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# Gene expression of ammonium transporters in *Ustilago maydis* and their role in pathogenicity and virulence.

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GENE EXPRESION OF AMMONIUM TRANSPORTERS IN *USTILAGO MAYDIS*  
AND THEIR ROLE IN PATHOGENICITY AND VIRULENCE

By

Kirsten Richardson  
B.A. Keuka College, 2015

A Thesis  
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

Masters of Science in Biology

Department of Biology  
University of Louisville  
Louisville, KY

August, 2017

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A Thesis Approved on

August 1<sup>st</sup>, 2017

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## ABSTRACT

### GENE EXPRESSION OF AMMONIUM TRANSPORTERS IN *USTILAGO MAYDIS* AND THEIR ROLE IN PATHOGENICITY AND VIRULENCE

Kirsten Richardson

August 1<sup>st</sup>, 2017

The fungus, *Ustilago maydis*, a plant pathogen of *Zea mays*, is capable of a dimorphic switch, e.g., a transition between a yeast-like form and a filamentous form. This switch is activated when *U. maydis* cells are starved for their preferred nitrogen source - ammonium. *U. maydis* has two important genes that play a role in this switch, *ump1* and *ump2*. Each gene is responsible for the uptake of ammonium and the latter is responsible for the filamentous response in the absence of ammonium. *Ump2* deletion mutants are unable to filament in response to low ammonium while *ump1* deletion mutants do not show this phenotype. The Ump2 protein is responsible, not only for ammonium transport, but also for sensing the ammonium in the environment around the cell; in contrast, *ump1* is solely a low-affinity, high-capacity transporter of ammonium and is not required for the filamentous response. Unexpectedly, single deletion strains and double deletion strains (i.e.,  $\Delta ump1 \Delta ump2$ ) are still able to grow in low ammonium conditions.

The present study investigates the roles of ploidy (haploid vs. diploid strains), promoter type (native promoter vs. constitutive promoter), gene structure (genomic copy vs. cDNA), and gene location (native location vs. ectopic location) on transcription levels

of both ammonium transporters under low ammonium conditions compared to high ammonium conditions. Each construct was normalized to their parent strains that served as the baseline of expression.

Across the different ploidy constructs, I found that the diploid solopathogenic strain D132 had decreased expression of *ump2* relative to the FB1 wild type strain under replete media conditions. D132 also increased its *ump2* expression 8log<sub>2</sub>-fold when under low ammonium conditions as compared to itself under high ammonium conditions. The haploid solopathogenic strain SG200 showed an increase in *ump2* expression compared to itself under low ammonium conditions and in high ammonium conditions in comparison to the FB1 wild type.

An SG200  $\Delta$ *ump2* mutant was used to test the roles of promoter type, gene structure, and gene location. Little difference in ammonium transporter transcription levels was observed compared to the FB1 wild type strain in replete media. Overexpression constructs had increased *ump2* expression, as expected, and yielded a filamentous phenotype when under low ammonium conditions. SG200  $\Delta$ *ump2* mutants complemented with the various promoters driving the *ump2* expression reestablished levels of expression, demonstrating that function was restored at the transcriptional level. When tested for ability to filament on low ammonium, such complemented strains restored the filamentation ability comparable to that of SG200 wild type strain. However, none of the strains expressing the over-expression constructs regained the fuzz phenotype on charcoal media or the ability to cause disease on maize, demonstrating lack of complementation of those phenotypes of the SG200  $\Delta$ *ump2* mutant.



## TABLE OF CONTENTS

LIST OF TABLES .....	vii
LIST OF FIGURES .....	viii
CHAPTER I .....	1
INTRODUCTION .....	1
CHAPTER II .....	7
METHODS.....	7
<b>Cells and Growth Conditions.....</b>	<b>7</b>
<b>Constructs for altering expression of <i>ump2</i> .....</b>	<b>7</b>
<b>PCR.....</b>	<b>8</b>
<b>RNA Purification and cDNA Synthesis .....</b>	<b>9</b>
<b>Primers for qRT-PCR.....</b>	<b>10</b>
<b>qRT-PCR Analysis .....</b>	<b>10</b>
<b>Mating Assay and Plant Pathogenesis. ....</b>	<b>12</b>
CHAPTER III.....	13
RESULTS.....	13
<b>Effects of ploidy on transcription levels of <i>ump2</i> and <i>ump1</i>.....</b>	<b>13</b>
<b>Effects on transcription levels of ammonium transporters of altered <i>ump2</i> transcription levels and expression in different genomic contexts .....</b>	<b>14</b>
CHAPTER IV.....	13
DISCUSSION.....	23
REFERENCES.....	27
CURRICULUM VITAE .....	29

## LIST OF TABLES

Table	Page
1. <i>U. maydis</i> Strains Used in this Study.....	8
2. Construct Primers.....	9
3. qRT-PCR Primers.....	10

## LIST OF FIGURES

Figure	Page
1. Tetrapolar Mating System.....	2
2. qRT-PCR Analysis of Ploidy on <i>ump2</i> and <i>ump1</i> .....	15
3. Filamentation on High vs Low Ammonium Conditions across overexpression and different genomic contexts.....	16
4. qRT-PCR Analysis of <i>ump2</i> and <i>ump1</i> in Different Genomic Contexts.....	19
5. SG200 Complementation: Cell Morphology under Low Ammonium Conditions and Mating Assay.....	21
6. qRT-PCR Verification of Complementation by SG200 $\Delta$ <i>ump2</i> Strains using $P_{otef}$ or the Native Promoter.....	22

## CHAPTER I

### INTRODUCTION

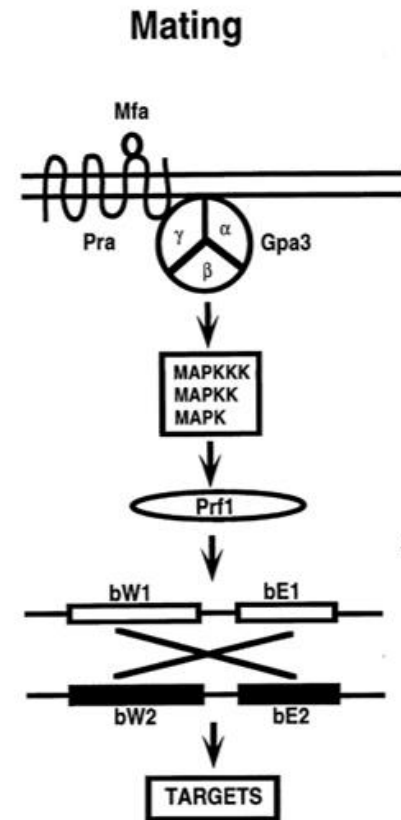
*Ustilago maydis* is a basidiomycete responsible for the smut disease and gall formation in maize. *U. maydis* has a tetrapolar mating system that consists of two mating loci that are required for haploid partners to mate with their corresponding counterpart before downstream events, such as host infection, can take place (Djamei A, 2016). The two mating loci are locus *a*, which is the initial step in mating, and governs cell-cell recognition, and the *b* locus which regulates further downstream events of sexual reproduction and pathogenic development. The *a* locus has two variants, or idiomorphs, called *a1* and *a2* (Regenfelder E, 1997). Haploid cells possessing two opposite idiomorphs must come together to form a sexually compatible interaction. Each cell is responsible for the production of a pheromone and a receptor for the pheromone of the opposite mating-type cell. These idiomorphs are able to do this because they contain the genes for the structural pheromone precursor and for a receptor that can receive a signal from an opposite pheromone type. Successful interaction due to the pheromone response is the first hurdle that must be passed so that the components of the *b* locus can check for additional aspects of compatibility.

