INVESTIGATING THE ROLE OF TRANSCRIPTION FACTORS *UST1* AND *MED1* IN MATING, PATHOGENICITY AND SPORULATION OF *USTILAGO MAYDIS*

by

NADIA CHACKO

(Under the Direction of Scott Gold)

ABSTRACT

The plant pathogenic fungus *Ustilago maydis* is a model organism for the study of obligate pathogenesis. Mating, dimorphic switch, growth *in planta* and sporulation of *U. maydis* are areas of considerable interest in the context of study of pathogenicity of this fungus. In the work presented here, two transcription factors *ust1* and *med1* involved in these vital processes in the life cycle of *U. maydis*, were studied. Transcription factors *ust1* and *med1* are orthologs of conidiation regulators *stuA* and *medA* genes of *Aspergillus nidulans*.

The role of Ust1 in regulation of genes differentially expressed during saprobic budding growth of *U. maydis* was studied using a reverse genetic approach. Several potential targets of Ust1 were identified in an earlier study of genes differentially expressed during budding growth of *U. maydis*. Deletion studies indicate that these genes are dispensable for mating, pathogenicity and budding growth. One exception was the *thi1* gene, which was auxotrophic for thiamine in culture but dispensable for mating and pathogenicity.

The Δ*ust1* mutant produces filaments and teliospore-like structures in culture. To assess the suitability of use of the Δ*ust1* mutant as a surrogate for study of sporulation of *U. maydis*, a

transcriptome analysis was conducted. Comparison of gene expression patterns of the Δ*ust1* mutant, wild type teliospore producing plant galls, plant galls of Δ*hgl1* which produces immature teliospores to that of wild type budding cells in culture showed that there is some overlap in the gene network involved in teliospore formation and the 'sporulation' of Δ*ust1*.

The functional characterization of the *U. maydis med1*, a transcription factor identified in the microarray study, by gene deletion showed that this gene is required for conjugation tube formation, mating *in vitro* and expression of normal virulence. The expression of mating associated genes *mfa1*, *pra1* and transcription factor *prf1* was lower in Δ*med1* compared to wild type suggesting an important role of *med1* in regulation of transcription of mating related genes in *U. maydis*.

INDEX WORDS: *Ustilago maydis*, *Aspergillus nidulans*, Dimorphic switch, Teliospores, Transcriptional analysis, Microarray, Pathogenicity, Galls

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DEDICATION

To my parents

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

The Basidiomycotan order Ustilaginales consists of about 1200 species in 50 genera including the economically important smut fungi. Smut fungi infect over 4000 species of plants causing up to 25% yield loss. Of the *Ustilago* species*, U. maydis* and *U. hordei,* that cause smut of maize and barley, respectively, are important in the U.S. Even though the average yield loss due to corn smut is below 2%, this is substantial due to the high value of the crop in the U.S. (Martinez-Espinoza et al., 2002). In 2009 with the maize crop estimated at a value of $48.7 billion, a 2% loss would be approximately $1 billion USD (National Corn Growers Association). In addition to its economic importance, *U. maydis* is an important model for the study of dimorphism and biotrophic pathogenesis.

The dimorphic switch from yeast to hypha is a central initiator of the pathogenic life cycle of *U. maydis*. In nature this happens when cells of compatible mating types fuse. The dikaryon formed, survives only if it infects the host, maize. With successful infection, galls are produced which, upon maturation, become filled with spores. The spores formed are called teliospores and are diploid and melanized. Several signaling pathways and numerous genes and regulators have either been shown or are presumed to be involved in the dimorphic switch and telisopore formation. Identification of the key regulators of the dimorphic switch, pathogenicity development and spore development of *U. maydis* in host tissue will give better understanding of these processes.

One putative regulator of dimorphism and sporulation in *U. maydis* is the transcription factor *ust1*. The deletion phenotype of the Δ*ust1* mutant is a conversion from budding growth to the production of filamentous cells and formation of teliospore-like structures in culture (Garcia-Pedrajas et al., 2010a). The *ust1* gene is the ortholog of the transcriptional regulator *stuA* from *Aspergillus nidulans*. The *ust1* gene was initially identified after the observation that potential StuA binding sites were present in the promoter regions of 13 among the 37 genes found to be down-regulated in filamentous *U. maydis* cells (Garcia-Pedrajas et al., 2004). Transcription factor-encoding genes, *ust1* and *stuA*, are part of a group of fungal-specific transcription factors called the APSES domain proteins. The APSES domain proteins are characterized by a basic helix-loop-helix domain and have been shown to regulate major morphogenetic changes like the yeast-hypha transition and sporulation in several fungi (Aramayo et al., 1996; Gimeno and Fink, 1994; Stoldt et al., 1997; Ward et al., 1995).

A. nidulans stuA functions as a developmental modifier during asexual reproduction and also regulates sexual reproduction (Wu and Miller, 1997; Sheppard et al., 2005). The StuA protein regulates gene expression by directly binding to promoter elements (Dutton et al., 1997). The Ust1 protein in its APSES domain bears high similarity to StuA and therefore, like other APSES protein, is likely to bind to the 8 base sequence A/TCGCGT/ANA/C in the promoter regions of its target genes. One of the genes repressed by *stuA* is the developmentally regulated *awh11* gene (Dutton et al., 1997). The ortholog of *awh11* in *U. maydis*, um00205 is up-regulated in budding vs filamentous stage and has the StuA specific binding sequence in its promoter. The expression of the gene is also de-repressed in Δ*ust1* mutant indicating a similar mode of regulatory action between Ust1 and StuA (Garcia-Pedrajas et al., 2010). Its deletion phenotype suggests that *ust1* acts as a repressor of filamentous growth and sporulation.

The primary goal of this study was to identify the specific regulatory role of *ust1* in morphogenesis and sporulation in *U. maydis*. The specific objectives were to:

1. Determine the role of Ust1 in the regulation of genes with putative Ust1 binding sites that are up-regulated in the budding compared to the filamentous stage.
2. Determine, via transcriptome analysis, the suitability of culture grown *ust1* deletion mutants as axenic surrogates of wild type *in planta* teliospore development.

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CHAPTER 2

LITERATURE REVIEW

The phylum Basidiomycota in the kingdom fungi is host to important plant pathogens like rusts and smuts. Smut fungi belong to the order Ustilaginales which includes over 1200 species in 50 genera including the maize pathogen *Ustilago maydis*. *U. maydis* has a very narrow host range infecting only maize and its wild progenitor, teosinte. Due to its genetic tractability, it has become a model system for the study of biotrophic interactions between a fungal pathogen and its plant host (Brefort et al., 2009a). Unlike small grain smut fungi that sporulate only in flower-derived tissues, *U. maydis* can infect and cause symptoms on all above ground parts of the plant. The symptoms of infection vary from mild chlorosis to large tumors which are filled with teliospores at maturity.

The morphogenenetic swtich from a saprobic yeast form to a filamentous form is essential for pathogenicity of *U. maydis* (Feldbrugge et al., 2004). This switch occurs when haploid yeast cells of compatible mating type fuse. Compatibility is determined by the presence of different alleles at the *a* and *b* mating type loci. The *a* mating locus has genes that code for lipopeptide pheromones and receptors of pheromones produced by cells with its compatible *a* allele (Bölker et al., 1992). The dikaryon formed by the fusion of yeast cells further develop when the transcription factors bE and bW, encoded by different alleles dimerize to form an active heterodimeric transcription factor (Gillissen et al., 1992; Kahmann et al., 1995; Kamper et al., 1995; Yee and Kronstad, 1993). The dikaryon directly penetrates the plant tissue and spreads

inter and intracellularly, subsequently fragmenting to produce teliospores (Banuett and Herskowitz, 1996). Teliospores are produced only *in planta* and on maturation are diploid and melanized. The spores are produced inside the galls on the host. Teliospores serve as overwintering structures and can survive un-germinated for years. Under suitable environmental conditions teliospores germinate and concomitantly undergo meiosis. The resulting haploid yeast cells begin anew the life cycle.

Signaling pathways and regulators of dimorphism and sporulation in *U. maydis*

During mating, as the pheromone signal is perceived by the corresponding receptor, two signaling pathways, MAPK and cAMP, transfer this signal. The core components of the MAPK pathway include Ubc4/Kpp4 (MAPKKK), Fuz7/Ubc5 (MAPKK) and Ubc3/Kpp2 (MAPK). The genes in the MAPK cascade are required for conjugation tube formation, filamentation and pathogenicity of *U. maydis* (Banuett and Herskowitz, 1994; Mayorga and Gold, 1999; Muller et al., 2003). Downstream of Ubc3 is the transcription factor Prf1, an essential regulator of mating and pathogenicity. Prf1 is regulated by phosphorylation by the Ubc3/Kpp2 MAPK and Adr1 the catalytic subunit of the cAMP-dependent protein kinase, PKA (Durrenberger et al., 1998; Gold et al., 1994; Kaffarnik et al., 2003). In turn the activated Prf1 induces the expression of the *mfa* and *pra* genes that encode pheromone and pheromone receptor, respectively over and above the basal level.

The components of the cAMP pathway of *U. maydis* have also been shown to be essential for the morphogenetic switch, pathogenic activity and sporulation. The important components of this pathway include the G protein α subunit, Gpa3, adenylyl cyclase, Uac1 and PKA comprised of the regulatory subunit Ubc1 and catalytic subunit Adr1 (Kronstad et al., 1998). The *ubc1* gene

is not necessary for initial colonization but is required for later production of galls and teliospores (Gold et al., 1997). Uac1 and Adr1 are essential for infection of plant tissue and proliferation thereafter (Durrenberger et al., 1998; Gold et al., 1994). As cAMP is required for the maintenance of the budding phenotype in *U. maydis*, the deletion of the *uac1*or *adr1* converts cells to constitutively filamentous in culture (Durrenberger et al., 1998; Gold et al., 1994).

By comparing differential gene expression between the budding wild type and the filamentous *uac1* mutant, several genes differentially expressed in each of these morphogenetic stages were identified (Andrews et al., 2004; Garcia-Pedrajas et al., 2004). Search for a common regulator of genes differentially expressed in the budding stage led to the identification of a putative transcriptional regulator with an APSES domain, *ust1* (Garcia-Pedrajas et al., 2010c). Thirteen of the 37 genes differentially expressed in the budding stage had the putative Ust1 binding site in their 5' 1kb regions. The deletion of the *ust1* gene makes *U. maydis* filamentous and defective in mating and pathogenicity. In addition the Δ*ust1* strain produces teliospore-like structures in culture (Garcia-Pedrajas et al., 2010c).

APSES domain transcription factors and their functions

APSES domain proteins are fungal-specific transcription factors that regulate major morphogenetic changes like the yeast-hypha transition, pathogenicity and sporulation (Aramayo et al., 1996; Gimeno and Fink, 1994; Lysøe et al., 2011; Noffz et al., 2008; Stoldt et al., 1997; Ward et al., 1995). The name APSES was derived from the transcription factors, Asm1 (*Neurospora crassa*), Phd1 and Sok2 (*Saccharomyces cerevisiae*), Efg1 (*Candida albicans*) and StuA (*Aspergillus nidulans*), that have a basic helix-loop-helix (bHLH) domain (Aramayo et al., 1996; Gimeno and Fink, 1994; Stoldt et al., 1997; Ward et al., 1995). While Phd1, Sok2 and

Efg1 are regulators of the yeast-hypha switch, other APSES proteins like StuA, Mstu1 (*Magnaporthe grisea*) and FgStuA (*Fusarium graminearum*) are involved in the regulation of asexual and/or sexual reproduction (Lysøe et al., 2011; Nishimura et al., 2009; Pan and Heitman, 2000; Sheppard et al., 2005; Stoldt et al., 1997; Wu and Miller, 1997). APSES proteins GcSTUA (*Glomerella cingulata*) and Mstu1 are needed for pathogenicity and their loss causes reduction in turgor pressure of appressoria and consequently, a reduction in penetration of host cells by the fungi (Nishimura et al., 2009; Tong et al., 2007). StuA (*A. fumigatus*) and FoStuA have also been found to be required for appropriate regulation of secondary metabolism (Lysøe et al., 2011; Twumasi-Boateng et al., 2009). To date, *ust1* is the only the APSES domain protein gene studied in the Basidiomycota.

The bHLH domain of the APSES proteins are highly conserved domains binding to sequence-specific DNA. The binding of StuA and Efg1 to their corresponding response elements has been shown experimentally (Dutton et al., 1997; Leng et al., 2001). The basic region of the first helix determines DNA binding sequence specificity with further preferences established by residues constituting the loop and through partner dimerization. The bHLH proteins generally bind to an E box (CANNTG) as seen in *C. albicans* Efg1 (Leng et al., 2001). The *A. nidulans* StuA on the other hand was shown to bind to the MluI cell cycle box (MCB) sequence characterized by 4 base (CGCG) conserved core. The StuA binding site extends to a degenerate 8 base sequence A/TCGCGT/ANA/C (Dutton et al., 1997). More recently APSES proteins Efg1 and Efh1 of *C. albicans* have been shown to bind to the MCB sequence indicating the possibility of general affinity of APSES proteins for the MCB sequence (Noffz et al., 2008). Efg1 has also been shown to bind to, and dimerize with, other transcription factors like Czf1 and Flo8 through

regions outside the APSES domain which might contribute to its ability to bind to more than one specific binding site (Giusani et al., 2002; Noffz et al., 2008).

The identification of genes regulated by APSES proteins has primarily been accomplished by searching for the 8 base binding site of the protein in the promoter regions of these genes. The *A. nidulans awh11* gene has been shown to be repressed by StuA by direct binding of the protein to the binding site in the promoter of this gene (Dutton et al., 1997). The corresponding ortholog of *awh11* in *C. albicans, wh11,* though not known to be directly regulated by an APSES protein, is differentially expressed in the white budding stage of the fungus. The homolog of this gene in *U. maydis* um00205 has the putative Ust1/ StuA binding site in its promoter, is differentially expressed in budding phase and its expression is de-repressed in the Δ*ust1* mutant (Garcia-Pedrajas et al., 2010c). This gene may therefore be a direct target of the *U. maydis* APSES protein, Ust1.

The dramatic phenotype of Δ*ust1*, of filamentation and production of spore-like structures in culture, suggests that Ust1 may act as a repressor of filamentation and teliospore formation during the saprobic stage of the life cycle of the fungus. The roles of StuA and Efg1 as repressors support the hypothesis that Ust1 is a transcriptional repressor. Another APSES protein, Phd1, however, acts as an activator of genes required for invasive and non-invasive pseudohyphal growth in *S. cerevisiae* (Pan and Heitman, 2000). This could mean that *ust1* can act both as a repressor and an activator. More studies are required to understand the role of *ust1* in morphogenesis and sporulation in *U. maydis*.

Genes associated with sporulation in *U. maydis*

Teliospore production in *U. maydis* occurs only after mating, in the presence of the host plant. The processes that occur during infection, subsequent spread of the fungus in the host and sporulation have been studied (Banuett and Herskowitz, 1996). Several genes including *gpa3*, *rum1*, *hgl1*, *fuz1* and *hda1* are required for the development of teliospores. Hgl1 is a putative transcription factor, the deletion of which causes the arrest of teliospore development resulting in production of immature and un-pigmented spores (Durrenberger et al., 2001). The development of the Δ*fuz1* mutant stops before the accumulation of mucilaginous matrix containing enzymes needed for the fragmentation of hyphae to teliospores (Banuett and Herskowitz, 1996). Deletion of *hda1* and *rum1* prevents the rounding off of teliospores (Reichmann et al., 2002). The loss of Ust1 prevents mating and sporulation indicating a larger role in regulatory control for this protein in the pathogenic life cycle of the fungus. The events from infection to sporulation and subsequent teliospore maturation must be under the control of several regulators responding to signals that may be coming from the host. Considering how similar the Ust1 protein is to *A. nidulans* StuA in structure and function, *A. nidulans* can serve as a model for the search for other regulators in *U. maydis*.

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CHAPTER 3

CHARACTERIZATION OF PUTATIVE TARGETS OF *UST1* IN *USTILAGO MAYDIS*

INTRODUCTION

Dimorphism in fungi refers to the ability to switch between unicellular yeast cells and filamentous forms. In the pathogenic fungus *Ustilago maydis*, this switch from yeast to filament is also associated with a switch from saprobic to pathogenic lifestyle. Mating is one way this process occurs in *U. maydis*. Other environmental stimuli like low nitrogen, acidic pH and lipids in growth medium also cause filamentation of *U. maydis* sporidia. Filamentation by mating or in response to environmental signals is regulated by cAMP and MAPK pathways. The loss of genes coding for adenylate cyclase (*uac1*) and the catalytic subunit of PKA enzyme, *adr1*, causes the cells to filament constitutively due to low PKA activity (Durrenberger et al., 1998; Gold et al., 1994).

A morphogenetic switch is a complex process involving expression of several genes. A suppressive subtractive hybridization study comparing budding cells and the filamentous *uac1* mutant identified 37 genes up-regulated in the budding stage and 26 genes in the filamentous stage (Andrews et al., 2004; Garcia-Pedrajas et al., 2004). From inspection of the promoter regions of the genes up-regulated in the budding stage, the binding site consensus sequence for the *Aspergillus nidulans* conidiation regulator, *stuA*, and potentially for its *U. maydis* ortholog *ust1* was identified in 13 of them (Garcia-Pedrajas et al., 2010b). The ortholog of transcription factor, *ust1* in other fungi like *Candida albicans* (*efg1*) and *Saccharomyces cereviseae* (*phd1* and

sok2) are regulators of yeast-hypha transition (Pan and Heitman, 2000; Stoldt et al., 1997). The deletion of *ust1* causes filamentation and formation of spore like structures in culture (Garcia-Pedrajas et al., 2010b). Taking the deletion phenotype of Δ*ust1* together with the function of orthologs of this gene in other fungi, it can be speculated that the gene is another regulator of the dimorphic switch in *U. maydis*.

Fungal-specific transcription factors that regulate morphological transformations and have a conserved bHLH domain and DNA binding domain are classified into a group called APSES proteins. APSES domain proteins bind to sequence-specific regions in the promoter elements of the genes they regulate. The response element or binding site of *A. nidulans stuA* is an eight base sequence (AT/CGCGT/ANA/C). Thirteen genes up-regulated in the budding stage have the *stuA* response element in their promoter suggesting that these genes are regulated by *ust1*. The filamentous phenotype of the Δ*ust1* mutant suggests that this gene is required for the maintenance of the budding phenotype, possibly by repressing activity of genes required for budding growth.

To test the hypothesis that *ust1* is a regulator of the genes with the *stuA* response factor, we conducted deletion studies. Three (um01080, um00118 and um00205) of the thirteen genes up-regulated in the budding stage, were deleted in previous studies. The loss of the GABA aminotransferase gene (um01080) decreased the ability of the fungus to grow on media containing alanine as sole nitrogen source (Straffon et al., 1996). The loss of the gene UDP glucose dehydrogenase (um00118), caused cell wall defects resulting in difficulty in cell separation (Garcia-Pedrajas, unpublished). In this study we deleted six more genes that are potential targets of *ust1*. The deletion of thiamine biosynthetic enzyme (*thi1*) (um11140) causes thiamine auxotrophy that could be rescued by the addition of thiamine hydrochloride to the

medium. None of the gene deletions yielded phenotypes that were informative of function of the Δ*ust1* gene and all mutants produced filaments in response to lipids. All deletion mutants mated normally and displayed wild type pathogenicity levels.

MATERIALS AND METHODS

Strains and growth conditions

U. maydis strains 1/2 (*a1b1*), 2/9 (*a2b2*) and SG200 were grown in liquid potato dextrose broth (PDB) (Sigma-Aldrich, St Louis, MO, USA) or solid potato dextrose agar (PDA) medium at 30°C. Selection of transformed *U. maydis* strains was carried out on YEPS-sorbitol (1% yeast extract, 2% peptone, 2% sucrose and 1M sorbitol) plates containing carboxin (3µg/ml). Transformants of *Escherichia coli* strain DH5α (Bethesda Research Laboratories, Gaithersberg, MD, USA) were selected on Luria Bertani (LB, Difco) agar or LB broth with kanamycin (50µg/ml).

