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Coordinate regulation of *Ustilago maydis* ammonium transporters and genes involved in mating and pathogenicity☆

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ABSTRACT

The dimorphic switch from budding to filamentous growth is an essential morphogenetic transition 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 differ. Here, we explore the relationship between filamentation and expression levels of ammonium transporters (AMTs) that also sense low ammonium for *Ustilago maydis*, the pathogen of maize. Overexpression of the high affinity ammonium transporter, Ump2, under normally non-inducing conditions, results in filamentous growth. Furthermore, *ump2* expression levels are correlated with expression of genes involved in the mating response pathway and in pathogenicity. Ump1 and Ump2 transcription levels also tracked expression of genes normally up-regulated during either filamentous growth or during growth of the fungus inside the host. Interestingly, haploid strains deleted for the *b* mating-type locus, like those deleted for *ump2*, failed to filament on low ammonium; they also shared some alterations in gene expression patterns with cells deleted for *ump2* or over-expressing this gene. Deletion of *ump2* either in both mating partners or in a solopathogenic haploid strain resulted in a dramatic reduction in disease severity for infected plants, suggesting some importance of this transceptor in the pathogenesis program.

1. Introduction

Many fungi have the ability to switch from budding growth to filamentous growth, and several pathogenic fungi utilize this ability to cause disease. One of the cues for such a dimorphic switch is the availability of nutrients. In the presence of abundant carbon and nitrogen, fungal cells (like *Saccharomyces cerevisiae* and *U. maydis*) grow by budding. However, under conditions of nitrogen limitation, the cells undergo pseudohyphal or filamentous growth. Ammonium transporter (AMT) genes encode proteins that are essential for uptake of ammonium as a nitrogen source and are conserved across a wide variety of taxa. 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 (Lo et al., 1997; Lorenz and Heitman, 1998; Smith et al., 2003; Biswas and Morschhauser, 2005). The high affinity AMT proteins of *S. cerevisiae*, *U. maydis* and *Candida albicans* are required for filamentous growth under low ammonium conditions (Lorenz and Heitman, 1998; Smith et al., 2003; Biswas et al., 2007). While only the haploid invasive growth in *Schizosaccharomyces pombe* and *S. cerevisiae* on low ammonium is dependent on these ammonium permeases, both mating and haploid invasive growth in *Cryptococcus neoformans* are induced by ammonium limitation and specifically require the high affinity ammonium transporter (Mitsuzawa, 2006; Rutherford et al., 2008a).

Abbreviations: AMT, ammonium transporter; qRT-PCR, real time quantitative reverse transcription PCR; High, nitrogen−replete medium, e.g., containing 30 mM ammonium; Low, low ammonium medium containing 50 μM ammonium.

☆ All sequence data for this study are available in NCBI GEO under GSE64993 and GSM1585543-GSM1585550.

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Mutational studies conducted on the high affinity AMT, Mep2, in *S. cerevisiae* and *C. albicans* reveal that Mep2 not only has a transport function but also has an independent role for the induction of filamentous growth. 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., 2008b). In *S. cerevisiae*, Mep2 dependent pseudohyphal growth is not restricted to low ammonium conditions but is dependent on the expression level of Mep2 (Rutherford et al., 2008b). In *S. cerevisiae*, the expression level of Mep2 is important for its regulatory functions, whereby induction of Mep2 leads to pseudohyphal growth under non-limiting ammonium conditions. This led the authors to hypothesize a role for Mep2 interacting with signal transduction pathway(s) to activate downstream effectors. The study revealed differential expression of genes predicted to be involved in pseudohyphal growth, and presented evidence for differential activation of the MAPK pathway in a Mep2-dependent manner.