Unlike the *a* locus, for which there are only two different forms, the *b* locus has more than twenty different alleles that can match up. If the allele in one cell is different than that of its partner, sexual reproduction will occur (Wahl R, 2010). If cells are

successfully mated, the *b* locus will lead to production of proteins that form a heterodimer and cells will produce a filamentous dikaryon. The two homeodomain proteins, bE and bW, that form the heterodimer are required to maintain this filamentous dikaryon structure and to allow the fungus to infect its host (Banuette, 1994). As a whole, this complex (shown in Figure 1) is essential in the production of the heterodikaryon and to sustain cell proliferation to further the pathogenicity.

Like all organisms, *U. maydis* requires a variety of nutrients that it can obtain from its environment. One of these essential nutrients is nitrogen. Ammonium is a vital nitrogen source for fungi and plants, alike (Thomas, 2012). Each cell must have the ability to take up ammonium in order to carry out cellular functions required for life, not to mention pathogenicity and virulence, in the case of

pathogenic yeast and fungi. Initially, the transmembrane ammonium transporters, Mep1, Mep2 and Mep3, were characterized in the model yeast, *Saccharomyces cerevisiae*. Each has its own affinity for ammonium, but Mep2 in particular is an ammonium permease with the highest affinity for ammonium of the three transporters (Julian Rutherford, 2008;



**Figure 1: Tetrapolar Mating System:**

This schematic represents the pheromone mating pathway in general terms. From the top, the receptor receives a signal from the pheromone, in the environment that had been released from another *Ustilago maydis* cell. Once the opposite idiomorphs come together, the *b* locus interacts and, if compatible, will encode for transcription factor necessary for pathogenicity and virulence.

Marini, 1997). Mep3 has the lowest affinity, with no significant difference in ammonium uptake than a triple *mep1mep2mep3* deletion strain, although it does transport enough ammonium to sustain the life of the species in very low ammonium conditions. Of the three, Mep2 has been termed a “transceptor,” since it is not only able to transport ammonium, but is also responsible for sensing the available ammonium in the environment around the cell. Due to this sensing ability, Mep2 is also required for the induction of filamentous growth as a response to low nitrogen. Neither Mep1 nor Mep3 have the ability to sense the available ammonium around the cell, so in *mep2* deletion strains, there is no transition to a filamentous growth stage.

Similar to the yeast ammonium transporters in *S. cerevisiae*, *U. maydis* has two ammonium transporters that behave similarly to the yeast model, *ump2* and *ump1* (Smith, 2003). The response to low ammonium of haploid cells (i.e., filamentous growth) only requires one of these transporters, Ump2, the high-affinity transporter. *ump2* deletion strains are unable to filament when starved for ammonium as opposed to the wild type cells that do filament when in starvation conditions. As for *ump1* deletion strains, there is no phenotypic change. Interestingly, it has been shown that the wild type phenotype for *S. cerevisiae* strains deleted for *mep1*, *mep2* and *mep3* can be restored when *ump2* is expressed in such mutants, allowing for ammonium transport and sensing to occur (Smith, 2003). The exact mechanism behind *ump2* driving filamentous growth is still not fully understood, but the mitogen-activated protein kinase (MAPK) and cAMP-dependent PKA (protein kinase A) signaling pathways are known to affect filamentous growth. It has been theorized that *ump2* interacts with the PKA signaling pathway, so this may be a lead into how this mechanism works (Pham, 2009).

Ploidy refers to the number of chromosome sets within a cell of an organism. The number of chromosome sets can change for various reasons such as during fertilization, where this number would double, or during meiosis, in which ploidy is halved (Galitski, 1999). Characteristically, cells with different ploidy levels will show different morphological and physiological characteristics. In *S. cerevisiae*, when opposite mating type cells, i.e., *MATa* and *MAT $\alpha$* , come together and mate, the result is a *MATa/MAT $\alpha$*  diploid cell (Haber, 2012). The original haploid cells and the newly mated cells each have different phenotypes, likely the direct result of their different genotypes which control expression of specific genes in diploids. There may be additional effects on phenotype due specifically to ploidy.

Most of our understanding of the importance and consequences of ploidy has been obtained through plant models and some animal models (Albertin, 2012). Interestingly, the effects of ploidy in most other eukaryote kingdoms, including fungi, still remain mostly unknown. In maize, global gene expression was analyzed across strains/cultivars with a variety of different total number of ploidy levels and found few significant differences on the gene expression within the plant (Riddle, 2010). The change in ploidy affects a large portion of expression for the maize genome, but at very low levels, i.e., typically under a two-fold change.

One goal of this thesis is to further investigate how expression of the ammonium transporter genes, *ump1* and *ump2*, changes in various ploidy backgrounds of *U. maydis*. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was used to quantify gene expression at the level of transcription in mutant strains in comparison to the FB1 wild type strain.

The second goal of this study was to further investigate the gene expression of the ammonium transporters, *ump1* and *ump2*, in *U. maydis* in association with various overexpression constructs and genetic backgrounds. qRT-PCR analysis was again used to quantify gene expression in mutant constructs in comparison to the wild type progenitor strain. Plant infections were also used to examine any phenotypic difference in pathogenicity in the FB1 and SG200 backgrounds.

As part of this second goal, I was also interested in examining how gene structure and genomic context might influence gene expression. For this reason, I compared *ump2* constructs using cDNA versus gDNA to determine the difference in the ammonium transporter gene expression. cDNA is reverse transcribed from the mRNA of interest (Candy Taylor, 2016). These constructs would lack the intron region of the initial transcript which can sometimes play a role in regulating gene expression. Conversely, gDNA (genomic DNA) would include both the exons and the introns. Since gDNA contains the entire genomic sequence, there are other factors that can play a role in gene expression (James Yun, 2006). Such factors that can affect gene expression or be used as critical component of regulation include the 5' untranslated region (UTR) and 3'-UTR. The 5'-UTR, the region just upstream of the initiation codon, has been shown to affect expression in *S. cerevisiae* (Hoshida H., 2016). Within this region, there is potential for an intron promoter to exist, that can change expression from the native promoter. In *S. cerevisiae*, an intron promoter was found to increase expression from one of the strongest promoters, TDH3p, when tested. As for 3'-UTR, the region following the termination codon, there may be additional regulatory units present. This includes, but is not limited to, regulatory units that may influence translation efficiency, binding sites that, when



bound, can decrease gene expression or degrade the transcript, and alternative polyadenylation sites. In mouse Thy1+ germline cells, it has been found that particular sequence patterns in the 3' UTR length can affect gene expression both by upregulation or downregulation (Huang, 2015). This change can have an impact on the stability of the RNA, which would, in turn, affect gene expression. Thus, the roles of 5'- and 3'-UTRs were also examined in *ump2* constructs, so as to assess their importance in both transcription levels of ammonium transporters and also their ability to confer full phenotypic function associated with wild type expression of the *ump2* gene.

