. violaceum* genome sequence (http://www.broadinstitute.org/annotation/genome/Microbotryum_violaceum). Predicted sequence analysis of MepA and MepC revealed that both of these putative proteins have domains consistent with other ammonium transporters as determined by PROSITE (http://ca.expasy.org/tools/scanprosite/). Sequence comparison of MepA and MepC revealed that, MepA has 55% sequence identity to the *U. maydis* high affinity

ammonium transporter Ump2 at the amino acid level, while MepC has 54% identity to *U. maydis* Ump2 (See Fig. 8).

To gain insight into the function of MepA and MepC, we carried out complementation analyses in the heterologous hosts *U. maydis* and *S. cerevisiae.* Both the genes were constitutively expressed from the P_{otef} promoter in *U. maydis* lacking the *ump2* gene (this strain fails to filament when grown on SLAD (low ammonium) agar (Smith et al., 2003)). MepA when expressed failed to rescue the filamentation defect of the *ump2* deletion mutant (Fig. 9). We did not have access to the cDNA sequence of MepC, therefore the genomic copy was used for expression study. MepC showed partial rescue of the filamentation defect of the *ump2* mutant under nitrogen limiting conditions (Fig. 9). For expression in *S. cerevisiae*, MepA and MepC were cloned into the pYES yeast expression vector where the expression of the gene is under the control of the GAL1 promoter. MepA when expressed was able to rescue the growth defect of the *Δmep1Δmep2Δmep3* mutant but not the filamentation defect (Smith et al., 2003). However, MepC, when similarly expressed did not recue the growth defect (data not shown).

***B. cinerea* Meps are able to rescue the filamentation defect of the *ump2* knockout mutant to varying degrees.** Three genes encoding Meps *(bcmep1, bcmep2, bcmep3)* were identified and cloned from *B. cinerea* (R. Martín-Dominguez, personal communication). The predicted amino acid sequences of the three genes bear similarity to the Meps from *Aspergillus nidulans,* MeaA, MepA and MepB, respectively. They also show similarity to *U. maydis* Ump2 and to the *S. cerevisiae* Meps 1, 2, and 3 (Table 2). All the three Meps show variable expression under experimental growth conditions

(Martín-Dominguez et al., 2010). *B. cinerea* Meps were expressed in mutants of *U. maydis* and *S. cerevisiae* to determine whether they could functionally replace the missing transporters in these heterologous systems.

Mep1, Mep2 and Mep3 of *B. cinerea* were expressed from the P_{otef} promoter in the *U. maydis* strain lacking the *ump2* gene. All the three Meps were able to rescue the filamentation defect of the *ump2* KO mutant to varying degrees (Fig. 11). Mep1, Mep2 and Mep3 were also expressed from the GAL1 promoter present in the pYES yeast expression vector. None of the three Meps were able to rescue the filamentation defect of the triple mutant of Mep in *S. cerevisiae*, Mep1 showed the strongest response in rescuing the growth defect of the Δ*mep1, 2, 3* mutant of *S. cerevisiae*, while Mep2 and Mep3 did not rescue the growth defect as efficiently as Mep1 (Figs. 12 and 13). This difference in phenotype was not due of the difference in the expression levels of Mep1, Mep2 and Mep3 in *S. cerevisiae* compared with *U. maydis* (Fig. 10), as demonstrated by RT-PCR.

Figure 8: Sequence alignment of MepC with other known ammonium transporters. Sequence alignment using Clustal W (http://www.genome.jp/tools/clustalw/) showing the presence of conserved amino acid residues in MepC. Comparisons are made with the *M. violaceum* MepA (Accession No: AAD40955), *U. maydis* Ump1 (Accession No: XP_760670) and Ump2 (Accession No: XP_76036), and *S. cerevisiae* Mep1 (Accession No: NP_011636.1), Mep2 (Accession No: NP_014257.1) and Mep3 (Accession No: NP_015464.1)

	***U. maydis* Ump2**	***S. cerevisiae* Mep1**	***S. cerevisiae* Mep2**	***S. cerevisiae* Mep3**
***B. cinerea* Mep1**	50%	55%	45%	54%
***B. cinerea* Mep 2**	54%	50%	50%	49%
***B. cinerea* Mep 3**	47%	37%	44%	40%

Table 2: Percentage similarity between Amt proteins of *U. maydis*, *S. cerevisiae* and *B. cinerea*.

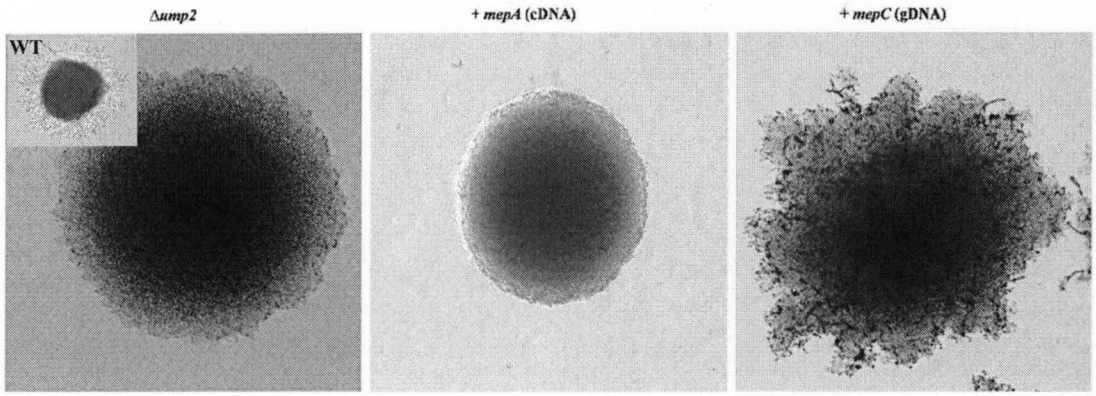

Figure 9: Expression of *M. violaceum* Meps in *U. maydis:* cDNA for *mepA,* and gDNA for *mepC* from *M. violaceum* were cloned into the P_{otef} vector and integrated at the *ip* locus in the the *ump2* mutant strain. Colony morphology, observed after 4 days of growth on SLAD at 30° C, shows that only *mepC* can partially rescue the filamentation defect of Δ*ump2* mutant. For comparison, inset show wild type (WT) grown under the same conditions as the mutants.

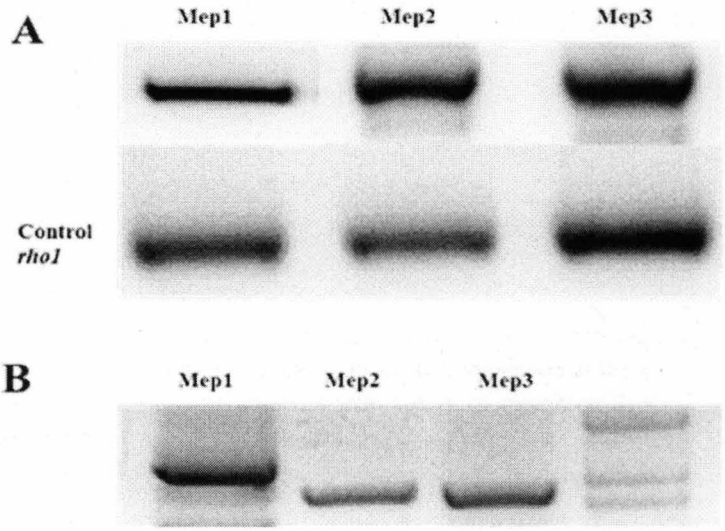

Figure 10: RNA levels of BcMep1, BcMep2 and BcMep3 in (A) *U. maydis* and (B) *S. cerevisiae.* RT-PCR was carried out on total mRNA isolated from *U. maydis* and *S. cerevisiae* strains transformed with Mep1 (1.6 kb), Mep2 (1.4 kb) and Mep3 (1.4 kb) from *B. cinerea,* to demonstrate that the three genes were expressed in the two heterologous hosts. For (A), image for Mep1 was from a separate agarose gel.

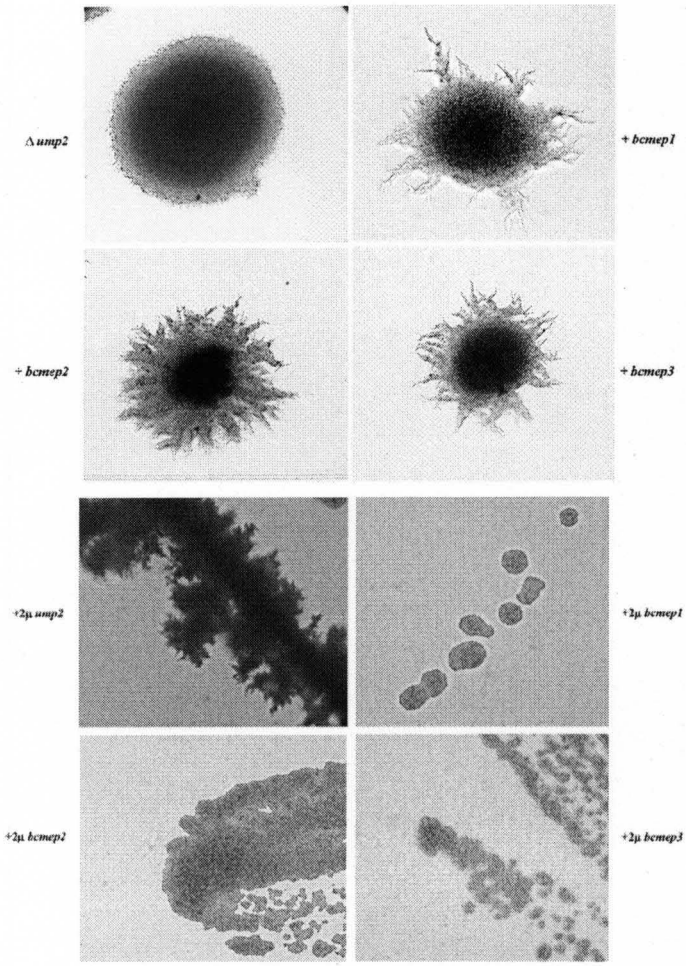

Figure 11: Expression of *B. cinerea* Meps in *U. maydis.* Each of the cDNAs for *mep1, mep2* and *mep3* from *B. cinerea* were cloned into the P_{otef} vector and integrated at the *ip* locus in the *ump2* mutant strain. Colony morphology, observed after 4 days of growth on SLAD at 30° C, shows rescue of the filamentation defect of the ump2 deletion mutant by all the three *B. cinerea* Meps to different extents.

Figure 12: Expression of *B. cinerea* Meps in *S. cerevisiae.* Each of the cDNAs for *mep1, mep2* and *mep3* from *B. cinerea* were cloned into the pYES2.1 expression vector (Invitrogen), as was that for *ump2* from *U. maydis.* Colony morphology, on SLADG was observed after 4 days of growth at 30°C, shows rescue of growth and filamentation defect by *U. maydis ump2* and only rescue of growth defect by the *B. cinerea* Meps.

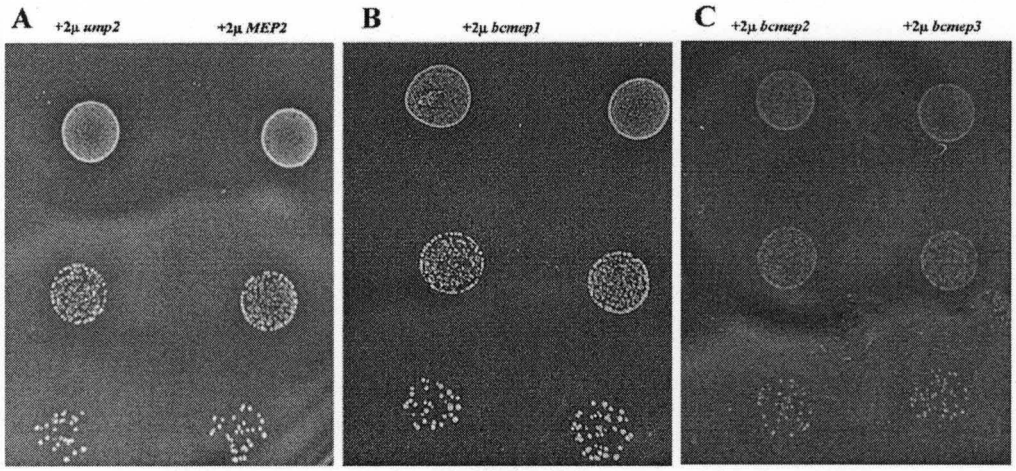

Figure 13: Expression of fungal Meps in *S. cerevisiae*. Only Mep1 from *B. cinerea* **(B)** was able to rescue the growth defect of the strain MLY131 a/α (Δ*mep1*Δ*mep2*Δ*mep3*) to the wild type level **(A).** *bcmep2* and *bcmep3* from *B. cinerea* were barely able to rescue the growth defect **(C).** To determine the growth morphology, the transformants were transferred to SLADG and observed for 4 days at 30°C and compared to the *ump2* from *U. maydis* and *MEP2* from *S. cerevisiae* **(A).**

DISCUSSION

The ability of many fungi to cause disease is attributed to the ability to switch from a budding yeast-like form to a filamentous form. Evidence suggests that one of the factors affecting this transition is the ability to sense the availability of nutrients, specifically nitrogen. Members of the ammonium transporter family have been identified from different realms of life including bacteria, fungi, plants and animals, and homologues have been identified in humans. These proteins are highly conserved and share a similar protein structure. The possession within a species, of multiple Amts, all with different kinetic properties, is not an uncommon phenomenon in fungi. For example, two Amts each have been identified from *A. nidulans* and *U. maydis*, while three each have been found in *S. cerevisiae*, *S. pombe*, *Fusarium fujikuroi* (Costanzo et al., 2001; Marini et al., 1997; Mitsuzawa, 2006; Monahan et al., 2002; Smith et al., 2003; Teichert et al., 2008).

The *S. cerevisiae* Mep1, Mep2 and Mep3 proteins have been well characterized. *S. cerevisiae* cells that lack all the three Mep proteins, do not grow well under conditions where ammonium is provided as the sole nitrogen source and its concentration is less 5 mM (Marini et al., 1997). This suggests that the Mep proteins function to transport limiting concentrations of ammonium to be used as a nitrogen source (Mitsuzawa, 2006). Although, Mep1p transports the majority of ammonium, Mep2p is the highest affinity ammonium transporter and Mep3p is a low affinity ammonium transporter. Moreover, under ammonium limiting conditions, Mep2p shows the highest level of expression (Marini et al., 2006; Marini et al., 1997). Typically, under conditions of nitrogen limitation, diploid *S. cerevisiae* cells differentiate to form pseudohyphae. Cells lacking

the Mep2p neither have any growth defect nor defect in their ammonium uptake ability under conditions of low ammonium. However, these cells lose their ability to differentiate to form pseudohyphae in low ammonium, but retain this ability when limiting concentrations of glutamine or proline are the nitrogen source (Lorenz and Heitman, 1998). This observation led to the proposal that Mep2 is the sensor of low ammonium, and this generates the signal required for pseudohyphae formation.

Structure-function analysis of different members of the Mep/Amt family continues to be conducted using the above-mentioned, well-characterized expression tool in yeast. Using yeast as the heterologous host, many of the newly discovered Mep/Amts were confirmed to function as ammonium transporters. One such example is the characterization of the Ump proteins of *U. mayids* (Smith et al., 2003). Also using this system, the sensor function of *S. cerevisiae* Mep2p was extended to Mep/Amt homologues from other fungi. Examples include Ump2 from *U. maydis*, Mep2 from *Candida albicans*, Amt1 from *Hebeloma cylindrosporum*, which are required for filamentous growth under low nitrogen conditions (Javelle et al., 2003; Smith et al., 2003).

In this study we investigated the role of the ammonium permeases present in plant pathogens, *M. violaceum* and *B. cinerea*. Smith et al (2003) reported the presence of two ammonium transporters in *M. violaceum*, MepA and MepC. Expression analysis of MepA and MepC under ammonium limiting conditions showed an up-regulation of both these genes, consistent with the expression of Mep2 from *S. cerevisiae* and *ump2* from *U. maydis* under low nitrogen condition (Hughes and Perlin, 2005). Hughes and Perlin (2005) further demonstrated the up-regulation of the Meps during mating. Although, no

reports are available yet for the specific role of ammonium permeases in mating in fungi, it is reported that low nutrient availability, specifically nitrogen, along with mating pheromones are essential for mating in *S. pombe* (Leupold, 1987). Furthermore, mating by *C. neoformans* is also induced by ammonium limitation and requires the high affinity ammonium permease, Amt2 (Rutherford et al., 2008).

Our investigation of MepA and MepC in the heterologous hosts *S. cerevisiae* and *U. maydis* revealed that, although MepA can rescue the growth defect of the Δ*mep1, 2, 3* mutant of *S. cerevisiae*, it cannot rescue the filamentation defect of either this mutant or the *ump2* mutant of *U. maydis*. Although, expression of MepC in the *ump2* mutant of *U. maydis* can rescue the filamentation defect to some extent, this was not the case when it was in *S. cerevisiae* (data not shown). We believe that this could be because the construct used the genomic copy of the MepC gene, *i.e.*, containing introns.

Our collaborators in Spain (R. Martín-Dominguez and E. Benito; Universidad de Salamanca) have identified and cloned three ammonium transporters, *bcmep1*, *bcmep2*, and *bcmep3* from the plant pathogen *B. cinerea*. Initial experiments conducted by this group suggest variable expression patterns for the three Meps during saprophytic growth. Under experimental conditions, it was observed that Bcmep1 is expressed at all developmental stages; Bcmep3 was detected after all ammonium was extinguished in the media, whereas Bcmep2 was not all detected. Based on these results it is suggested that sensing of essential nutrients might be considered as a signal for the fungus to be present on a suitable host (Martín-Dominguez et al., 2010). To confirm the functioning of Bcmep1, Bcmep2 and Bcmep3 as ammonium transporters, the three genes were expressed *S. cerevisiae* and in *U. maydis*.

Expression of the three *B. cinerea* Meps in *U. maydis* restored the filamentation of the *ump2* mutant under low ammonium conditions. Nevertheless, the extent of filamentation was distinct for each *bcmep1*, *bcmep2* and *bcmep3*. None of the three Meps were able to rescue the pseudohyphal defect of the *S. cerevisiae* triple mutant. Further, only BcMep1 provided substantial rescue of even the growth inhibition on low ammonium. This could be due to a closer similarity of the *B. cinerea* Meps (Mep2 and Mep3) to the *U. maydis* Ump2 at the amino acid level than to ammonium transporters in *S. cerevisiae* (Table 2). Also, the ability of *B. cinerea* Mep1 to rescue the growth defect of *S. cerevisiae* triple *mep* mutants to a greater extent than Bcmep2 and Bcmep3 could be because of its higher similarity to the *S. cerevisiae* Mep1 and Mep3 (Table 2). Although heterologous expression could provide understanding of the basic function of a gene, the *B. cinerea mep* expression need not mimic all the regulatory events or expression levels that would occur in the natural host, as observed in other studies, like *F. fujikuroi* (Teichert et al., 2008).