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 comparable developmental pathways (Sánchez-Martínez and Pérez-Martín, 2001). In *U. maydis*, the pathogen of maize, the growth form of wild-type cells and pathogenicity on maize are inextricably linked because the filamentous, dikaryotic stage is the natural pathogenic cell type. The haploid strains of the fungus are saprophytic, budding cells. Cell fusion is controlled by the *a* mating-type locus, encoding pheromone precursors and receptors, similar to those of yeast. The *b* mating-type locus encodes two homeodomain proteins, bE and bW, that interact when produced from different alleles. Heterozygosity at the multiallelic *b* locus is required for the production and maintenance of a stable filamentous dikaryon, and for pathogenicity (Banuett and Herskowitz, 1994). The *b* heterodimer controls post cell fusion events required for the production of a stable filamentous dikaryon and regulates the transcription of a set of target genes controlling morphogenetic transitions and pathogenicity (Banuett and Herskowitz, 1994). However, little is known about the genes directly involved in the transition from budding to filamentous growth, though it clearly is influenced by the control exerted by the *b* locus, in addition to environmental conditions (*e.g.*, lipids, including corn oils; low pH; and nitrogen availability (Kahmann et al., 1999; Klosterman et al., 2007)) that can lead to a similar filamentous morphology *in vitro*, even in the absence of a mating partner. Global gene expression differences for each cell type have been observed (Andrews et al., 2004; Garcia-Pedrajas and Gold, 2004; Babu et al., 2005), strengthening the idea that elucidating the 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 fact, *U. maydis* is one of the few examples where an AMT has been linked to pathogenicity (Smith et al., 2003; Schnaiderman et al., 2013).

For *U. maydis*, *in vitro* phenotypic changes in response to low ammonium require only one of the ammonium transporters, Ump2. Deletion of *ump1* (encoding the other, low affinity ammonium transporter) does not yield any discernible difference in phenotype from the wild type (i.e., filamentous growth under ammonium limiting conditions). In contrast, cells deleted for *ump2* (encoding the high affinity ammonium transporter) were unable to produce filaments under similar growth conditions (Smith et al., 2003). 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; van den Berg et al., 2016). Such control of *ump2* expression similarly could be the case in *U. maydis*. MAPK and cAMP-dependent PKA signaling pathways are both implicated in affecting filamentous growth in *U. maydis*, but their role in affecting filamentation in response to low nitrogen availability, specifically ammonium, mediated by *ump2*, has yet to be fully elucidated. Earlier investigations hypothesized that the connection of *ump2* to the PKA signaling pathway occurs at multiple levels. More recently, Ump2 has been found to interact physically with Rho1 (Paul et al., 2014), a small G protein required for viability in haploid *U. maydis* cells (Pham et al., 2009). This provides additional evidence for a role of the Ump2 protein in coordinating with signaling pathways.

In the current study, we address the role of *ump2* in modulating transcription of key sets of genes to initiate the appropriate morphogenetic response. We show that filamentation facilitated by *ump2* can occur on nitrogen-replete media and this is dependent on the expression levels of *ump2* as seen in the case of *S. cerevisiae*, suggesting that ammonium limitation as such might not be solely responsible for induction of the filamentous response by *ump2*. The results of the transcriptional profiling revealed that AMT proteins affect transcription of genes shown to be essential in causing disease on the host. Moreover, haploid cells deleted for the *b* mating-type locus, similar to *ump2* deletion mutants, lost their ability to filament on low ammonium, and shared some transcriptional alterations with the *ump2* mutant. Finally, our study also reveals the requirement for AMT proteins in causing disease on the host.

2. Materials and methods

2.1. 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 h. In order to assure that any filamentation observed on the latter medium was not due to acidification of the media, the strains were also tested on AM with low ammonium that had been buffered with Tris−HCl, pH 7.0; filamentation was comparable in all cases to what was observed on unbuffered AM low ammonium plates. *U. maydis* strains used are listed in Table 1. All mutants were generated in either the FB background (Banuett and Herskowitz, 1989), or the SG200 background (Kämper et al., 2006).

2.2. 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 obtained from Eurofins MWG Operon [Huntsville, AL].

2.3. 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 min, followed by 34 cycles of a three-step process of denaturation at 94 °C for 30 s, annealing at 60 °C for

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