Generation of gene deletions

All gene deletions were constructed using the DelsGate technique (Garcia-Pedrajas et al., 2008). The deletion construct was made using the plasmid vector pDONR-cbx with the 5' and 3' flanks of each gene cloned at the *attB* sites. Deletion constructs were verified by PCR and sequencing. The open reading frames of the genes were replaced by the deletion construct by homologous recombination.

U. maydis transformations were done in compatible haploid strains 1/2, 2/9 and the solopathogenic strain SG200 according to the previously described method (Barrett et al., 1993). Confirmations of gene deletions were made using PCR and Southern hybridization. The DNA

from all strains and the wild type was digested with *Eco*RV, *Eco*RI and *Hin*dIII enzymes for genes um11574, um11400, um04478 and um01840, respectively and separated on a 0.8% agarose gel. The DNA was transferred to Hybond XL nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using 0.4M NaOH as transfer solution. The 5' flank of the gene was labeled with DIG using the DIG-High Prime labeling and detection kit (Roche, Indianapolis, IN, USA). Hybridization and subsequent development of the blot was done according to the manufacturer's instructions.

Mating assays

Plate mating assays were done to determine the effect of loss of genes studied. The deletion strains in compatible mating (1/2 and 2/9) backgrounds were grown overnight in PDB. Equal volumes of compatible strains were co-spotted on charcoal mating plates (YEPS medium with 1% charcoal), sealed with parafilm and incubated at room temperature in the dark for 24h. Wild type control strains were co-spotted along with the mutants and observed for the production of dikaryotic filaments indicative of a positive mating reaction.

Pathogenicity assays

Seven-day-old seedlings of maize variety Golden Bantam (Athens Seed Co., Watkinsville, GA, USA) were used to test the pathogenicity of the deletion mutants. Mixtures of mutant and wild type cells (10^6 cells /ml) in compatible genetic backgrounds were injected at the culm of 7 day old plants. The plants were kept in a growth chamber with daily cycles of 16h light at 28°C followed by 8h of dark at 20°C. Symptoms were scored 7, 10 and 14 days post inoculation on a scale of 0 to 5 (0- no symptoms, 1-chlorotic lesions/anthocyanin production, 2-leaf galls, 3-small stem galls, 4-large stem and basal galls and 5-plant death). The results were analyzed using a non-parametric statistical test (Shah and Madden, 2004).

Growth assay for auxotrophic mutants

A growth assay to test thiamine auxotrophy was carried out using minimal medium (MM) with 1% glucose. Mutants and the wild type in 1/2 and 2/9 backgrounds were initially grown in PDB and 10^6 cells were inoculated into MM and grown at 30°C with constant shaking at 200 rpm. Rescue of auxotrophy was determined using a growth assay in MM supplemented with 0, 20, 40, 60 and 80 µM of thiamine hydrochloride. Cell counts were taken 24 and 48h post inoculation. The cultures were photographed and the means of cell counts for each genotype were plotted.

Filamentation in response to lipids

To determine if the loss of putative *ust1* targets would affect their ability to switch to the filamentous state in presence of lipids, 10^6 cells of each mutant and wild type strains were inoculated into MM with 1% glucose, tween 20 or tween 80 as the sole carbon source. Cultures were grown at 30°C with constant shaking and cells were microscopically observed and photographed 4 days post inoculation.

RESULTS

Colony morphology and growth rate of deletion strains of putative *ust1* targets

To understand the role of Ust1 as a regulator of genes up-regulated during budding growth, we conducted gene deletions using the Delsgate technique. The genes selected for deletion had the putative Ust1 binding sites in their promoter and were previously found to be up-regulated in the budding stage (Garcia-Pedrajas et al., 2004). The deletion construct for 10 of these genes were made by the Delsgate technique and analyzed by PCR and sequencing. Six of the constructs

were successfully used in *U. maydis* transformations in 1/2, 2/9 or SG200 backgrounds (Table 3.1). No viable *U. maydis* knockouts were obtained for the remaining 4 among the 10 genes indicating a probable essential nature of these genes in survival of the fungus. The gene deletions were confirmed by PCR and/or Southern hybridizations. Positive transformants were grown on potato dextrose agar (PDA) and in potato dextrose broth (PDB) media for phenotypic observation. The mutant strains of all genes studied produces white sticky colonies that resemble the wild type. The growth rates of the mutants in all genetic backgrounds were comparable to those of the wild type when grown in potato dextrose broth (data not shown).

The deletion of the gene encoding thiamine biosynthetic enzyme (*thi1*), UM11400, caused thiamine auxotrophy which manifested as a significant reduction in rate of growth in minimal medium without an external source of thiamine (Figure 3.1A). Thiamine auxotrophy of Δ*thi1*, was remedied by the addition of 20 µM of thiamine hydrochloride (Figure 3.1B and C).

Filamentation of the mutants in response to lipids

Lipids induce a switch in morphology from yeast to what resembles the infectious filament in *U. maydis* (Klose et al., 2004). We studied the ability of mutants in the putative Ust1 target genes to switch to filamentous growth in response to lipids in the external environment. Wild type filamentation in response to lipids was observed in five out of six mutants. The Δ*thi1* deletion mutant was unable to filament in the presence of lipids unless the medium was amended with thiamine hydrochloride. Filamentation was observed in wild type and mutants only in the presence of Tween 80 (data not shown).

Mating and pathogenicity assays

To test the role of the deleted putative Ust1 target genes on mating, compatible haploid mutants were co-spotted on YPD charcoal plates. Mating was observed as white fuzzy filaments formed 24- 48h after inoculation (data not shown). The deletion mutants for; putative protein (um00027), proton nucleoside co-transporter (um01840), thiamine biosynthetic enzyme (um11400) and carboxymuconolactone decarboxylase (um11574) genes did not show a mating defect when co-spotted with wild type or a compatible mating type mutant. For the deletion mutants for phosphate transporter (um04478) and myo-inositol co-transporter (um04478) genes, where deletions were made only in the *a1b1* mating type, the positive mating reaction was observed when co-spotted with wild type *a2b2* strain. These results suggest that the tested genes were dispensable for mating.

To further determine if the genes um00027, um01840, um11400 and um11574 are required for pathogenicity, maize plants were inoculated with compatible strains of the mutants and the disease index was calculated 7, 10 and 14 days post inoculation (dpi). The mutants induced typical disease symptoms and the rate of disease development was comparable to that of the wild type indicating that these genes are not required for pathogenicity. The disease indices of mutants did not show any significant differences from the wild type (Tables 3.2 to 3.4).

DISCUSSION

APSES proteins are regulators of the fungal morphogenetic switch between yeast and hyphal forms. In the human pathogen *Candida albicans*, the APSES domain transcription factor Efg1 is required for normal budding growth and formation of filaments in response to serum (Noffz et al., 2008; Stoldt et al., 1997). APSES proteins of *Saccharomyces cerevisiae* Phd1 and

Sok2 and *Wangiella deramtitidis* WdStuA also regulate the yeast-hypha transition (Gimeno and Fink, 1994; Wang and Szaniszlo, 2007; Ward et al., 1995). Other functions of APSES proteins deduced by mutation studies include regulation of sexual and asexual sporulation, formation of appressoria and secondary metabolism (Aramayo et al., 1996; Lysøe et al., 2011; Nishimura et al., 2009; Tong et al., 2007; Twumasi-Boateng et al., 2009).

In this study, we analyzed the deletion mutant phenotype of six genes that are potentially regulated by the *U. maydis* putative APSES domain transcription factor Ust1. The genes selected for the study were a subset of the thirteen genes earlier found to be up-regulated in the budding stage of *U. maydis* (Garcia-Pedrajas et al., 2004) and which had the putative Ust1 binding site (*stuA* response element) in their promoter region. The deletion of genes putatively regulated by *ust1* did not affect their morphological, mating or pathogenicity phenotypes. The deletion of the *thi1* gene however made the strain auxotrophic for thiamine when grown in minimal medium. Despite the very high expression of the genes during budding growth, their deletion did not affect saprobic growth or the budding phenotype. Similarly the ability of the mutants to switch to the filamentous form in the presence of lipids was not affected except for the Δ*thi1* mutant that required externally applied thiamine for this switch. This observation suggested that the gene is not necessary for the switch.

stuA, the ortholog of *ust1* in *A. nidulans* ensures the proper development of conidiophores and sterigmata by regulating the localization of two transcription factors, *brlA* and *abaA* (Miller et al., 1992). *stuA* is also required for the expression of several genes in the biosynthesis of secondary metabolites in *A. fumigatus* (Twumasi-Boateng et al., 2009). The deletion phenotype of Δ*ust1*, includes significant change in filamentation and formation of pigmented spore like structures in culture (Garcia-Pedrajas et al., 2010b). This phenotype suggests that, like *stuA*, the

ust1 gene functions as a regulator of morphogenesis and sporulation through regulation of specific transcriptional regulators, by direct regulation of several genes involved in morphogenesis, or by both. If the deletion phenotype is the cumulative effect of lack of expression of several genes and/or transcription factors required for morphogenesis, the deletion of individual genes may not be reflective of the Δ*ust1* phenotype. This could explain the lack of informative phenotypes with the deletion of the bud up-regulated genes putatively controlled by *ust1*.

Transcriptional profiling of APSES transcription factor deletion mutants of *stuA* and *fostuA* indicate regulation either directly or indirectly of large numbers of genes (Lysøe et al., 2011; Sheppard et al., 2005; Twumasi-Boateng et al., 2009). However evidence for direct regulation of these genes by APSES protein binding to promoter elements, is limited. The *A. nidulans* APSES protein *stuA* regulates the *awh11* gene by directly binding to its promoter. The ortholog of *awh11* in *U. maydis*, heat shock protein 12 (um00205) is up-regulated in the budding stage and has the putative Ust1/StuA binding site in its promoter. The expression of this gene is highly de-repressed in the Δ*ust1* mutant (Garcia-Pedrajas et al., 2010b). Since the presence of binding sites in the promoter of a gene does not always mean it is actively bound by the corresponding transcription factor under all conditions, the six genes studied here may not be direct targets of Ust1. Microarray analysis of Δ*ust1* versus the wild type showed the altered expression of 7 of these genes (um00011, um00027, um00205, um00336, um00455, um00496 and um03522) indicating a possibility of direct or indirect regulation of these genes by *ust1*.

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Figures and Tables

Table 3.1. Deletion status and mutant phenotypes of bud up-regulated putative Ust1 targets

um no.	Name of the gene	Deletion status	No. of Ust1 binding sites	Deletion phenotype
um00011	Acetamidase-A	Not deleted	2	NA
um00027	Putative protein	Deleted (this study)	1	No observable phenotype
um00118	UDP glucose dehydrogenase	Deleted (Garcia-Pedrajas, unpublished)	1	Cell separation defects
um00205	Heat Shock Protein 12	Deleted (Garcia-Pedrajs, unpublished)	1	No observable phenotype
um00336	Putative protein	Not deleted	6	NA
um00455	Phosphate transporter	Deleted in one mating type (this study)	2	No observable phenotype
um00496	Cruciform DNA binding protein	Not deleted	1	NA
um01080	GABA amino transferase	Deleted (Stratton et al., 1996)	1	Decreased growth in media with alanine as sole nitrogen source
um01840	Proton nucleoside co-transporter	Deleted (this study)	4	No observable phenotype
um03522	Amino acid permease	Not deleted	1	NA
um04478	Myo-inositol transporter	Deleted in one mating type (this study)	1	No observable phenotype
um11400	Thiamine biosynthetic enzyme	Deleted (this study)	2	Thiamine auxotrophy
um11574	Carboxy muconolactone decarboxylase	Deleted (this study)	1	No observable phenotype

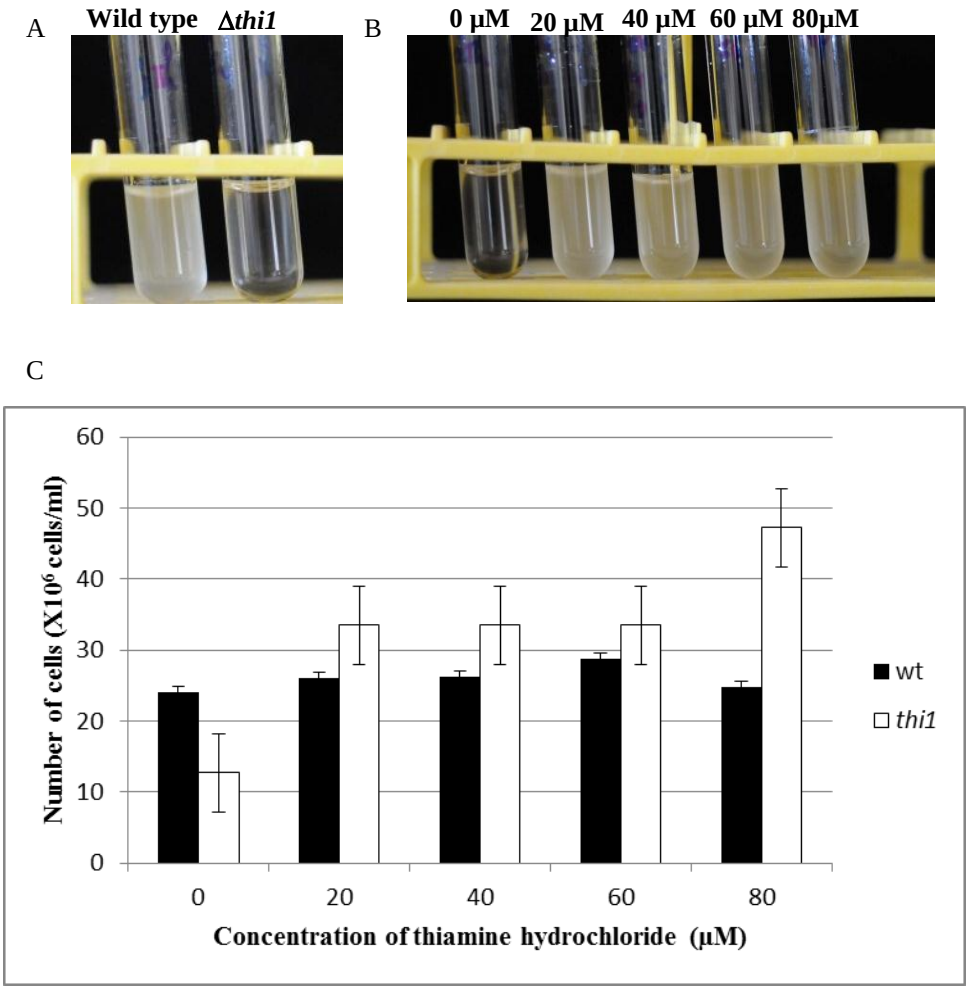

Figure 3.1. Thiamine auxotrophy in Δ*thi1* mutant and its remediation by addition of thiamine hydrochloride. (A) The growth of Δ*thi1* mutant in minimal media is reduced compared to wild type. (B) Thiamine auxotrophy is remedied by the addition of 20, 40, 60 and 80µM of thiamine hydrochloride. (C) Growth of wild type and *thi1* mutant in absence and presence of thiamine hydrochloride in the medium 24h after inoculation.

Table 3.2. Pathogenicity assay for um00027 deletion mutant (putative protein)

Crosses	Disease Index[1]		
	7d	10d	14d
Δum00027 X Δum00027	2.17[a]	2.71[a]	3.21[a]
Δum00027 X wt	2.20[a]	2.78[a]	3.12[a]
wt X Δum00027	2.27[a]	2.75[a]	3.07[a]
wt X wt	2.15[a]	2.65[a]	3.15[a]

[1]Disease index values are average of 60 plants in three replications

Table 3.3. Pathogenicity assay for um01840 (proton nucleoside co-transporter)

Crosses	Disease Index[1]		
	7d	10d	14d
Δum01840 X Δum01840	2.57[a]	3.05[a]	3.25[a]
Δum01840 X wt	2.43[a]	2.75[a]	3.07[a]
wt X Δum01840	2.57[a]	3.00[a]	3.62[a]
wt X wt	2.53[a]	3.12[a]	3.72[a]
um01840 SG200	1.15[a]	1.67[a]	1.47[a]
wt SG200	1.65[a]	1.78[a]	1.93[a]

[1]Disease index values are average of 60 plants in three replications

Table 3.4. Pathogenicity assay for um11400 (thiamine biosynthetic enzyme)

Crosses	Disease Index[1]		
	7d	10d	14d
Δum11400 X Δum11400	2.13[a]	2.65[a]	3.15[a]
Δum11400 X wt	2.30[a]	2.85[a]	3.27[a]
wt X Δum11400	2.60[a]	3.03[a]	3.68[a]
wt X wt	2.20[a]	2.60[a]	3.11[a]

[1]Disease index values are average of 60 plants in three replications

Table 3.5. Pathogenicity assay for um11574 (carboxy muconolactone decarboxylase)

Crosses	Disease Index[1]		
	7d	10d	14d
Δum11574 X Δum11574	1.88[a]	2.31[a]	2.83[a]
Δum11574 X wt	2.02[a]	2.55a	2.93[a]
wt X Δum11574	1.83[a]	2.45[a]	3.21[a]
wt X wt	2.02[a]	2.18[a]	2.91[a]

[1]Disease index values are average of 60 plants in three replications

CHAPTER 4

APSES DOMAIN GENE DELETION STRAIN Δ*UST1* OF *USTILAGO MAYDIS* SERVES AS A PARTIAL *IN VITRO* SURROGATE FOR *IN PLANTA* SPORULATION

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ABSTRACT

The basidiomycete fungus *Ustilago maydis* requires the presence of its maize host to produce spores and complete its life cycle. The study of sporulation and identification of the genes involved in this process is limited by this requirement. Deletion of the *ust1* gene, the ortholog of the *Aspergillus nidulans* transcription factor *stuA*, generates a mutant that produces spore-like structures in culture but is non-pathogenic. A previously identified presumptive transcription factor, *hgl1*, is required for the completion of *in planta* spore development at a late stage. In this study we compared the transcriptome profile of wild type *U. maydis in vitro* and *in planta* to that of these two sporulation mutants, Δ*ust1* (*in vitro*) and Δ*hgl1* (*in planta*) to identify the changes that occur during sporulation. The gene expression profile of Δ*ust1* grown *in vitro* includes a subset of differentially expressed genes with the strains that sporulate *in planta*. In particular, genes that are likely involved in pigment production in wild type (*in planta*) produced teliospores and the spore-like structures produced by the Δ*ust1* mutant are similarly expressed. Several genes, the protein products of which facilitate the biotrophic growth of the fungus, showed different expression patterns *in planta* compared to Δ*ust1 in vitro*. Several transcription factors that may be crucial for *in planta* growth and the development of teliopsores also were expressed differently in the *in planta* samples and the Δ*ust1* mutant. In comparing the gene expression patterns between Δ*hgl1* and wild type galls, we found that the two differ in expression of secreted proteins, cell wall components and pigmentation genes.

INTRODUCTION

The fungal pathogen *Ustilago maydis* is the causal agent of common smut of corn. The production of large quantities of dark diploid teliospores marks the culmination of pathogenic

development of *U. maydis*. These spores are produced in galls that can develop on all aerial parts of the host plant, maize. The fungus is biotrophic, requiring the host for the completion of its life cycle. In its haploid, saprobic stage, the fungus exists as budding yeast. Fusion of cells of compatible mating type generates a dikaryotic hypha which invades the host and ramifies inter- and intracellularly, eventually leading to the production of enlarged galls and ultimately teliospores. Teliospores are disseminated by wind and splash and serve as survival structures. Upon teliospore germination, meiosis occur leading to regeneration of the haploid yeast cells which are recombinant for parental traits.