To summarize, our study shows that the genes identified as putative ammonium transporters from *M. violaceum* and *B. cinerea* share some functional homology with those of both *S. cerevisiae* and *U. maydis*. Ability of MepC from *M. violaceum* and Mep1, Mep2 and Mep3 from *B. cinerea* to rescue the filamentation defect of *U. maydis* suggests that these proteins act as sensors of low ammonium at least in the heterologous host. Although heterologous systems provide an initial insight into the role of these proteins under inducing conditions (low ammonium), specific roles of each of these proteins, *i.e.*, the ability of each of these proteins to transport ammonium and/or be involved in sensing conditions of low ammonium, needs to be established in their natural

host. Such work for *B. cinerea* Meps is currently being pursued by our collaborators in Spain (R. Martín-Dominguez and E. Benito, personal communication).

CHAPTER III

PHYSICAL AND GENETIC INTERACTION BETWEEN AMMONIUM TRANSPORTERS AND SIGNALING PROTEIN, RHO1.

SUMMARY

Ammonium transporter proteins (Amts) mediate the transport of ammonium across the cell membrane to be used as a nitrogen source. *E. coli* AmtB is known to associate and form trimers and such association in the membrane is an integral aspect of function. Similarly, evidence suggests that fungal Amts (or Meps) similarly form homo- and/or heterodimers (*i.e.*, among different paralogues), and that such associations are required for regulating the activity of the Amt proteins. We were able to demonstrate the formation of homo- and hetero-oligomers of the ammonium transporter proteins, Ump1 and Ump2, in the maize pathogen, *U. maydis*. We also demonstrate the interaction of Ump1 and Ump2 proteins with the signaling molecule, Rho1 GTPase. Our data further suggest that the interaction of the Ump proteins with Rho1 occurs during growth of cells in low ammonium, a condition required for expression of the Umps. We suggest that the interaction relieves the negative effect of Rho1 on another GTPase, Rac1, the master controller of filamentation. This is the first evidence of the physical interaction of ammonium transporters in eukaryotes with a specific component of a signaling cascade, providing a critical connection between ammonium transport/sensing and the signal transduction pathway leading to filamentation.

INTRODUCTION

Formation of oligomers of transporter subunits is a typical feature of secondary active transporters and channels, as evidenced in the case of the lactose transport proteins, sucrose transporters and ABC transporters (Bhatia et al., 2005; Reinders et al., 2002; Schulze et al., 2003; Veenhoff et al., 2001). It has been proposed that some transporters oligomerize to form pores, whereas for others, such as the ammonium transporters, each subunit of the oligomer forms a functional pore (Loque et al., 2007). The association to form oligomers in the lipid bilayer is suggested to be driven by hydrophobicity (Grasberger et al., 1986).

Experimental analysis for estimation of the mass of the native protein using density centrifugation and using denaturing SDS PAGE for determining the mass of the polypeptide monomer of *Escherichia coli* Amt protein, suggested that it forms trimers (Blakey et al., 2002). However, the very distantly related erythrocyte Rh proteins are predicted to form hetero-oligomeric tetramers (Eyers et al., 1994; Hartel-Schenk and Agre, 1992; Ludewig et al., 2003). The functional importance of the trimeric state of the Amt in *E. coli* was determined by the identification of a physically interacting small trimeric PII protein GlnK, This interaction confers a nitrogen-dependent regulation of the ammonium transport activity of AmtB (Conroy et al., 2007; Coutts et al., 2002; Javelle et al., 2004).

Following the identification of the three Amt/Meps from *S. cerevisiae*, it was observed that a particular strain that had complete deletion of the *MEP2* gene, a single point mutation of *MEP1*, *mep1-1* (glycine412aspartate), but still retained an intact *MEP3* gene was unable to grow under conditions of low ammonium. This was contrary to the

observation that complete knockout of both *MEP1* and *MEP2*, in the presence of an intact *MEP3*, left the cells still able to grow under low ammonium conditions (Marini et al., 2000; Marini et al., 1997). The particular mutation in *MEP1* (*mep1-1*) was trans-dominant and inhibited the activity of *MEP3* by affecting the ammonium transport function of Mep3 (Marini et al., 2000). This cross inhibition between Mep transporters pointed to a direct interaction between these proteins. Such an interaction could take place at the plasma membrane in a complex of Mep proteins. Additional biochemical analyses using a FLAG epitope fused to the Mep2 protein also showed that Mep2 monomers interact to form trimeric complex (Rutherford et al., 2008). Analogous results were obtained when a similar point mutation was introduced into the *meaA* gene of *Aspergillus nidulans* which changed the glycine residue at 447 (near the C-terminal end) to aspartate in the MeaA protein. This mutation was also trans-dominant to the wild type MeaA or to the second Amt homologue in *A. nidulans,* MepA protein (Monahan et al., 2002). Equivalent mutations when made in the plant ammonium transporters, specifically the tomato *LeAMT1;1* and Arabidopsis AMT1;1, were non-functional and trans-inactivated wild type transporters (Loque et al., 2007; Ludewig et al., 2003). These results illustrate an interaction between the Amt monomers in *S. cerevisiae*, *A. nidulans, Arabidopsis* and tomato. These results also suggest that the interaction among the monomers involve the C-terminus of the protein. Determination of the crystal structure of the Amt from *Archaeoglobus fulgidus* provided further evidence of the importance of C-terminus in maintaining physical interaction with cytosolic loops of the neighboring subunits (Andrade et al., 2005).

Mutations affecting the functionality of Amt oligomers suggest that the C-terminus is important for the function and regulation of the complex. Also, co-expression studies combined with extensive study of the C-terminus mutants reveal that the assembly of Amt subunits plays a central role in cooperative regulation (Loque et al., 2007). Studies conducted in *Arabidopsis* by Loque et al, (2007) show the carboxy terminus of the oligomeric Amt serves as an allosteric regulator and that a productive interaction is necessary for allosteric activation of the complex. Phosphorylation of conserved residues at the C-terminus is considered as the cognate control mechanism (Nuhse et al., 2004). Allosteric regulation is shown to be also mediated by the threonine residue at amino acid position 460 in the C-terminal trans-activation domain in *Arabidopsis*. Phosphorylation of this residue (affected by availability of ammonium), disrupts the transactivation of the neighboring Amt subunit, thereby inhibiting Amt activity (Lanquar et al., 2009). Furthermore, investigation of the N terminal cysteine residues in the tomato orthologue revealed that this residue is important for increasing the stability of the Amt protein complex (Graff et al., 2010).

The formation of hetero-complexes is not a requirement for the transport function of ammonium transporters. This is supported by experiments conducted in *S. cerevisiae* and *A. nidulans*, where cells expressing a single functional ammonium permease can support growth on low concentrations of ammonium (Marini et al., 1997; Monahan et al., 2002). The authors also suggest that hetero-interaction may serve to modulate different transport activities of the permeases. Furthermore, in both *S. cerevisiae* and *A. nidulans,* the sum of the permease activity of an individual permease was not equal to that of wild type (Marini et al., 2000; Monahan et al., 2002).

Our investigation of the *U. maydis* ammonium permeases provides evidence of homo- as well as heterodimer formation. In the present study we also confirm the findings of Pham et al (2009) that Ump2 interacts with the signaling protein Rho1, belonging to the conserved family of Rho/Rac GTPase proteins . Yeast two hybrid experiments to determine the interacting partners of the *U. maydis* Rho1 proteins, identified the high affinity ammonium transporter Ump2 from a cDNA library. This interaction between Rho1 and Ump2 was also confirmed using co-immunoprecipitation (Pham et al., 2009). Our study confirms and provides the first evidence for the physical interaction of ammonium transporters with a signaling component in eukaryotes.

MATERIALS and METHODS

Strains and Growth Conditions. The yeast reporter strain utilized in this study is NMY 32 (*MAT a his3Δ200 trp1-901 leu2-3, 112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-LacZ ade2::(lexAop)$_8$-ADE2 GAL4*; a gift from J. Heitman, Duke University) provided in the dual membrane kit [Dualsystems Biotech, Zurich, Switzerland]. *U. maydis* strains used in this study are listed in Table 3. *E. coli* strains DH5α [Bethesda Research Laboratories] and TOP10 [Invitrogen, Carlsbad, CA] were employed for cloning and ORF amplification requirements.

TABLE 3. *U. maydis* strains used in this study.

Strain	Genotype	Reference
Fungal Strains		
1/2 WT	*a1b1*	*Gold, et al, 1997*
Δump2 a1	*a1b1 ump2::hyg^R*	This Study
rho1^{crg} a1	*a1b1 rho1::Pcrg-rho1-cbx^R*	*Pham et al., 2009*
rho1^{crg} Δump2 a1	*a1b1 rho1::Pcrg-rho1-cbx^R, ump2::hygR*	This Study

U. maydis cells were grown at 25-28°C in YEPS (1% yeast extract, 2% each of peptone and sucrose and/or 1.5% agar) and SLAD (synthetic low ammonium) as described previously (Smith et al., 2003). Induction or suppression of the P_{crg1} promoter was carried out by growing cells in either YP (1% yeast extract and 2% peptone) or minimal media (0.17% yeast nitrogen base (YNB) and ammonium sulfate adjusted to a final concentration of 50 μM) supplemented with 2% arabinose or 2 % dextrose, SLAD and SLAA, respectively. For the Split-Ubiquitin assay the yeast cells were grown on SD medium (0.17% YNB and 1X amino acid drop-out solution) supplemented with dextrose. The yeast cells were kept at 30°C for 3-4 days. The NMY32 strains were maintained on YPAD plates (1% yeast extract, 2% peptone, 2% dextrose, 0.004% adenine sulfate and 2% agar)

DUALmembrane Screen for Split-Ubiquitin Assay. The DUALmembrane screen was performed following the DUALmembrane kit user manual [Dualsystems Biotech, Zurich, Switzerland]. pCCW-STE was used as the bait vector, which is recommended to be used with integral membrane proteins that lack a leader sequence. For each known target protein the respective cDNA was cloned between the two *Sfi I* sites in the multiple cloning site, in frame with the upstream STE sequence (provided to help increase expression of bait in yeast) and the downstream Cub-LexA-VP16 cassette. The pDL2xN-STE prey vector was chosen for the study, and each target protein cDNA was cloned between the two *Sfi I* sites in the multiple cloning site. The bait was specifically selected to test for interaction with a particular prey in a directed split ubiquitin assay. This method was utilized to specifically to look at the interaction of *U. maydis* ammonium permeases with each other and among themselves and also to confirm the interaction with

Rho1 detected by Pham et al (2009). Specifically, *ump1*, *ump2* and *rho1* genes from *U. maydis* were each cloned into the bait and the prey vector at the *Sfi I* sites following cloning of the cDNA fragments into the pCR 2.1 TOPO vector [Invitrogen, Carlsbad, CA].

For directed two hybrid assay, bait and prey plasmids were introduced into *S. cerevisiae* strain NMY 32 [Dualsystems Biotech] by co-transformation. Growth was assessed after 5 days on TDO (SD/-Trp/-Leu/-His) and QDO (SD/-Trp/-Leu/-His/-Ade) to determine the strength of the interactions. The strength of the interaction was further assessed by replica plating cells onto plates containing β-X-gal to test the activity of β-galactosidase. Yeast cells expressing β-galactosidase turned blue within minutes to hours depending on the strength of the interaction.

Vector Construction for Bimolecular Fluorescence Complementation Assay. Genetic manipulations involving switching the endogenous copy of the gene with the copy of the gene fused to YFP or the gene fused to the N terminus or C terminus of the YFP in *U. maydis*, were accomplished through homologous recombination as described previously (Brachmann et al., 2004). The complete or partial YFP sequence was fused to the three genes of interest (*ump1*, *ump2* and *rho1*) following fusion PCR and cloning into the pCR 2.1 TOPO vector [Invitrogen]. Another fragment, approximately 1 kb in size, from downstream (down flank) of the gene of interest was amplified and cloned into pCR 2.1 TOPO. This down flank was digested and cloned into the vector containing the gene of interest with the YFP fusion (up flank). The 3' end of the up flank and the 5' end of the down flank are complementary to the 5' and 3' regions of the carboxin and hygromycin resistance cassettes, respectively. The replacement constructs were amplified using high

fidelity Phusion DNA polymerase [Finnzymes, Lafayette, CO], purified and used to transform *U. maydis* protoplasts (Brachmann et al., 2004). Transformants with the desired replacements were identified and confirmed by PCR.

The YFP plasmid obtained from the Fungal Genetics Stock Center [Kansas City, Missouri] was used in an earlier study conducted in *N. crassa* (Bardiya et al., 2008). Though not codon optimized for *U. maydis*, the YFP sequence was analyzed to determine the codons constituting the YFP protein and the frequency of their usage in *U. maydis*. Furthermore, to ensure expression of the YFP protein in *U. maydis*, the intact YFP protein was fused to the Ump1 and Ump2 proteins and its expression was detected in cells carrying the respective fusion protein when grown under low ammonium conditions (Fig. 14 A and B). Additionally, the cells carrying only one half of the YFP were scanned to detect any background florescence. In cases where we could detect background florescence, this was set as "0" and florescence above this was considered positive for interaction.

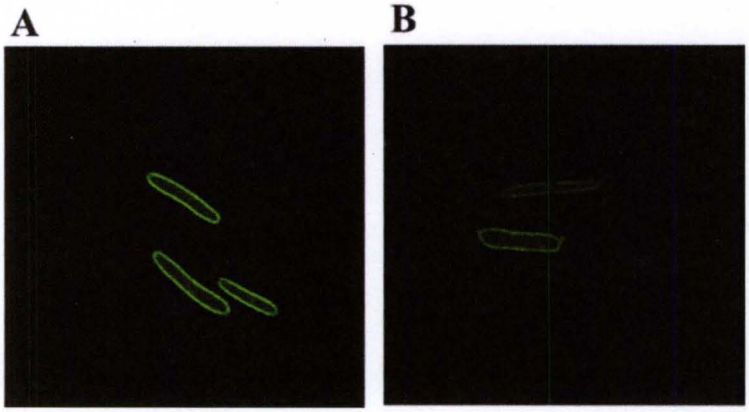

Figure 14: Confirmation of YFP expression in *U. maydis*. Cells expressing Ump2-YFP **(A)** and Ump1-YFP **(B)** grown in liquid SLAD for 24 hrs.

Microscopy. Images of cells carrying the complete and partial YFP fusions were acquired using an Olympus Fluoview FV-1000 confocal scanner coupled to an Olympus 1X81 inverted microscope, a PlanApoN 60× objective, and FV-10 ASW 2.1.

Imaging Protocol. A single channel scanning configuration with line scanning was set up for acquisition of complete or partial YFP-tagged proteins of interest, using the 488 nm line of an Argon laser. A transmitted light image was acquired during scanning for visualization of cell outline in the plane of scanning, by grouping of the transmitted detector with the argon laser. The optimal brightness setting for each channel was configured by determining the HV setting yielding maximal intensity without saturation. Each of the settings was tested against samples with cells not expressing tagged proteins to ensure exclusion of non-specific emission from cells.

RESULTS

Ump1 and Ump2 interact with each other and among themselves. The interaction between the two ammonium transporters was first tested using a directed two hybrid screen. This modified assay in yeast showed that Ump1 interacts with itself and so does Ump2. This assay also confirmed the hypothesis that the two ammonium transporters in *U. maydis* would form hetero-oligomers. All the three tested interactions were strong, as indicated by growth on the most stringent media, QDO and also by development of color on plates containing β-X-gal. The interaction between Ump1 and Ump2 was also confirmed by switching the bait and prey for the respective protein (Fig. 15A).

To examine interaction of these proteins *in vivo* in *U. maydis*, we used the bimolecular fluorescence complementation assay (BiFC). In this assay both Ump1 and Ump2 proteins were fused to the N-terminus and C-terminus of the YFP protein, respectively, and examined for fluorescence under both nutrient rich and low ammonium conditions. The N- and C- terminus of the YFP was swapped between Ump1 and Ump2

to assure that the fluorescence observed was consistent with a true interaction (Fig. 16A and B). Fluorescence from YFP could only be detected when cells carrying the fusion protein were grown under low ammonium conditions and not under rich nutrient conditions. The fluorescence confirmed the localization of the ammonium transporters to the membrane, also suggesting that fusing the Ump proteins with YFP did not interfere with their localization. The cells carrying the fusion proteins also grew like wild type cells, filamentously, under low ammonium conditions, signifying no loss of function, as a result of the fusion (data not shown).

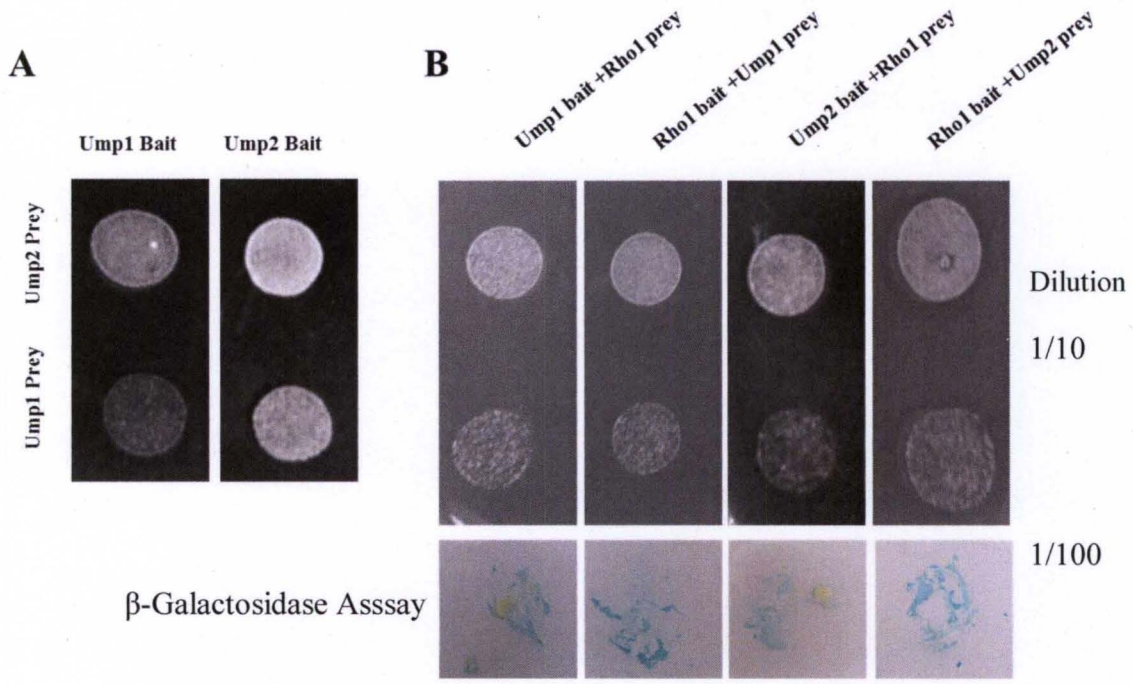

Figure 15: Split-Ubiquitin Assay. (A) Interaction between and among Ump1 and Ump2 as indicated by growth on QDO. **(B)** Interaction between ammonium transporters and Rho1 as indicated by growth on QDO and color change for β-galactosidase assay.