## CHAPTER II

### METHODS

#### **Cells and Growth Conditions**

*U. maydis* strains used in this study are listed in Table 1. *U. maydis* strains were grown in YPS or on high ammonium Array Media [AM] (6.25% Holliday Salt Solution [20], 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  $\mu$ M ammonium sulfate and 2% agar) at room temperature. All mutant strains were generated in either the FB1 or SG200 backgrounds. FB1 is a wild type haploid strain, while SG200 is a genetically engineered haploid strain that constitutively expresses the bE1/bW2 heterodimer, and is thus solopathogenic (Kamper, 2006).

#### **Constructs for altering expression of *ump2***

When overexpressing and deleting *ump2* in *U. maydis*, constructs were obtained via homologous recombination as previously described (Brachmann, 2001). The  $\Delta$ *ump2* construct was created using the primers Ump2KOup5' and Ump2\_Dn\_TapR (sequences listed in Table 2 below). As for the *ump2* overexpression construct, the *ump2* ORF cDNA copy was amplified using the primers Ump2\_CGO5' and Ump2\_CGO3'. This fragment was then cloned into pCR2.1 TOPO (Invitrogen). Further strains were generated to provide constitutive expression of ammonium transporter genes via the

**Table 1. *U. maydis* Strains Used in this Study**

Strain	Genotype	Source
FB1 WT	<i>a1 b1</i>	(Banuette, 1994)
SG200	<i>a1 mfa2 bW2bE1</i>	(Kamper, 2006)
D132	<i>a1/a2 b1/b2</i>	(Garcia-Pedrajas, 2010)
SG200 $\Delta$ <i>ump2</i>	<i>a1mfa2 bW2bE1 ump2::hygR</i>	This Study
SG200 $\Delta$ <i>ump2</i> P <sub><i>otef</i></sub> <i>ump2</i>	<i>a1mfa2 bW2bE1 Potef-ump2</i>	This Study
SG200 $\Delta$ <i>ump2</i> P <sub><i>ump2</i></sub>	<i>a1mfa2 bW2bE1 Pump2</i>	This Study
FB1 $\Delta$ <i>ump2</i> P <sub><i>ump2</i></sub> cDNA	<i>a1b1 Pump2</i>	This Study
FB1 $\Delta$ <i>ump2</i> P <sub><i>otef</i></sub> <i>ump2</i> gDNA	<i>a1b1 Potef-ump2 gDNA</i>	This Study
FB1 P <sub><i>ump2</i></sub> + Coding Region	<i>a1b1 Pump2 + CR</i>	This Study
FB1 P <sub><i>otef</i></sub>	<i>a1b1 Potef-ump2</i>	This Study
FB1 $\Delta$ <i>ump2</i> P <sub><i>otef</i></sub> <i>ump2</i>	<i>a1b1 Potef-ump2</i>	This Study
SG200 $\Delta$ <i>ump2</i> P <sub><i>ump2</i></sub> + CR + 3' UTR	<i>a1mfa2 bW2bE1 Pump2</i>	This Study

constitutively active P<sub>*otef*</sub> promoter. *Ump2* was cloned downstream of the P<sub>*otef*</sub> promoter in the p123 (*Otef*) vector which is the vector used to confer resistance to carboxin after first linearizing the vector using the *SspI* restriction enzyme. *U. maydis* was then transformed and plated on selectable marker media to find recombinants that had carboxin resistance. One construct was designed using genomic DNA instead of cDNA as the source of the *ump2* gene. Mutants were complemented via cloning the *ump2* ORF into the *Otef* vector either with the native promoter or by having transcription of *ump2* driven by the constitutive P<sub>*otef*</sub> promoter already present in the vector. PCR was performed after each construct was generated to ensure to verify the construct size.

## PCR

PCR was performed using either the PTC-100 Programmable Thermal Controller or the Bio Rad T100 Thermal Cycler. The cycle conditions utilized a denaturation temperature of 94 °C for 4 minutes, proceeded by 34 cycles of a three-step process beginning with a

temperature of 94 °C for 30 seconds, annealing at a temperature between 55 °C to 64 °C and ending with an extension period at 72 °C for 1 minute. A final extension was performed at 72 °C for 10 minutes to end this reaction. Ex-Taq Hot Start DNA polymerase (Takara) was used to catalyze these reactions.

**Table 2: Primers used for overexpression construct**

Primer Name	Sequence
Ump2KOup5'	GGCAAGACAAGACGAGAAGA
Ump2_Dn_TapR	TGCGTGTCTCAAACCTCCTCT
Ump2_CGO5'	ATTAACCGCGGAAATGGTTAACGCCAGCTAC
Ump2_CGO3'	TGATTGCGGCCGCTTAGACAGCAGTAGGCTG

### **RNA Purification and cDNA Synthesis**

Methods used to extract RNA from cells were based on the RNeasy Plant Mini Kit (Qiagen, cat no. 74903) with a few variations for more purified yield of RNA. Cells were grown on appropriate plates for 48 hours before they were scraped off the plate and ground into a powder on ice in a mortar and pestle that had been chilled at least overnight at -80 °C. All steps followed the RNeasy Plant Mini Kit protocol until the wash steps; to ensure a pure and uncontaminated yield, three gentle 'flicks' towards the bottom of the column were performed to remove residual ethanol. Additionally, a ten minute drying period was added before elution of the RNA to allow for less ethanol contamination. Sample purity was assessed with the NanoDrop 2000 UV-Vis Spectrophotometer. Concentrations above 80 ng/ml were considered high enough to continue processing. cDNA was synthesized using SuperScript III First-Strand Synthesis System (Invitrogen,

Cat no. 18080-51). Sample purity was assessed again with the NanoDrop 2000 UV-Vis Spectrophotometer. Protein and organic contamination was also measured with an ideal range of 1.8 – 2.2 (260/280 and 260/230 ratio).

### Primers for qRT-PCR

Primers used for qRT-PCR are shown in Table 3 below. These were designed using ABI Primer Express software version 3.0. All other primers were designed using Primer3 software. Primers were ordered from and synthesized by Eurofins Genomics (Louisville, KY).