The morphological transition from yeast to hypha in *U. maydis* is very crucial as it is coupled with a switch to the pathogenic stage in its life cycle. In a study to identify regulators of this morphological switch, the APSES domain transcription factor, Ust1, was analyzed [1]. APSES transcription factors in fungi like *Aspergillus nidulans* (StuA), *Candida albicans* (Efg1) and *Neurospora crassa* (Asm1) are known to be regulators of sexual and asexual sporulation and in the bud-hypha transition in dimorphic species [2,3,4,5]. The deletion of the *ust1* gene in *U. maydis* produced a haploid mutant that grows as filaments [1]. The *ust1* gene was also found necessary for mating and pathogenicity [1]. Interestingly, the mutant also produces dark round structures that resemble teliospores 48 h post inoculation in liquid culture. The number of these spore-like structures increased to a maximum at about 72 h of *in vitro* growth. The expression of a gene encoding the spore-specific protein, *ssp1*, which is highly expressed in teliospores, is de-repressed in the Δ*ust1* mutant suggesting an overlap of gene expression in teliospores and the Δ*ust1* spore-like structures. The phenotype of the Δ*ust1* mutant suggests that *ust1* is a repressor of genes involved in sporulation and its function may be to prevent premature sporulation during saprobic growth. Identification of the sporulation genes repressed by *ust1* should provide a better

idea of the developmental processes leading to teliosporogenesis and thereby may provide new targets for control of this fungus.

Transcriptional profiling is a powerful technique to identify genes involved in specific developmental processes by comparing changes in gene expression patterns across multiple developmental stages. In *U. maydis* mutants of genes representative of various developmental processes leading to sporulation can be useful to identify genes involved in sporulation. To assess the value of the Δ*ust1* mutant as a surrogate for *in planta* teliosporogenesis and to identify genes uniquely required for *in planta* teliosporogenesis, we employed microarray analysis to compare the gene expression pattern of the Δ*ust1* mutant at the stage when it produces spore-like structures to the expression pattern of the wild type dikaryon during normal *in planta* teliospore formation. We also included the Δ*hgl1* mutant that produces un-pigmented immature teliospores *in planta* as a developmental check. Hgl1 is a protein required for teliospore formation. However its exact function is not known, although it has been suggested to be a transcription factor [6].

To answer the question of whether the Δ*ust1* mutant can be used as a surrogate system in the study of *U. maydis* sporulation, we compared the gene expression patterns of the *in vitro* grown Δ*ust1* mutant to wild type sporulating tumors *in planta.* To identify genes uniquely required for *in planta* teliosporogenesis but not required for 'sporulation' of Δ*ust1*, we analyzed the genes that show similar expression patterns in wild type and Δ*hgl1* galls but differed in the Δ*ust1* mutant. We also compared the gene expression patterns between fully developed plant infections yielding mature teliospores produced by the wild type *U. maydis* dikaryon and the immature spores produced in galls generated by the Δ*hgl1* dikaryon to identify genes expressed during the later stages of teliosporogenesis. We show that gene expression patterns are suggestive of

similarities in the processes leading to 'sporulation' in Δ*ust1* and teliospore development, especially in the pigmentation of spores. Several genes that facilitate biotrophic growth of *U. maydis* showed similar trends in gene expression pattern in the *in planta* strains. These could be potential targets for further study of biotrophic growth and sporulation of *U. maydis*. We also show that the major difference in gene expression between Δ*hgl1* and wild type galls lies in pigmentation genes, secreted proteins and genes encoding enzymes involved in the production of fungal cell wall components.

RESULTS AND DISCUSSION

Microarray analysis

To assess the suitability of using Δ*ust1,* as a surrogate for sporulation studies in *U. maydis*, we performed transcriptome analysis comparing the gene expression of 48 h *in vitro* cultures of strain 1/2 (also known as 521, the sequenced strain), a wild type *a1b1* haploid, and strain 14/25, the Δ*ust1* strain derivative of strain 1/2. The time point 48 h was selected for the collection of the cultures as it coincided with the production of spore-like structures in the Δ*ust1* mutant. Additionally, we compared *in planta* gene expression in the sporulating tumors produced by wild type and Δ*hgl1* dikaryons at 14 days post inoculation (dpi). 6332 out of the total 6866 genes in the genome showed 2-fold or greater expression variation in at least one experimental condition compared to the wild type grown *in vitro*. Of these, 5685 genes were up-regulated (82.7%) and 647 (9.4%) genes were down-regulated ≥2-fold (Fig. 4.1. A and B). At the higher level of differential expression of ≥4-fold, 4480 genes (65.2%) were up-regulated and 158 genes (2.3%) were down-regulated (Fig. 4.2. A and B). At a cut off of ≥8-fold change, the total number of genes up-regulated was narrowed to 2498 (36.3%), with 81 genes (1.1%) down-regulated (Fig.

4.3. A and B). The *in planta* samples from wild type and Δ*hgl1* share the most genes with similar trends in gene expression as compared to wild type in culture. qPCR analysis of select genes confirmed their microarray expression values (Fig. S 4.1.).

Analysis of genes with common expression patterns in the Δ*ust1* mutant and in the *in planta* grown strains

To assess the similarity in the production of spore-like structures by Δ*ust1* to sporulation *in planta*, we identified the genes that showed a common pattern of expression in the Δ*ust1* mutant, wild type galls and Δ*hgl1* galls. Among the 5685 genes that were up-regulated ≥2-fold, 599 were similarly expressed in all three genotypes. Of the 647 genes that were ≥2-fold down-regulated, 48 genes were commonly down-regulated in all three genotypes. These genes that showed similar expression patterns were classified into functional categories using the MIPS FunCat database (Fig. 4.4) (http://mips.helmholtz-muenchen.de/genre/proj/ustilago/Search/index.html). Most of the genes in both the up- and down-regulated categories were those required for metabolism. In the up-regulated category, 301 out of 599 genes were related to various metabolic pathways. The FunCat database assigns 2686 genes in the *U. maydis* genome to metabolic functions. Therefore, the 301 up-regulated genes in this category represent 11.2% of the metabolic genes in the genome. This suggests that all three genotypes were metabolically active at the time their cells were harvested, unlike mature teliospores, which are known to be lacking in enzymes for certain pathways like the TCA cycle for glucose catabolism [7]. The induction of these metabolic genes may have been due to the shared presence of filamentous cells that were forming teliospores or spore-like structures in all 3 experimental conditions.

The similarity in the expression patterns of the above mentioned 301 metabolic genes also indicates similarity in some metabolic processes between the three experimental strains. The general trend of down-regulation of most of the genes required for iron uptake in the three experimental conditions compared to the wild type haploid is one such aspect. Iron uptake in *U. maydis* involves two systems, a permease-based system that requires the activity of *fer1* and *fer2* and a siderophore-based system [8,9]. The expression of *fer1* and *fer2* was down-regulated over 6-fold *in planta*. The biosynthesis of the siderophores ferrichrome and ferrichromeA, which sequester iron upon their secretion, requires the expression of enzymes Sid1, Sid2 and Fer3-5 [8,10,11]. The expression of genes involved in the biosynthesis of ferrichrome (ornithineN5 oxygenase-*sid1*) and ferrichromeA (*fer3*-5) was down-regulated in all three experimental conditions, while that of *sid2* was down-regulated 8-fold in Δ*ust1* alone. Iron uptake by the permease system is essential for virulence and biotrophic growth in *U. maydis*. The siderophore system is also said to be required for storage of iron in spores and, consistent with this, the expression of this pathway is up-regulated during spore formation [12]. In our study however, we found that the central genes in this pathway were down-regulated over 4-fold in all three experimental conditions.

The expression of versicolorin b synthase (um11112), another gene involved in metabolism was 8.9-fold and 6.2-fold lower in wild type galls and Δ*ust1*, but only 2.5-fold down-regulated in Δ*hgl1*. In other fungi, versicolorin b synthase is an enzyme in the biosynthetic pathway leading to the production of aflatoxin B1. Aflatoxins are highly poisonous, secondary metabolites produced by some filamentous fungi, including several species of *Aspergillus*. *U. maydis* is not known to produce any mycotoxins and consumption of corn smut galls is a customary delicacy in Latin America. In *U. maydis*, versicolorin is believed to function as a glucose oxidase, and is

postulated to help modify host metabolism to make fungal hyphae a nutritional sink tissue [13]. If so, the down-regulation of this gene in both types of *in planta* samples, which were dependent on the host tissue for nutrition, is surprising.

Two other functional groups of particular interest during sporulation were cell fate and cell cycle/DNA processing. The cell fate category includes genes that are involved in morphogenesis/directional cell growth and cell death. Ninety five genes in this category showed common patterns of expression in the three experimental samples. Of these, 92 were up-regulated and 3 down-regulated. Among the up-regulated genes that were involved in directional growth, were the members of the pheromone responsive MAPK pathway *ubc3/kpp2* (um03305) and *fuz7/ubc5* (um01514), a GTP binding protein related to *ras* (um05654) and several other kinases (um01180, um03446, um10496). Genes encoding G proteins Gpa2, 3 and 4 and two genes encoding the motor protein kinesin (um00896 and um04727) were also up-regulated. Since all three experimental conditions used in the microarray produced filamentous cells under the conditions tested as opposed to the yeast form of the wild type haploid grown *in vitro*, up-regulation of genes for directional growth was not unexpected.

Cell death by autophagy and apoptosis is required for normal development and reproduction in fungi [14]. Thirteen of 28 genes annotated as being involved in authophagy (http://pedant.gsf.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t23631Ust_maydi) were up-regulated ≥2-fold in all three experimental samples. Among them, autophagy genes *atg1* (um06363) and *atg8* (um05567) are required for survival, as well as gall and teliospore formation in *U. maydis*. The deletion of *atg8* had a greater effect than deletion of *atg1*, with an additive effect in the double mutant [15]. The expression of *atg8* was up-regulated over 10-fold in wild type galls and Δ*hgl1* galls, and over 2 fold in Δ*ust1*. While the expression of *atg1* was up-

regulated in wild type (3.5-fold) and Δ*hgl1* (2.9-fold) gall tissue, it was down-regulated (2.4-fold) in Δ*ust1 in vitro.*

Morphogenesis and virulence in *U. maydis* are closely associated with the regulation of the cell cycle [16]. The main components of cell cycle regulation in *U. maydis* are the cyclins *clb1* and *clb2, cdk1* and *wee1*. Variation in levels of expression of *clb1* and *clb2* and consequently the induction and release of cell cycle arrest are required during various developmental processes like mating, filamentation, plant penetration and pathogenesis. The expression of *clb1* was up-regulated 9.1-fold and 7.4-fold in wild type galls and Δ*hgl1* galls, respectively, and 2.3-fold in Δ*ust1*. Cyclin *clb2* was up-regulated 21.7 and 14.3-fold in wild type and Δ*hgl1* galls, respectively, and 3.2-fold in Δ*ust1*. The expression of the *biz1* protein that suppresses expression of *clb1* to induce G2 arrest during plant infection was up-regulated 9.6 and 20-fold in wild type and Δ*hgl1* galls, respectively, and unchanged in Δ*ust1*. The cell cycle arrest induced by action of Biz1 *in planta* is relieved by another protein, Clp1 [17]. Expression of the *clp1* gene in wild type galls and Δ*hgl1* galls was up-regulated 102.3-fold and 153-fold, respectively, and was unchanged in Δ*ust1*. The expression of *clp1* is dependent on the *b* mating type locus. It is required for the formation of clamp connections and thereby, for nuclear distribution and proliferation of the dikaryotic hypha in the plant [18]. The up-regulation of this gene *in planta* and its unchanged expression in Δ*ust1* is another gene expression difference in the filaments formed by the dikaryotic strains *in planta* and the haploid Δ*ust1*mutant *in vitro*.

Among the unclassified genes, *mig2-6* (um06126; a previously identified maize-induced gene) was up-regulated in Δ*ust1,* and in wild type galls and Δ*hgl1* galls. Maize-induced genes are not expressed in wild type haploid cells *in vitro* but are highly induced during plant infection

[19]. The other *mig* proteins also were highly up-regulated *in planta*, but unchanged in Δ*ust1*. Mig2-6 is a secreted protein with unknown function [20]. The expression of *mig2-6* was very high (291-fold induction) in the Δ*hgl1* galls and in the wild type galls (234-fold induction) suggesting that its expression may be enhanced by the presence of the host. It also was induced in Δ*ust1*, but not to the same extent (20-fold) as *in planta*. The up-regulation of *mig2-6* in all 3 experimental samples suggests that it may be a useful indicator of teliospore development.

In summary, a comparison of the gene expression patterns in Δ*ust1*, wild type galls and Δ*hgl1* galls revealed some similarities in important processes like basic metabolism, cell cycle regulation and autophagy. Even though the expression values for specific genes differed among the genotypes, similar trends were observed in all three cases for genes involved in iron uptake and for particular secreted proteins, like *mig2-6*.

Expression patterns of genes previously shown to be required for or expressed during normal *in planta* gall production by *U. maydis*

Several *U. maydis* signal transduction pathways are necessary for infection and teliospore formation. The deletion of various signal transduction components (Table 4.1) blocks the sporulation pathway at different stages, and thus their gene expression patterns were of interest. The expression of *ubc1*, the regulatory subunit of the cAMP dependent protein kinase, was not significantly altered in the wild type and Δ*hgl1* galls but was down-regulated over 2-fold in Δ*ust1*. Another gene connected to the cAMP pathway, *ukb1*, was up-regulated in the *in planta* strains but did not change significantly in expression in Δ*ust1*. Both genes are needed for gall induction and aggregation of hyphae in galls but are not essential for hyphal proliferation [21,22]. Among the G proteins, Gpa3 was previously shown to be the only gene to generate an observable mutant phenotype and was necessary for mating and pathogenic development [23].

Its role, along with that of other components of the cAMP pathway, is in part, believed to be repression of teliospore production in culture [24]. The expression of *gpa3* was up-regulated >2-fold in all three experimental strains contrary to a function in repression of sporulation. Hda1 and Rum1 proteins are needed for rounding up after formation of the mucilaginous matrix in the plant. The expression of *rum1* and *hda1* was not significantly altered in Δ*ust1* but was up-regulated over two fold in wild type and Δ*hgl1 in planta*.

The most striking expression pattern was noted for the gene *ssp1*. *ssp1* is expressed at a low level in the haploid sporidial stage but was highly induced in mature teliospores [25]. The expression of *ssp1*was up-regulated in *in planta* wild type galls (20.6-fold) and Δ*ust1* (14.3-fold) but was down-regulated (2.3-fold) in Δ*hgl1* galls. This result is consistent with the fact that teliospores produced by Δ*hgl1* do not reach maturity.

PKA and MAPK activity during *in vitro* and *in planta* growth has been determined to be different involving different components. The presence of an active bE/bW heterodimer in the *in planta* strains contributes to these differences [12]. This could explain the differences in the expression levels of genes belonging to these pathways like *ubc1*, *adr1* and *gpa3* in the *in planta* strains and in Δ*ust1* (Table 4.1). The function of the protein products of *hda1* and *rum1* are to suppress transcription of certain genes during haploid growth that are targets of the bE/bW heterodimer [26]. The lack of significant difference in expression of these genes between the *in vitro* haploid strains Δ*ust1* and wild type budding cells (control) could be due to the lack of the active *b* heterodimer. In general the activities of genes earlier found to be required for teliospore development seem to be contingent on the presence of an active *b* heterodimer and/or the presence of the host. This makes the process of formation of spore-like structures in Δ*ust1*

mutant different. However, the strong up-regulation of expression of *ssp1*, the gene highly expressed in mature teliospores but expressed at a very low level in haploid sporidia, in Δ*ust1* indicates some similarity of the spore-like structures to teliospores. The exact function of *ssp1* is unknown but it co-localizes with lipid bodies and therefore is thought to be involved either in the mobilization of storage lipids during germination of teliospores or in lipid metabolism [25]. The expression of *ssp1* is de-repressed in Δ*ust1* only at the time of formation of the spore-like structures and not during its early filamentous stage [1]. The lower expression of *ssp1* in the Δ*hgl1* mutant, which does not produce mature teliospores, compared to wild type (galls) and Δ*ust1* also points towards a possibility of some physiological similarity between mature teliospores and the spore-like structures produced by Δ*ust1*.

Expression patterns of *U. maydis* orthologs of fungal sporulation genes

Since the number of identified sporulation markers in *U. maydis* is limited, we compared the expression pattern of orthologs of known sporulation genes from other fungi, in the three experimental conditions. In Table 4.2, we list the expression of 10 *U. maydis* genes that were similar to sporulation genes from other fungi. The expression of all 10 genes were up-regulated in the *in planta* produced wild type and Δ*hgl1* galls to varying degrees. However, the expression patterns in Δ*ust1* showed varying trends. Among the *A. nidulans* sporulation genes, the three genes of interest that have orthologs in *U. maydis* were those required for asexual reproduction or conidiation. The expression of *abaA*, a component of the central regulatory network of conidiation in *A. nidulans*, was up-regulated in *in planta* conditions but did not significantly change in Δ*ust1*. In *A. nidulans*, the expression of *abaA* is dependent on the expression of another component of the conidiation pathway, *brlA* which in turn is modulated by *stuA*, the

ortholog of *ust1*. The ortholog of *brlA* was not found in *U. maydis*, indicating the sporulation pathways in these divergent fungi are different.

Two other genes needed for conidiation in *A. nidulans* are *flbA* and *fadA*. FlbA is a RGS protein the direct target of which is the GTP-Gα subunit FadA. Its activation causes increase in GTPase activity leading to the inactivation of the PKA pathway which is needed for maintaining vegetative growth and thereby promotes sporulation [27]. The *flbA* and *fadA* orthologs were up-regulated in *in planta* conditions but their expression was unchanged in Δ*ust1*.

In the *U. maydis* genome, four genes with similarity to the *veA* gene of *A. nidulans* exist. *veA* positively regulates sexual sporulation in *A. nidulans* [28]. Two of the genes identified in *U. maydis* (um01146 and um04203) were up-regulated in all three experimental conditions (Table 4.2). The expression of um00893 and um10556 was up-regulated in wild type and Δ*hgl1* galls but was unchanged in Δ*ust1*.

Many of the important sporulation markers from other fungi did not have any close homologs in *U. maydis* indicating the uniqueness of its sporulation pathway. The expression levels of most of the markers identified in *U. maydis*, were up-regulated to varying degrees in the *in planta* conditions. In Δ*ust1* the expression levels of most of these genes were unchanged. Teliospore development is a complex process occurring within the host and the extent of the involvement of the host in this process is not fully understood. A large number of host and pathogen genes are differentially expressed in an organ specific manner during pathogenesis and sporulation of *U. maydis* [29]. The sporulation markers from other fungi therefore may be good models for teliospore development in a more natural setting such as in the plant.

Expression patterns of pigmentation genes during spore production

Development of pigmentation in the spore-like structures produced by Δ*ust1* is a major characteristic suggesting its ontological similarity to teliospores. In the host, the immature spores, produced by the rounding off of hyphae, develop pigmentation gradually as the teliospores mature [30]. In Δ*ust1*, the hyphae are initially un-pigmented and develop spore-like structures that become pigmented only after growth in culture for over ≥48 h [1]. The pigment produced by *U. maydis* spores is melanin [31], however, the biosynthetic pathway of melanin in *U. maydis* has not been studied in detail. The difference in pigmentation of spores produced by Δ*hgl1* from those produced by wild type and Δ*ust1* prompted us to look at the expression patterns of pigmentation related genes among these conditions. We found five genes that appeared to be orthologs of genes in melanin and other pigment synthesis pathways in fungi. Laccase I precursor (um05361) and FET5 multicopy oxidase (um05861) share high similarity with melanin synthesis genes laccase1 (CNLAC1) and laccase2 (CNLAC2) of *Cryptococcus neoformans,* respectively. The transcript of um05361 (*lac1*) shows an over 20-fold increase in the wild type galls and in Δ*ust1* but was reduced by 2-fold in Δ*hgl1* (Table 4.3). The expression of um05861 (*lac2*) was up-regulated in wild type galls and Δ*ust1* to a greater extent than in Δ*hgl1* galls. Laccases are required for melanin biosynthesis and virulence in *C. neoformans* [32]. In *C. neoformans,* melanin is synthesized from DOPA that it obtains from an external source like the human host [33]. The DOPA melanin pathway is the most common melanin synthesis pathway in the Basidiomycota. The up-regulation of the *lac1* gene in the pigmented wild type galls and Δ*ust1* and down-regulation in the developmentally restricted Δ*hgl1* strain may indicate a role of *lac1* in melanin synthesis.