Ump1 and Ump2 interact with Rho1 GTPase protein. The interaction between Ump2 and Rho1 was first identified in the yeast two hybrid screens for interacting partners of Rho1. Directed split ubiquitin assay was used to confirm this interaction and also to test

the interaction of the low affinity ammonium transporter, Ump1, with Rho1. Interaction of Ump1 or Ump2 with Rho1 was indicated by growth of yeast cells expressing these proteins on QDO stringent medium and the strength of the interaction was also confirmed by the β-galactosidase assay (Fig. 15B). The *in vivo* interaction of both Ump1 and Ump2 with Rho1 was also confirmed in *U. maydis* using the BiFC. Fluorescence due to reconstitution of the YFP, when tested between Rho1 and Ump1 or Ump2 was detected only when the cells were grown under low ammonium conditions (Fig. 17A and B). Fluorescence as a result of interaction between Ump2 and Rho1 was detected at the membrane, whereas fluorescence was dispersed mostly throughout the cell when interaction was tested between Ump1 and Rho1.

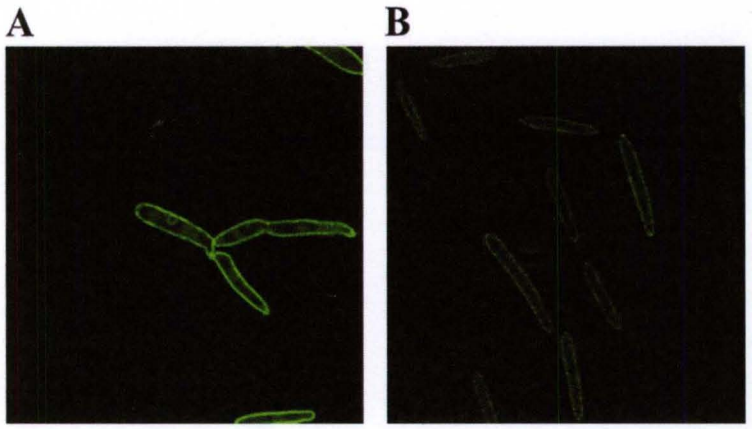

Figure16: Interaction between Ump1 and Ump2 under low ammonium. Cells expressing Ump1 tagged to N-terminus of YFP and Ump2 tagged to C terminus of YFP **(A)** and Ump1 tagged to C-terminus of YFP and Ump2 tagged to N terminus of YFP **(B)** grown for 24 hrs in liquid SLAD.

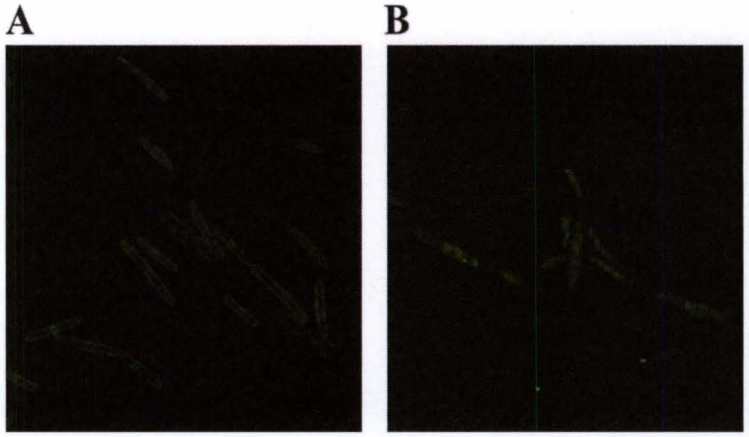

Figure17: Interaction between Ump1 and Ump2 and Rho1. Cells expressing Rho1 tagged to N-terminus of YFP and Ump2 tagged to C terminus of YFP **(A)** and Rho1 tagged to N-terminus of YFP and Ump1 tagged to C terminus of YFP **(B)** grown for 24 hrs in liquid SLAD.

Evidence of genetic interaction between *rho1* and *ump2*. To determine any interaction between *rho1* and *ump2* at the genetic level, the *ump2* gene was deleted in the haploid *U. maydis* strain containing *rho1* under the control of the *crg1* promoter. In this strain, the expression level of *rho1* depend on the growth condition, specifically the carbon source used for growing the cells (*i.e.,* induced with arabinose; repressed with glucose (Bottin et al., 1996; Brachmann et al., 2001)). Cells over-expressing *rho1* (*i.e.,* grown on SLAA) show reduced filamentation (Fig. 18C) under low ammonium conditions, compared to the wild type cells (Fig. 18A). However, deletion of *ump2* in P_{crg} *rho1,* obliterated the ability of these cells to filament under similar growth conditions.

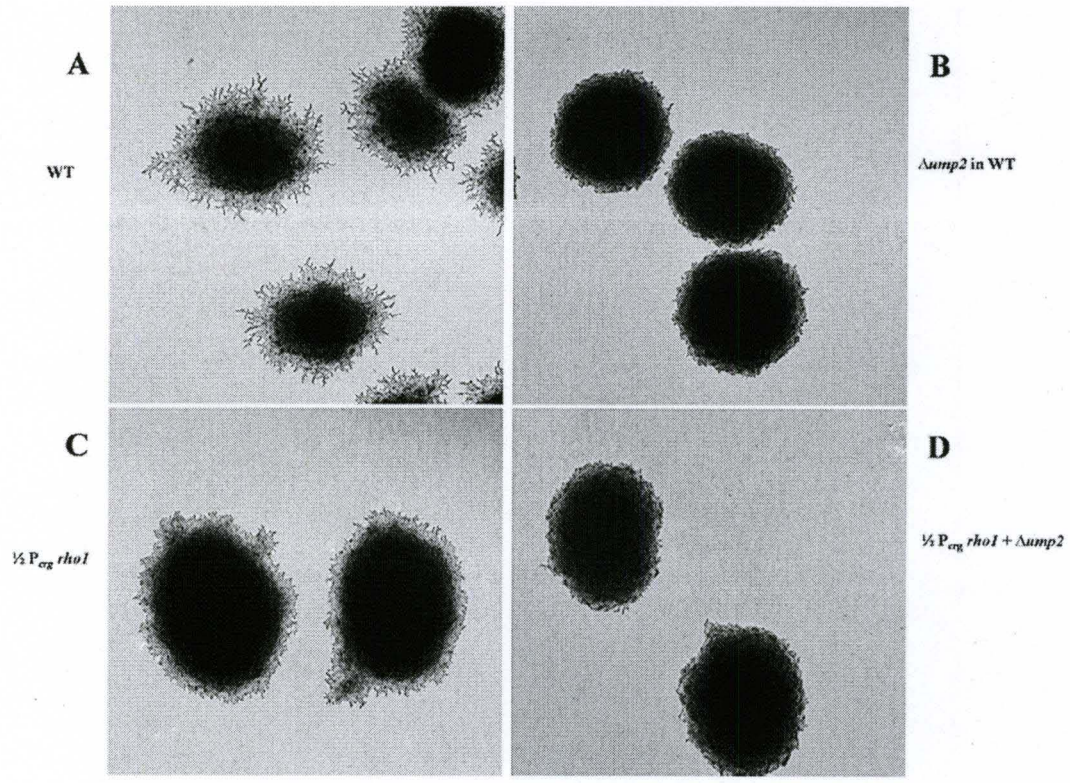

Figure 18: Haploid cells **(A)** WT; **(B)** Δ*ump2*; **(C)** *rho1* under the control of the *crg1* promoter and **(D)** *ump2* deletion in P_{crg} *rho1* background, were grown on SLAA (synthetic low ammonium supplemented with arabinose) and colony morphology was observed after 4 days. *ump2* deletion abolishes the reduced filamentation of the *rho1* over-expression strains.

DISCUSSION

Regulation of metabolic functions at the membranes is important as they contain transport molecules that help catalyze the first step in a pathway. These transport molecules are required for membrane stability and are often linked to the signal transduction components which help the cells communicate with their environment. It has been proposed that multimerization of these membrane proteins could provide a mechanism for specific regulation and membrane structure stability (Veenhoff et al., 2002). Moreover, membrane proteins could form oligomers as volume exclusion (volume of a protein molecule inaccessible to other proteins as a result of the presence of the first protein) and the likelihood of self-association is enhanced because of the way in which the protein is localized and oriented in the two dimensional space in the membrane (Grasberger et al., 1986; Veenhoff et al., 2002).

Two lines of evidence suggest interaction between and among the two ammonium transporters identified in *U. maydis*. Firstly, the split ubiquitin assay suggested the interaction between the two ammonium transporters and it also confirms that both Ump1 and Ump2 each interact to form homo-oligomers and also, that they interact with each other, to form hetero-oligomers. The physical interaction between Ump1 and Ump2 was confirmed using the bimolecular fluorescence assay in *U. maydis*. Although the functional importance of the interaction between Ump1 and Ump2 is not evident at present, it is not necessary for the transport of ammonium across the membrane in *U. maydis*. No evidence of allosteric regulation between the two ammonium transporters has yet been identified in *U. maydis,* in contrast to what has been observed in other organisms like yeast and *A. nidulans* (Marini et al., 2000; Monahan et al., 2002).

Our study also confirms and provides the first evidence of a physical interaction between an ammonium transporter and the signaling protein, Rho1. Rho1 belongs to a conserved family of Rho/Rac GTPase proteins, shown to control filamentation. Their activity is controlled by guanine exchange factors (GEFs) which replace GDP with the GTP molecule, thereby activating the Rho/Rac GTPase; additionally, GTPase activating proteins (GAPs) inactivate the Rho/Rac GTPase by stimulating intrinsic GTPase activity (Schwartz, 2004). Following activation, the Rho-GTPases stimulate downstream effector proteins. The Rho/Rac GTPases control an array of cellular processes like actin organization, cell integrity, cytokinesis, pathogenicity, signal transduction and cell migration (Köhli et al., 2008; Leveleki et al., 2004; Mahlert et al., 2006; Nakano K, 1997; Wadsworth, 2005). Rho GTPase is also implicated in controlling filamentation in both filamentous and dimorphic fungi. For example a *rho1*-null mutant of *F. oxysporum*, lacks the ability to grow filamentously on solid media (Martínez-Rocha et al., 2008).

In *U. mayids*, Rho1, is the only member of its family of proteins required for cell viability. Rho1 is involved in controlling cytokinesis and cell polarity. Although, over-expression of Rho1 led to reduced filament production in *U. maydis* cells, cells depleted for Rho1 showed non-polarized growth followed by cell death, suggesting a role for Rho1 in the dimorphic transition (Pham et al., 2009). The lethality of the *rho1* deleted strain can be rescued when another gene, *rac1,* is deleted in this background. Rac1 is another GTPase protein and has been shown to be the master regulator controlling filamentation in *U. maydis*. A constitutively active allele of Rac1 causes the cells to undertake isotropic delocalized cell extension and eventually cell death (Mahlert et al., 2006). Epistasis analyses suggest that Rho1 acts upstream and negatively regulates Rac1

to control cytokinesis and cell polarity. According to the model suggested by Pham et al (2009), Rho1 could be interfering with the localization of Rac1 at the polar tips thereby preventing polar growth. Alternatively, it could be sequestering away the effector of Rac1, Cla4 (a p21 activated kinase) or utilizing the common GEF (Cdc24) of both Rho1 and Rac1, to prevent polar growth.

We demonstrate here the interaction of Rho1 with the ammonium transporters, Ump1 and Ump2. This interaction between the proteins seems to occur under low ammonium conditions, as evidenced by the presence of fluorescence only under ammonium limiting conditions. We hypothesize that this interaction would be functionally relevant because the interaction of Rho1 with the ammonium transporters under low ammonium conditions could inhibit Rho1 from negatively regulating Rac1, the master controller of filamentation, thereby causing filamentation under low nutrient conditions. The observed reduced filamentation of P_{crg}*rho1* on SLAA media (Fig. 18C) could be due to the altered stoichiometry of the Rho1 protein, in excess compared to what is present in wild type; therefore, interaction with Ump2 is not sufficient to avoid inhibition of Rac1, thereby affecting filamentation. However, when *ump2* was deleted in P_{crg}*rho1*, the filamentation associated with low ammonium was completely lacking (Fig. 18D). This maybe be because now Rho1 is free to block Rac1. Furthermore, under rich nutrient conditions, the expression of ammonium transporters is much reduced (also see chapter 4), therefore we cannot detect the interaction of Umps with Rho1. Under such conditions Rho1 is likely free, and inhibits the activity of Rac1, thereby blocking filamentation. Interestingly, this could also explain the filamentation phenotype observed under rich nutrient conditions when Ump2 is over-expressed (Paul et al., 2009). Under

these conditions, Ump2 would be expressed and therefore would be available to interact with Rho1, relieving the repression on Rac1, thereby causing filamentation. Hence, we propose that Rho1 acts downstream of the ammonium transporter proteins and coordinates with Rac1 to form filaments under conditions of nutrient limitation.

CHAPTER IV

COORDINATE REGULATION BY AMMONIUM TRANSPORTERS OF *USTILAGO MAYDIS* GENES INVOLVED IN MATING AND PATHOGENICITY

SUMMARY

The switch to a filamentous growth form is an essential morphogenetic transition that many fungi utilize to cause disease in the host. Although different environmental signals can induce filamentous growth, the developmental programs associated with transmitting these different signals may be the same at one or more levels. Filamentous growth induced in response to low ammonium availability by ammonium transporter proteins is not yet shown to be essential for pathogenicity. In our present study we show that over-expression of the high affinity ammonium transporter, *ump2*, in *Ustilago maydis* under normally non-inducing conditions results in filamentous growth. Our experiments to determine the role of ammonium transporter expression in affecting signal transduction pathway genes or downstream effector genes to control filamentation revealed that *ump2* expression levels are correlated with the expression of genes involved in the mating response pathway and in pathogenicity. The expression of these genes is further affected by changes in the expression of *ump1* (the low affinity ammonium transporter). *ump1* and *ump2* also affect expression of genes shown to be expressed during either filamentous growth or during growth of the fungus inside the host. Finally, we show that the expression of both ammonium transporters is essential for the pathogen

to cause infection on the host.

INTRODUCTION

Ammonium transporter proteins (Amts), essential in the uptake of ammonium from the environment, are conserved across many taxa. Studies conducted to date in fungi reveal that each species contains at least one high affinity and one low affinity ammonium transporter (Biswas and Morschhäuser, 2005; Javelle et al., 2003; Marini et al., 1997; Mitsuzawa, 2006; Smith et al., 2003). Amt proteins in the different fungal species are essential for a variety of processes: *e.g.,* initiation of the dimorphic transition required for completion of the sexual life cycle in some, pathogenicity in others where the filamentous form is infectious, while in others it appears to be a means of foraging for nutrients (Biswas and Morschhäuser, 2005; Lo et al., 1997; Lorenz and Heitman, 1998; Smith et al., 2003). The high affinity ammonium transporter proteins of *S. cerevisiae*, *U. maydis* and *C. albicans* are required for filamentous growth under low ammonium conditions (Biswas et al., 2007; Lorenz and Heitman, 1998; Smith et al., 2003). While the haploid invasive growth in *S. pombe* and *S. cerevisiae* on low ammonium is dependent on the ammonium permeases, both mating and haploid invasive growth in *C. neoformans* are induced by ammonium limitation and specifically require the high affinity ammonium transporter (Mitsuzawa, 2006; Rutherford et al., 2008). The high affinity ammonium transporters are therefore hypothesized to function as ammonium sensors.

Mutational studies conducted on Mep2 in *S. cerevisiae* and *C. albicans* reveal that the roles of Mep2 in transport and induction of filamentous growth can be separated. An additional function attributed at least to the *S. cerevisiae* Mep2 is the function of a

transceptor, activating the cAMP dependent PKA pathway after the addition of ammonium to starved cells (Van Nuland et al., 2006). Although the exact connection between Mep2 signaling and the signal transduction pathway is not known, epistasis studies suggest RAS-cAMP being a downstream target of Mep2 (Lorenz and Heitman, 1998; Rutherford et al., 2008). Mep2 dependent pseudohyphal growth was not restricted to low ammonium conditions but was also shown to be dependent on the expression level of Mep2 in both *S. cerevisiae* and *C. albicans* (Biswas and Morschhäuser, 2005; Rutherford et al., 2008). In *S. cerevisiae*, the expression level of Mep2 is important for its regulatory functions, whereby induction of Mep2 leads to ammonium responsive dimorphic switch under non-limiting ammonium conditions. This led the authors to hypothesize a role for Mep2 as interacting with signal transduction pathway(s) to activate downstream effectors. This study revealed differential expression of genes already predicted to be involved in pseudohyphal growth, and presents evidence of differential activation of the MAPK pathway in a Mep2 dependent manner (Rutherford et al., 2008).

Although many different signals can induce filamentous growth, the strategy for responding to these extracellular signals by cellular differentiation has been proposed to be conserved among fungi, *i.e.*, induction of a comparable developmental pathway (Sánchez-Martínez and Pérez-Martín, 2001). The ability to switch between different morphological states is an important virulence determinant is *U. maydis*. It was shown that conjugation tube formation and true filaments formation, under the control of *a* and *b* mating loci are induced under nitrogen starvation conditions (Banuett and Herskowitz, 1994). Furthermore, mating in haploid *U. maydis* cells is induced under conditions of nutrient deprivation in the presence of cells of opposite mating type and this is under the

control of the cAMP–PKA pathway. However, in the absence of compatible mating partners, haploid cells grow filamentously under similar growth conditions (Kahmann et al., 1999). While the usual pathogenic form of *U. maydis*, the dikaryon, is formed as a result of the fusion of compatible mating types, diploid solopathogenic strains are also found. These are produced from teliospores that germinate without meiosis and about 1-3% of the teliospores that undergo germination result in diploid culture in the laboratory (Babu et al., 2005; Kojic et al., 2002) . Although these different cell types are pathogenic to the host, the pathogenicity levels of these different strains are different. Gene expression studies reveal a global variation in the developmental program associated with each cell type (Andrews et al., 2004; Babu et al., 2005; Garcia-Pedrajas and Gold, 2004). Under laboratory conditions, filamentous growth of *U. maydis* is observed in response to factors like nitrogen limitation, pH, lipids, air and polyamines (Klosterman et al., 2007). Developmental programs triggered by the morphogenetic transition in response to various signals *in vitro* may reveal normal interactions between the pathogen and the host *in vivo* (Sánchez-Martínez and Pérez-Martín, 2001).