**Table 3: qRT-PCR Primers**

Primer Name	Forward/Reverse	Sequence
eIF2	Forward	5'-CAAATGCGATCCCGAACAG-3'
	Reverse	5'-TGGGACACCACTTGTCAAGCA-3'
ump2	Forward	5'-TGGGTCCCGTTCTCATTTTC-3'
	Reverse	5'-AGGGGATGGGATTGTAGACAA-3'
ump1	Forward	5'-CGGTCTCACCTGGATGTTCT-3'
	Reverse	5'-AGCCAACGACGGACCACTT-3'

### qRT-PCR Analysis

qRT-PCR was performed using the AB Applied Biosystems StepOne Real-Time PCR System. Non-specific detection was observed using SybrGreen as the fluorescence. qRT-PCR utilized an initial denaturation temperature of 95 °C for 10 minutes, followed by 35 2-step cycles beginning with a temperature of 95 °C for 15 seconds, 60 °C for 1 minute, followed by the dissociation curve with settings at 95 °C for 15 seconds, 60 °C for one minute and 95 °C for another 15 seconds. 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* (unchanged in RNA-Seq analysis), encoding the translation initiation factor eIF2. Changes in the gene expression are averages of at least three biological replicates and are displayed as log<sub>2</sub>-fold changes relative to expression of wild type FB1 under high ammonium condition or as a comparison of the respective mutant at high and low ammonium. For statistical analysis of qRT-PCR results, first the transcript level of each target for each mutant in low vs high ammonium conditions was compared, using Kruskal Wallis (Daniel, 1990). Second, the absolute transcript level for each mutant under each conditions was compared to that for FB1 wild type under the same condition. So, all the mutants on high ammonium were compared to wild type on high ammonium and then all the mutants on low ammonium were compared to the wild type on low ammonium. This analysis again used Kruskal-Wallis, as we assume these do not vary normally. Third, analysis of covariance on all of the changes for high to low ammonium. This analysis corrects for any variations within biological replicates of each mutant and sets the “control” (in our case high Ammonium) to zero, and then looks at the absolute increase or decrease in transcript level based on these corrections. All gene expression was normalized against FB1 wild type on high ammonium or to SG200, depending on whether the comparison was for ploidy effects or relative expression for mutants derived from a particular background progenitor strain.

### **Mating Assay and Plant Pathogenesis.**

Strains were grown in YPS overnight and then densities were measured with a spectrophotometer. Cultures were diluted to an absorbance at 600 nm. Strains then continued to grow for approximately 4 additional hours to an absorbance at 600 nm of between 0.5 and 0.7. Golden Bantam corn seedlings were grown to 8 to 10 days and then infected via a syringe into the stem. Virulence was measured on a scale of 0 to 5 depending on the symptoms present after 7 days, 9 days, and 14 days post-infection. Scale ratings were 0 meaning a healthy plant after infection, 1 for chlorosis, 2 for small galls present on the plant leaves, 3 for small galls on both the leaves and plant stem, 4 for larger galls and 5 representing a dead plant. Ratings were averaged among each group infected and Kruskal-Wallis Test with Multiple Comparison (Daniel, 1990) was used for statistical analysis of the infection rating.

## CHAPTER III

### RESULTS

#### **Effects of ploidy on transcription levels of *ump2* and *ump1***

*U. maydis* strains with different chromosomal ploidy levels showed differences in gene expression of ammonium transporters. The FB1 wild type (Banuette, 1994), a haploid strain, was used as the comparison strain against two solopathogenic strains. These solopathogenic strains have the ability to infect their host plant in the absence of mating with a compatible haploid partner. D132 (Garcia-Pedrajas, 2010), a diploid solopathogenic strain, and SG200 (Kamper, 2006), a haploid solopathogenic strain, were used for this comparison.

qRT-PCR provides the opportunity to explore differential gene expression, in this case, of the ammonium transporters in *U. maydis*, *ump2* and *ump1*. These data allowed me to evaluate transcription levels of these two target genes against the FB1 wild type strain in both high and low ammonium conditions.

High ammonium conditions resulted in differential gene expression of both ammonium transporters in the diploid solopathogenic strain, D132 and the haploid solopathogenic strain, SG200 compared to wild type FB1 under the same conditions. The expression of *ump2* in D132 was substantially reduced, roughly 6 log<sub>2</sub> fold lower compared to the FB1 wild type strain (Figure 2A). As for *ump1* in D132, gene expression was also significantly reduced, a 5 log<sub>2</sub> fold change (p<.05). Compared with



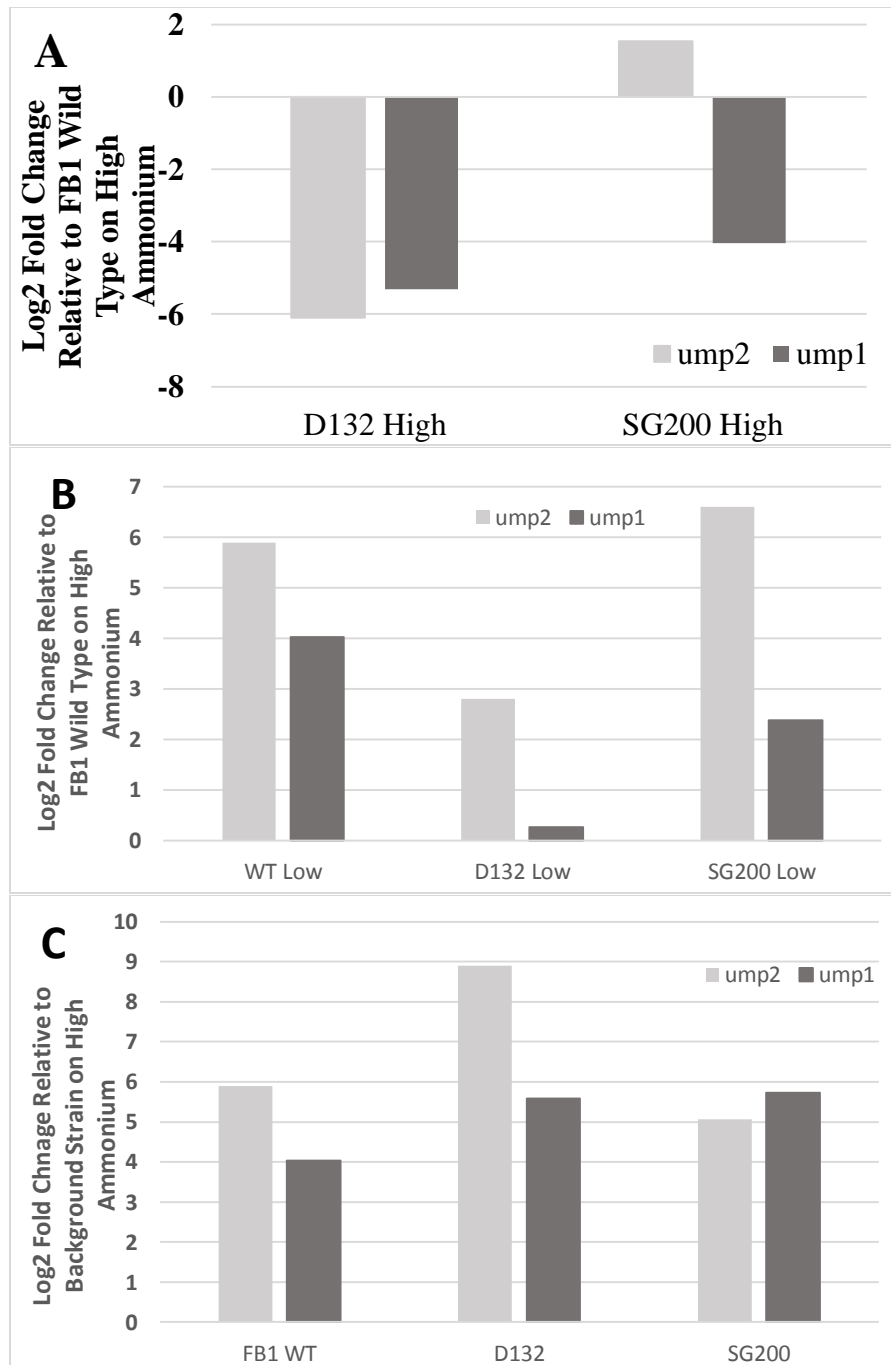
FB1 wild type in high ammonium conditions, SG200 had increased expression of *ump2* and reduced expression of *ump1*, both of which were statistically significant ( $p=.05$ ).