Another melanin synthesis pathway predominantly found in ascomycetous fungi is the DHN pathway [34,35]. In this pathway, a polyketide synthase enzyme converts acetate molecules to 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) [36]. Two polyketide synthase genes of *U. maydis* (um06414 and um06418) were highly up-regulated in wild type and Δ*ust1* strains but also to a lesser extent in Δ*hgl1*

In *Aspergillus fumigatus* the *ayg1* gene product converts the heptaketide naphtopyrone to 1,3,6,8-THN in the second step of melanin biosynthesis from acetyl-CoA and Malonyl-CoA [37]. The *ayg1* gene is part of a cluster of 6 genes any of which if disrupted, results in the alteration of *A. nidulans* conidial coloration. The ortholog of *ayg1* was up-regulated in all three experimental conditions with the wild type showing over 100-fold increased expression. In the Δ*hgl1* strain, which produces hyaline spores, the up-regulation of this gene was lowest (Table 4.3). The up-regulation of two polyketide synthase genes and *ayg1* in wild type galls and Δ*ust1* to a greater level than Δ*hgl1*, begs the question as to whether the DHN pathway is present in *U. maydis*.

Comparison of gene expression pattern of *in planta* strains with Δ*ust1*

The genes that show a similar pattern of expression in the *in planta* strains but differ from the *in vitro* sporulating Δ*ust1* mutant may be important for sporulation *in planta*. To identify these potential sporulation genes, we looked for genes that were up- or down-regulated in the *in planta* strains but followed the opposite trend in Δ*ust1* when compared to wild type haploid.

The expression of 253 genes was up-regulated in the *in planta* Δ*hgl1* and wild type tumors and down-regulated ≥2-fold in the Δ*ust1* mutant when compared to the wild type haploid. These

genes when classified into FUNCAT categories, the greatest number of genes was in the category proteins with binding function or cofactor requirements (Fig. 4.5). The genes falling in this category included those encoding transcription factors, enzymes and proteins that bind specific ligands. Other highly represented FUNCAT categories among the genes up-regulated in the *in planta* strains alone included metabolism, cellular transport, biogenesis of cellular components, protein fate and cell cycle and DNA processing.

Further analysis of 54 genes that showed high expression (≥8-fold) in at least one *in planta* strain shows that a large number of these gene products are involved in the facilitation of biotrophic growth of *U. maydis*. Among the 54 genes highly up-regulated in the *in planta* strains are transporters and permeases that encode proteins with potential roles in the transport of water and nutrients. In biotrophic pathogens like *U. maydis* the unidirectional transfer of nutrients and water from the host is expected [38]. The gene ontology classification of all genes in the *U. maydis* genome assigns 563 genes the function of transport. The expression of 369 genes with transport function were up-regulated ≥2-fold in the *in planta* strains and down-regulated or not significantly altered in the *ust1* mutant compared to the wild type haploid strain *in vitro*. A few selected genes that encode transporters and permeases that facilitate the transportation of sugars, amino acids and water that were up-regulated in the *in planta* strains are listed in Table 4.4. Among the transporters, *srt1,* a sucrose specific transporter was considerably up-regulated in the *in planta* strains. This transporter is known to be essential for full virulence of the fungus and is expressed only during infection [39]. The possibility of other fungal transport proteins functioning as virulence factors needs to be investigated further.

The expression of an invertase *suc2* was up-regulated in the *in planta* strains compared to Δ*ust1* mutant (Table 4). The expression of this gene was earlier shown to be high 6dpi in leaf

galls of maize plants [40]. The high fructose and glucose content of infected maize tissue is attributed to the activity of invertase *suc2* [40]. Our expression data indicates that the fungus maintains its dependence on the host for nutrients through activity of this invertase 14 dpi.

Another gene of interest that was up-regulated in wild type (12.03-fold) and Δ*hgl1* galls (16.04-fold) and down-regulated in the Δ*ust1* (9.85-fold) mutant was um05731. This gene encodes a secreted protein that shows high similarity to chorismate mutase. Chorismate mutase is an enzyme in the shikimate pathway for synthesis of the aromatic amino acids phenylalanine and tyrosine. Chorismate derived secondary compounds in plants include indole acetic acid, salicylic acid and phytoalexins. Chorismate mutase produced by the nematodes *Meloidogyne javanica* and *Heterodera glycines* are said to alter the host tissue on colonization [41,42]. Similarly the secreted chorismate mutase enzyme produced by the human pathogenic bacteria *Mycobacterium tuberculosis* is involved in host-pathogen interactions [43]. In addition, to chorismate mutase several genes in the shikimate/quinate pathway and transporters of their protein product (quinate permeases) were up-regulated in the *in planta* strains without significant change in expression in the Δ*ust1* mutant. There may be a potential role for these fungal genes in the IAA and auxin synthesis contributing to the formation of host galls.

Another set of genes of interest that were up-regulated in wild type and Δ*hgl1* galls and down-regulated ≥2-fold in Δ*ust1* were transcription factors. Fifty genes with FUNCAT assigned function of transcriptional control belonged to this set. The gene encoding a zinc finger protein related to transcription factor AreA of *A. nidulans* was up-regulated in the *in planta* strains (9.88-fold and 6.66-fold in wild type and Δ*hgl1*) and down-regulated in the Δ*ust1* mutant (2.12-fold). AreA is responsible for glutamine mediated nitrogen repression in *A. nidulans* [44]. The ortholog

of transcription factor SamB of *A. nidulans* um02587 which codes for the Fuz1 protein was up-regulated in the *in planta* strains (5.41 and 4.54-fold in wild type and Δ*hgl1*) and down-regulated (2.89-fold) in Δ*ust1*. This transcription factor is required for polarized cell growth and other developmental processes during conidiation of *A. nidulans* [45]. In *U. maydis* Fuz1 is required for polarized growth of the dikaryon and the accumulation of the mucilaginous matrix prior to hyphal fragmentation during telioporogenesis [30].

Another gene um11810, the ortholog of the bZIP transcription factor AtfA of *A. nidulans* and Atf1 of *Schizosaccharomyces pombe* was up-regulated 6.62 and 6.54-fold in wild type and Δ*hgl1* galls respectively and down-regulated 2.28-fold in Δ*ust1* mutant. The AtfA and Atf1 proteins regulate genes that are part of oxidative and osmotic stress defense system including catalase and glycerol-3-phosphate dehydrogenase [46]. The resistance response of host plant in response to pathogens is often accompanied by the release of reactive oxygen species. The up-regulation of ortholog of *atfA* in the *in planta* strains may be to counter this response of the host.

The entire set of the 50 transcription factors up-regulated in the *in planta* strains and down-regulated in the Δ*ust1* mutant are listed in the supplementary material. These genes may be good potential targets for study of pathogenesis and *in planta* sporulation of *U. maydis*.

Genes differentially expressed in pair-wise comparison of wild type galls and Δ*hgl1* galls

To better understand the spore development checkpoint encountered in the Δ*hgl1* mutant we made a pairwise comparison of its *in planta* gene expression pattern to gene expression in wild type *in planta*. Employing the Student's t-test with a stringency of 95% confidence, we found the expression of 166 genes down-regulated and 154 genes up-regulated in galls produced by Δ*hgl1* mutant compared to those produced by the wild type at a cut off of ≥2-fold. FUNCAT

classification of genes differentially regulated in the Δ*hgl1* mutant is shown in Fig. 6. One category that was represented in both up and down-regulated genes was cell rescue, defense and virulence. Forty-nine genes in this category were down-regulated and 24 up-regulated in the Δ*hgl1* mutant compared to wild type galls. The down-regulated genes include several transporters, oxidoreductases, laccase and the multicopy oxidase related to FET5.

Since the spores produced by the Δ*hgl1* mutant do not fully mature, we looked in detail at the FUNCAT category, biogenesis of cellular components. Thirty-four genes in this category were down-regulated and 18 were up-regulated in the Δ*hgl1* mutant. Among the down-regulated genes in this category, 17 were those required for the synthesis of cell wall components including several glucanases, chitinases and three genes encoding cytochrome P450, spore specific protein *ssp1*, laccase and pigmentation genes. The expression levels of two genes, *lac1* (um05361) and *ssp1* (um12271) are indicative of teliospore maturation in *U. maydis*. Laccase is known to be a part of melanin synthesis pathway in Basidiomycota such as *Cryptococcus*. The expression of *ssp1*, a lipid dioxygenase is highly up-regulated in mature teliospores and it is thought to be required for the mobilization of storage lipids [25]. The expression of laccase is down-regulated 171.8-fold and *ssp1* is down-regulated 8.7-fold in Δ*hgl1* galls compared to wild type galls. Differential expression of these genes in mature galls and teliospores produced by wild type as compared to galls containing immature telisospores produced by Δ*hgl1* suggests that laccase and *ssp1* are useful indicators of teliospore maturity.

Eight genes required for cell wall biogenesis were up-regulated in Δ*hgl1* compared to the wild type strain *in planta*. These include two glucan synthases (um00857 and um10364), the orthologs of which are known to be required for cell wall synthesis in ascomycetes [47,48].

Together this expression pattern of cell wall synthesis genes suggests that Δ*hgl1* teliospores are in their early stage of spore wall synthesis.

Analysis of the *U. maydis* genome revealed the presence of a large number of genes that encode secreted proteins, many specific to this fungus [49]. TargetP analysis of all proteins in the genome identifies 754 genes encoding proteins with signal peptide sequences indicating likely secretion (http://mips.helmholtzmuenchen.de/genre/proj/ustilago/Search/listTargetP.html?target=Secretory%20pathway). Among the 154 genes up-regulated in Δ*hgl1* compared to wild type plant galls, 56 encoded secreted proteins. Most of the up-regulated proteins were unique to *U. maydis* and designated hypothetical proteins. Some of the genes encoding secreted proteins up-regulated in Δ*hgl1* (um03615, um03744, um03745, um03746, um05299, um05311 and um05314) are putative effectors that were earlier found to be expressed differentially *in planta* [29]. This differential expression of effectors may mean that in addition to organ specificity, developmental stage specificity is also a regulator of fungal effector secretion. Other genes encoding secreted proteins up-regulated in Δ*hgl1* included maize induced genes *mig1*, *mig2-2, 2*-3 and *2-4*.

Thirty-two genes, the expression of which was down-regulated in Δ*hgl1* as compared to the wild type galls, were classified by TargetP as secreted proteins. Ten of the down-regulated genes (Table 4.5) code for enzymes that are believed to modify the fungal cell wall during infection in order to adapt to host conditions [20]. Four other genes (um00466, um03138, um11403 and um11839) that encode secreted proteins with sequence repeats were also down-regulated in Δ*hgl1*. No enzymatic function is attributed to these proteins [20]. Another highly down-regulated gene, um01788 (87.7-fold), was not similar to genes from other fungi (Table 4). Four genes that were up-regulated in the Δ*hgl1* strain, um01788 (4.14-fold), um03416 (5.23-fold), um06075

(18.78 fold) and um11111 (2.35-fold) are also potentially involved in modification of the fungal cell wall during infection.

CONCLUSIONS

In conclusion, our results show that the spore-like structures produced by Δ*ust1* in a number of cases show transcriptional similarities with galls containing *in planta* produced teliospores. Prime examples are the genes encoding pigmentation related enzymes. As previously observed the high expression level of *ssp1* indicates that spore-like structures in Δ*ust1* share at least some physiological similarities with mature teliospores [1]. Therefore this mutant and the spore-like structures it produces can be useful models for the study of pigmentation in *U. maydis*. In comparison with wild type cells in culture, wild type galls and Δ*hgl1* galls share more similarity in gene expression with each other than with Δ*ust1*. This similarity can be ascribed to biotrophic growth of these strains in the host plant. The spore-like structures produced by Δ*ust1* share some developmental similarities with teliospores but in the presence of the host, the fungus has to behave differently. Being a biotroph the fungus has to avoid detection by the host and use the host resources for its growth. The broad scale difference in expression pattern of *in planta* grown strains from Δ*ust1* may, to a large part, be due to niche exploitation. More detailed *in planta* studies are thus essential to generate a complete picture of the sporulation process. The characterization of the *in planta* up-regulated genes identified in this study may give more insight into the pathogenesis and sporulation processes. In comparison between galls of Δ*hgl1* and wild type, the main differences in gene expression involved genes encoding components of cell wall biogenesis, pigmentation and several putative secreted effectors.

MATERIALS AND METHODS

Strains and growth conditions

U. maydis haploid strains including wild type 1/2 (*a1b1*) and 2/9 (*a2b2*) and Δ*ust1* strain 14/25, a derivative of strain 1/2 [1], and Δ*hgl1* strains 6/19 and 6/20 in -*a1b1* and *a2b2* backgrounds, respectively [6] were grown and maintained on Potato Dextrose Agar (PDA) medium (Sigma-Aldrich, St. Louis, MO, USA). For experiments using samples grown *in vitro*, wild type strain 1/2 and Δ*ust1* mutant strain 14/25 were grown in Potato Dextrose Broth (PDB) (Sigma-Aldrich) for 48 h at 30°C. The cells were then transferred to liquid array medium (6.25% Holliday salt solution, 30mM L-glutamine, 1% glucose, pH adjusted to 7.0 and filter sterilized) [50] and grown at 30° C for 24 and 48 h with constant shaking at 200 rpm. For *in planta* experiments, compatible mating strains in wild type and Δ*hgl1* backgrounds were grown in PDB overnight and 10^6 cells per ml of each strain were mixed and injected into ears of the maize dwarf variety 'Tom Thumb' (Seed Savers Exchange, Decorah, IA, USA). Galls were collected 14 dpi. Samples were frozen in liquid nitrogen and stored at -80°C until RNA extraction. Two independent biological replicates were analyzed for each sample.

RNA extraction and microarray analysis

RNA was extracted and purified from galls and in culture samples using the Sigma Spectrum Plant Total RNA kit (Cat. No. STRN50). The RNA was treated with DNaseI (New England Biolabs), precipitated, quality assessed on a bioanalyzer (Agilent Technologies, CA, USA) and sent to NimbleGen (Madison, WI, USA). At NimbleGen, the RNA was reverse transcribed to cDNA and Cy3 labeled. cDNA samples were hybridized on chips with multiple

perfect match probes for 6866 gene models of *U. maydis*. The raw array scan data was normalized with appropriate controls.

The normalized data returned by NimbleGen was processed further on ArrayStar v4.0 software (DNASTAR, Madison, WI, USA). The expression values from the two biological replicates of each strain were averaged and compared between the various genotypes. Expression results for genes with less than 95% confidence interval were excluded from further study. Pairwise expression comparisons between Δ*hgl1* and wild type galls was done using Student's t test with a cut off of 95% significance. The Benjamini Hochberg post hoc correction was applied to reduce false discovery rate [51]. The expression values of culture grown Δ*ust1*, Δ*hgl1* galls and wild type galls were compared to the wild type strain in culture using F-test (ANOVA).

qPCR confirmation of microarray detected differential gene expression

cDNA was synthesized from total RNA, using the SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) using the oligo-dT primer following the manufacturer's protocol. Message abundance was measured by quantitative real time PCR using SYBR-GREEN methodology (BioRad, Hercules, CA) with gene-specific primers, on a Cepheid SmartCycler (Cepheid, Sunnyvale, CA, USA). The relative expression level for each gene was calculated using the $2^{-\Delta\Delta CT}$ method [52] after normalization with the reference gene, *cpr1* (um03726).

Acknowledgments

We thank Dr. James Kronstad for providing strains of Δ*hgl1* mutant used in this study.

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Figures and Tables

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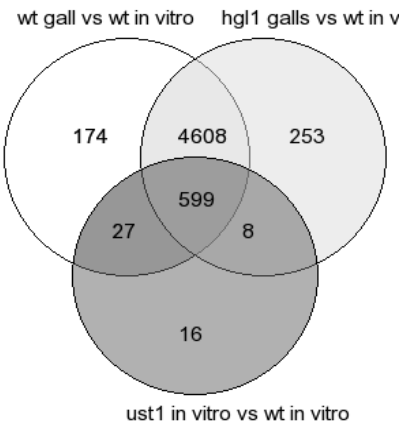

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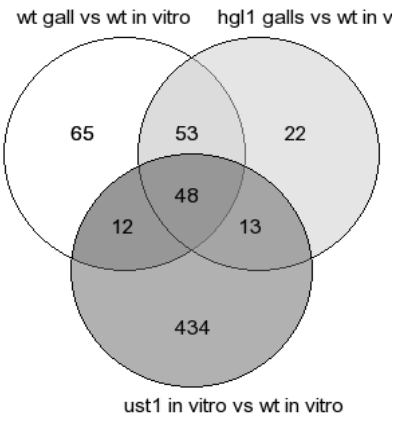


Figure 4.1. The comparison of gene expresssion pattern of *in planta* strains wild type galls and Δ*hgl1* galls, *in vitro* sporulating strain Δ*ust1* compared to *in vitro* haploid wild type.
A. Genes up-regulated ≥2 fold in wild type galls, Δ*hgl1* galls and Δ*ust1* mutant compared to wild type *in vitro.* B. Genes down-regulated ≥2 fold in wild type galls, Δ*hgl1* galls and Δ*ust1* mutant compared to wild type *in vitro.*

A B

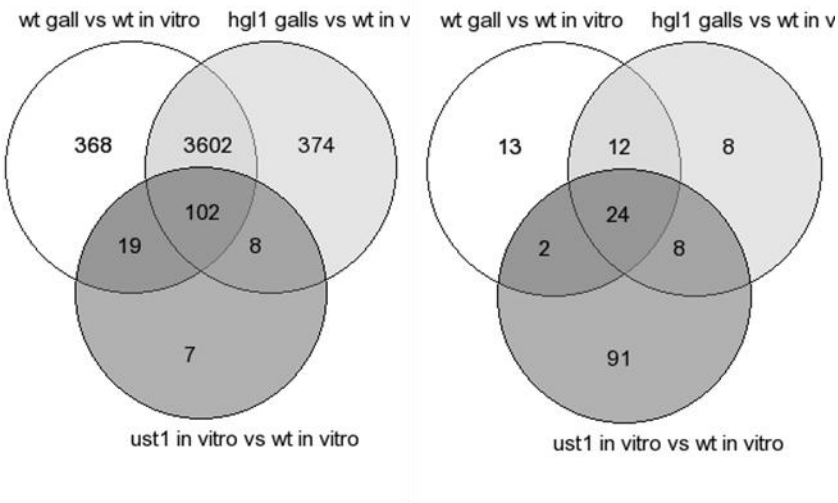


Figure 4.2. The comparison of gene expresssion pattern of *in planta* strains wild type galls and Δ*hgl1* galls, *in vitro* sporulating strain Δ*ust1* compared to *in vitro* haploid wild type.
A. Genes up-regulated ≥4 fold in wild type galls, Δ*hgl1* galls and Δ*ust1* mutant compared to wild type *in vitro.* B. Genes down-regulated ≥4 fold in wild type galls, Δ*hgl1* galls and Δ*ust1* mutant compared to wild type *in vitro*

A B

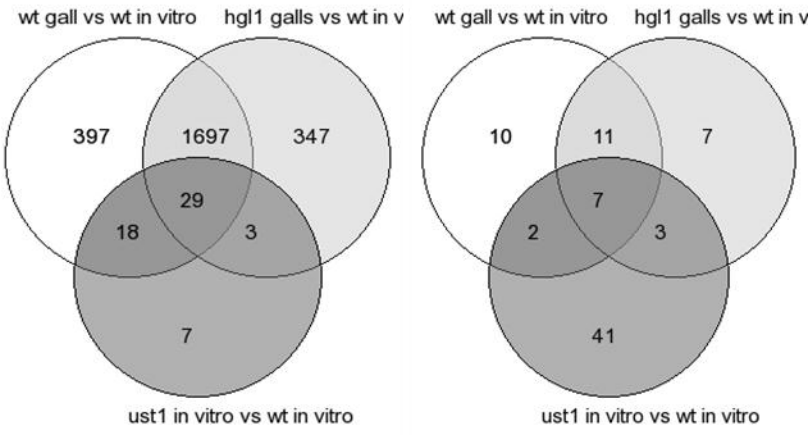


Figure 4.3. The comparison of gene expresssion pattern of *in planta* strains wild type galls and Δ*hgl1* galls, *in vitro* sporulating strain Δ*ust1* compared to *in vitro* haploid wild type.
A. Genes up-regulated ≥8 fold in wild type galls, Δ*hgl1* galls and Δ*ust1* mutant compared to wild type *in vitro.* B. Genes down-regulated ≥8 fold in wild type galls, Δ*hgl1* galls and Δ*ust1* mutant compared to wild type *in vitro.*