In *U. maydis* the ammonium transporters, referred to as Umps, are required for phenotypic changes in response to low ammonium conditions. Whereas deletion of *ump1* (low affinity ammonium transporter) does not yield any discernable difference in phenotype from the wild type (filamentous growth) under ammonium limiting conditions, cells deleted for *ump2* (encoding the high affinity ammonium transporter) were unable to produce filaments on SLAD (synthetic low ammonium media). This inability to produce filaments under low ammonium conditions did not impair the ability of the *ump2* mutants to be pathogenic on the host (Smith et al., 2003). While the wild type and *ump1* mutants

are able to grow filamentously on other nitrogen sources, the *ump2* mutant also lacks the ability to grow filamentously on limiting nitrogen sources other than ammonium, except histidine (Klosterman et al., 2007). The *ump1ump2* double mutants, though unable to transport any observable amount of ammonium in *in vitro* uptake assays, were still able to grow on SLAD and displayed tangled filaments. In *S. cerevisiae* transcriptional control of ammonium permeases occurs in response to a particular nitrogen source and this ensures expression of appropriate pathways (Cooper, 2002). Such control of *ump2* expression could be possible in *U. maydis*. Both MAPK and PKA signaling pathways are implicated in affecting filamentous growth in *U. maydis*, but their role in affecting filamentation in response to low nitrogen, specifically ammonium, mediated by *ump2* has yet to be explored in detail. Preliminary studies reveal that the connection of *ump2* to the PKA signaling pathway may occur at multiple levels (Garcia-Pedrajas and Gold, 2004; Klosterman et al., 2007; Smith et al., 2003).

In the present study we show that filamentation facilitated by *ump2* can occur on nitrogen replete media and this is dependent on the expression levels on *ump2* as seen in the case of *S. cerevisiae*. This result suggests that ammonium limitation, per se, might not be responsible for induction of the filamentous response by *ump2*. To explore the role of *ump2* in control of gene expression associated with filamentation, the transcriptional profile of cells deleted for *ump2* was compared to that of cells over-expressing *ump2;* these analyses were conducted under both nutrient replete and nutrient limitation conditions. A number of genes previously reported to be important for pathogenicity were identified to be differentially expressed between the two mutants. The result of the

transcriptional profiling was further confirmed for a small subset of the identified genes by qRT-PCR.

MATERIALS and METHODS

Strains and Growth Conditions. *U. maydis* cells were grown at 30°C on Array Media [AM] (6.25% Holliday Salt Solution (Holliday, 1974), 1% glucose, 30 mM Glutamine/ 50 mM Ammonium sulfate and 2% agar) and Array Medium [AM] with low ammonium (6.25% Holliday Salt Solution, 1% glucose, 50 µM ammonium sulfate and 2% agar) for 48 hrs. *U. maydis* strains used are listed in Table 4. All mutants were generated in either the FB background (Banuett and Herskowitz, 1989) or the 2/14 or 2/11 backgrounds (Smith et al., 2004).

TABLE 4. *U. maydis* strains used in this study.

Strain	Genotype	Reference
Fungal Strains		
FB1 WT	*a1b1*	*Banuett, et al, 1989*
FB2 WT	*a2b2*	*Banuett, et al, 1989*
Δump2 a1	*a1b1 ump2::hyg^R*	This Study
Δump2 a2	*a2b2 ump2::hyg^R*	This Study
ump2^{Otef} a1	*a1b1 P$_{otef}$-ump2, cbx^R*	This Study
2/14 WT	*a1b2*	*Smith, et al, 2004*
2/11 WT	*a2b1*	*Smith, et al, 2004*
Δump1Δump2 a1	*a1b2 ump2::hyg^R, ump1::cbx^R*	*Smith, et al, 2003*
Δump1Δump2 a2	*a2b1 ump2::hyg^R, ump1::cbx^R*	*Smith, et al, 2003*

Primer Design. Primers, other than for the Real Time PCR, were designed using the Primer 3 program available at [http://frodo.wi.mit.edu/primer3/] (Rozen and Skaletsky, 2000). Primers were ordered from Eurofins MWG Operon [Huntsville, AL].

PCR. PCR reactions were run on a PTC100 thermal controller [MJ Research Inc., San Francisco, CA] and a DNA Engine thermal cycler [Bio Rad Laboratories, Hercules, CA]. PCR cycling conditions utilized an initial denaturation temperature of 94°C for 4 minutes, followed by 34 cycles of a three step process of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute per 1 kb of anticipated product length. A final extension at 72°C for 10 minutes was used to complete all products. For most reactions Ex-Taq™ Hot Start DNA polymerase (Takara, Madison, WI) or Apex DNA polymerase [Genesee Scientific, San Diego, CA] was used. For high fidelity reactions, Phusion DNA polymerase [Finnzymes, Lafayette, CO] was used.

Genetic Manipulation and Vector Construction. Deletion and over-expression of *ump2* in *U. maydis* were obtained by homologous recombination as described previously (Brachmann et al., 2004). The *ump2* deletion construct was created using a 6 kb PCR product from strain um2h-2, Δ*ump2 a1b1* (Smith et al., 2003) generated using primers Ump2KOup5' (GGCAAGACAAGACGAGAAGA) and Ump2_Dn_TapR (TGCGTGTCTCAAACTCCTCT). The *ump2* over-expression construct was produced by amplifying the *ump2* ORF from the plasmid pUmp2 303 (Smith et al., 2003) using the Ump2_CGO5' (ATTAACCGCGGAAATGGTTAACGCCAGCTAC) and Ump2_CGO3' (TGATTGCGGCCGCTTAGACAGCAGTAGGCTG) primers and cloning the product into pCR2.1 TOPO (Invitrogen). To provide constitutive expression, the *ump2* ORF was cloned after the P_{otef} promoter between the *SacII* and *NotI* sites of the p123 vector (Weber et al., 2006). The Otef expression vector was linearized using the restriction enzyme *Ssp1* before transforming *U. maydis* to select for recombinants at the *ip* locus, providing carboxin resistance (Brachmann et al., 2001).

RNA Isolation and Expression Analysis. RNA isolation from *U. maydis* for the transcriptional profile, used cells grown on AM-glutamine and AM-low ammonium plates for 48 hours. Cells were scraped off and homogenized under liquid nitrogen in a mortar and pestle. These cells were further processed using the RNeasy plant mini kit [Qiagen, Valencia, CA] following the manufacturer's instructions. The integrity of the RNA samples was checked using an Agilent Bioanalyser 2100. The RNA samples were treated with DNaseI [New England Biolabs, Ipswich, MA] before proceeding to the preparation of the cDNA samples. The double stranded cDNA samples were prepared with Super Script III [Invitrogen, Carlsbad, CA] using 25 μg of RNA as the starting material and following manufacturer's instructions. 10 μg of cDNA was sent to Nimblegen [Rejkavik, Iceland] for hybridization experiments.

For Real-Time PCR experiments, RNA was isolated from *U. maydis* strains using the TRIzol reagent protocol from Invitrogen, with modifications. *U. maydis* cells were grown on AM-glutamine, AM-ammonium and AM-low ammonium agar plates for 2 days. The cells were scraped off the plates and homogenized under liquid nitrogen in a mortar and pestle. Roughly 100 mg of the homogenate was treated with 1 ml of TRIzol and processed further for RNA extraction. 5 μg of total purified RNA samples were treated with Turbo DNase [Ambion Inc., Foster City, CA] before being used for synthesizing cDNA using the cDNA synthesis kit [Superscript II, Invitrogen]. The cDNA was used as a template for RT-PCR and real-time PCR reactions.

Quantitative-RT PCR. To verify differences in expression levels of a subset of genes identified by microarray data, qRT-PCR was employed. Primers for the different genes were designed using the ABI primer express software version 3.0, ensuring that all the

primers sets investigated had the same amplification efficiency, since comparison of gene expression using real-time PCR assay assumes that the efficiency of amplification for all the primer pairs is equal. Primers used in the study are specified in Table 5. The generated cDNA was diluted 5-20 fold depending on the starting concentration of RNA that was used for making cDNA. All real-time PCR reactions were performed in a 20 µl mixture containing 1/10 volume of the diluted cDNA preparation, 1X Power SYBR green PCR master mix [Applied Biosystems, Foster City, CA, USA], 1 µl each of the 5 µM forward and reverse primers, making up the reaction volume to 20 µl with water. Quantifications of RNA expression levels were performed in an Applied Biosystems Step-One thermocycler using the following PCR conditions: 95°C for 15 mins followed by 95°C for 15 secs and 60°C for 1 min for 40 cycles. Melting curve analysis was performed at the end of each cycle to ensure specificity of the reaction. The concentration was determined by the comparative CT method (threshold cycle number at the cross point between amplification plot and threshold) and values were normalized to expression of the constitutively expressed gene, *eif2B*, encoding the guanine-nucleotide exchange factor for translation initiation factor eIF2. Changes in the gene expression are averages of at least two biological replicates and are displayed relative to expression under high ammonium condition. Negative and positive values were considered as down-regulation or up-regulation of expression of gene of interest, respectively. Error bars indicate standard deviation of mean expression values of at least two biological replicates.

TABLE 5. Genes and the Primers used for qRT-PCR.

Gene Name	Primers	Sequences (5' → 3')
guanine nucleotide exchange factor (um04869)	rt-eIF-2B-F N	ATCCCGAACAGCCCAAAC
	rt-eIF-2B-R N	ATCGTCAACCGCAACCAC
ump1 (um04523)	rt-Ump1-F	CGGTCTCACCTGGATGTTCCT
	rt-Ump1-R	AGCCAACGACGGACCACTT
ump2 (um05889)	rt-Ump2-F	TGGGTCCCGTTCTCATTTTC
	rt-Ump2-R	AGGCGATGGGATTGTAGACAA
prf1 (um02713)	rt-Prf1-F new	TCGGTAGAACGAGCTGTGATG
	rt-Prf1-R new	CTGTTGGACGATGTTGGAGTTG
mfa1 (um02382)	rt-Mfa1-F	ATGCTTTCGATCTTCGCTCAG
	rt-Mfa1-R	TAGCCGATGGGAGAACCGT
bE (um00577)	b2Elft164	CTACCCGAACTTTTCCCTCAC
	b2Ert451	TTCAAGGCTTTGCTTGTGTCT
bW (um00577)	b1Wlft1559	TCGAGTCTGCCTCAATTCCT
	b1Wrt1827	CTCTCCTATGCTGGCTCCAC
kpp6 (um02331)	rt-Kpp6-F new	GGTACCGTGCTCCGGAGATT
	rt-Kpp6-R new	CATCCGACTGCCCATACAT
actin (um11232)	rt-Actin-F	CTCGGGTGACGGTGTTACG
	rt-Actin-R	AGTGCGGCAGCGAGTAACC
rep1 (um03924)	rt-Rep1-F	TGGTCTCGAAGAACTCGAACAA
	rt-Rep1-R	CCGAAAAGACCGTCCAGGAT
egl1 (um06332)	rt-Egl1-F	TGCAGCAAACTGCCCAAA
	rt-Egl1-R	TCCGAGAAGCGCCACTTG
mig2-6 (um06126)	rt-Mig2-6-F	GGCCAGTTGCAAGTTCCAA
	rt-Mig2-6-R	GCTGCCGCCGTGTTCTAC
pten (um3760)	rt-PTEN-F	CGGACGTACGGGTGTCAAG
	rt-PTEN-R	CACCCTGCAGAGGCAATGT
probable *hxt5*-hexose transporter (um02037)	rt-Hxt5-F new	TCTCGTCGGGTATCGGAAAG
	rt-Hxt5-R new	CGTAACCGAGAATGAAGAAGCA
chitin deacytelase (um01788)	rt-01788-F	TCATCCCTCAAGCGGTCAAC
	rt-01788-R	AGTGCGGGATCCGCTGTA
consered hypothetical (um03116)	rt-03116-F	AGAGGCAGATCTTTGGAAACGT
	rt-03116-R	TGGTTGAACGAAGCAGAAGCT
related to polyketide synthase (um04095)	rt-04095-F	TGGCCCGCGGATGAT
	rt-04095-R	TGCCGGGCTCGTTGTC
chitinase (um06190)	rt-06190-F	CGCACGTCCACGAATAAGCT
	rt-06190-R	GAGTCGAGGCTGTCCAATCC
probable high afinity glucose transporter (um11514)	rt-11514-F	TGCTGGCCGAGTACCATGA
	rt-11514-R	GCCTTCGCCAGAGCTCTCT

Mating Assay and Plant Pathogenesis. Cell densities of liquid cultures were measured spectrophotometrically. Liquid overnight cultures were diluted in fresh media to obtain an Absorbance at 600 nm (A_{600}) of 0.1. The newly inoculated culture was allowed to grow for an additional 4 hrs to obtain an A_{600} between 0.5 - 0.7 (exponential growth phase). Mating assays were performed using 10^7 cells / ml and spotting 10 µl onto PDA-charcoal and SLAD-charcoal plates as previously described (Gold et al., 1997). Plant infection using 8-10 day old Golden Bantam corn seedlings [Bunton Seed Co., Louisville, KY and W. Atlee Burpee & Co., Warminster, PA] was performed with a cell density of 10^8 cells / ml for pre-mixed haploid strains of opposite mating type as previously described (Gold et al., 1997). Virulence was rated by a disease index (DI) on a scale of 0 to 5 with 0 = no symptoms/ healthy plants, 1= chlorosis and / or anthocyanin production, 2 = small leaf galls, 3 = small leaf and stem galls, 4 = large galls and 5 = plant death. Diseases rating for the plants were obtained after 7, 10, 14, 17, and 21 days post infection (dpi) and the disease indices for each strain were averaged to get a DI measure per strain. Statistical analysis of the disease index measures were performed using a Kruskal-Wallis Test with a Multiple Comparison Test in R (Lovely et al., 2011).

RESULTS

Filamentation in response to Ump2 is correlated with transcription level of the *ump2* gene. Previous studies in *S. cerevisiae* and *C. albicans* revealed that increasing the expression of the respective high affinity ammonium transporter lead to filamentation even under non-inducing conditions (Biswas and Morschhäuser, 2005; Rutherford et al., 2008). Thus, we endeavored to explore the role of *ump2* expression in affecting

filamentation. Interestingly, over-expression of *ump2* causes filamentous growth of haploid cells of *U. maydis* under nutrient rich conditions. This is in contrast to wild type cells which grow by budding under similar nutrient rich conditions. When grown in low ammonium (SLAD), cells carrying the *ump2* over-expression construct showed extensive filamentous growth within 2 days as opposed to the wild type cells that display filamentous growth after 3 days (Fig. 19B and D). This suggests that ammonium limitation per se might not be the only trigger for filamentous response mediated by *ump2*. Additionally, the *ump2* deletion mutant displayed no filamentous growth either under nutrient rich or under ammonium limiting conditions (Fig. 19A and C).

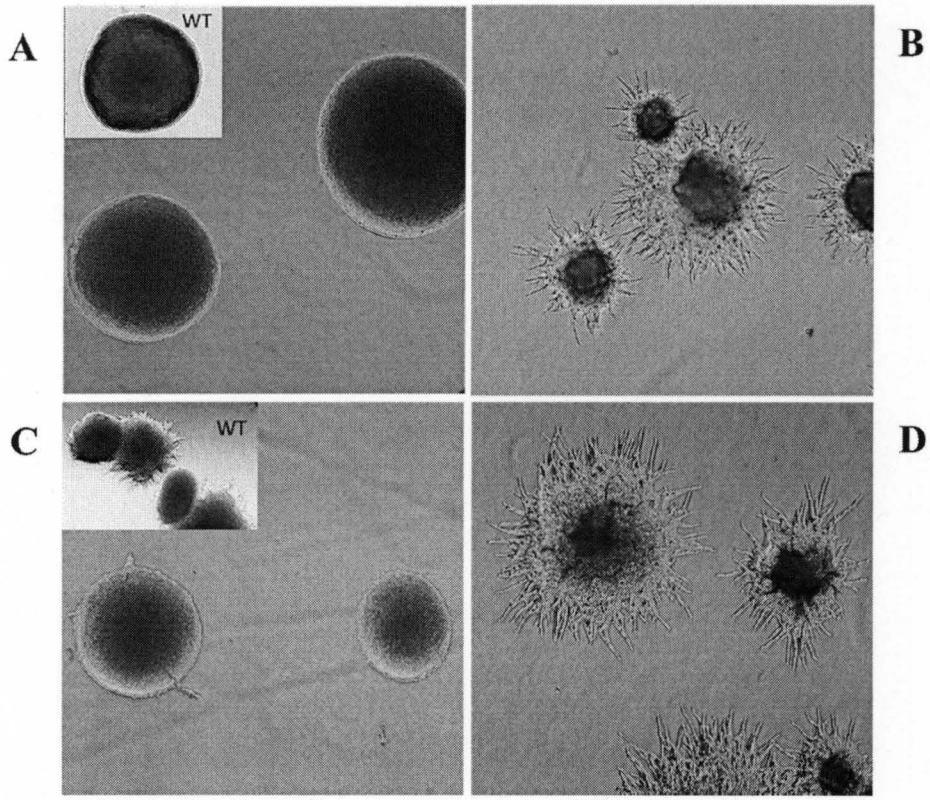

Figure 19: Haploid cells with *ump2* disruption (**A**) and *ump2* over-expression (**B**) grown on YPS (yeast, peptone and sucrose). Cells over expressing *ump2* showed filamentous growth in nitrogen and carbon replete media. Haploid cells with *ump2* disruption (**C**) and *ump2* over-expression (**D**) grown on SLAD (synthetic low ammonium) for 48 hrs. For comparison, insets (in **A** and **C**) show wild type (WT) grown under the same conditions as the mutants.