Low ammonium conditions in D132 resulted in a downregulation of *ump2* expression compared to the FB1 wild type under high ammonium conditions according to the Kruskal-Wallis one-way analysis of variance ( $p=.05$ ) (Figure 2B). As for the SG200, there was a 'trend towards' differential expression ( $p=.06$ ) for *ump2* but none of the observed changes were statistically significant.

When comparing each strain with itself when grown on low ammonium vs. replete media, D132 showed significant differences in gene expression ( $p=.05$ ) (Figure 2C). The log<sub>2</sub> fold change in *ump2* on low ammonium compared to replete medium was significant as was that for *ump1*. FB1 wild type showed this same trend, though the induction in D132 was greater than that in FB1. SG200 also showed statistical significance in its induction from high to low ammonium conditions with a 5 log<sub>2</sub> fold change, similar to that in the FB1 and D132 strains. Overall, both the diploid and haploid solopathogenic strains, D132 and SG200, respectively, exhibited changes in ammonium transporter gene expression due to different ploidy levels.

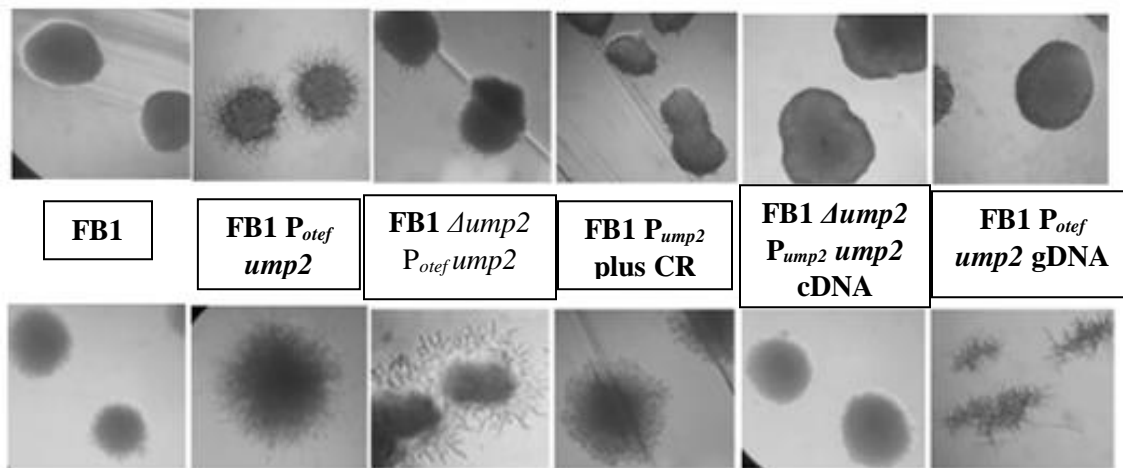
### **Effects on transcription levels of ammonium transporters of altered *ump2* transcription levels and expression in different genomic contexts**

Higher concentrations of ammonium block filamentation in *C. albicans* when the high affinity transporter was over-expressed (Biswas, 2014). Conversely, in *S. cerevisiae*, increasing the expression of the high affinity ammonium transporter, Mep2, led to pseudohyphal growth, even under non-inducing conditions (Julian Rutherford, 2008). In this portion of the study, I explored the role of *ump2* expression constructs on



**Figure 2: qRT-PCR analysis of ploidy on *ump2* and *ump1*.** The FB1 wild type strain shows increased expression of ammonium transporters from high to low ammonium conditions. D132, diploid solopathogenic strain, and SG200, haploid solopathogenic strain, show differences in transcript level in comparison to FB1 wild type strain. A) qRT-PCR Log<sub>2</sub> fold changes of *ump2* and *ump1* of solopathogenic strains grown in replete media for 48 hr; changes are shown relative to the haploid FB1 wild type strain. B) Log<sub>2</sub> fold changes of *ump2* and *ump1* of FB1 wild type and solopathogenic strains in ammonium deficient media relative to FB1 in replete media. C) Log<sub>2</sub> fold changes of *ump2* and *ump1* for each strain in ammonium deficient media relative to the same strain in replete media.

filamentation in *U. maydis*. In *U. maydis*, FB1 wild type cells grow via budding, yielding smooth colonies under replete media conditions (Figure 3). When grown in ammonium deficient conditions on solid media, FB1 cells produce filamentous colonies; FB1 cells with the *ump2* overexpression construct yielded a widespread filamentous growth within two days as opposed to four days in the FB1 wild type cells. Figure 3 shows that all strains had a smoother colony morphology under high ammonium, though the strain bearing *ump2* over-expressed from the constitutive  $P_{otef}$  promoter showed some filamentation, under even these, normally non-inducing conditions. On the other hand, while all strains filamented on low ammonium, strains with constructs in which  $P_{otef}$  was driving *ump2* expression showed hyperfilamentation under these conditions; this was true regardless of the structure of the *ump2* ORF provided (i.e., cDNA or gDNA).



**Figure 3: Filamentation on High vs Low Ammonium Conditions across overexpression and different genomic contexts.** Top row shows each strain in high ammonium conditions while the bottom row shows the same strains in low ammonium conditions (all images are after four days growth under the respective conditions). Strains are FB1 wild type, FB1  $P_{otef}$  *ump2*, FB1  $\Delta ump2$   $P_{otef}ump2$ , FB1  $P_{ump2}$  plus coding region (CR), FB1  $\Delta ump2$   $P_{ump2}$  *ump2* cDNA and FB1  $P_{otef}$  *ump2* gDNA, respectively.