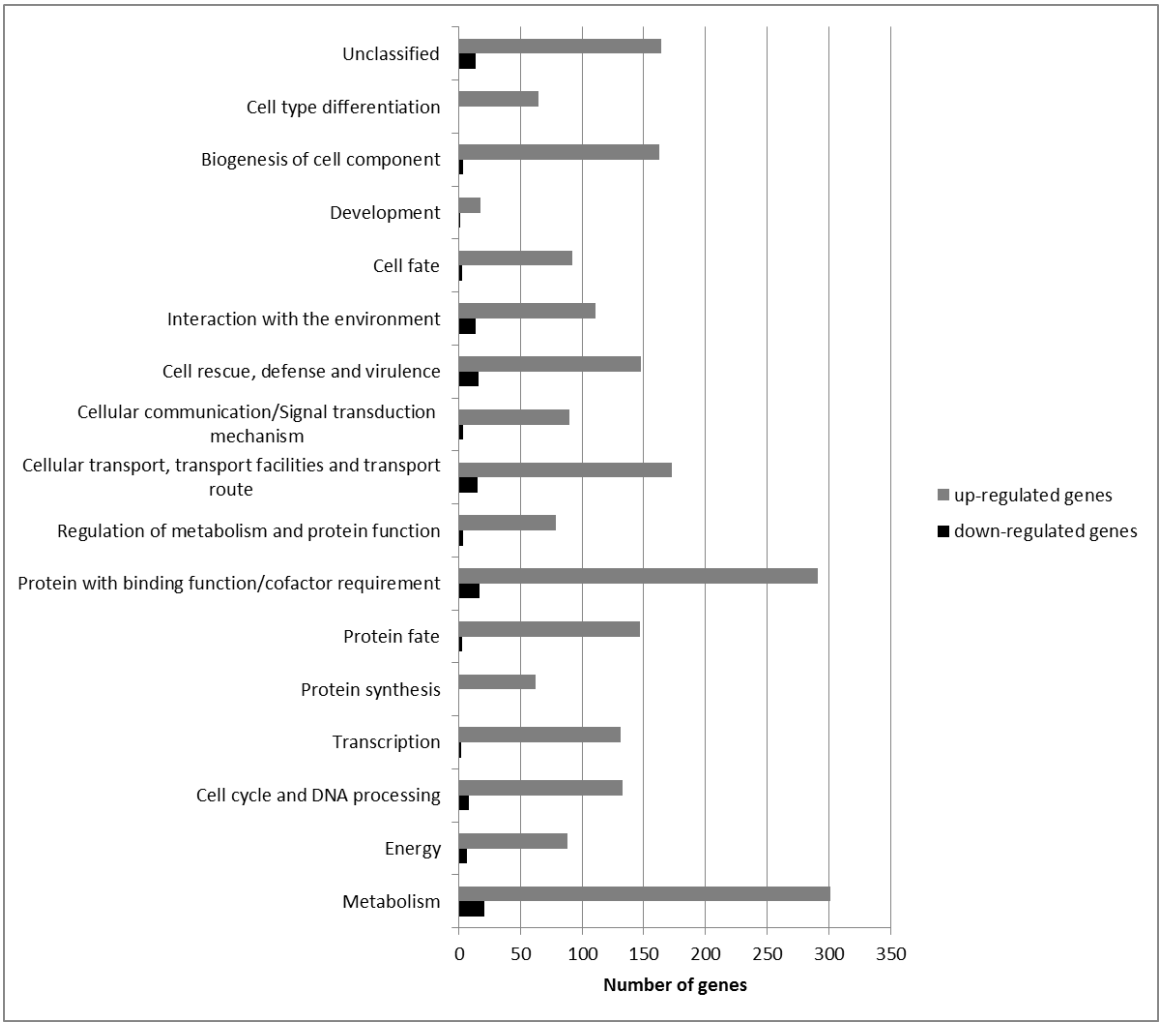


Figure 4.4. FunCat classification of genes sharing common expression patterns in *ust1* mutant and the *in planta* growth strains.

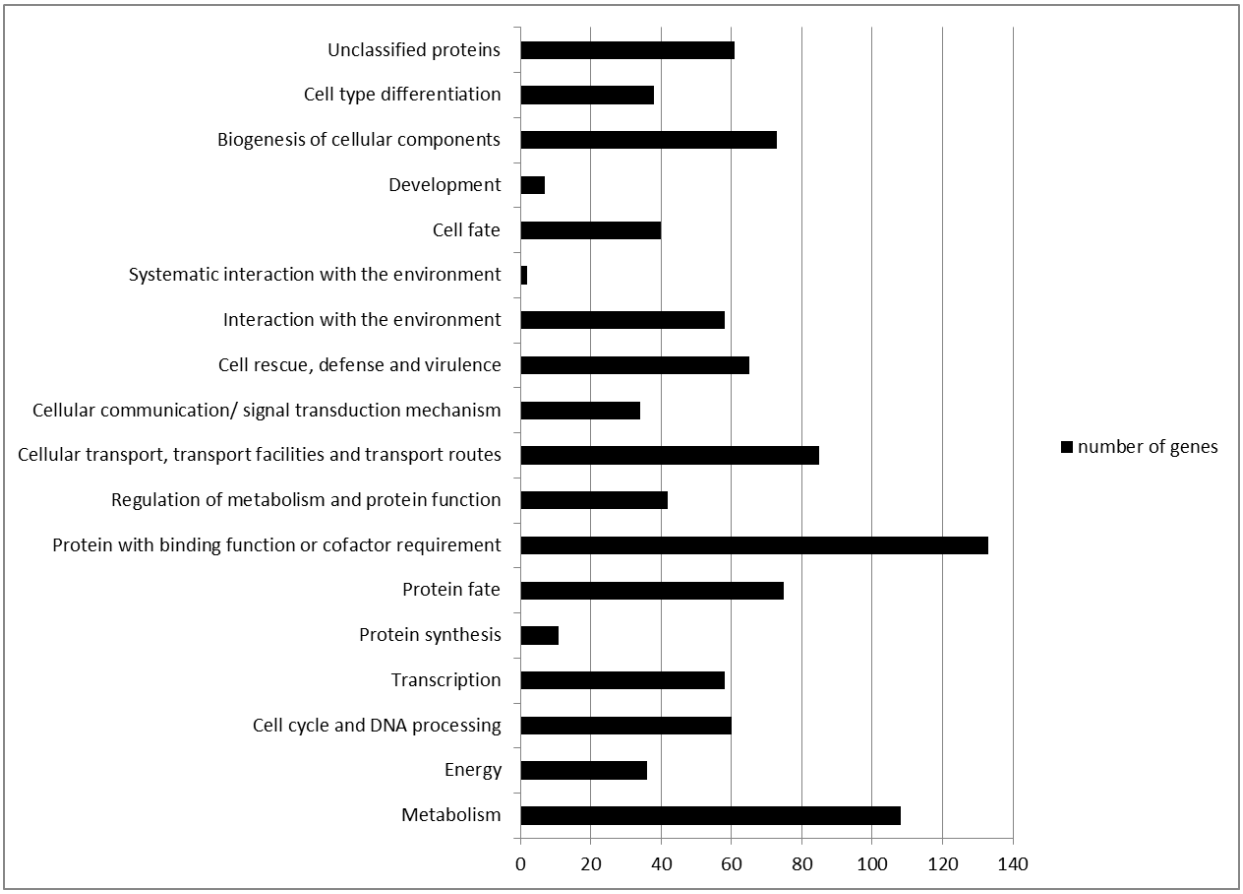


Figure 4.5. FunCat classification of genes up-regulated in the *in planta* strains and down-regulated in Δ*ust1*.

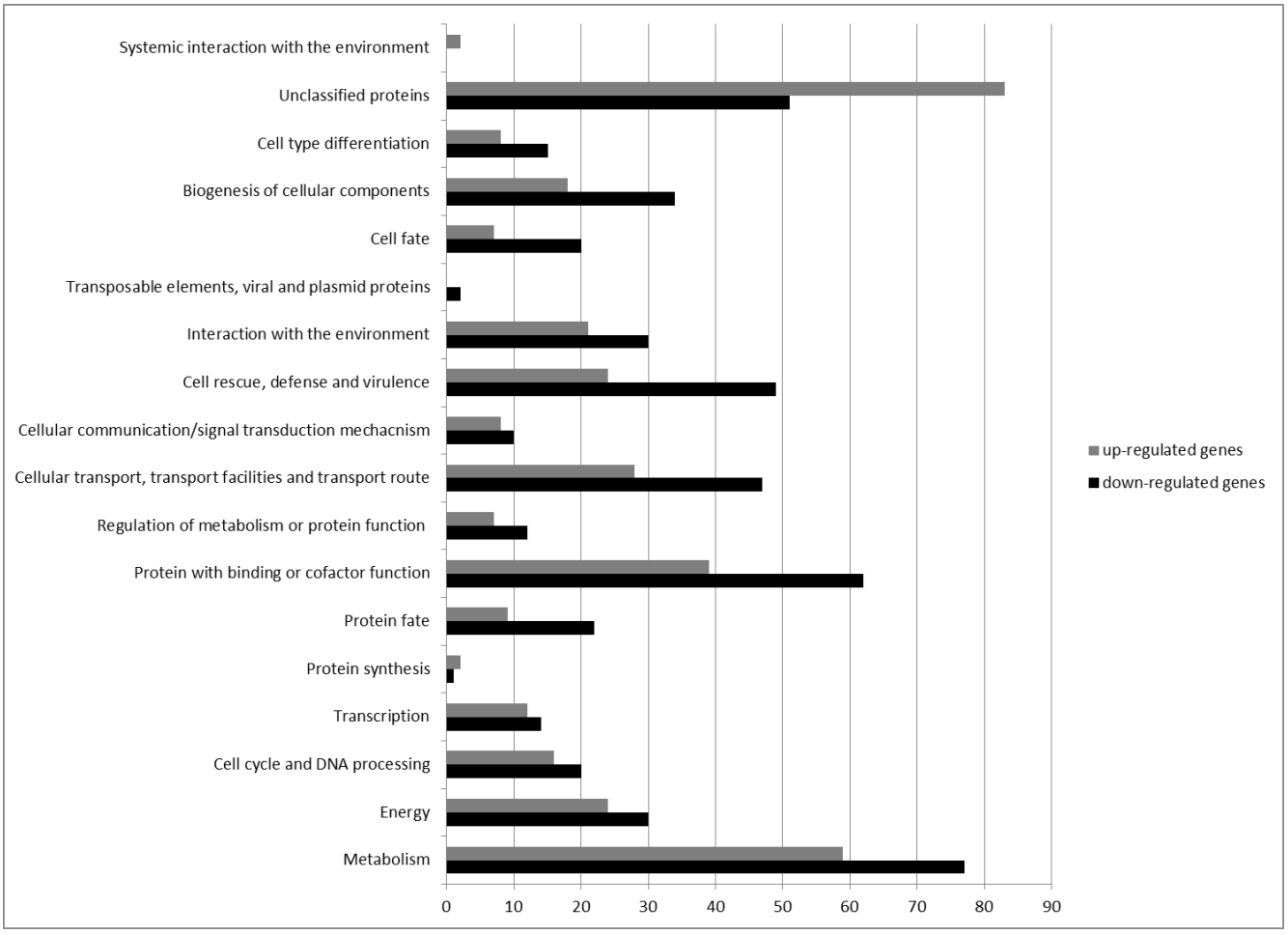


Figure 4.6. FunCat classification of genes differentially expressed in Δ*hgl1* galls compared to wild type galls

Table 4.1. Expression patterns of select signal transduction genes during spore production in *U. maydis*.

Gene Designation	Name of the gene in *U. maydis*	Wild type galls	Δ*hgl1* galls	Δ*ust1*
um10177	guanine nucleotide-binding protein alpha-1 subunit (*gpa1*)	5.11 up[1]	3.69 up	NC
um02517	Guanine nucleotide-binding protein alpha-2 subunit (*gpa2*)	26.63 up	26.70 up	4.12 up
um04474	Guanine nucleotide-binding protein alpha-3 subunit (*gpa3*)	42.18 up	26.51 up	4.38 up
um05385	Guanine nucleotide-binding protein alpha-4 subunit (*gpa4*)	57.79 up	51.29 up	2.44 up
um03315	Serine/Threonine kinase B-related (*ukb1*)	26.98 up	27.17 up	NC
um02065	Histone deacetylase (*hda1*)	5.94 up	6.39 up	NC
um02582	Regulator *Ustilago maydis* 1 protein (*rum1*)	3.49 up	3.79 up	NC
um06450	(PKAr - cAMP-dependent protein kinase type II regulatory chain (*ubc1*)	NC[2]	NC	2.37 down
um10537	Protein kinase A, catalytic subunit *(adr1)*	4.45 up	3.43 up	NC
um12271	Putative dioxygenase (*ssp1*)	20.66 up	2.37 up	14.31 up

[1] Differences in expression are in comparison to wild type grown in culture.

[2] Note: All expression changes below 2 fold are designated as no change (NC). All expression values included in the table are significant ($P \leq 0.05$).

Table 4.2. Expression patterns of *U. maydis* genes associated with sporulation in other fungal systems.

Gene Designation	Name of the gene in *U. maydis*	Name of the gene in other fungi	Wild type galls	Δ*hgl1* galls	Δ*ust1*
um02835	Conserved hypothetical protein	*abaA* (*A. nidulans*)	2.46 up	2.64 up	NC
um02104	Probable regulator of G protein signaling protein	*flbA* (*A. nidulans*)	5.78 up	7.11 up	NC
um10177	Guanine nucleotide-binding protein alpha-1 subunit	*fadA* (*A. nidulans*)	5.11 up	3.68 up	NC
um00893	Related to *veA*	*veA* (*A. nidulans*)	11.28 up	11.36 up	NC
um01146	Conserved hypothetical protein	*veA* (*A. nidulans*)	8.28 up	3.24 up	2.29 up
um04203	Conserved hypothetical protein	*veA* (*A. nidulans*)	29.32 up	15.45 up	2.62 up
um10556	Conserved hypothetical protein	*veA* (*A. nidulans*)	4.30 up	5.17 up	NC
um04258	MAPKK kinase *ubc4*	Sporulation specific protein 1 (*Magnaporthe oryzae*)	2.39 up	NC	3.58 down
um05922	Related to RMD8-cytosolic protein needed for sporulation	Sporulation protein RMD1 (*Neurospora crassa*)	18.97 up	15.33 up	NC
um02994	Related to ELAV-like protein 2	Sporulation specific protein (*Verticillium albo-atrum*)	2.71 up	NC	NC

Table 4.3. Expression pattern of genes putatively involved in pigmentation.

Gene Designation	**Name of the gene in *U. maydis***	**Wild type galls**	**Δ*hgl1* galls**	**Δ*ust1***
um05361	Related to Laccase I precursor	21.383 up	8.037 down	24.946 up
um05861	Related to FET5-multicopper oxidase	22.060 up	2.474 up	15.680 up
um02035	Related to yellowish-green 1 (*ayg1*)	117.399 up	4.594 up	22.213 up
um06414	Related to polyketide synthase	97.423 up	9.419 up	58.620 up
um06418	Related to polyketide synthase	175.345 up	13.594 up	58.940 up

Table 4.4. Expression pattern of genes that associate with facilitation of biotrophic growth of *U. maydis* in maize.

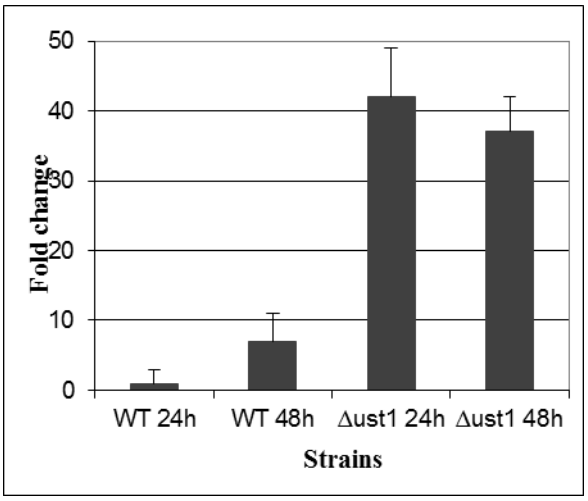
Gene Designation	**Name of the gene in *U. maydis***	**Wild type galls**	**Δ*hgl1* galls**	**Δ*ust1***
um01476	Related to HXT1-low affinity hexose facilitator	5.10 up	8.45 up	6.08 down
um02273	Putative protein	3.59 up	8.03 up	2.08 down
um2374	Related to monosaccharide transporter (*srt1*)	40.50 up	59.81 up	NC
um03034	Conserved hypothetical protein-	15.34 up	22.27 up	6.40 down
um03888	Related to multidrug resistant protein	3.37 up	9.74 up	4.11 down
um04079	Hypothetical protein	8.51 up	6.08 up	2.08 down
um04186	Probable general amino acid permease	14.87 up	17.13 up	4.02 down
um10365	Related to YBT1- vacuolar ABC protein transporting bile acids	6.63 up	8.85 up	2.06 down
um11212	probable QCR9 - ubiquinol--cytochrome-c reductase subunit 9	5.28 up	8.16 up	2.32 down
um00056	Probable neutral amino acid permease	26.24 up	24.011 up	3.80 down
um06012	Probable amino acid permease	51.91 up	69.76 up	4.33 down
um01945	Probable *suc2*-invertase	47.71 up	38.14 up	3.00 down

Table 4.5. Relative expression of down-regulated genes encoding secreted proteins in Δ*hgl1* galls compared to wild type gall tissue.

Gene Designation	Name of the gene in *U. maydis*	Fold change in Δ*hgl1* vs wild type tumors
um00235	probable EXG1 - exo-beta-1,3-glucanase (I/II), major isoform	3.74 down
um00445	Conserved hypothetical protein	11.43 down
um00876	Related to SPR1-exo-1,3-beta-glucanase precursor	7.43 down
um01888	Probable serine-type carboxypeptidase F precursor	2.97 down
um03024	Related to subtilisin-like serine protease	5.16 down
um05229	Related to beta-mannosidase precursor	21.41 down
um05361	Related to Laccase I precursor	171.86 down
um05861	Related to FET5- multicopy oxidase	8.19 down
um06118	Related to tripeptidyl-peptidase I precursor	6.62 down
um11908	Related to cathepsin d (lysosomal aspartyl protease	3.28 down
um00466	Hypothetical protein	31.62 down
um03138	Hypothetical protein	27.70 down
um11403	Conserved hypothetical protein	7.91 down
um11839	Hypothetical protein	3.66 down
um01778	Putative protein	87.72 down

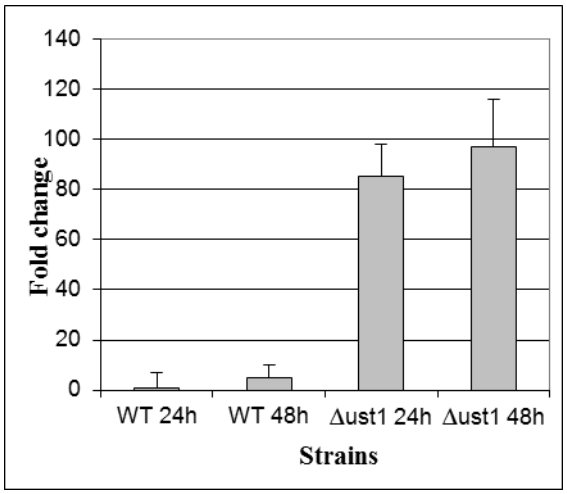
A

ssp1 gene expression



B

laccase gene expression



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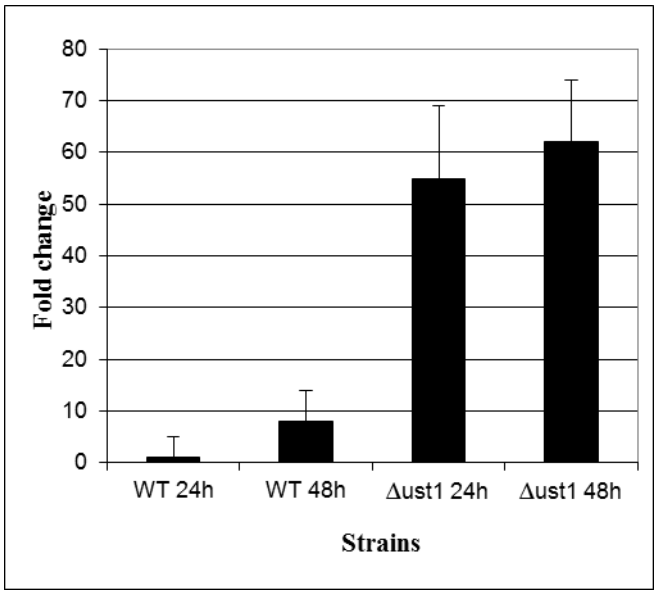
Polyketide synthase gene expression



Supplementary Figure 4.1 qPCR expression assay of microarray experimental samples: expression pattern of A. *ssp1* gene (um12271) B. laccase (um05361) and C. polyketide synthase (um06418) in in vitro samples of wild type and Δ*ust1*. The values are consistent with the expression values from microarray analysis

CHAPTER 5

DELETION OF THE *USTILAGO MAYDIS* ORTHOLOG OF THE *ASPERGILLUS* SPORULATION REGULATOR *MEDA* AFFECTS MATING AND VIRULENCE THROUGH PHEROMONE RESPONSE

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ABSTRACT

Mating of compatible haploid cells of *Ustilago maydis* is essential for infection and disease development in the host. For mating and subsequent filamentous growth and pathogenicity, the transcription factor, *prf1* is necessary. Prf1 is in turn regulated by the cAMP and MAPK pathways and other regulators like *rop1* and *hap1*. Here we describe the identification of another putative Prf1 regulator, *med1*, the ortholog of the *Aspergillus nidulans* medusa *(medA)* transcription factor and show that it is required for mating and pathogenicity in *U. maydis*. The Δ*med1* mutant produced excess quantity of secreted glycolipids in liquid culture. The fungus was unable to mate *in vitro* with the loss of the *med1* gene. The expression of *prf1* is down-regulated in *med1* compared to the wild type, suggesting that *med1* is upstream of *prf1*. The lack of complete abolition of pathogenicity of Δ*med1* is reflective of the phenotype of the Δ*rop1* mutant indicating *med1* might have a similar function to *rop1* in regulation of *prf1*.