***ump2* over-expression induces transcription of genes involved in mating and pathogenicity in *U. maydis*.** The observation that filamentation occurs under nutrient rich condition led us to investigate the role of *ump2* in altering the expression of downstream effectors of filamentation. To address this question we compared the transcriptional profiles of haploid cells overexpressing *ump2* with those of haploid cells carrying deletion of the *ump2* gene, when each mutant was grown under nutrient rich or low nutrient conditions. The over-expression of *ump2* under high ammonium conditions compared to the *ump2* disruption strain resulted in differential expression of 340 genes that exhibited a twofold or greater change in expression; however, only 116 genes showed differential expression consistently between the two biological replicates. Comparison between *ump2* over-expression and *ump2* deletion under low ammonium conditions showed differential expression of 528 genes, of which expression of only 110 genes were consistent between the two biological replicates. Genes grouped under the functional categories of metabolism, cellular transport, interaction with the environment, cell rescue, defense, and virulence were enriched in the comparisons. Interestingly, genes involved in the mating response pathway (*prf1* and *mfa1*) and pathogenicity (*kpp6*) were also differentially regulated between the two mutant strains. At least 2 fold up-regulation of *prf1*, *mfa1* and *kpp6* was observed when *ump2* was overexpressed under low ammonium conditions compared to the *ump2* deletion strain under similar growth condition.

Our next aim was to confirm the findings of microarray, for which we chose a set 17 genes for qRT-PCR analysis (Table 5). The expression of these genes was determined in four strains of *U. maydis:* haploid wild type cells (*a1b1*), haploid cells of the same

genetic background except with *ump2* over-expression (*ump2*^{otef} *a1b1*), haploid cells with *ump2* deletion (Δ*ump2 a1b1*) and haploid cells with deletion of both *ump1* and *ump2* genes (Δ*ump1*Δ*ump2 a1b1)*. In the qRT-PCR, gene expression was normalized to the constitutively expressed gene *eif2B.* The summary of changes in expression levels for these genes is found in Table 6.

qRT-PCR analysis of *ump1* and *ump2*. From qRT-PCR analyses, we found that *ump1* was 70-fold up-regulated under low ammonium conditions in comparison to high ammonium conditions. *ump2* deletion affects the expression level of *ump1*, as under these circumstances *ump1* is only 27-fold up-regulated under low ammonium conditions (Fig. 20A). However, *ump1* expression further decreases when *ump2* is overexpressed. This is because *ump2* over-expression results in increase in *ump1* under high ammonium so that the increase relative to nitrogen-limiting conditions is only 3-fold (Appendix II fig. 28 E). By comparison, for wild type cells *ump2* is only 30-fold up-regulated under low ammonium condition in comparison to high ammonium and expression level of *ump2* is similar under low and high ammonium condition (Fig. 20B).

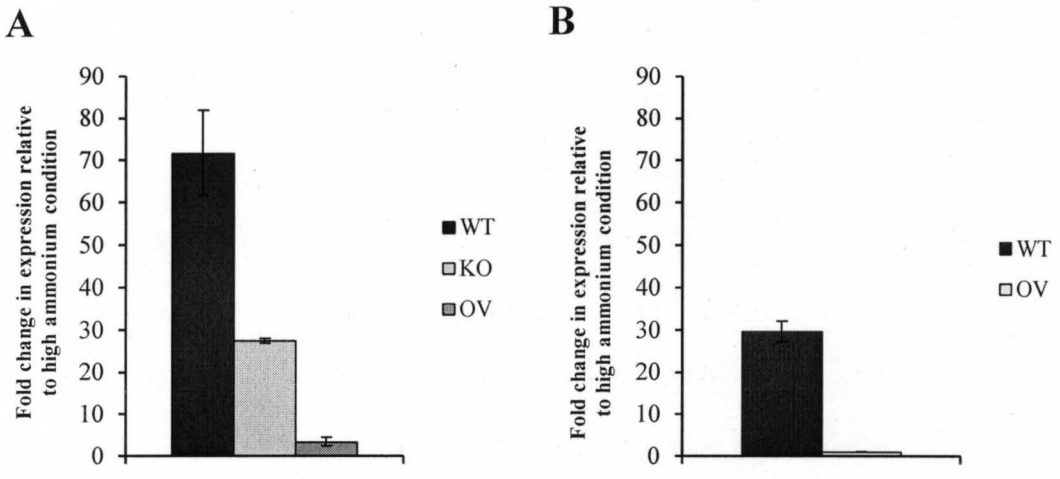

Figure 20: Relative expression under low ammonium conditions of **(A) *ump1*** and **(B) *ump2*** compared with expression under high ammonium conditions. WT=Wild type; KO= (Δ*ump2*); OV=*ump2* over-expression

Changes in *ump1* and *ump2* expression affect the expression of genes in the mating response pathway and pathogenicity. Prf1 is a transcription factor required for the mating program in *U. maydis*. Expression of the *prf1* gene was not significantly affected by the over-expression of *ump2* (~3.9-fold down-regulation under low ammonium condition *vs* 3-fold down-regulation for wild type, see Fig. 21A). In contrast, deletion of *ump2* decreased the magnitude to which *prf1* was down-regulated under low ammonium (~1.6 fold). Interestingly, *prf1* expression has an opposite trend in *ump1ump2* double deletion strain compared to the wild type, *i.e.* expression was 2-fold up-regulated under low ammonium conditions. Our results show that ammonium transporters negatively affect the expression of *prf1*. In *U. maydis prf1* controls the expression of mating type genes, particularly those of the *a* and *b* loci (Hartmann et al., 1999). So we next examined the expression of *mfa1* (mating pheromone precursor) (Fig. 21B) and *bW1/bE1* (*b* locus) (Fig. 21C). As a result of *ump2* deletion, *mfa1* expression was further reduced under low ammonium condition (~31-fold) compared to that of wild type, where *mfa1* was only 16-fold down-regulated under similar growth conditions. On the other hand, in the *ump2* over-expression strain or the *ump1ump2* double deletion strain the induction under high ammonium decreased to about 4-fold and ~2.5-fold, respectively. Expression levels of *ump1* and *ump2* also affected *bE/W* expression. While wild type haploid cells exhibit a 25-fold induction of *bW/bE* under low ammonium conditions, this level was dramatically reduced to ~4-fold in the *ump2* deletion strains. In strains deleted for both *ump1* and *ump2*, *bW1* expression was actually reduced in low ammonium compared to high ammonium (~3-fold).

kpp6, a MAPK gene essential for pathogenicity on the host, was determined to be differentially expressed in the microarray screen. Expression analysis of *kpp6* in the different mutants of ammonium transporters revealed that while this gene is down-regulated under low ammonium conditions in the wild type cells, the *ump* double mutants displayed a decrease in the magnitude of down-regulation under low ammonium conditions (Fig. 21D).

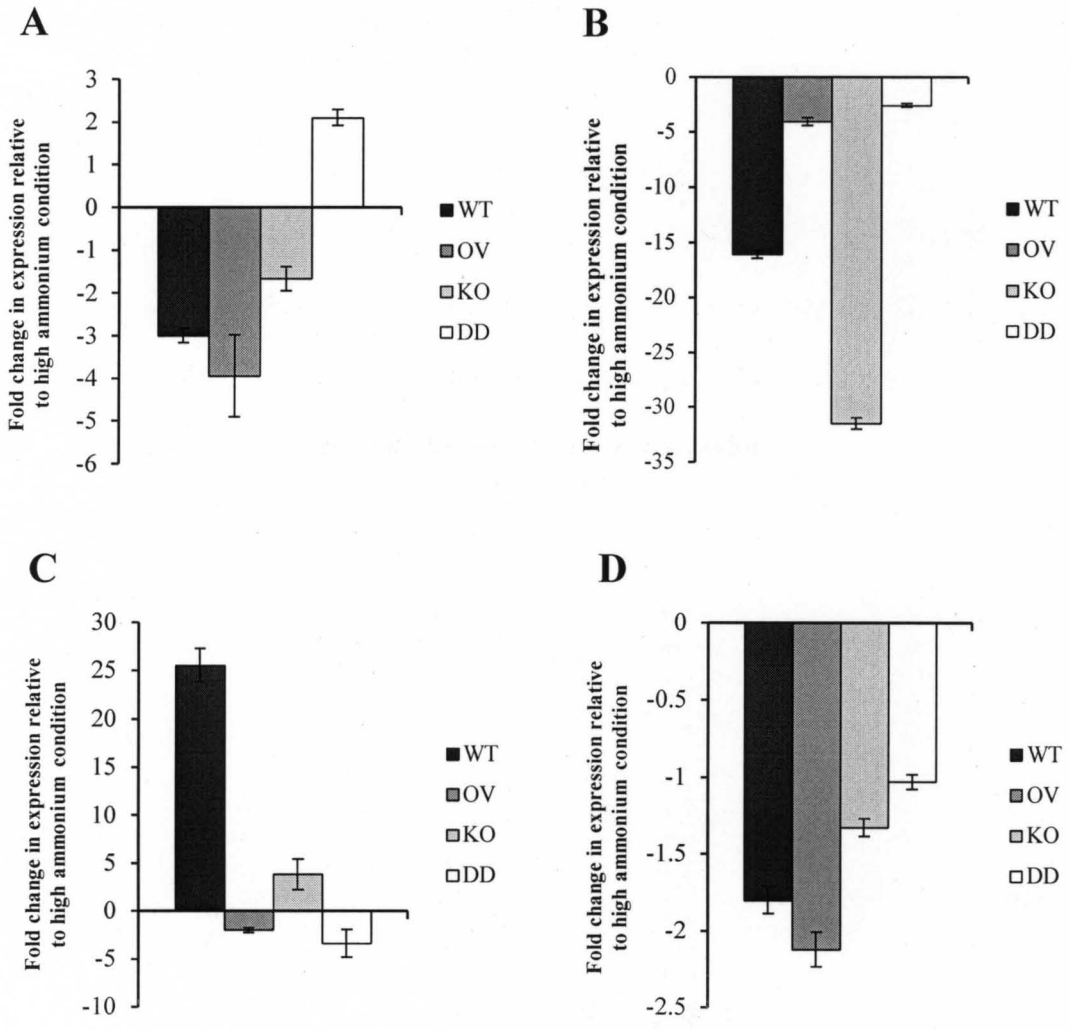

Figure 21: Relative expression under low ammonium conditions of **(A)** ***prf1,*** **(B)** ***mfa1,*** **(C)** ***bE/W*** and **(D)** ***kpp6*** compared with expression under high ammonium conditions. WT= Wild type; KO= Δ*ump2*; OV= *ump2* over-expression; DD= Δ*ump1*Δ*ump2*

TABLE 6. Fold change [a] in expression relative to high ammonium condition

Genes of Interest	Wild type	Δ*ump2*	Δ*ump1* Δ*ump2*	*ump2* OvEx[b]
ump1 (um04523)	71	27	NA	3
ump2 (um05889)	29	NA	NA	1
prf1 (um02713)	-3	-1.6	2	-3.9
mfa1 (um02382)	-16	-31	-2.5	-4
bE/W (um00577/um00578)	25	3.7	-3.4	-2
kpp6 (um02331)	-1.8	-1.3	-1	-2
rep1 (um03924)	-158	-92	-60	-60
egl1 (um06332)	9	2.5	-3.9	1.4
mig2-6 (um06126)	-371	-18	-1.8	-10
chitin deacytelase (um01788)	-1.5	-1.4	-1.7	-140
chitinase (um06190)	-374	-241	-8	NA
actin (um11232)	-2	-1.7	-18.8	-128
pten (um03760)	7	1	4	-4
probable *hxt5*-hexose transporter (um02037)	1.2	-1	-1.5	2.6
probable high affinity glucose transporter (um11514)	-12	-5.9	6	-40
related to polyketide synthase (um04095)	-10	4.7	NA	-37
conserved hypothetical (um03116)	-23	-112	-7.5	-3

[a] positive value means up regulation and negative value means down regulation under low ammonium

[b] *ump2* OvEx, over-expression strain for *ump2*, *ump2*^{otef}*a1*

Differential expression of the *U. maydis* genes coding for secreted enzymes in the various ammonium transporter mutants. It has been proposed that for the biotrophic development and establishment of *U. maydis* in the host, secreted protein effectors are important (Mueller et al., 2008). Several genes encoding such secreted proteins (endoglucanase (egl1), *rep1* gene coding for repellent, maize induced gene-*mig2-6*, chitinase and chitin deacetylase) were differentially expressed when comparing the *ump2* over-expression and *ump2* deletion strains in the microarray analysis. The expression of these were also confrmed using qRT-PCR (Fig. 22). The changes in expression in each mutant is listed in Table 6, but we highlight a few of the findings here. The expression of the gene coding for repellent protein, *rep1*, in *U. maydis* is dependent on the expression of *ump2* (Fig. 22A). Under low ammonium conditions *rep1* is down-regulated (~150-

fold), however deletion, of *ump2* alone or together with *ump1* decreases the extent of this down-regulation. *egl1,* which encodes a cellulase, is specifically expressed in the filaments in *U. maydis* (Teertstra et al., 2009; Wösten et al., 1996). The deletion of *ump2* reduces the magnitude of induction under low ammonium conditions, whereas the double deletion mutant actually experiences an overall decrease in steady state mRNA levels of *egl1* under similar growth conditions (Fig. 22B).

mig2-6 expression was identified as occuring during plant infection (Farfsing et al., 2005). Interestingly, our experiments suggest that under low ammonium conditions *mig2-6* is highly down-regulated (~300-fold; Fig. 22C). Changes in the expression levels of the ammonium transporters affect the expression of this gene: all the mutants examined had strongly reduced the degree to which this gene was down-regulated in low ammonium compared to the observations with the wild type. Another gene which shows an interesting expression pattern is the chitin deacetylase gene, which is proposed to be involved in fungal cell wall modification (Mueller et al., 2008). This gene is highly down-regulated (~140-fold) under conditions of low ammonium when *ump2* is over-expressed, whereas the *ump2* single and *ump1ump2* double disruption mutants showed expression patterns similar to those of wildtype (Fig. 22 D).

Another interesting finding was the effect of *ump2* expression on the expression level of the *actin* gene. Actin is often used as a reference gene in *U. maydis* real-time PCR analysis (Heimel et al., 2010). However, our study demonstrates that over-expression of *ump2* severely affected the expression of actin under low ammonium conditions (~125-fold down-regulated) while deletion of both *ump1* and *ump2* was

associated with a greater fold reduction for expression of actin when compared to the wild type (~18-fold; Fig. 22E).

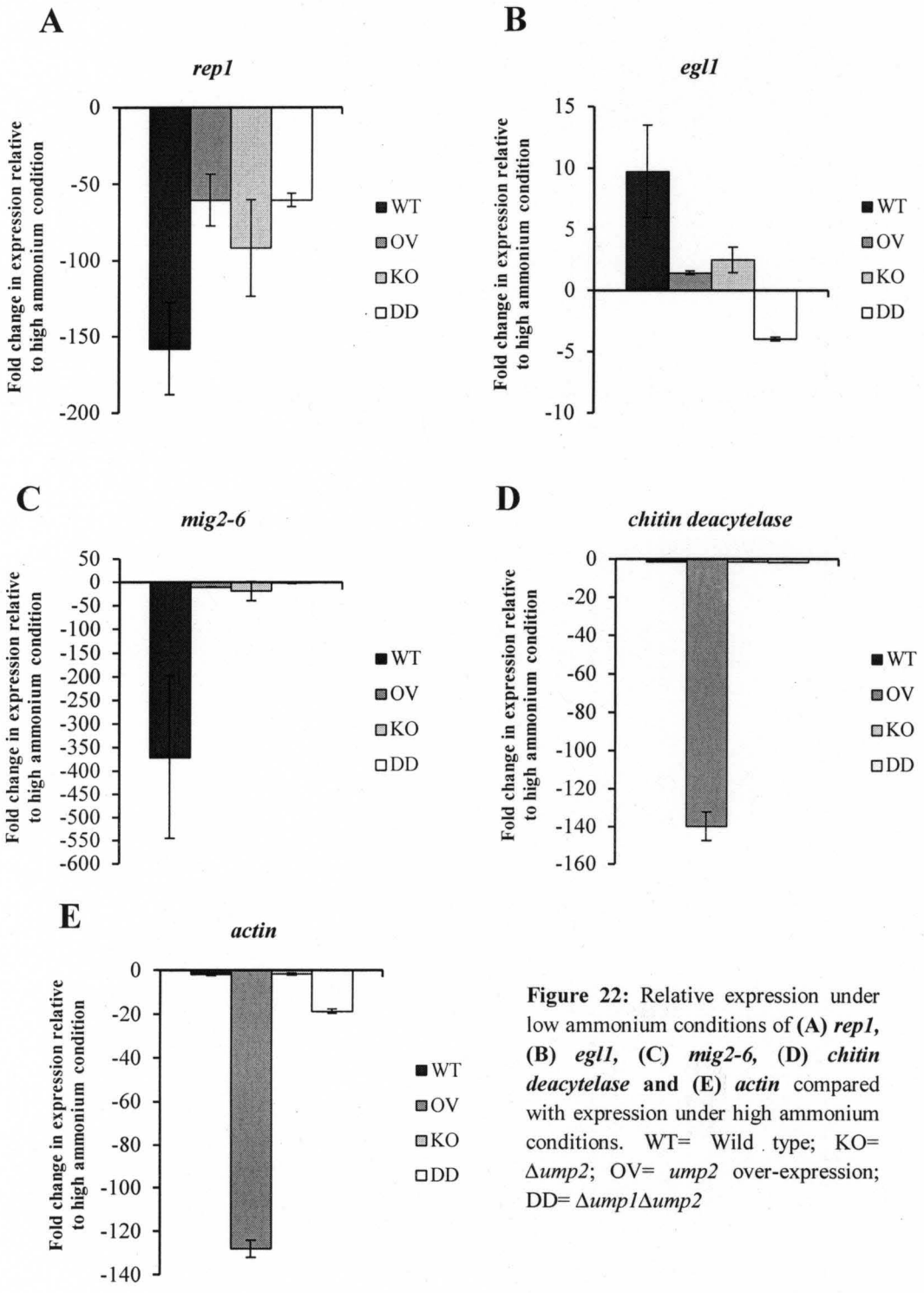

Figure 22: Relative expression under low ammonium conditions of **(A)** ***rep1,*** **(B)** ***egl1,*** **(C)** ***mig2-6,*** **(D)** ***chitin deacytelase*** **and (E)** ***actin*** compared with expression under high ammonium conditions. WT= Wild type; KO= Δ*ump2*; OV= *ump2* over-expression; DD= Δ*ump1*Δ*ump2*

Changes in the expression of several other genes are listed in Table 6 and graphically represented in the supplementary figures (see Appendix II).