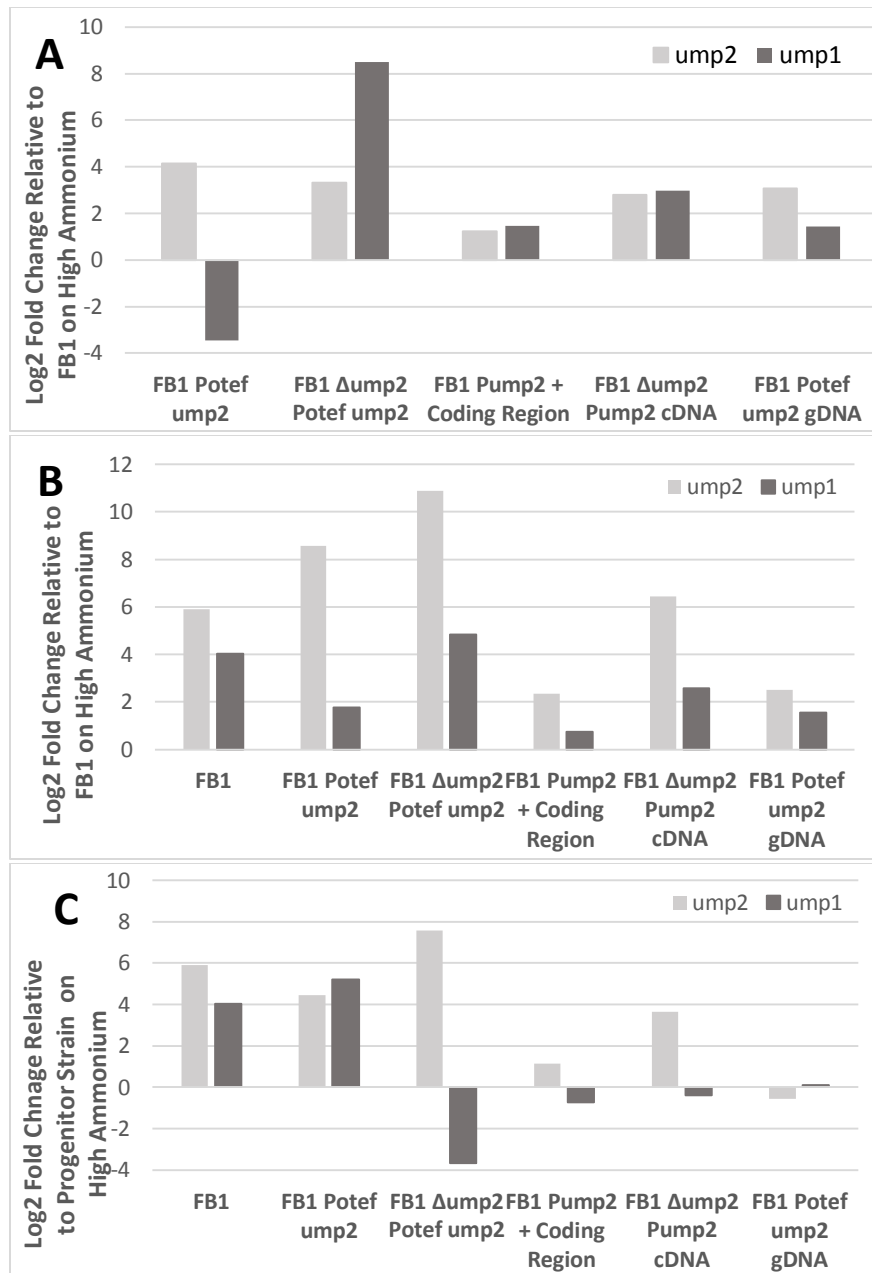
Furthermore, I explored the role of *ump2* transcription levels in various overexpression constructs and different genomic contexts using qRT-PCR analysis. Three overexpression constructs were designed: FB1  $P_{otef} ump2$  containing the native copy of *ump2* as well as a copy at the *ip* locus driven by the constitutively active promoter,  $P_{otef}$ ; FB1  $\Delta ump2 P_{otef} ump2$  containing only one copy of *ump2* cDNA at the *ip* locus driven by the constitutively active  $P_{otef}$ ; FB1  $P_{ump2} +$  coding region, containing two copies of *ump2*, one at the native locus and a second cDNA coding region driven by the native promoter as well, again integrated at the *ip* locus. qRT-PCR results showed that all three constructs upregulate *ump2* gene expression in replete medium (Figure 4A). *Ump1* expression varied slightly, as it was upregulated in all but the FB1  $P_{otef} ump2$ , in which it was down regulated in comparison to FB1 wild type. When low ammonium conditions were compared to FB1 wild type under high ammonium conditions (Figure 4B), there was an upregulation in *ump2* across all the strains. When each mutant construct under low ammonium conditions was compared to itself in high ammonium conditions, there was an increase in *ump2* in each construct. The biggest difference of note is the strain FB1  $\Delta ump2 P_{otef} ump2$  (Figure 4C), in which *ump1* was downregulated in comparison to the other overexpression constructs. This suggests that when the  $P_{otef}$  promoter was the sole promoter driving *ump2*, that *ump1* may be more downregulated.

Two different genomic contexts were investigated to explore other possible regulators outside of the coding region of the construct. FB1  $\Delta ump2 P_{ump2}$  cDNA strain lacked its native copy of *ump2* while the cDNA copy of *ump2* driven by its native promoter was cloned into the *ip* locus. Conversely, an overexpression gDNA construct was designed, FB1  $P_{otef} ump2$  gDNA, which contained the exons and intron of *ump2*

driven by the constitutively active promoter. Similarly, qRT-PCR revealed that both ammonium transporters were upregulated on high ammonium in comparison to the FB1 wild type strain. *ump2* and *ump1* were both upregulated in low ammonium conditions as well. FB1  $\Delta ump2$   $P_{ump2}$  cDNA strain showed nearly 4log<sub>2</sub>-fold increased transcript levels for *ump2* on low ammonium relative to the same strain grown on replete ammonium conditions. The data suggest that the cDNA construct actually induced *ump2* expression more than the gDNA copy did. This suggests that there may be some other regulatory component within the introns that affects the expression of *ump2*. When each construct under low ammonium conditions was compared to itself under high ammonium conditions (Figure 4C), the cDNA construct had an upregulation of *ump2* and very little change in *ump1*. In the strain using the ectopic gDNA copy, *ump2* expression was downregulated, though this low of a fold change actually suggests no change in expression under high and low conditions. This also applies to the small up regulation in *ump1* which suggests no change in expression.