INTRODUCTION

In corn smut disease development, a first step requires the fusion of sexually compatible yeast cells to produce dikaryotic hyphae. These dikaryotic hyphae are under cell cycle arrest and are consequently short lived unless they infect the host. On infection, cell cycle arrest is relieved and the hyphae proliferate inter- and intracellularly to produce tumors in aerial parts of the plant. The hyphae later become embedded in a mucilaginous matrix and undergo fragmentation to produce dark, diploid spores called teliospores (Banuett and Herskowitz, 1996).

U. maydis has a tetrapolar mating system made up of the biallellic *a* locus and multiallelic *b* locus. At the *a* locus are the genes *mfa* and *pra* that encode mating pheromone and receptor, respectively, that are required for the recognition of and response to opposite mating type cells. The *b* locus encodes homeodomain proteins *bE* and *bW* that are the subunits of a non-self

recognition heterodimeric transcription factor that regulates filamentation, dikaryon maintenance and pathogenicity. The expression of *a* and *b* mating type genes is regulated by the transcription factor Prf1. The expression of genes at the *a* mating type locus occurs only if Prf1 is phosphorylated by the cyclic-AMP dependent protein kinase (PKA) while the expression of genes from the *b* locus requires phosphorylation of Prf1 via PKA and the pheromone responsive MAP kinase. The expression of mating genes is also dependent on the regulation of *prf1* by two other factors, *rop1* and *hap2* and MAPKs Ubc3/Kpp2 and Crk1 (Brefort et al., 2005; Garrido et al., 2004; Hartmann et al., 1999; Mendoza-Mendoza et al., 2009). Morphogenesis that follows mating and the subsequent development of the fungus in the host leads to sporulation and is regulated by several other transcription factors like *rum1*, *sql1, hgl1* and *ust1* (Durrenberger et al., 2001; Garcia-Pedrajas et al., 2010a; Loubradou et al., 2001; Quadbeck-Seeger et al., 2000). Rum1 is required for teliospore development; Sql1 for filamentation and Hgl1 regulates both filamentaton and teliopsore maturation (Durrenberger et al., 2001; Loubradou et al., 2001; Quadbeck-Seeger et al., 2000). The APSES domain transcription factor encoding gene, *ust1,* is a major regulator of morphogenesis and virulence. The haploid *ust1* mutant shows a dramatic phenotype of *in vitro* filamentation and production of teliospore-like structures (Garcia-Pedrajas et al., 2010a). Ust1 and other fungal specific transcription factors with the characteristic APSES domain are known to be regulators of morphogenetic changes like yeast-hypha transition and sporulation (Borneman et al., 2002; Ohara and Tsuge, 2004; Pan and Heitman, 2000; Stoldt et al., 1997; Wu and Miller, 1997). *ust1* is the only APSES domain encoding gene in the *U. maydis* genome and is an ortholog of the *A. nidulans* APSES domain transcription factor *stuA.*

There is considerable conservation in the major pathways and molecular mechanisms that regulate morphogenesis and sporulation in fungi. Much is known regarding sporulation in *A.*

nidulans and it serves as a useful model. A complex central genetic pathway that includes transcription factors *brlA, abaA* and *wetA* regulates asexual reproduction or conidiation in *A. nidulans* (Boylan et al., 1987; Mirabito et al., 1989). Transcription factors *stuA* (stunted) and *medA* (medusa) regulate the proper temporal and spatial expression of the regulatory genes in the conidiation pathway of *A. nidulans* (Aguirre, 1993; Busby et al., 1996; Miller et al., 1993). *stuA* and *medA* also regulate sexual reproduction in *A. nidulans* ((Busby et al., 1996; Wu and Miller, 1997).

To investigate the regulatory role of *ust1* in *U. maydis*, an expression microarray study was conducted comparing the mutant to the wild type strain at time points when the mutant existed as filaments and when it produced 'spore-like' structures (Chacko et al., in preparation). One of the identified differentially expressed genes was the ortholog of the *A. nidulans medA* which was down-regulated 4.5-fold in Δ*ust1*. The gene (um03588) is annotated in *U. maydis* as "related to transcription factor medusa" and the protein sequence shows over 50% identity to the *medusa gene* from various fungi including *A. nidulans*. Additionally, um03588 was the only gene identified in a BLASTp search of the *U. maydis* genome (E value of 8e-61) with the *A. nidulans medA* protein sequence. The putative Med1 protein did not have any recognizable domains aside from the DNA binding region characteristic of transcription factors.

The main objective of this study was to characterize the role of *med1* (um03588), the *U. maydis* ortholog of the *A. nidulans*, *medA* gene. In this study we deleted *med1* and found that the gene is required for *in vitro* mating, pheromone response and expression of full virulence.

MATERIALS AND METHODS

Fungal and bacterial strains and growth conditions

U. maydis strains (Table 5.1) were grown on solid potato dextrose agar supplemented to 2% agar (2PDA) (Sigma, St Louis, MO, USA) at 30 °C. Transformants were selected on YEPS plates (1% yeast extract, 2% peptone and 2% sucrose) containing 1M sorbitol and 3 μg/ml carboxin. The transformant colonies were transferred and grown on PDA plates containing carboxin (3 μg/ml). Potato dextrose broth (PDB, Sigma) was used to grow liquid cultures of the fungus.

The *Escherichia coli* strain DH5α (Bethesda Research Laboratories, Gaithersberg, MD, USA) used for making the deletion construct, was grown at 37 °C in liquid and on solid Luria Bertani (LB) media containing kanamycin (50 μg/ml) after transformation.

Nucleic acid manipulations

The *med1* deletion construct was made using the DelsGate method (Garcia-Pedrajas et al., 2008). The *med1* ORF of strains *a1b1*, *a2b2* and SG200 were replaced completely with the DelsGate deletion construct plasmid containing a carboxin resistance marker (Table 1). *U. maydis* transformation was carried out as previously described (Barrett et al., 1993). Gene deletion was analyzed and confirmed using PCR and Southern hybridization, respectively. The DNA from all mutant strains and the wild type were digested with *Bgl*I (New England Biolabs, Ipswich, MA, USA) and separated on a 0.8% agarose gel. The DNA was transferred to a Hybond XL nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using 0.4 M NaOH as the transfer solution. The 1231 bp 5' flank ending 150 bp away from the start of the open reading frame of the *med1* gene was used as the probe. The probe was prepared by PCR amplification with the primers *med1*-probe-P1 (TGTACTGTGGCTGTACTGTGCTGT) and *med1*-probe-P2

(AGAGCGTTGAGTGAGAAAGCGAGA), purification of the PCR product and labeling using the DIG-High Prime labeling and detection kit (Roche, Indianapolis, IN, USA). Hybridization and subsequent development of the blot was done according to the manufacturer's instructions.

Plate mating assays

U. maydis strains of opposite mating type were grown in PDB overnight at 30 °C and cultures of comparable OD were used for mating assays. The compatible strains were co-spotted on charcoal mating plates (YEPS medium with 1% charcoal) as previously described (Holliday, 1974), sealed with parafilm and incubated at room temperature for 24 h.

Pheromone stimulation

A pheromone stimulation experiment was conducted with modifications to previously described methods (Brefort et al., 2005; Weber et al., 2003). Wild type and Δ*med1* strains in *a2b2* mating type background were grown in Complete Medium (CM) (Holliday, 1974) to an OD_{600} of 0.6. Synthetic non-farnesylated *a1* pheromone peptide (Abgent, CA, US) was dissolved in water, and added to the cells at a final concentration of 2.5 µg/ml. Cells were incubated for 8 h at room temperature at 100 rpm. Controls consisted of treating strains with an equal volume of water instead of pheromone under similar conditions.

Virulence assays

Maize seedlings of variety Golden Bantam (Athens Seed Co., Watkinsville, GA, USA) were grown in potting soil and injected after 7 days with cell suspension mixtures of 10^6 cells per ml for each strain. Solo pathogenic strains were inoculated at 10^6 cells per ml. Plants were grown in a Conviron E15 growth chamber (Mannitoba, Canada) with daily cycles of 16 h light at 28 °C followed by 8 h dark at 20 °C. Three replications of 20 plants each were used for each cross. The symptoms were scored at 7, 10 and 14 days post inoculation (dpi) based on the disease scale of 0,

no symptom; 1, chlorosis/anthocyanin production; 2, leaf galls; 3, small stem galls; 4, large stem galls and 5, plant death (Gold et al., 1997). The results were analyzed using the non-parametric statistical test (Shah and Madden, 2004).

Plants of the dwarf maize variety Tom Thumb (Seed Savers Exchange, Decorah, IA, USA) were grown for 40 days in the greenhouse and ears were injected with mating mixtures of 10^6 cells per ml of Δ*med1* mutants or wild type cells. Development of galls and teliospore maturation was observed. The mature teliospores were plated on PDA and incubated at 30 °C to observe germination.

Real time PCR

To study the expression of mating associated genes, cells of compatible mating types of Δ*med1* and wild type strains were co-spotted on charcoal mating plates and incubated for 24 h. The cells were scraped from the plates and RNA was extracted using the Spectrum^{Tm} Plant Total RNA Kit (Sigma) according to the manufacturers' instructions.

For glycolipid expression studies, the wild type and Δ*med1* mutant strains were grown in PDB for 24 h at 30 °C for tissue collection and RNA was extracted. RNA was also extracted from wild type and mutant strains first grown in nitrogen starvation medium containing 1.7 g/l of yeast nitrogen base and 5% glucose and supplemented with 0.2% ammonium sulfate for 15 h at 30 °C with constant shaking at 200 rpm, until they reach logarithmic phase and transferred to the medium without ammonium sulfate. The cells were further cultured for 12h under similar conditions and harvested.

cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) using oligo dT primers according to the manufacturer's instructions. qPCR was conducted using the SYBR-GREEN Supermix kit (Bio-Rad, Hercules,

CA, USA) on a Cepheid Smartcycler I (Cepheid, Sunnyvale, CA, USA). All real time PCR primers used in the expression studies were designed using the Intergrated DNA Technology (IDT) website. The sequences of gene specific primers for mating associated genes are as follows: um02713 (*prf1*) (AACGCATCAAGATGAGCTTGGCAC, ATGCGAAGGTGTCAAAGGATTGCG), um02382 (*mfa1*) (ATGCTTTCGATCTTCGCTCAGACCAC, AACAACACAGCTGGAGTAGCCGAT) and um02383 (*pra1*) (TCTTCGCAATGTTTGGCCTTGGTC, TCGAATGTGTCGTGAGACCGGAAA). Primers for study of expression of genes required for glycolipid biosynthesis were as follows: um06458 (*rua1*) (GCTCTTCGGTCTCTGCTTTG, CTTGTCCGTGATTGTGAAGC) and um06463 (*cyp1*) (AGCACCTTCTGAGCATAGTTG, CACCGTTTTCAACCTGCAAG). The reference gene used was cyclophilin um03726 (*cpr1*) amplified using primers, ACGCCGATTCACTTCGTC and AACGACGATCCCTCGTAACCGAAA). The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

RESULTS

Identification of *med1*

In *A. nidulans*, transcription factors *medA* and *stuA* are critical regulators of the pathway leading to asexual sporulation (conidiation) (Clutterbuck, 1969; Martinelli, 1979; Miller et al., 1993). Deletion of the ortholog of *stuA* in *U. maydis* (*ust1*) generated a filamentous strain that produced pigmented teliospore-like structures in culture, suggesting its possible role in regulation of sporulation (Garcia-Pedrajas et al., 2010a). In the transcriptome analysis of *ust1* compared to the wild type, a gene (um03588) with high similarity to the ortholog of *A. nidulans* transcription factor *medA* was identified by virtue of the fact that in was down-regulated 4.5-fold

in the *ust1* deletion mutant. BLASTp search of *A. nidulans medA* against the *U. maydis* genome database also identified the same gene with an expected value of 8e-61 and 57% identity. Bioinformatic analysis of the protein sequence of this gene (designated *med1*), like the *A. nidulans* protein did not have an identifiable functional domain other than a DNA binding region.

Δ*med1* secretes excess quantities of glycolipids in culture

To study the function of *med1*, deletions in the gene were made in 1/2, 2/9 and SG200 backgrounds of *U. maydis* (Table 5.1). Deletions were confirmed by PCR and Southern hybridization (Supplementary Fig. 5.1). The deletion strains were similar to the wild type in colony morphology and growth rate. However, when grown in liquid culture the mutant secreted copious amounts of a crystalline product. The crystals were produced by the mutant when grown in potato dextrose broth, minimal medium and low nitrogen media, including nitrogen starvation medium containing 1.7 g/l of yeast nitrogen base without ammonium sulfate. Wild type *U. maydis* produces small amounts of extracellular glycolipids like ustilagic acid, ustilipids and mannosylerythritol lipids that appear as crystals in culture (Boothroyd et al., 1956). The production of glycolipids by Δ*med1* is greater and earlier than in wild type (Fig. 5.1 A and B). To understand the role of *med1* in glycolipid production, the expression of *rua1*, the transcriptional activator of the gene cluster responsible for ustilagic acid synthesis and genes required for mannosylerythritol synthesis was analyzed in Δ*med1* and wild type backgrounds. The expression of *rua1* was not significantly different between Δ*med1* and the wild type strain (data not shown). However, the expression of *cyp1*, the cytochrome P450 monoxygenase essential for ustilagic acid biosynthesis was up-regulated 8.6-fold in Δ*med1* over the wild type under nitrogen starved conditions (Fig. 5.1C).

med1 is required for *in vitro* mating and filamentation

To test the Δ*med1* mutants for defects in mating or post-mating filamentation, deletion mutants were spotted on YPD charcoal plates (Fig. 5.2). On mating wild type compatible haploids, typical dikaryotic hyphae that appeared as white fuzzy growth were produced (Fig. 5.2 A). However, when compatible haploid *med1* deletion strains were co-spotted, fuzzy growth was not observed (Fig. 5.2 A). Absence of filamentation was also observed when mutants were co-spotted with a compatible wild type mating partner, suggesting a defect in fusion ability in Δ*med1* strains. The solopathogenic haploid strain SG200, which has the active, *bE/bW* heterodimer required for filamentation, is capable of filamentation on YPD charcoal plates (Fig. 5.2 B). The *med1* deletion mutant in the SG200 background, however, failed to filament on these plates (Fig. 5.2 B) indicating that *med1* is critical for post-mating filamentation.

Δ*med1* mutant does not produce conjugation tubes *in vitro*

To investigate the requirement of *med1* for the production of conjugation tubes, the Δ*med1* mutant and wild type (2/9) in the *a2b2* background were treated with synthetic *a1* pheromone dissolved in water. After 8 h of incubation, Δ*med1* cells treated with pheromones did not produce conjugation tubes. The Δ*med1* cells appeared to have a multiple budding phenotype in the presence of artificial pheromones that is reminiscent of the *ubc1* mutant or a wild type in the presence of cAMP (Fig. 5.3A). Wild type cells after 8 h of treatment with pheromones produced visible conjugation tubes (Fig. 5.3B).

Δ*med1* mutants are reduced in expression of *prf1, pra1* and *mfa1*

The inability of the Δ*med1* mutant to produce functional conjugation tubes suggests potential reduction or absence in production of pheromones and/or pheromone receptors. To

investigate the reduction in expression of pheromone and receptor genes and their regulator *prf1*, in the Δ*med1* mutant, RT-PCR experiments were conducted using RNA extracted from the compatible cells co-spotted on YPD charcoal plates. Control RNA was extracted from wild type cells similarly co-spotted on YPD charcoal plates. The average Ct values from three biological replications each with two technical replications, normalized against the reference gene *cpr1* (um03726) showed a relative down-regulation in expression of *mfa1*, *pra1* and *prf1* genes in the *med1* mutant when compared to the wild type strain (Fig. 5.4). The 19-fold reduction in the expression of *prf1*, in the *med1* mutant suggests that *med1* may be upstream of *prf1* in the pheromone response pathway.

Δ*med1* mutants are significantly reduced in virulence

To assess the effect of deletion of the *med1* gene on pathogenicity of *U. maydis*, various pair-wise combinations of compatible mutant and wild type cells were injected into 7 day old maize plants. The disease index measured on 7, 10 and 14 days after inoculation from three separate replications was recorded. The mutant was found to be significantly reduced in virulence compared to wild type and wild type and mutant combinations (Table 5.2 A and B). The plants infected with Δ*med1* mutants displayed symptoms including chlorosis and leaf, stem and basal galls but the symptoms were reduced and progressed at a slower rate than wild type. Moreover, unlike wild type plants, plant death was not observed when inoculated with *med1* mutants. The Δ*med1* mutants in the SG200 background also showed statistically significant reduction in virulence compared to those inoculated with wild type SG200 (Table 5.3). The plants inoculated with Δ*med1* SG200 generally did not show advanced symptoms like stem and basal galls. Despite the reduction in virulence of the Δ*med1* mutant, it produced galls when

injected into cob of adult cv Tom Thumb maize plants. The teliopsores produced developed normally and were able to germinate and produce sporidia (data not shown).

DISCUSSION

In this work we showed that the *U. maydis* ortholog of the *medA* gene of *Apsergillus nidulans* is required for mating *in vitro* and to confer full virulence to the fungus. The gene also directly or indirectly represses the expression of the *cyp1* gene required for the synthesis of ustilagic acid, a secondary metabolite produced by *U. maydis*.

Function of the *medA* gene of *A. nidulans* and its orthologs in other fungi

In *A. nidulans* transcription factors *brlA*, *abaA* and *wetA* coordinate the expression of genes required for the development of conidiophores and uninucleate structures like sterigmata and conidia. *A. nidulans medA* is classified as a developmental modifier along with another transcription factor *stuA* (Adams et al., 1998). Together these genes ensure that the conidiophores exhibit precise spatial pattern formation through the regulation of correct temporal and spatial expression of transcription factor *brlA* (Busby et al., 1996; Miller et al., 1993). In addition to its role in conidiation, in *A. nidulans medA* is also required for the production of the sexual fruiting body called the cleistothecium (Clutterbuck, 1969). In *Fusarium oxysporum*, Ren1, a protein with high similarity to MedA is required for normal conidiogenesis but not for normal vegetative growth (Ohara et al., 2004).