Mating efficiency of *U. maydis* is mostly unaffected in mutants of ammonium transporters. Differences in the expression of *mfa1* and *bE/W* in the *ump2* deletion strain compared to the wild type strain led us to examine mating efficiency, as measured by aerial hyphae "fuz" of these strains on charcoal agar (Banuett, 1995). The mating assay was conducted on both nutrient rich (PDA) plates (Fig. 23) and SLAD plates (Fig. 24) containing charcoal.

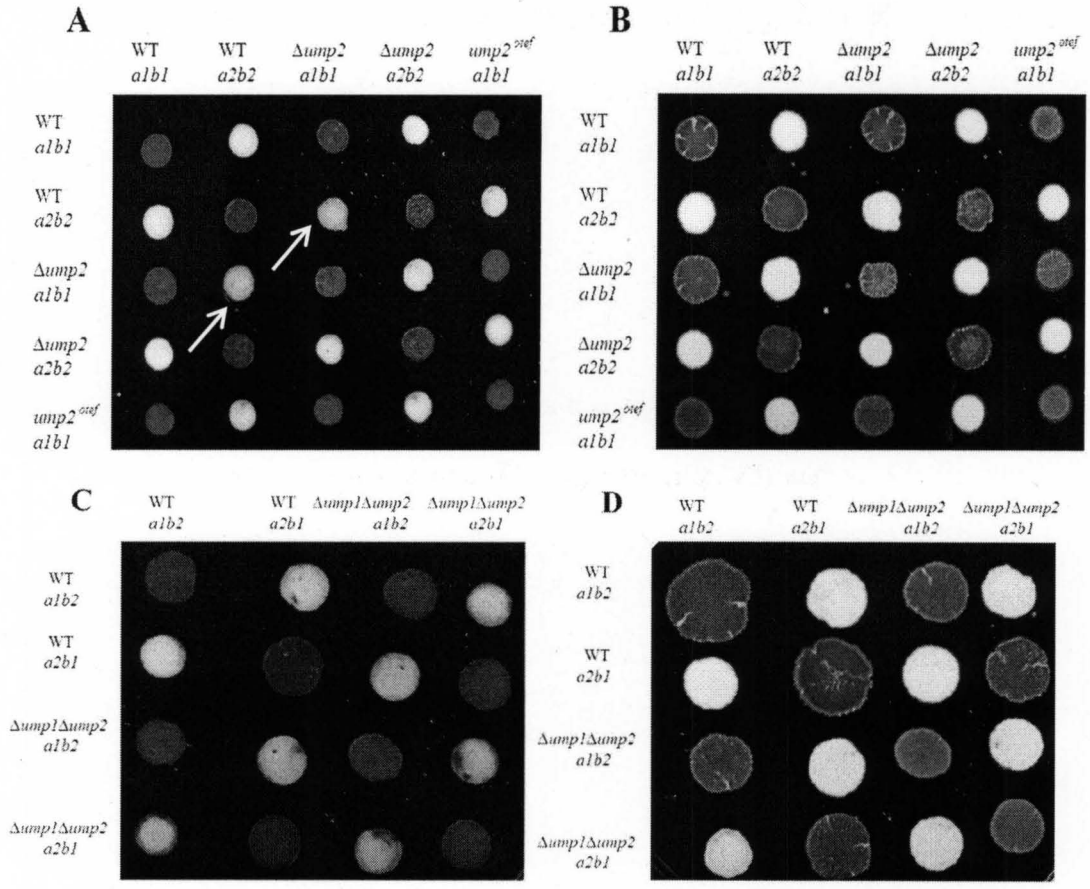

Figure 23: Plate mating assay under nutrient rich conditions for WT, *ump2* over-expression, *ump2* deletion **(A and B)**, *ump1ump2* double deletion strains **(C and D)**. Equal mixtures of haploid strains of opposite mating-type background were plated onto PDA plates containing activated charcoal and were grown for 24 hrs **(A and C)** and 48 hrs **(B and D)**. A positive mating reacting produced a white "fuz" phenotype of aerial hyphae production.

Plate mating assay revealed that although the strain with *ump2* deletion in the *a1b1* displayed a slight reduction in mating on nutrient rich PDA plates after 24 hours (Fig. 23A, indicated by arrows), this difference was no longer observed when the strains were allowed to grow for an additional 24 hours (Fig. 23B).

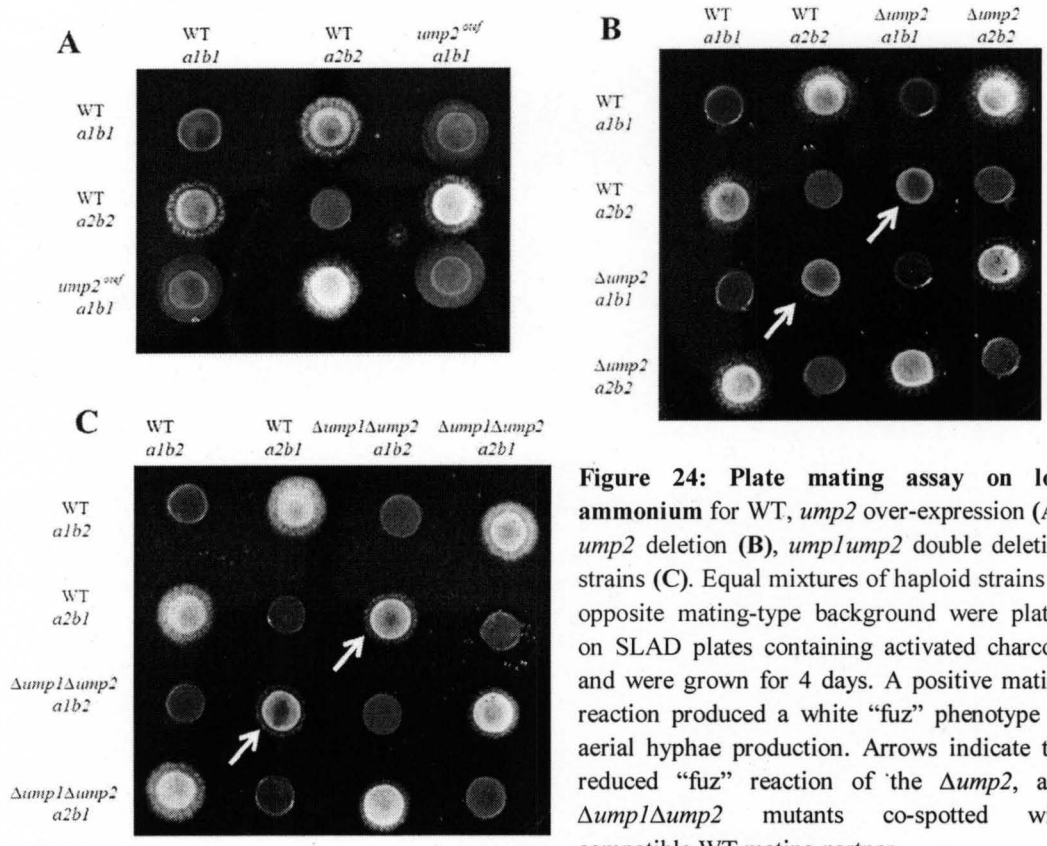

Figure 24: Plate mating assay on low ammonium for WT, *ump2* over-expression **(A)**, *ump2* deletion **(B)**, *ump1ump2* double deletion strains **(C)**. Equal mixtures of haploid strains of opposite mating-type background were plated on SLAD plates containing activated charcoal and were grown for 4 days. A positive mating reaction produced a white "fuz" phenotype of aerial hyphae production. Arrows indicate the reduced "fuz" reaction of the *Δump2*, and *Δump1Δump2* mutants co-spotted with compatible WT mating partner

Mating efficiency was observed for 4-5 days on SLAD plates containing charcoal, as the cells are slower to grow under these conditions. The mating between the wild type strains (*a1b1* X *a2b2*) displayed the white "fuz" phenotype resulting from aerial hyphae production and they also produced filaments around the colony resulting from co-spotting cells of opposite mating type. These filaments were specific to the mating reaction and not similar to the filaments observed under low ammonium condition as individual

mating type cells did not display this phenotype. A drastic reduction in the mating efficiency was observed when cells deleted for *ump2* in the *a1b1* were co-spotted with wild type cells of the opposite mating type (*a2b2*; see Fig. 24B, arrows). However, this reduction was limited to deletion of *ump2* in the *a1b1* background and not observed when *ump2* was deleted in the *a2b2* background. A slight reduction in the mating efficiency was also observed in the *ump1ump2* deletion strains and this reduction was also limited to one strain, with *a1b2* background (see Fig. 24C, arrow). These results would imply that the decrease in mating is dependent on the *a* locus, specifically *a1*, and is probably affected by *ump2* expression levels. However, effects of mating-type background on phenotypic differences associated with gene disruption or over-expression, have been observed in earlier studies and is yet an unexplained phenomenon (Pham et al., 2009).

Δ*ump1*Δ*ump2* mutant strains are attenuated for virulence. To determine the role of ammonium transporters in virulence, compatible mutant strains of opposite mating type were mixed and co-injected into 8-10 day old maize seedlings. Measures of virulence were taken at regular intervals (7, 10, 14, 17, and 21 days post infection). The virulence for each infection was measured by a disease index, where the severity of the disease was assessed on a scale of 0-5.

TABLE 7. Pathogenicity of mutant strains.

Treatment	Strains	Plants Tested	Disease rating by number of affected plants						Disease Index
			0 Points	1 Point	2 Points	3 Points	4 Points	5 Points	
1	*WT*	32	1	12	8	1	0	10	2.53
2	Δ*ump2*	32	4	9	3	5	4	7	2.53
3	$ump2^{Otef}$	33	2	15	3	1	2	10	2.42
4	Δ*ump1*Δ*ump2*	32	10	20	1	0	0	1	0.84**

** significant compared to all strains tested, Kruskal-Wallis Test with a Multiple Comparison Test with p value < 0.05

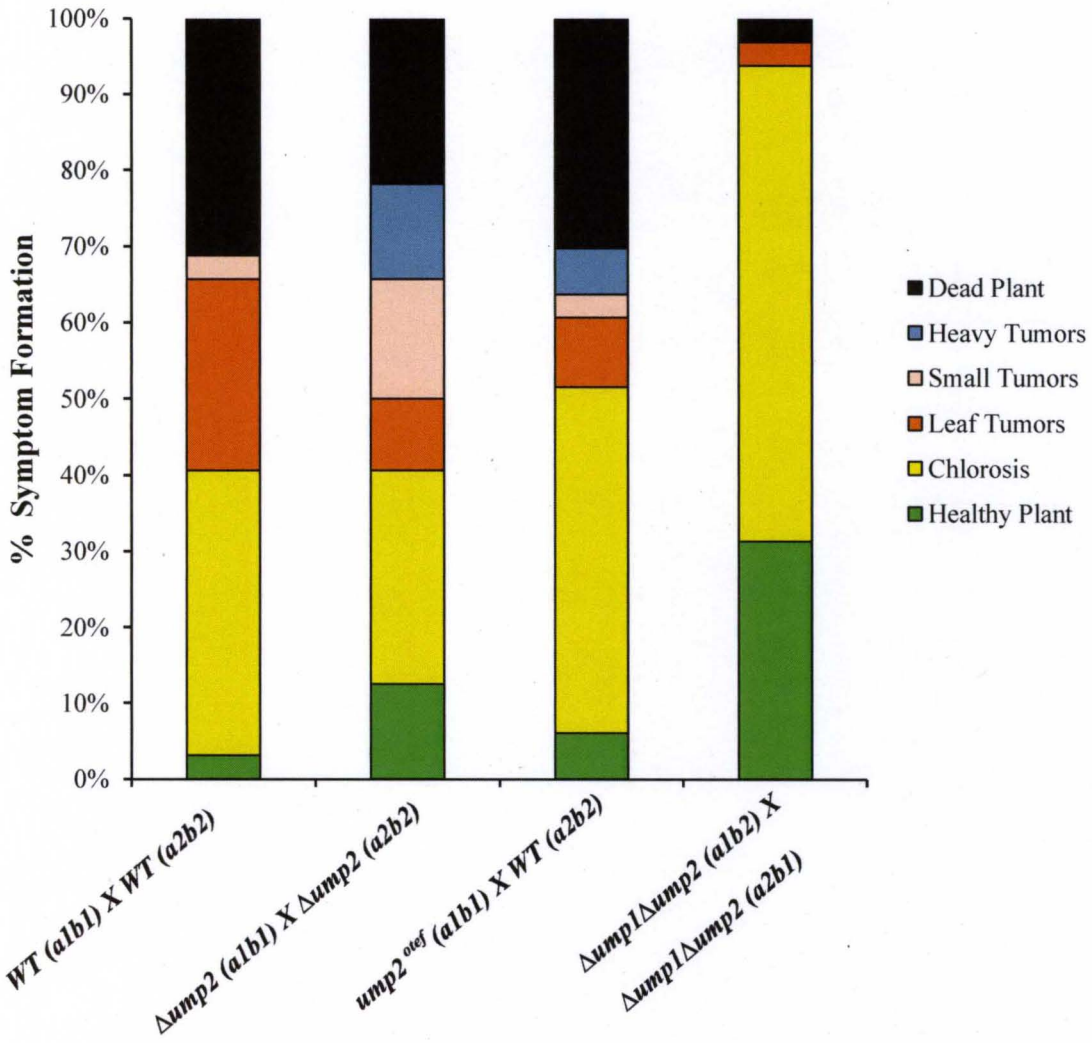

Figure 25: Disease symptom formation in the various *U. maydis* mutant strains. All strains were rated at 21 days. Plants were inoculated with one paired background, indicated on the X-axis. The graphs display the percentage of plants with specific symptoms of infection. *ump1ump2* double deletion strain displayed reduced pathogenicity. This was statistically significant compared to the wild type and to the other mutants used in this study.

The results presented in Table 7, indicate that although the deletion of the high affinity ammonium transporter did not reduce virulence compared to the wild type, the absence of both the high and the low affinity ammonium transporters considerably decreased the disease symptoms on the host and this reduction was statistically significant compared to the other infections. The results are also presented in a percent of

symptom formation graph (Doehlemann et al., 2009), Figure 25. In the *ump1ump2* double mutant, there were significantly more healthy plants surviving at the end of the study and in most of the plants (~60%), the fungus was unable to progress in infection beyond stage 1 (chlorosis) (see Fig. 25).

DISCUSSION

The transition from yeast-like growth to filamentous growth is an essential strategy that many fungi utilize to cause disease on the host. In the basidiomycete fungus, *U. maydis*, this dimorphic transition changes the life style of the fungi from being saprophytic to being pathogenic. The transition in the life style occurs in response to sensing different environmental conditions (Bahn et al., 2007). One of the factors responsible for the transition is nutrient limitation, specifically for nitrogen. One of the most preferred sources of nitrogen is ammonium and in *U. maydis* under condition of low ammonium (SLAD), the yeast-like cells differentiate to form filaments. The filamentous growth of haploid cells observed in response to low ammonium is functionally distinct from filaments formed as a result of mating followed by the formation of dikaryon. Therefore it has not been clear that the developmental program associated with filamentous growth under ammonium limiting condition in the laboratory is of any functional importance with respect to the ability of *U. maydis* to be pathogenic.

The observation that genes induced during infection were also induced under nitrogen limiting condition led Snoeijers et al. (2000) to propose that the pathogen would face similar growth conditions in the host. Furthermore in *C. lindemuthianum*, there was reduction in pathogenicity and in *M. grisea* reduction in the expression of the

pathogenesis genes, due to mutations in genes controlling nitrogen metabolism. This led Ho et al. (2007) to suggest the importance of characterizing genes involved in nitrogen metabolism and its control for a more complete understanding of *U. maydis* pathogenicity (Ho et al., 2007; Lau and Hamer, 1996; Pellier et al., 2003).

In different fungi the developmental processes like filamentous growth, invasive growth and mating in response to low ammonium are dependent on the Amt family of ammonium permeases. For example, haploid invasive growth in response to low ammonium in *S. pombe*, *S. cerevisiae* and *C. neoformans* and mating the response in *C. neoformans* are dependent on ammonium transporters (Mitsuzawa, 2006; Rutherford et al., 2008; Rutherford et al., 2008). The two ammonium permeases of *U. maydis* are involved in transporting ammonium into the cell and an additional 'sensor of ammonium' function is assigned to the high affinity ammonium transporter (Ump2). In this present study we present evidence for the role of *ump2* in controlling differentiation under both nitrogen limiting and nitrogen replete conditions by affecting gene expression of several possible direct or indirect target genes.

Typically, the *U. maydis* cells grow by budding under nutrient rich conditions, but under conditions of nutrient limitation the cells are filamentous. Mutants of *ump2,* while retaining the same non-filamentous phenotype as the wild type cells under nutrient rich conditions, display lack of filamentous growth under conditions of low ammonium. Similar to the findings in *S. cerevisiae* and *C. albicans,* we were able to link the ability of *U. maydis* to grow filamentously to the expression levels of the high affinity ammonium transporter (Biswas and Morschhäuser, 2005; Rutherford et al., 2008). We established that an increase in the expression of *ump2* increased the degree of filamentation.

Increased expression of *ump2* made the cells filament faster under low ammonium conditions, within 2 days, and more interestingly, it triggered filamentous growth under nutrient rich conditions. This suggests a possible role of *ump2* in dimorphic transition independent of low ammonium. To determine additional roles of *ump2* in regulating filamentous growth under non-inducing conditions, we compared the transcriptional profiles of cells over-expressing *ump2* to those of cells deleted for *ump2*, under both nutrient rich and nutrient limited conditions, followed by qRT-PCR analysis.

Transcript comparison of *ump1* and *ump2* under low ammonium revealed that although *ump1* is the low affinity ammonium transporter, the expression of *ump1* is higher than that of *ump2* under low ammonium conditions. Perhaps this reflects that the capacity of Ump1 for ammonium transport is not as good as that of Ump2 and therefore *ump1* needs to attain higher expression levels to perform its function of ammonium transport. Moreover, the expression of *ump1* is dependent on the expression of *ump2*, as we observe a decrease in *ump1* expression when *ump2* is deleted. The impact of Ump2 on Ump1 expression is also evident when *ump2* is overexpressed. Although when *ump2* is overexpressed, a dramatic induction of *ump1* under low ammonium condition is not evident, this could be attributed to the fact that *ump1* expression will increase under high ammonium condition under the influence of a constitutively active *ump2* and the differences in expression under high and low ammonium would be reduced further (see Fig. 28E). These results suggest a direct correlation between the expression levels of the two ammonium transporters in *U. maydis*.