**Deletion of *ump2* in a solopathogenic strain eliminates filamentation on low ammonium, fuzz on charcoal medium, and pathogenicity.**

Deletion of *ump2* (or both *ump1* and *ump2*) in haploid strains (i.e., FB1 and FB2) had previously been observed to have negative effects on filamentation on low ammonium and on pathogenicity (Jinny Paul, 2014). Given these observations, we wanted to examine whether these effects would be also seen in a haploid strain whose pathogenicity does not require mating and fusion of compatible haploids. When the *ump2* deletion mutant was produced in the solopathogenic haploid strain SG200 (Kamper,

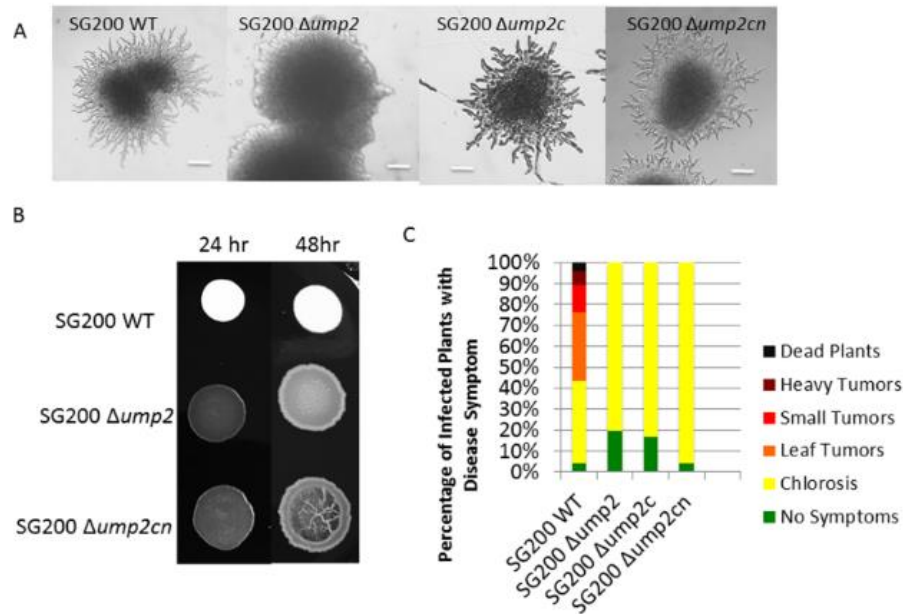


**Figure 4: qRT-PCR analysis of *ump2* and *ump1* in different genomic contexts.** *Ump2* overexpression constructs and different genetic backgrounds show an increase in transcript level in comparison to FB1 wild type strain. All strains were grown in ammonium replete or deficient media, as indicated, for 48 hr. A) qRT-PCR Log<sub>2</sub> fold changes of transcription relative to the wild type FB1 strain, of *ump2* and *ump1* for different *ump2* genomic contexts grown in replete medium. B) Log<sub>2</sub> fold changes relative to FB1 in replete media of *ump2* and *ump1* of FB1 wild type and strains with different *ump2* genomic contexts when grown in ammonium deficient media. C) Log<sub>2</sub> fold change of *ump2* and *ump1* for strains with different *ump2* genomic contexts in ammonium deficient media relative to that for the same strain in replete media.

2006), this strain also failed to filament on low ammonium. (Fig. 5a). The SG200 strain is a solopathogenic strain commonly used for plant pathogenicity experiments as it allows infectious filament formation and successful completion of the lifecycle in planta. This strain normally produces the “fuzz” reaction on charcoal media. Surprisingly, when the *ump2* gene was deleted in this background, the resulting strain failed to display aerial hyphae on charcoal plates (Fig. 5b). Two independent strains complemented for *ump2* in the SG200 *Dump2* mutant were produced using either the native promoter or *Potef* to drive *ump2* expression. Both strains showed complementation of the filamentation defect on low ammonium (Fig. 5a). However, in contrast, neither strain displayed wild type levels of aerial hyphae production on charcoal media (Fig. 5b).

To evaluate whether the apparent defect in pathogenicity observed in the dikaryon experiments above could be replicated in strains that did not require mating prior to infection, again the SG200 strain was used. In this case, defects in pathogenicity due to *ump1* or *ump2* could be assessed independently of the initial mating process. The results are presented in a percent of symptom formation graph (Fig. 5c). Significantly more healthy plants survived at the end of the study in the group that was infected with the SG200 *Dump2* mutant or the SG200 *ump1 ump2* double mutant (not shown) and in most of the plants (~63-83%), the fungus was unable to progress in infection even to stage 1 (chlorosis) (Fig. 5c). Two independent strains complemented for *ump2* in the SG200 *Dump2* mutant with either the native promoter or the *Potef* to drive *ump2* expression both failed to generate wild type levels of virulence (Fig. 5c).

Figure 6 provides evidence that both constructs yielded wild type or above expression levels of transcription for *ump2*, thus ruling out the possibility that the complemented strains were not expressing the introduced trans copies of that gene.



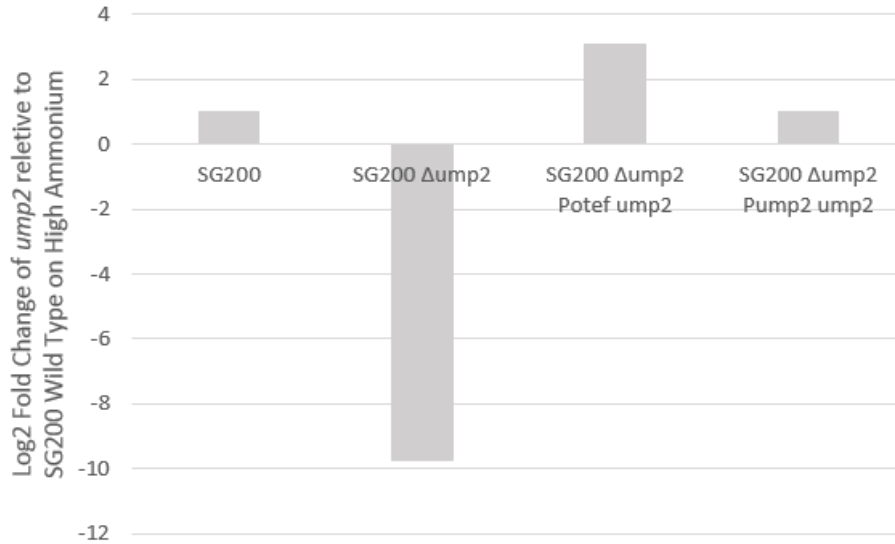
**Fig. 5. SG200 Complementation: Cell Morphology under Low Ammonium Conditions and Mating Assay.** Deletion of *ump2* in a solopathogenic haploid strain eliminates filamentation due to low ammonium, but also the fuzz phenotype on charcoal, and is associated with loss of virulence on maize. (A) SG200, SG200 $\Delta$ *ump2*, SG200  $\Delta$ *ump2c* complemented strains. On AM low ammonium, for 48 h. Size bars, 200  $\mu$ m. (B) Approximately  $10^6$  cells from mid-log phase cultures of either SG200, SG200 $\Delta$ *ump2* mutants, or the SG200 $\Delta$ *ump2c* and SG200 $\Delta$ *ump2cn* derivatives were spotted onto PDA charcoal medium and allowed to grow for 24 h and 48 h at 26 °C. (C) Disease symptom formation in the *U. maydis* SG200 mutant strains. All strains were rated at 10 days post-infection. Plants were inoculated with one background, indicated on the X axis. A disease index (DI; [30]) was used initially to rate virulence of each infection, 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. The graphs display the percentage of plants with specific symptoms of infection.