In *U. maydis*, *med1* is needed for observable plate mating reactions. However, since the fungus retained pathogenicity in compatible matings with strains with loss of *med1* it can be deduced that the gene is not absolutely required for mating at least *in planta*.

The role of *med1* in mating and virulence in *U. maydis*

Mating in *U. maydis* requires the production and perception of lipopeptide pheromones by the cells of complementary mating types. The *mfa* genes (*mfa1* and *mfa2*) encode the pheromone precursors while the *pra* genes (*pra1* and *pra2*) encode their corresponding receptors. The inability of Δ*med1* strains to form conjugation tubes can be explained by the observed dramatic reduction in expression of the *mfa1* and *pra1* genes which indicates reduced pheromone production and /or reception by the mutant.

The expression of the *a* and *b* mating genes was earlier shown to be regulated by the HMG domain transcription factor *prf1* (Hartmann et al., 1999; Urban et al., 1996). Prf1 was in turn found to be regulated transcriptionally by the HMG domain protein Rop1 and post-translationally by cAMP and pheromone signaling (Brefort et al., 2005; Hartmann et al., 1999). The complexity and size of the promoter of *prf1* leaves open the possibility of it being regulated by several more regulators like *med1*. Since the expression of *prf1* is significantly reduced in the Δ*med1* mutant, *prf1* appears to be downstream of *med1* in the mating pathway. The possibility of Med1 regulating *prf1* expression by direct binding like Rop1 and Hap2 proteins requires further analysis.

The mutation in *ubc1*, the gene encoding the regulatory subunit of PKA, or addition of a high external cAMP concentration to the wild type haploid, triggers a multiple budding phenotype (Gold et al., 1994). Δ*med1* in response to pheromone stimulation resembles a weak recapitulation of this cAMP induced phenotype. The transfer of pheromone signal requires the normal functioning of the cAMP pathway and high cAMP can stimulate the expression of pheromone gene *mfa1* indicating a crosstalk between cAMP and pheromone pathways (Kruger et al., 1998). This high expression of *mfa1* in response to cAMP does not however induce

conjugation tube formation. A constitutively high cAMP level is, on the other hand, known to cause diminished response to pheromone and in turn to reduce conjugation tube formation in the wild type (Muller et al., 2004). Therefore in Δ*med1,* activation of the cAMP pathway may yield multiple budding and repress conjugation tube formation. However, complete loss of conjugation tube formation occurs in mutants in which Pra1, the pheromone receptor, or the pheromone responsive MAP kinase pathway are inactivated (Muller et al., 2003). Thus the complete loss of conjugation tube formation in Δ*med1* also makes it plausible for the gene to be a positive regulator of the pheromone response pathway. Therefore *med1* may function as a point of convergence of both cAMP and MAPK pathways with respect to pheromone related morphogenesis.

The activity of the *prf1* gene is required for mating, both *in vitro* and *in planta* (Hartmann et al., 1999). When the direct regulator of *prf1*, *hap2,* is deleted, the mutant loses the ability to mate and is non-pathogenic (Mendoza-Mendoza et al., 2009). Hap2 binds to a CCAAT box in the promoter of the *prf1* gene and regulates its expression. A similar regulatory role was shown for the HMG domain protein Rop1. The *rop1* gene is required for pheromone induced gene expression and plate mating reactions but not for pathogenicity, indicating that *rop1* mutants can mate *in planta* (Brefort et al., 2005). This result can be explained by another factor influencing the regulatory role of Rop1 on *prf1 in planta*. However, the Δ*rop1* mutant shows reduced filamentation when co-spotted with its corresponding compatible wild type strain indicating a reduced mating reaction even though it is incapable of forming conjugation tubes in response to pheromones (Brefort et al., 2005). The Δ*med1* mutant on the other hand is incapable of forming conjugation tubes and mating *in vitro*. Since *prf1* is required for cell fusion and filamentous growth (Hartmann et al., 1999) the phenotype of Δ*med1* taken together with that of Δ*rop1*

suggests that *med1* gene is a regulator of *prf1 in vitro*. The loss of *med1* also reduces the virulence of *U. maydis,* another characteristic regulated by *prf1*. The reduction of virulence in Δ*med1* could indicate at least a partial regulatory role of *med1* over *prf1 in planta*.

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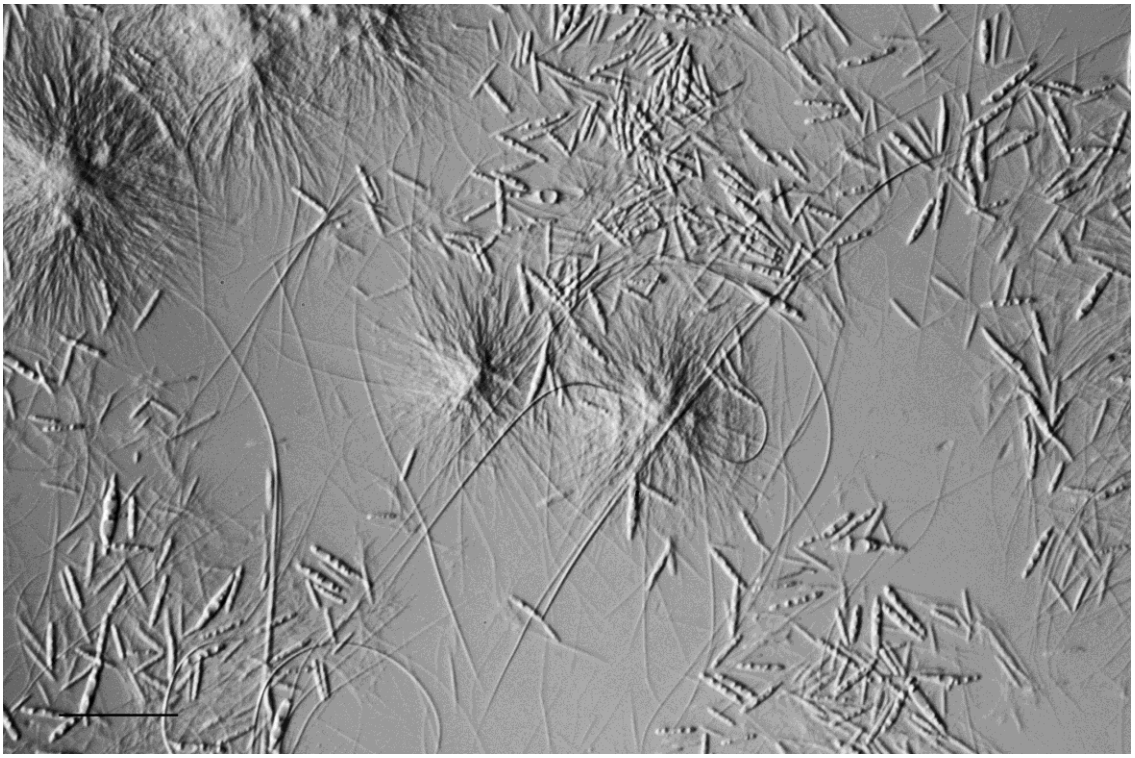
Figures and Tables

A



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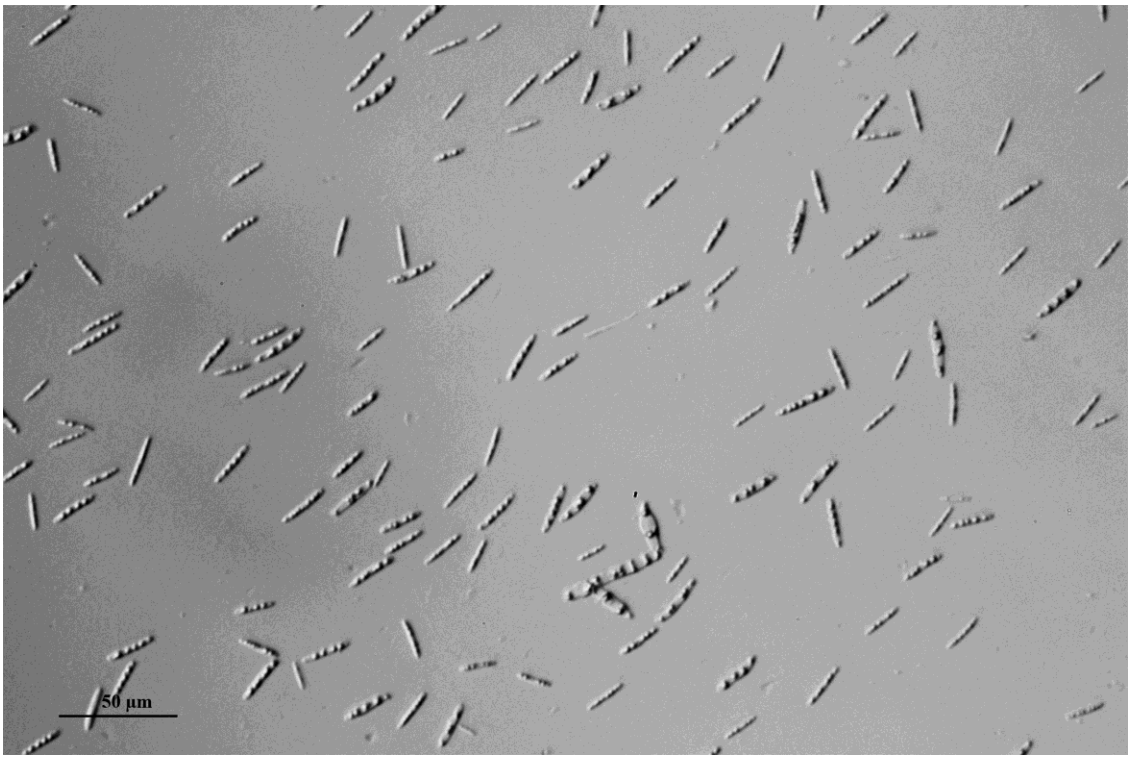

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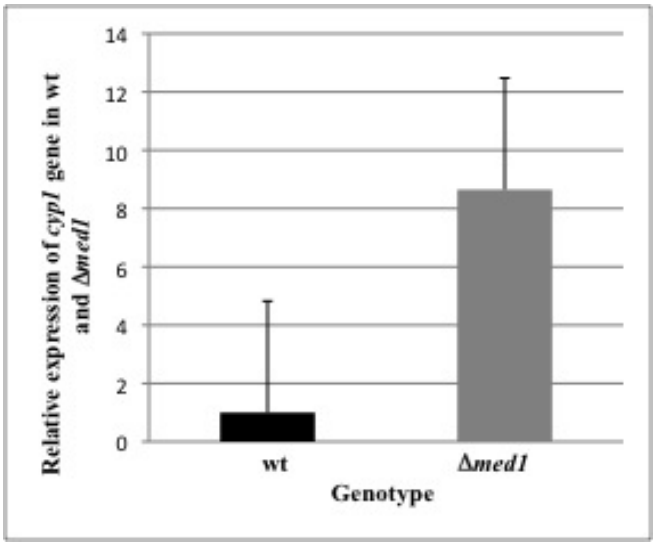


Fig. 5.1. Over-production of secreted glycolipids by Δ*med1*. (A) Δ*med1* cells in potato dextrose broth (PDB) culture 48 hours post inoculation (hpi) showed the presence of large quantities of secreted glycolipids that appeared as elongated and star shaped crystals that were not visible in 48 hpi cultures of in PDB (B). (C) The expression of the *cyp1* gene was up-regulated in Δ*med1* compared to the wild type in glycolipid inducing low nitrogen medium conditions. The values

are an average of three biological replicates with two technical replicates each. The *cpr1* gene was used as control and the expression of *cyp1* in Δ*med1* is relative to *cyp1* expression in wild type when it is arbitrarily fixed at 1.

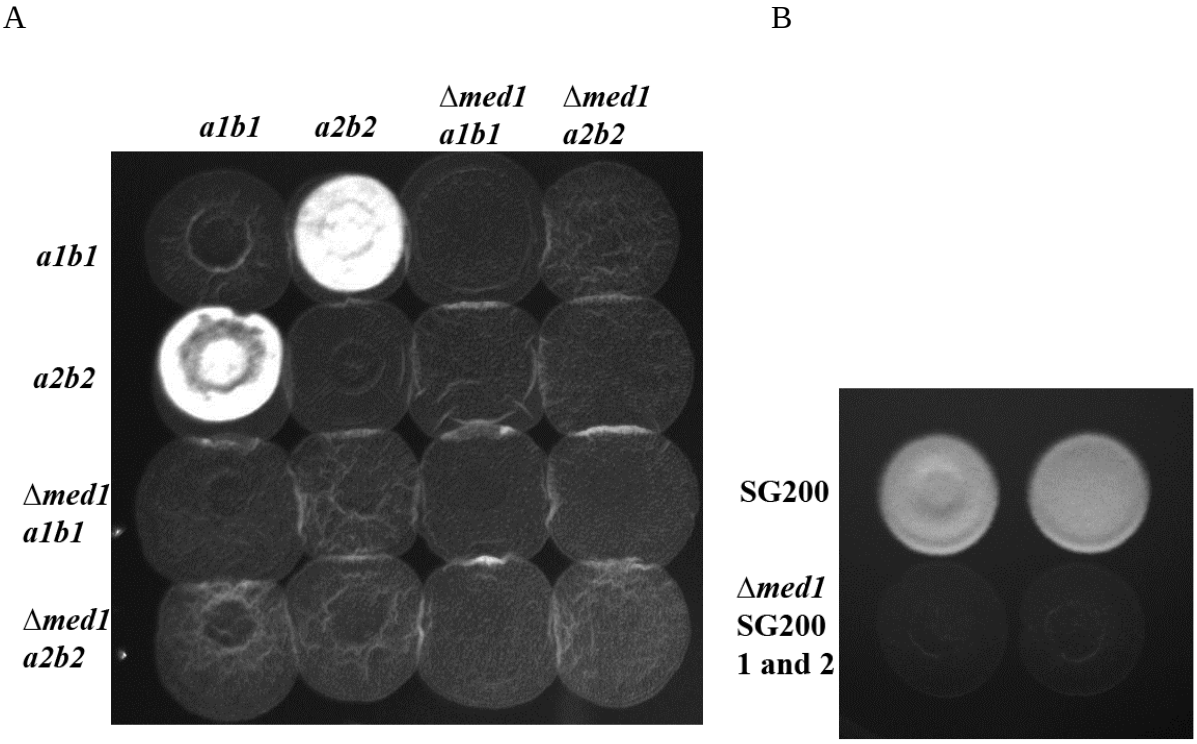
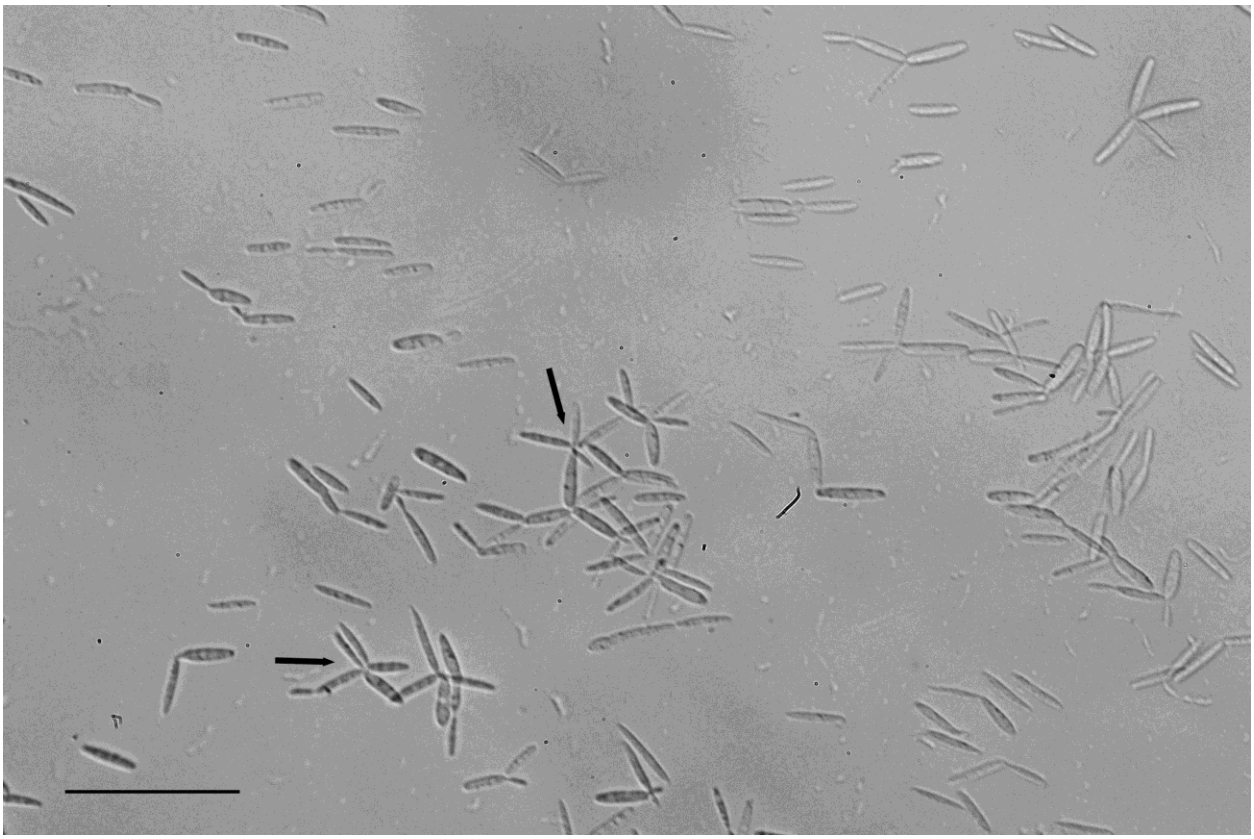


Fig. 5.2. Impairment of *in vitro* mating in Δ*med1*. (A) Plate mating assay with wild type and Δ*med1* spotted cell suspensions. Positive mating reactions appear as white fuzzy growth. (B) The filamentation of SG200 strain on charcoal plates was affected when *med1* gene was deleted in this genetic background. The loss of filamentation was consistent in two independent Δ*med1* mutants in the SG200 background.