In *U. maydis* it is proposed that mating is induced in response to nutrient deprivation under the control of cAMP-PKA pathway, which represses hyphal growth

and induces the mating pathway (Sánchez-Martínez and Pérez-Martín, 2001). However, in the absence of a compatible mating partner, the cells undertake filamentous growth under similar growth condition (Kahmann et al., 1999; Sánchez-Martínez and Pérez-Martín, 2001). Nitrogen starvation also induces the formation of conjugation tubes and true filaments, although, the formation of the former is dependent on the presence of compatible *a* mating loci and the latter is dependent on active *a* and *b* loci (Banuett and Herskowitz, 1994). The transcription factor Prf1 is involved in integrating the environmental signals and affects the morphological outcomes of *U. maydis* development. However the role of *prf1* for filamentous growth in haploid cells is unclear (Sánchez-Martínez and Pérez-Martín, 2001). Expression analysis of *prf1* reveals that this gene is down-regulated under low ammonium conditions in wild type haploid cells. Similar results were also obtained for *mfa1* expression, whereas *bE/W* was induced under low ammonium conditions. We hypothesize that the down-regulation in the expression of *prf1* and *mfa1* could be associated with the absence of a compatible mating partner, and the induction of *bE/W* is consistent with the role of this gene in filamentation; but, in this case the filamentation concerns haploid cells under low ammonium.

Monitoring the expression levels of *prf1*, *mfa1* and *bE/W* with the different mutants *ump* backgrounds revealed that these genes are not only affected by *ump2* expression but also by *ump1* expression. *prf1* expression is negatively regulated by the ammonium transporters, as deletion of *ump2* up-regulates the expression of *prf1* under low ammonium and the *ump1ump2* further induces the expression of *prf1* under low ammonium in comparison to the wild type. Based on the expression data we propose a role for the ammonium transporter expression in transmitting information regarding

surrounding environmental conditions to the developmental programs to help decide the fate of the cells under nutrient limiting conditions. Conditions of nutrient limitation, specifically ammonium, are sensed by Ump2 which relays the signal to downstream effectors. In response to nutrient limitation, the cells sense the presence/ absence of a mating partner. In the absence of a compatible partner, there is reduction in the expression of genes involved in the mating pathway, specifically *prf1* and *mfa1*. Furthermore, in the absence of *ump2* expression (*i.e.*, *ump2* deletion), the cells are unable to sense the nitrogen starvation. They, therefore, do not initiate the mating response pathway and further decrease the expression of *mfa1* under low ammonium in comparison to the wild type. *ump1* expression also affects *mfa1* expression as deletion of *ump1* in the *ump2* deletion strains increases the expression of *mfa1* under low ammonium. The decrease in the expression of *mfa1* could be a factor responsible for the reduction in the mating response displayed by the *ump2* deletion strains under low ammonium condition. However, this phenotype was much more dramatic in the *a1b1* back ground in comparison to the *a2b2* background. A subtle difference in mating efficiency was also observed in mating between *a1b1* and *a2b2*, when both strains carried the *ump2* deletion. Again, the reduction seemed to be associated specifically with cells bearing the *a1* idiomorph.

The induction in the expression of *bE/W* under low ammonium suggests the involvement for this mating locus in filamentous growth under nitrogen starvation. Our data suggest that the expression of *bE/W* under such growth conditions is controlled by expression of both the high and the low affinity ammonium transporter. Lack of filamentous growth in the *ump2* deletion strain, is associated with reduced induction of

bE/W and deletion of both *ump1* and *ump2* further decreases the expression of *bE/W* under low ammonium conditions. This also suggests that the tangled filaments formed under low ammonium condition by the *ump1ump2* double mutant are not dependent on *bE/W* expression. The inability of the double mutants to maintain the expression of bE/W could be one of the possible reasons for the inefficiency of the double mutants to cause disease on the host plant. The inability of the double mutants to cause disease on the plants could also be attributed to the changes in the expression level of the MAPK, Kpp6, in these mutants compared to the wild type. *kpp6* has previously been shown to be important for pathogenicity, as *kpp6* mutants strains were attenuated in pathogenicity (Brachmann et al., 2003). The role of ammonium transporters in pathogenicity for the double mutants could be through their inability to transport ammonium under limiting conditions or by inappropriate control of bE/W or Kpp6 which would, in turn, regulate other genes essential for causing disease to the host. Another possibility for the role of ammonium transporters in affecting pathogenicity is by directly or indirectly affecting the expression of other genes (not identified in our screens) essential for pathogenesis.

Ammonium transporter expression also affects expression of other transporter proteins and expression of genes coding for secreted enzymes. Some of these secreted proteins are expressed during growth of the fungus inside the host, while others are expressed specifically during filamentous growth (Farfsing et al., 2005; Schauwecker et al., 1995; Teertstra et al., 2009; Wösten et al., 1996). Yet, none of these genes has been shown to be essential for pathogenicity on the host plant. This would suggest that ammonium transporters might play a role in assessing the nutrient situation inside the

host during infection and transmit this signal to the pathogen for effective colonization and progression inside the host.

In conclusion, ammonium transporters play a role in determining the morphogenetic fate of the cells under low ammonium condition by affecting more than one signaling pathway. At one level, interaction of Ump2 with Rho1 protein affects the filamentation ability under nutrient rich and nutrient limiting condition (see Chapter 3); at another level, *ump2* helps signal to the cell the environmental milieu in which it is present, and with the help of the cAMP-PKA pathway to decide whether to undergo mating (in the presence of a compatible mating partner) or filamentous growth in response to nitrogen starvation. Ammonium transporter proteins affect the pathogenicity of *U. maydis* by either affecting the ability of the cells to sense and transport ammonium in nutrient limiting environments, which could be essential for growth in the plant. Alternatively, Ump1 and Ump2 could affect, directly or indirectly, control transcription of genes essential for morphogenesis and pathogenicity, as a response to particular environments.

CHAPTER V

GENERAL CONCLUSION

Dimorphic transition from a yeast-like budding growth form to a filamentous growth form is an essential strategy that many animal and plant pathogens undertake to cause disease to their hosts. This transition occurs in response to a myriad of environmental cues, which could be a measure undertaken to adapt to new conditions. This transition could also occur in response to host cues, as a trigger to cause infection. Although the result of sensing the different cues is filamentous growth, the filaments that are created are not necessarily similar in function. Moreover, the control of the different filamentous growth responses, or the developmental programs associated with the morphogenetic transitions would be different with very little overlap. This study focuses on understanding the functioning of ammonium transporter proteins (Amts) in sensing and affecting growth under nitrogen replete and nitrogen limiting conditions. All Amts transport ammonium, while some also sense the limiting ammonium conditions in the environment. In doing so, these fungal Amts are able to sense the ammonium limiting condition and in response to such an environment induce the yeast-like cells to grow filamentously.

Amts are conserved across many taxa and they are also well characterized among fungi (Wirén and Merrick, 2004). Among fungi, Amt proteins

are characterized as high affinity and low affinity ammonium transporters. Some of the high affinity Amts "sense" the condition of low ammonium and transmit this signal, followed by morphogenetic transition of the cell to grow filamentously. The high affinity ammonium transporter of *S. cerevisiae*, Mep2, is essential for the pseudohyphal growth response to low ammonium in this organism. Similarly, in *U. maydis*, the high affinity ammonium permease, Ump2, is essential for the cells to grow filamentously under similar nutrient conditions (Smith et al., 2003). To determine the conservation of function of the putative ammonium transporters in *M. violaceum* and *B. cinerea*, we utilized the ammonium transporter mutants of *S. cerevisiae* and *U. maydis*.

Expression of the two Meps from *M. violaceum* in the *U. maydis ump2* mutant revealed that only MepC and not MepA can rescue, to some extent, the filamentation defect in *U. maydis*. This could suggest that MepC also possesses the sensor function associated with high affinity ammonium transporters. However, this could not be confirmed in *S. cerevisiae* Mep mutants. The ability of MepA to rescue the growth defect of *S. cerevisiae* Mep mutants suggests a role for MepA in ammonium transport. Additional experiments to confirm the role of these proteins in ammonium sensing and transport needs to be done in *M. violaceum*. Meps from *B. cinerea* were also expressed in Mep mutants of *S. cerevisiae* and *U. maydis*. Although all the three *B. cinerea* Meps were able to rescue the filamentation defect in *U. maydis ump2* mutant, none of them were able to rescue the pseudohyphal growth defect in *S. cerevisiae*. Nevertheless, all three Meps of *B. cinerea* were able to rescue the growth defect to different degrees in *S. cerevisiae*. While heterologous systems are useful for the initial characterization of genes with unknown function, the real function of the gene can only be assigned after they are

examined in the respective organisms. That is, to determine whether any of the three Meps from *B. cinerea* acts as a sensor of low ammonium and which of these are only involved in ammonium transport, it would be essential to study these genes in their natural host, *B. cinerea*. Such work is currently underway by our collaborators (R. Martín-Dominquez and E. Benito, personal communication).

To further characterize the functioning of ammonium transporters in *U. maydis*, we determined the interaction partners of Ump1 and Ump2. Studies in *E. coli*, *A. fulgidus* and *S. cerevisiae* suggest that ammonium transporter proteins interact with each other to form oligomers (Andrade et al., 2005; Blakey et al., 2002; Rutherford et al., 2008). Although the functional importance of the self-association among ammonium transporters is not clear, a few studies indicate that hetero-dimeric association helps modulate the transport function of one member by the other (Marini et al., 2000). Moreover, the only crystal structure of an Amt, that of AmtB from *E. coli*, suggests that it is the interface of the homotrimer that actually serves as the channel through which NH_4^+/NH^3 passes into the cell (Zheng et al., 2004). Our study indicates that *U. maydis* ammonium transporter proteins physically interact among themselves and with each other to form homo- as well as heterodimers. Our study also demonstrates for the first time the physical interaction of an ammonium transporter with a signaling protein. After a yeast two-hybrid analysis initially suggested that Ump2 was an interacting partner of Rho1 (Pham et al., 2009), we were able to show that Rho1 interacts physically with both Ump1 and Ump2. Based on the experiments conducted to determine the consequence of the interaction at the genetic level, we hypothesize that interaction of Rho1 with Ump2 might be to sequester Rho1 away from Rac1, thereby positively affecting filamentation. In

situations when *ump2* is not expressed, *i.e.*, growth under nutrient rich conditions or when *ump2* is deleted, Rho1 would be free to block Rac1 activity and thereby suppress filamentous growth. These results suggest that ammonium transporter proteins interact with members of the signaling pathway to affect downstream effectors to control filamentous growth response.

Analogous to the observation in *S. cerevisiae* and *C. albicans*, it was found that filamentation due to *ump2* expression was dependent on its expression level (Biswas and Morschhäuser, 2005; Rutherford et al., 2008). Over-expression of *ump2* resulted in filamentous growth even under non-inducing conditions (nutrient rich conditions). This suggested that *ump2* could be involved in filamentation response even under non-inducing conditions and might interact with or affect signal transduction pathways to initiate the appropriate morphogenetic response. To further determine the role of ammonium transporters in affecting filamentation by controlling downstream effectors we compared the transcriptional profile of cells deleted for *ump2* and cells overexpressing *ump2* grown under both nutrient rich and nutrient limiting conditions. The results obtained were confirmed for several genes by qRT-PCR analysis. Our study reveals the role Ump2 in controlling the expression of genes involved in mating response pathway (*prf1*, *mfa1* and *b* loci genes) and in pathogenicity (*kpp6*). It was observed that while *ump2* negatively regulates the expression of *prf1*, *ump1* deletion further affects the expression of *prf1*. *ump1*, along with *ump2*, affects the expression of *mfa1* and also the *b* mating locus genes. While the decrease in the expression of *ump2* (*ump2* deletion) decreased the expression of *bE/W* under low ammonium conditions, it increased the magnitude to which *mfa1* is down-regulated under similar growth conditions. It has been

proposed that nitrogen starvation induces mating in *U. maydis* and that such conditions are essential for the formation of conjugation tube and true filaments (Banuett and Herskowitz, 1994; Sánchez-Martínez and Pérez-Martín, 2001). A role of ammonium transporters in affecting mating has also been suggested in *S. pombe* and *C. neoformans* (Leupold, 1987; Rutherford et al., 2008). Taken together, this suggests a role of *ump2* in transmitting the signal of the prevailing environmental conditions to the cell, which can thereby decide whether to undertake mating or not. The cells under nutrient limiting conditions are ready to undergo mating, but in the absence of a compatible mating partner these cells repress the expression of genes associated with mating. In the absence of mating, they instead undertake the morphogenetic response associated with low ammonium, *i.e.,* filamentous growth. An induction in the expression of bE/W in the haploid under such conditions suggests that that this gene might be involved in the filamentous growth associated with low ammonium.

ump1 and *ump2* also affect the transcription of genes that are specifically shown to be expressed during filamentous growth or only previously found to be expressed during the growth of the fungus inside the host. Ump2 also affected the transcription of genes that are associated with the modification of the fungal cell wall. In short, Ump2 affects the transcription of genes coding for secreted enzymes. Although none of these proteins are known to be essential for pathogenicity, they are expressed once the fungus establishes itself inside the host. This possibly suggests a role of ammonium transporter proteins in assessing the environment the fungus faces during different stages of infection and this helps regulate corresponding gene expression.

The ability of the fungi to succeed inside the host depends on their ability to utilize the host nutrients efficiently. Studies conducted to identify pathogenicity genes in fungi have revealed genes associated with fungal nutrition. For example, mutations in genes coding for enzymes in the glyoxylate pathway show that this pathway is essential for pathogenicity in many organisms (Dunn et al., 2009). Additionally, mutations affecting fatty acid oxidation and production of secondary metabolites in *Alternaria alternate* led to loss of pathogenicity in this organism (Imazaki et al., 2010; Van De Wouw and Howlett, 2011). Studies conducted in *U. maydis* have identified the importance for virulence of iron accumulation genes, where mutations associated with these genes led to reduced virulence of the fungus (Eichhorn et al., 2006). Studies have also revealed the expression of a high affinity sucrose transporter only during infection, and mutation in this gene renders *U. maydis* non-pathogenic (Wahl et al., 2010). These studies show the importance of these different processes for pathogenicity. Our study reveals the importance of ammonium transporter proteins in pathogenicity of *U. maydis*. We show that while the deletion or over-expression of the high affinity Amt, Ump2, did not obviously impair pathogenicity, the *Δump1Δump2* double deletion mutants were dramatically attenuated in their ability to infect the host. This could be due to the inability of the double mutant to transport ammonium and/ or the role of both *ump1* and *ump2* in affecting transcription of genes essential for pathogenicity.

Studying the role of Amts is important for understanding how the signals from the external environment are transmitted into the cell and how this indirectly helps to decide the fate of the cell. It is interesting to understand how a single extracellular signal can activate multiple pathways to converge and control morphogenesis and development.

More work in the future could further dissect the control or regulation of these ammonium transporter proteins in promoting morphogenetic transition and their role in signal transduction to control dimorphism associated with virulence and pathogenicity.

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APPENDIX I

Microarray data: Genes listed in the tables show at least two fold differential expression, and the data are consistent between the two biological replicates used in the study.

Table 8: *ump2* over-expression compared to *ump2* deletion under high ammonium conditions.

Table 9: *ump2* over-expression compared to *ump2* deletion under low ammonium conditions.

Table 10: genes up-regulated as a result of *ump2* over-expression compared to *ump2* deletion under both high and low ammonium condition.

Table 11: genes up regulated due to *ump2* deletion compared to *ump2* over-expression under both high and low ammonium condition.

Table 8: *ump2* over-expression compared to *ump2* deletion under high ammonium condition

Gene number		Regulation	Gene number		Regulation
um00037		up	um05809		up
um00132		up	um05889	Ump2	up
um00133		up	um05981		up
um00466		up	um06332	Egl1	up
um00529	JEN1	up	um06440		up
um00738		up	um10301		up
um01237		up	um10432		up
um01656		up	um10472		up
um01793		up	um10529		up
um01949		up	um10706		up
um02037	HXT5 -Hexose transporter	up	um10731		up
um02137		up	um10942	probable Protein kinase C	up
um03006		up	um11063		up
um03116		up	um11112		up
um03117		up	um11162		up
um03619		up	um11163		up
um03908		up	um11514	High-affinity glucose transporter	up
um03924	Rep1 - repellent protein 1	up	um11554		up
um04095	related to polyketide synthase	up	um11558		up
um04096		up	um11587		up
um04097		up	um11763		up
um04104		up	um12009		up
um04105		up	um12010		up
um04106		up	um12015		up
um04107		up	um12023		up
um04109		up	um12253		up
um04362		up	um12290		up
um04481		up	um12316		up
um04482		up	um00102		down
um04683		up	um00384		down
um04686		up	um00407		down
um04813		up	um00840		down
um04922		up	um00842		down
um04923		up	um01025		down
um04933		up	um02193		down
um04939		up	um03392		down
um04942		up	um03397		down
um04954		up	um04092		down
um04976		up	um04309		down
um04977		up	um04526		down
um04992		up	um04538		down
um04993		up	um04693		down
um05002		up	um05182		down
um05038		up	um05443		down
um05104		up	um05548		down
um05300		up	um05600		down
um05495		up	um05603		down

um05514		up	um05888		down
um05528		up	um10996		down
um05593		up	um11244		down
um05636		up	um11420		down
um05642		up	um12030		down
um05656		up	um12332		down
um05659		up	*um2382	Mfa1	up
um05704		up			
um05724		up			
um05731		up			
um05737		up			
um05739		up			
um05746		up			
um05764		up			
um05798		up			
um05799		up			

* Differential expression only in one replicate

Table 9: *ump2* over-expression compared to *ump2* deletion under low ammonium condition

Gene number		Regulation	Gene number		Regulation
um00025		up	um05737		up
um00056	neutral amino acid permease	up	um05746		up
um00309		up	um05753		up
um00465		up	um05764		up
um00466		up	um05799		up
um00738		up	um05889	Ump2	up
um00844	Sdh2 - Succinate dehydrogenase	up	um05917		up
um00876		up	um06126	Mig2-6	up
um00981		up	um06133		up
um01165		up	um06190		up
um01436		up	um06414		up
um01437		up	um06461		up
um01438		up	um10055		up
um01443		up	um10120	Chitin Synthase 3	up
um01854		up	um10211		up
um01949		up	um10324		up
um02215		up	um10419	Cts1 - Chitinase	up
um02314		up	um10545		up
um02321		up	um10636		up
um02629		up	um10676		up
um02758	related to Chitinase A precursor	up	um11063		up
um02793		up	um11112		up
um02804		up	um11763		up
um02981		up	um12024		up
um03024		up	um12253		up
um03115		up	um12300		up
um03116		up	um12315		up

um03117		up	um00529		down
um03138		up	um00664		down
um03411	probable endo-1,4-beta-xylanase	up	um00847		down
um03560		up	um01025		down
um03561		up	um01428		down
um03713		up	um01723		down
um03776		up	um01852		down
um03923		up	um03040		down
um03924	Repl - repellent protein 1	up	um03414		down
um04095	related to polyketide synthase	up	um03694		down
um04096		up	um03990		down
um04097		up	um04737		down
um04105		up	um05023		down
um04106		up	um05888		down
um04107		up	um05972		down
um04109		up	um06038		down
um04145		up	um06278		down
um04285		up	um06490		down
um04357		up	um10608		down
um04697		up	um10609		down
um04807		up	um10815		down
um04915		up	um11051		down
um04920		up	um11315		down
um05068		up	um12090		down
um05247		up	*um2382	Mfa1	up
um05248		up	*um2713	Prf1	up
um05436		up	*um2331	Kpp6	up
um05573		up	*um3760	PTEN	up
um05604		up			
um05626		up			
um05728		up			
um05731		up			

* Differential expression only in one replicate.