A

Strain	qRT-PCR Log <sub>2</sub> Fold Change of <i>ump2</i> Expression relative to SG200 WT on Low Ammonium <sup>a</sup>
SG200	1.00 ± 0.09
SG200 $\Delta$ <i>ump2</i>	-9.76 ± 0.13
SG200 $\Delta$ <i>ump2</i> <i>Potef ump2(c1)</i>	3.07 ± 0.06
SG200 $\Delta$ <i>ump2</i> <i>Pump2 ump2(c2)</i>	1.01 ± 0.03

B



**Figure 6. qRT verification of *ump2* transcription in SG200  $\Delta$ *ump2* strains provided the gene ectopically driven by *P<sub>otef</sub>* or the native promoter.** Both complement constructs, with *P<sub>otef</sub>* and with the native promoter driving *ump2* expression, demonstrated expression levels of *ump2* similar to that of the wild type when all three were tested under low ammonium conditions. A) qRT-PCR Log<sub>2</sub> fold changes of *ump2* of mutant strains relative to SG200 wild type on low ammonium<sup>a</sup>, plus/minus standard error across technical replicates. B) Graphical representation of qRT-PCR log<sub>2</sub> fold changes of mutants relative to SG200 wild type on low ammonium<sup>a</sup>.

<sup>a</sup> Low ammonium is 50  $\mu$ M ammonium and 1% glucose by volume.

## CHAPTER IV

### DISCUSSION

*U. maydis* is a basidiomycete fungus that exhibits a dimorphic switch from a yeast-like state to a filamentous growth state. This switch changes the fungus from a saprophytic stage to a pathogenic stage, infecting mainly *Z. mays*. This switch is triggered due to different environmental conditions, such as nutrient limitation. Nitrogen limitation, specifically ammonium, is one factor responsible for this switch, yielding either dikaryon formation for mated haploid cells, or a filamentous phenotype for haploid cells not engaged in mating. There are two genes encoding ammonium permeases in *U. maydis* responsible for transporting ammonium into the cell; *ump2* and *ump1*. *Ump2*, the high affinity transporter, has an additional role, acting as a sensor of the available ammonium outside of the cell and transmitting this signal to change cell morphology to the filamentous form. The current study investigated the roles of ploidy (haploid vs. diploid strains), promoter type (native promoter vs. constitutive promoter), gene structure (genomic copy vs. cDNA), and gene location (native location vs. ectopic location) on transcription levels of both ammonium transporters under low ammonium conditions compared to high ammonium conditions.

Ploidy refers to the number of chromosome sets within a cell of an organism. In past studies, it has been found that ploidy can play a role in gene expression, but typically at very low levels (i.e., 2-fold change). Three different strains, haploid, haploid

solopathogenic and diploid solopathogenic, were used to investigate the role of ploidy in *U. maydis*. Using qRT-PCR as a measure of gene expression, both the diploid and haploid solopathogenic strains, D132 and SG200, respectively, the expression of the ammonium transporter, *ump2*, was shown to be significantly affected. This suggests that being primed for pathogenic development was not the factor responsible for *ump2* induction, since there was already a functioning bE/bW heterodimer within the haploid solopathogenic strain SG200 and substantial induction of *ump2* was not observed in that strain. The diploid solopathogenic strain contains complete b1/b2 alleles, which code for the two pieces of the heterodimer (i.e., bE1/bW2) responsible for initiating the pathogenic program. However, in addition, D132 is diploid for the remainder of the genome as well, suggesting effects of the diploid state on *ump1* and *ump2* expression.

The promoter driving gene expression of *ump2* was investigated to see if this had an effect on *ump2* gene expression. Expression levels from the native promoter for *ump2* were compared to those from a constitutively active promoter,  $P_{otef}$ , in the FB1 background. Results showed that the use of  $P_{otef}$  increased the expression of *ump2* dramatically under both high and low ammonium conditions. Surprisingly, use of just one copy of the *ump2* gene via  $P_{otef}$  actually increased *ump2* expression more than having two copies (i.e., one via the native promoter and one via  $P_{otef}$ ) (Fig. 4a). In replete media conditions, the promoter did not seem to have as large of an effect on *ump2* expression as it did under low ammonium conditions. When *ump2* expression was compared when the gene was driven by  $P_{otef}$  to that from an overexpression construct containing two copies of *ump2* (FB1  $P_{ump2}$  + Coding Region), the constitutive promoter had a much larger effect on upregulating *ump2* than the construct containing two copies. Under low ammonium

conditions, the *P<sub>otef</sub>* promoter showed an 8 log<sub>2</sub>fold increase in expression under high ammonium conditions while the construct containing two copies of *ump2* showed less than a 3 log<sub>2</sub> fold change (Fig. 4b).

I also examined the role of gene structure and gene location in affecting expression of the ammonium transporters. Gene structure in this study refers to comparison of expression for the cDNA construct with that of the genomic DNA (gDNA) portion of *ump2* used to express *ump2*. The cDNA copy included only the coding region of *ump2* while the gDNA copy included both the exons and the introns. In comparison to the FB1 wild type strain in replete media conditions, both gDNA and cDNA constructs showed increased *ump2* expression. Under low ammonium conditions, the strain expressing the cDNA copy increased *ump2* expression slightly less than 4log<sub>2</sub>fold, whereas expression from the gDNA construct actually displayed a slight decrease in *ump2* transcription level relative to the same strain under replete conditions. This suggests that there may be some other regulatory components within the introns or the 5' or 3'UTRs that affect the expression of *ump2* by downregulating its expression under low ammonium conditions. Results in the FB1 background were determined under the assumption that the native promoter and gene expression are not effected due to their genomic location. When cloning into *U. maydis*, the *ip* locus was used as an ectopic location from the native copy of the *ump2* gene. When using the *ip* locus, we found that *ump2* expression can be restored when replaced with a copy of *ump2* using the native promoter and increases *ump2* expression when *ump2* is driven by the constitutively active promoter.

The use of the solopathogenic strain SG200 and its mutant derivatives suggests that the presence of at least *ump2* is required for full pathogenicity even in the absence of mating, as these mutants were also severely impaired in disease production. The most parsimonious explanation here is that lacking the high affinity ammonium transporter and therefore a way to sense and obtain ammonium, the mutants are not able to move to the next stage in the host, where nitrogen may initially be limiting. Additionally, while the SG200 mutants complemented with the wild type *ump2* cDNA regained the ability to filament on low ammonium (Fig. 5a), they failed to regain either wild type production of aerial hyphae on charcoal or virulence (Fig. 5b and 5c, respectively). It was confirmed that the complemented mutants had similar transcription levels of *ump2* as wild type (see Fig. 6). Since only cDNA of the coding region was used in these constructs, this suggests additional aspects of *ump2* regulation including, e.g., as-yet-unidentified regulatory signals in either the 5'- or 3'-UTR of the *ump2* mRNA that were not included in the complementation constructs used here.

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