Table 10: Genes up-regulated under conditions of high and low ammonium in *ump2* over-expression strain.

Gene Number	
um00466	hypothetical protein
um00738	putative protein
um01949	putative protein
um03116	conserved hypothetical protein
um03117	conserved hypothetical protein
um03924	Repl - repellent protein 1 precursor
um04095	related to polyketide synthase
um04096	hypothetical protein
um04097	related to polyketide synthase

um04105	related to Polyketide synthase
um04106	related to O-methyltransferase B
um04107	related to Phenol 2-monooxygenase
um04109	related to Cytochrome P450
um05731	conserved hypothetical protein
um05737	conserved hypothetical protein
um05746	related to arginine-tRNA-protein transferase
um05764	related to Malic acid transport protein
um05799	hypothetical protein
um05889	Ump2 - High Affinity Ammonium transporter
um11063	conserved hypothetical protein
um11112	related to Versicolorin B synthase
um11763	related to acetylxylan esterase
um12253	conserved hypothetical Ustilago-specific protein

Table 11: Genes up-regulated under conditions of high and low ammonium in *ump2* deletion strain

Gene Number	
um01025	conserved hypothetical protein
um05888	related to transcription elongation factor TFIIS

APPENDIX II

Expression analysis of additional genes confirmed using qRT-PCR

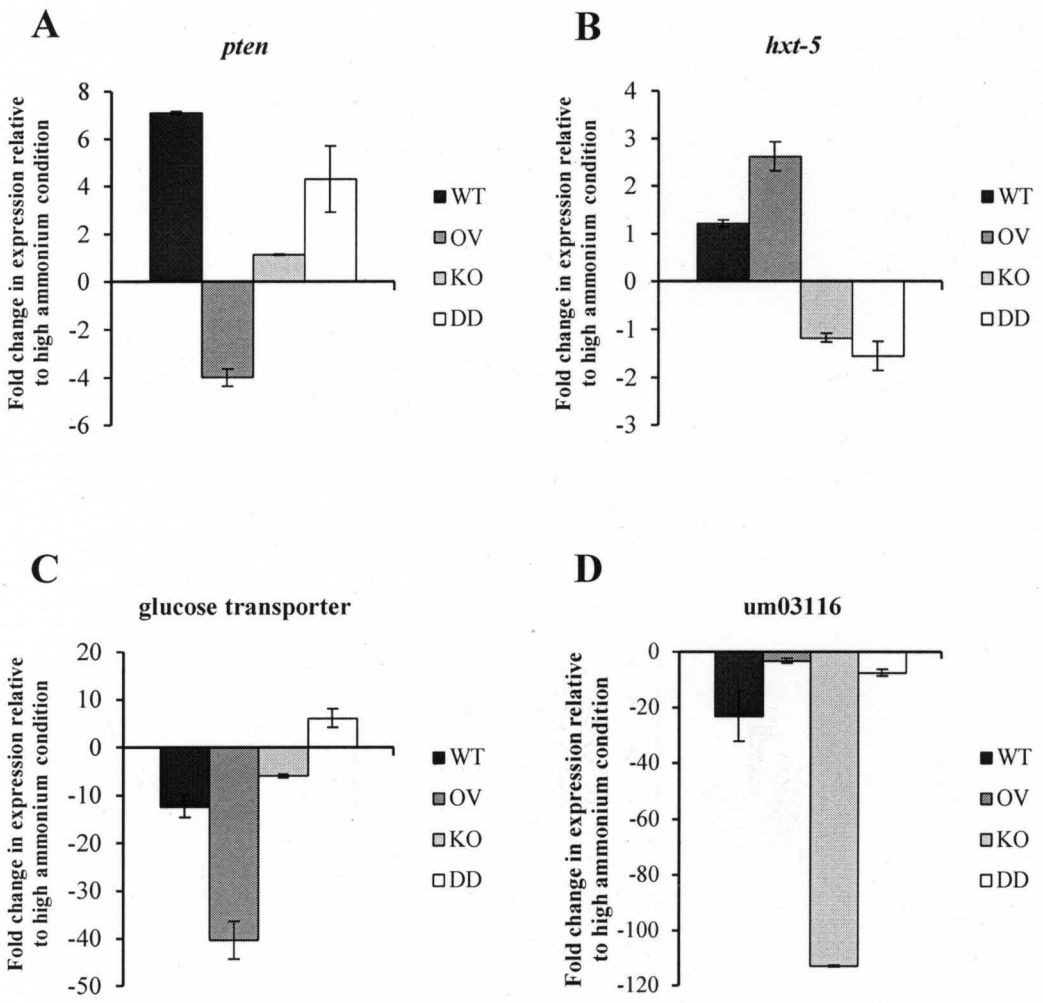


Figure 26: Relative expression under low ammonium conditions of **(A)** ***pten*****, (B) probable hexose transporter** ***hxt5*****, (C) probable high affinity glucose transporter, and (D) um3116-conserved hypothetical** compared with expression under high ammonium conditions. WT= Wild type; KO= Δ*ump2*; OV= *ump2* over-expression; DD= Δ*ump1*Δ*ump2*

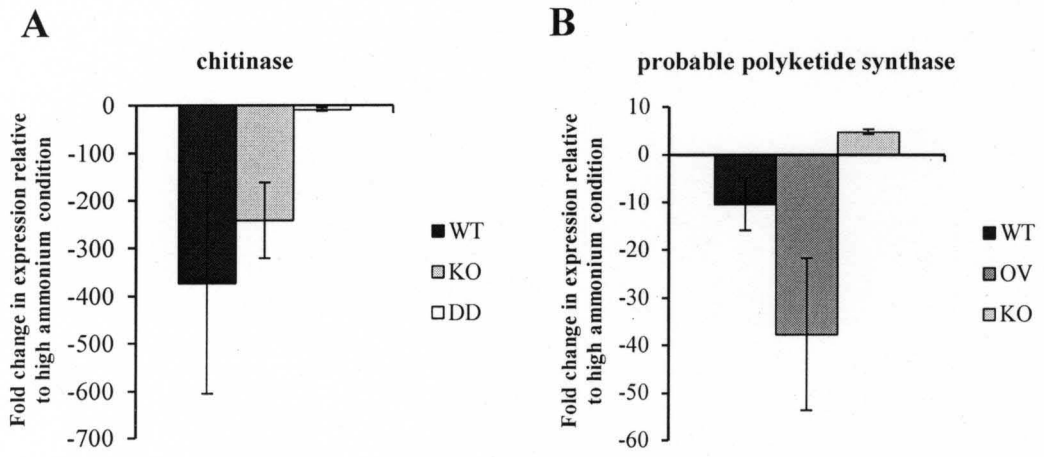
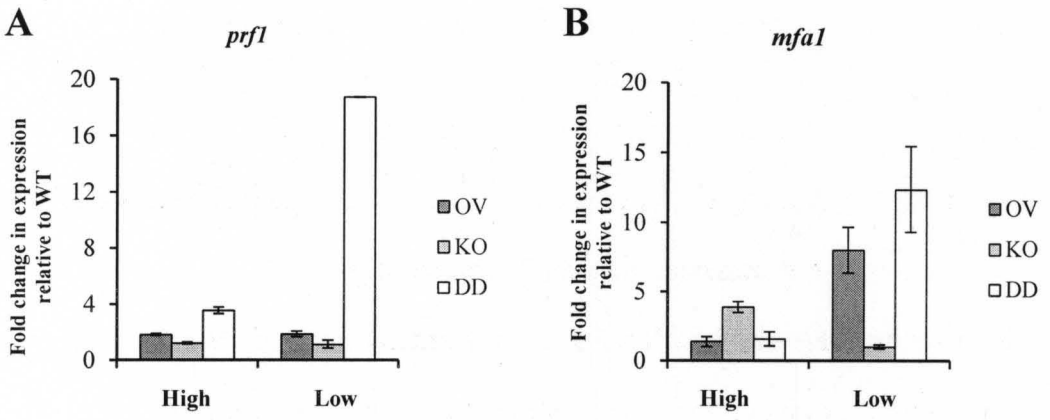
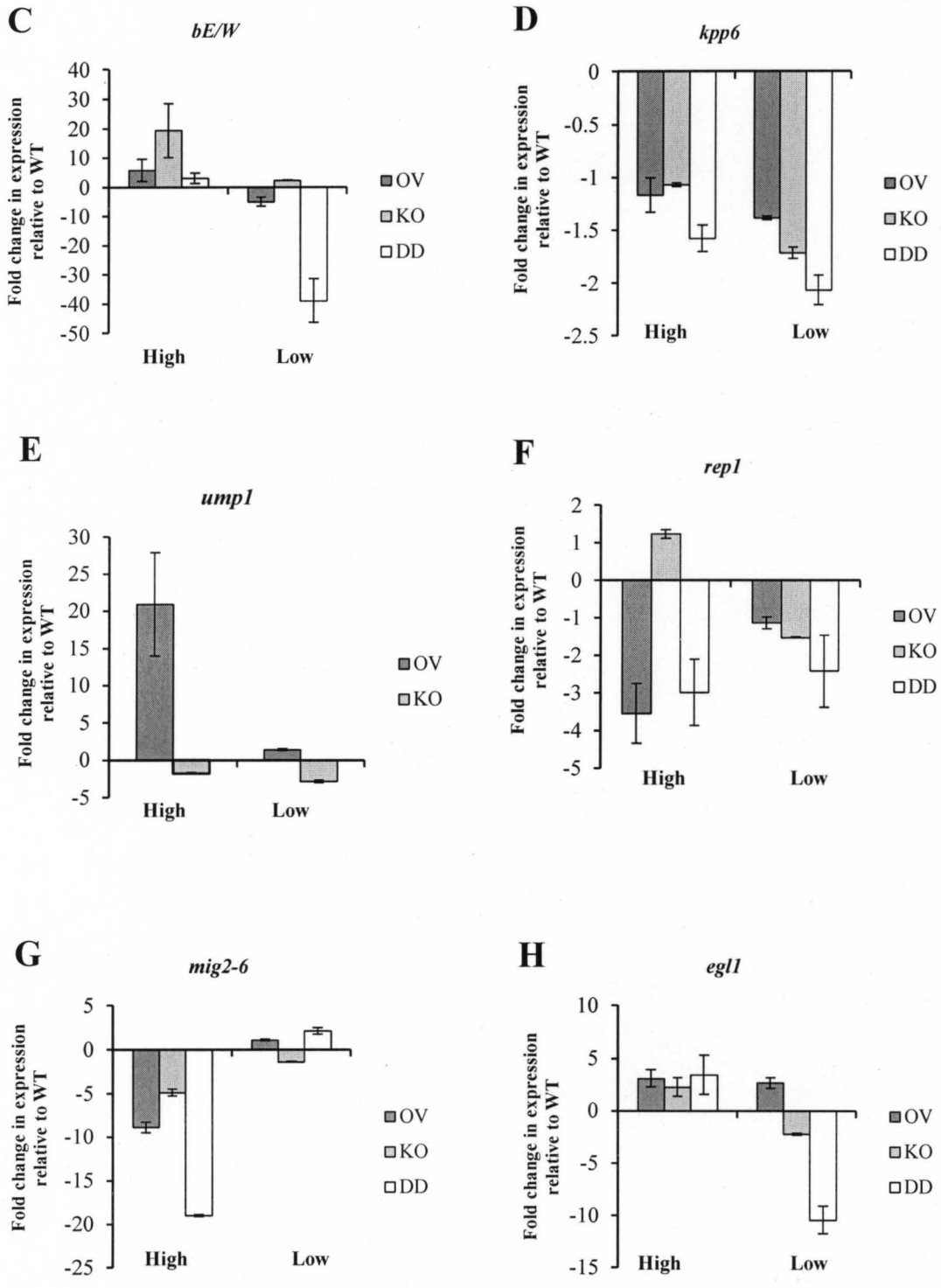


Figure 27: Relative expression under low ammonium conditions of **(A) um6190-probable chitinase, and (B) um4095-probable polyketide synthase** compared with expression under high ammonium conditions. WT= Wild type; KO= Δ*ump2*; OV= *ump2* over-expression; DD= Δ*ump1*Δ*ump2*

The changes in the expression of some of the genes were also determined by comparing the expression of the mutants to the wild type, separately under high and low ammonium conditions. Data represented in Figure 28, represent relative expression of the gene of interest in the respective mutant compared to the wild type. Positive value means up-regulated and negative value means down-regulated compared to the wild type under the respective nutrient condition.




C
bE/W
Fold change in expression relative to WT
40
30
20
10
0
-10
-20
-30
-40
-50
High
Low
OV
KO
DD
D
kpp6
Fold change in expression relative to WT
0
-0.5
-1
-1.5
-2
-2.5
High
Low
OV
KO
DD
E
ump1
Fold change in expression relative to WT
30
25
20
15
10
5
0
-5
High
Low
OV
KO
F
rep1
Fold change in expression relative to WT
2
1
0
-1
-2
-3
-4
-5
High
Low
OV
KO
DD
G
mig2-6
Fold change in expression relative to WT
5
0
-5
-10
-15
-20
-25
High
Low
OV
KO
DD
H
egl1
Fold change in expression relative to WT
10
5
0
-5
-10
-15
High
Low
OV
KO
DD

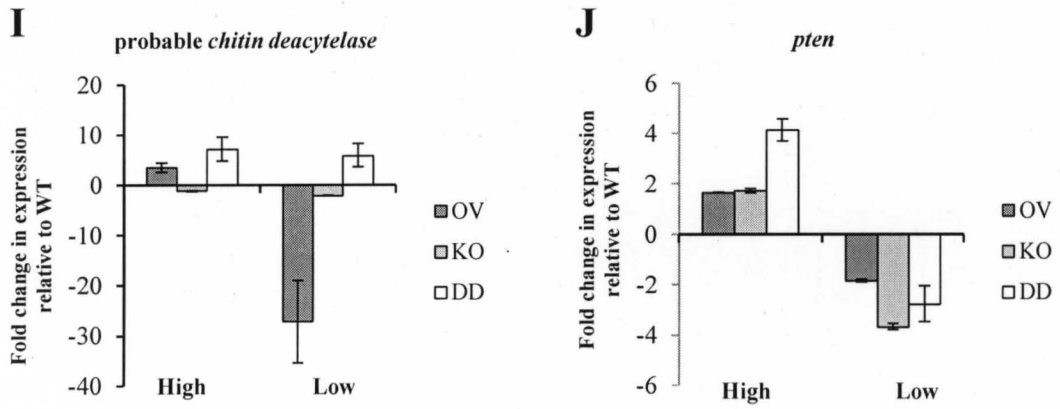


Figure 28: Relative expression of (A) *prf1*, (B) *mfa1* (C) *bE/W*, (D) *kpp6*, (E) *ump1*, (F) *rep1*, (G) *mig2-6*, (H) *egl1*, (I) chitin deacytelase and, (J) *pten*. Changes in the gene expression are displayed relative to expression to the wild type under both hi and low ammonium condition. Error bars indicate standard deviation of mean expression values. WT= Wild type; KO= Δ*ump2*; OV= *ump2* over-expression; DD= Δ*ump1*Δ*ump2*

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Publications

The role of ammonium transporters in the transcriptional control of genes involved in the pathogenicity of the plant pathogen *Ustilago maydis*. Jinny A. Paul and Michael H. Perlin. (*Manuscript in Preparation*)

Interaction of ammonium transporter proteins of *Ustilago maydis* with the signaling protein Rho1. Jinny A. Paul, Michelle Barati and Michael H. Perlin. (*Manuscript in Preparation*)

MtSNPscore: a combined evidence approach for assessing cumulative impact of mitochondrial variations in disease. Anshu Bhardwaj, Mitali Mukerji, Shipra Sharma, Jinny Paul, Chaitanya S Gokhale, Achal K Srivastava, Shrish Tiwari. BMC Bioinformatics 2009, 10(Suppl 8): S7

The Indian Genome Variation Consortium:

(a) The Indian Genome variation database (IGVdb): a project overview
Hum Genetics 2005 August, 118, 1-11

(b) Genetic landscape of the people of India: a canvas for disease gene exploration
Journal of Genetics 2008 April, 87, 1:1-20

Abstract/Posters Presentation:

Jinny A. Paul, Michelle Barati, and Michael H. Perlin. Biomolecular fluorescence and transcriptomics reveal physical and transcriptional interactions among ammonium transporters and signaling components of *Ustilago maydis*. 26th Fungal Genetics Conference, Asilomar, CA 03/11

Anna R. Hellmann, Jinny A. Paul and Michael H. Perlin. Cellular morphology as a response to nutrients and rapamycin in *Ustilago maydis.* 5th International meeting on *Ustilago maydis*, Guanajuato, Mexico, 12/10

Raul Martin-Dominguez, Jinny A. Paul, Michael H. Perlin and Ernesto P. Benito. The methylammonium permease (MEP) gene family of *Bortrytis cinerea:* expression and functional analysis. Abstract at XV International Botrytis Symposium. Cadiz, Spain 06/10

J. Paul, A. Hellman and M. H. Perlin. Exploring interactions among ammonium transporters. Abstract at the 15th Annual EPSCoR Conference, Lexington, KY 05/10

J. Paul, A. Hellman and M. H. Perlin. Exploring interactions among ammonium transporters. Abstract at the 25th Fungal Genetics Conference, Asilomar, CA 03/09

J. A. Paul and M. H. Perlin. Role of ammonium transporters in Smut Fungi. Abstract at KAS meeting, Louisville, KY 12/07

Raff, E., N. Sonderman, C. Pham, J. Paul, and M.H. Perlin. More research on Umps. Posters-at-the Capitol, Frankfort, KY 2/07.