A



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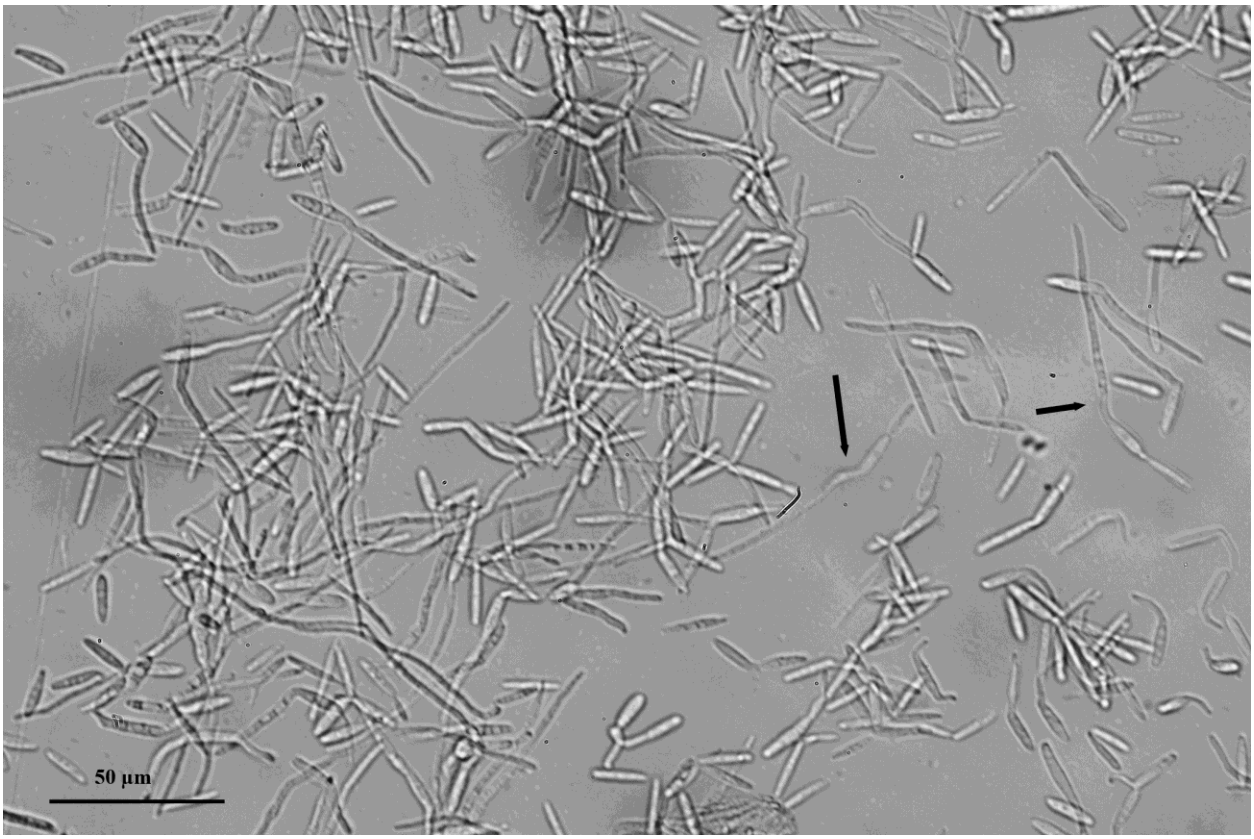
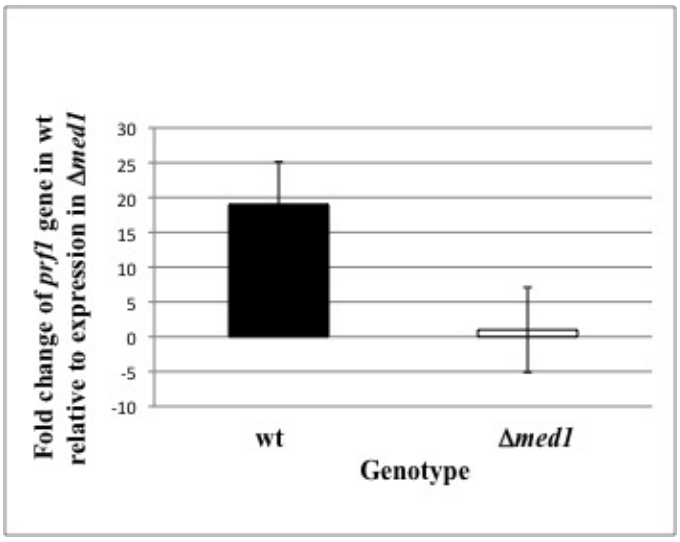
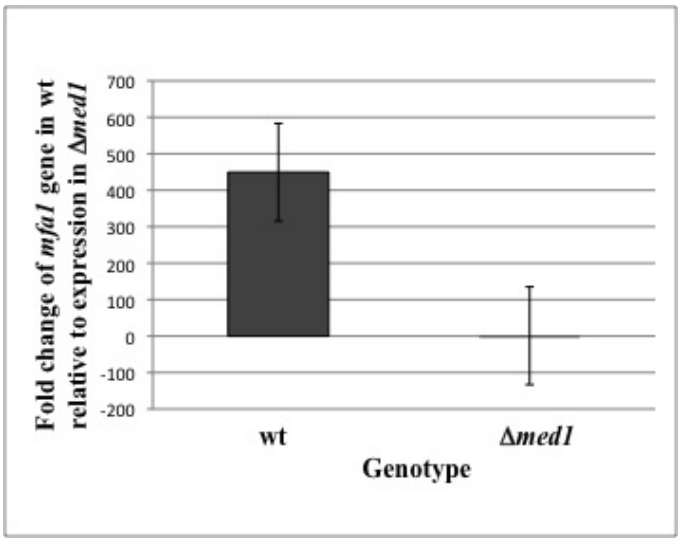


Fig. 5.3. The Δ*med1* mutant is unable to produce conjugation tubes in response to artificial pheromones. (A) The Δ*med1* mutant cells appeared to have multiple budding phenotype (shown by arrows) and did not produce conjugation tubes in response to pheromone stimulation. (B) After 8 h of treatment with artificial *a1* pheromones, wild type cells produced conjugation tubes (shown by arrows).

A


Fold change of prf1 gene in wt relative to expression in Δmed1
30
25
20
15
10
5
0
-5
-10
wt
Δmed1
Genotype

B


Fold change of mfa1 gene in wt relative to expression in Δmed1
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600
500
400
300
200
100
0
-100
-200
wt
Δmed1
Genotype

C

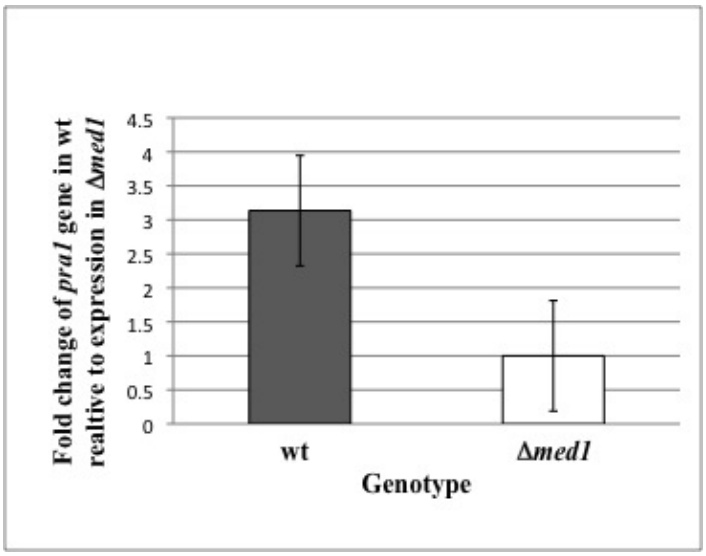


Fig. 5.4. Reduced expression of pheromone responsive genes in the Δ*med1* mutant. The expression levels of genes encoding (A) the transcription factor *prf1*, (B) the *a1* pheromone *mfa1*, and (C) the pheromone receptor *pra1*were all lower in Δ*med1* compared to the wild type.

Table 5.1. *U. maydis* strains

Strain	**Genotype**	**Reference**
1/2 (521)	*a1b1*	(Gold et al., 1997)
2/9 (518)	*a2b2*	(Gold et al., 1997)
7/20	SG200 (*a1::mfa2 bE1bW2*)	(Muller et al., 1999)
med1-1	*a1b1* Δ*med1::cbx*	This study
med1-2	*a1b1* Δ*med1::cbx*	This study
med1-3	*a1b1* Δ*med1::cbx*	This study
med1-4	*a2b2*Δ*med1::cbx*	This study
med1-5	*a2b2* Δ*med1::cbx*	This study
med1-6	*a2b2* Δ*med1::cbx*	This study
med1-7	SG200 Δ*med1::cbx*	This study
med1-8	SG200 Δ*med1::cbx*	This study

Table 5.2. *med1* is required for full virulence of mated haploids.

A. Virulence assay of Δ*med1*-1(*a1b1*) and Δ*med1*-4(*a2b2*) mutant dikaryons

Sl. No.	Cross	Disease Index		
		7d	10d	14d
1.	Δ*med1*-1 X Δ*med1*-4	0.92^{a}	1.40^{a}	1.82^{a}
2.	Δ*med1*-1X *2/9*	1.02^{a}	1.50^{ab}	2.08^{ab}
3.	*1/2* X Δ*med1*-4	1.20^{a}	2.07^{b}	2.61^{b}
4.	*1/2* X *2/9*	1.92^{b}	3.03^{c}	4.00^{c}

N=60 plants in 3 replications of 20 plants each

B. Virulence assay of Δ*med1*-2 (*a1b1*) and Δ*med1*-5 (*a2b2*) mutant dikaryons

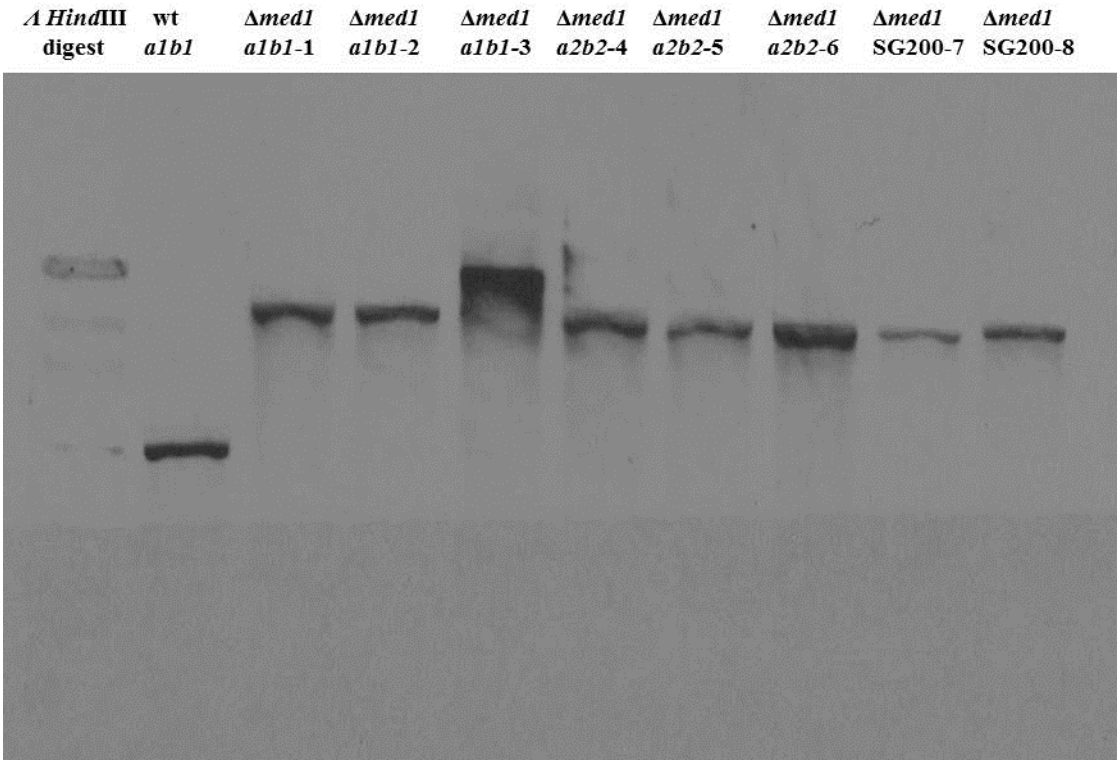
Sl. No.	Cross	Disease Index		
		7d	10d	14d
1.	Δ*med1*-2 X Δ*med1*-5	1.05^{a}	1.30^{a}	1.38^{a}
2.	Δ*med1*-2 X *2/9*	1.47^{b}	1.72^{ab}	2.02^{ab}
3.	*1/2* X Δ*med1*-5	1.68^{b}	2.08^{b}	2.47^{b}
4.	*1/2* X *2/9*	2.28^{c}	2.85^{c}	3.40^{c}

N=60 plants in 3 replicates of 20 plants each

Table 5.3. *med1* is required for full virulence of solopathogenic haploids

Sl. No.	Strain	Disease Index		
		7d	10d	14d
1.	Δ*med1* SG200	0.51^{a}	0.58^{a}	0.48^{a}
2.	SG200	1.45^{b}	1.55^{b}	1.63^{b}

N=60 plants in 3 replications of 20 plants each.



Supplementary Fig.5.1. Southern hybridization for testing *med1* gene deletion: *Eco*RI produces a 4274 bp band for the wild type and a 9738 bp band for the Δ*med1* mutant. Strain Δ*med1 a1b1*-3 in lane 5 was PCR positive and behaved similar to other mutants but was not further used in mating or virulence assays.

CHAPTER 6

CONCLUSIONS

The study of morphogenesis and sporulation in *Ustilago maydis* is important due to the link between these stages and virulence. *U. maydis* in addition to its importance as a pathogen is a model system for the study of obligate parasitism. The results obtained from this study are thus useful for study of other biotrophic fungal pathogens.

The sporulation process of *U. maydis* occurs in the host plant maize. The developmental stages in spore formation are well known although the regulatory networks involved in this process are not well characterized. The transcription factor *ust1* is part of a group of fungal specific proteins involved in major morphological changes like yeast-hypha transition and sporulation. The function of *ust1* deduced from deletion studies indicates it to be a suppressor of the dimorphic switch and sporulation during saprobic growth. This study was carried out to further investigate the role of *ust1* in morphogenesis and sporulation.

One of the objectives of the study was to identify the functions of putative targets of Ust1. The genes selected for characterization were also differentially expressed during the saprobic yeast phase of the pathogen. As an additional criterion for study the target genes were identified by the presence of putative binding sites of Ust1 in their promoters. The hypothesis for this objective was that the phenotype of Δ*ust1* is generated by the transcriptional silencing of one or more of these target genes. Six putative target genes were selected based on the above criterion and studied by deletion. None of these genes were required for saprobic growth, mating nor pathogenesis. The lack of observable phenotype for deletion mutants of these potential targets of

ust1 indicates that the dramatic phenotype of Δ*ust1* may be the effect of loss of function of several genes.

The Δ*ust1* mutant produces spore-like structures in culture that resemble teliospores that *U. maydis* produces *in planta*. To assess the possibility of using the Δ*ust1* mutant as a surrogate for sporulation studies, transcriptome analysis was conducted comparing expression patterns of Δ*ust1* in culture to wild type *in planta*. Another sporulation mutant Δ*hgl1* was used as a developmental check. The results indicated that the spore-like structures and teliospores share similar expression patterns of pigmentation genes. In addition *ssp1*, an indicator of teliospore maturation, showed similar pattern of expression in Δ*ust1* and wild type *in planta*. The difference in expression pattern of Δ*hgl1* and wild type was mainly in genes coding for secreted proteins and cell wall components. Results from transcriptional analysis indicate that *ust1* can be a useful model for study of specific processes during sporulation including development of pigmentation.

From the microarray study, another transcription factor required for mating and pathogenesis was identified. The ortholog of *medA*, a component of the central conidiation pathway of *Aspergillus nidulans* was identified and characterized. Transcription factor *med1* of *U. maydis* was found to be required for mating *in vitro*. The gene is also required for full virulence. The mating associated genes *mfa1, pra1* and *prf1* were found to be down-regulated in Δ*med1* mutant compared to wild type. The down-regulation of *prf1*, the transcription factor required for mating and pathogenicity in the *med1* mutant indicates the possibility of *med1* being a regulator of *prf1*. Prf1 is known to be regulated in a complex manner *in vitro* and *in planta*. The lack of complete abolition of virulence of the Δ*med1* mutant suggests to a partial regulatory role of *med1* over *prf1 in planta*.

Being a transcription factor, *ust1* regulates at least a few genes by direct binding. To confirm the ability of Ust1 to bind to its putative targets, assays such as chromatin immunoprecipitation can be used. A strain where the *ust1* gene is tagged with a single HA tag, made in this study could be useful for this purpose (Refer Appendix B). The UV suppressor mutants of ust1 mutant produced in this study also can be useful for further identification of partner proteins of *ust1*(Refer Appendix A). Further studies using these resources will be helpful in understanding the complete role of *ust1* as a master regulator of sporulation in *U. maydis*.

APPENDIX A

Introduction: The bHLH transcription factors usually have partner proteins that they dimerize with while binding to the promoter of genes they regulate. There is a possibility of Ust1 interacting with another protein physically. The *ust1* gene has a role in mating, sporulation and pathogenicity. The study of interaction of genes in these specific pathways is thus essential to get a clear picture of the function of *ust1*.

Methodology and Results: With the objective of identifying the interactors of the *ust1* gene, we generated 98 UV suppressor mutants of the Δ*ust1* mutant. The mutants were then classified into 10 phenotypic groups based on their colony morphology, growth in liquid culture and pigmentation. The Δ*ust1* mutant produces dark, dry colonies and is filamentous in culture. When grown for 48h or more, Δ*ust1* produces spore-like structures that resemble teliospores. The phenotypes of the suppressor mutants varied from wild type-like yeasty colonies with no pigmentation to dark filamentous cells that differed from *ust1* only in their inability to produce spore-like structures. From the UV mutants, two mutants that had wild type phenotype and mutants that produce filaments in culture but were un-pigmented were selected for attempted complementation with a genomic DNA library previously constructed in the plasmid pJW42 (Barrett et al., 1993).

The protoplasts produced from filamentous mutants were un-transformable. The loss of cell content and nuclei in interconnected filamentous cells during protoplasting could be a reason for this. The transformants obtained from the wild type-like mutants did not completely complement

the phenotype of the mutants. Several of the mutants that appeared complemented were not stable or were difficult to cure indicating the possibility of these being secondary mutations. Further attempts at complementation were abandoned due to the unstable nature of the transformants, difficulty in curing and difficulty in production of viable protoplasts from filamentous mutants.

Reference

Barrett, K.J., et al., 1993. Identification and complementation of a mutation to constitutive filamentous growth in *Ustilago maydis*. Molecular Plant-Microbe Interactions. 6**,** 274-283.

APPENDIX B

CONSTRUCTION OF A C-TERMINAL HA TAGGED *UST1* ALLELE *USTILAGO MAYDIS* REPLACEMENT STRAIN

Introduction: APSES domain transcription factors like StuA are known to bind to MluI cell cycle box sequences (MCB) with a recognition sequence of CGCG. The exact binding site of the *Aspergillus nidulans* transcription factor StuA has been determined to be the 8 base degenerate sequence A/TCGCGT/ANA/C (Dutton et al. 1997). In *A. nidulans* and several other fungi, this StuA response element (StRE) has been used to identify the genes regulated by their corresponding APSES proteins. In *U. maydis* also, the degenerate 8 base binding site has been used to find potaential genes regulated by *ust1*. To determine if Ust1 regulates genes by actually binding to consensus sequences, we proposed to perform Chromatin Immunoprecipitation (ChIP) experiments with a strain of *U. maydis* with the *ust1* gene tagged with an HA epitope. Construction of such a strain is described here.

Methodology and Results: To build a tagging construct, a 1kb region inside the open reading frame (ORF) and the 3' flank of the gene were PCR amplified separately. The primers used for PCR amplification of the ORF were: the forward primer ustHA-P1 (GGATCGCACACGCCCACCCATACC) including 5' *Kpn*I recognition sequence and the *ust1* ORF specific region; and the reverse primer ustHA-P2 (cGCttcgaaTTAAGCGTAGTCTGGGACGTCGTATGGGTAATGCCGCTGCCGTTGCCGAC AC) including (3' to 5') the reverse complement of the *ust1* ORF specific region from bases

followed by the HA epitope tag sequence with a stop codon and finally the 5' *Hind*III recognition sequence. Primers used for the *ust1* 3' flank region were: the forward primer ustHA-P3 (CCGgaattcGCATGCGTGCATCTTGTGGTCTTG) including (5' to 3') the *Eco*RI recognition sequence followed by 3' specific sequence; and the reverse primer ustHA-P4 (CCCgcggccgcGACGGGTGTATTTGGCGCTGAATG) including (3' to 5') the reverse complement of the gene specific bases followed by the recognition sequence for *Not*I site. The amplified ORF and 3' DNA were cloned into PCR 2.1-TOPO TA vector (Invitrogen, CA, USA). The ORF along with the HA tag was excised from PCR2.1-TOPO using *Kpn*I and *Hind*III and cloned into the polylinker 2 region of the vector pCbx-5 (Fig. 1) containing these sites. The 3' end was similarly excised from PCR 2.1-TOPO using *EcoR*I and *Not*I and cloned into polylinker 2 of pCbx-5. The entire construct consisting of the *ust1* partial open reading frame, HA tag, carboxin marker and *ust1* 3' sequence was excised using *Kpn*I and *Not*I, gel purified using the QIAquick gel extraction kit (Qiagen Inc., CA, USA) and used for *U. maydis* transformation.

U. maydis gene replacement transformants in the 1/2 strain background were selected on YEPS carboxin plates and tested for the integration of the tagging construct at the *ust1* locus by PCR. Four out of 12 transformants possessed the tagged *ust1* allele replacement. To ensure that the transformants were not altered phenotypically by the presence of the construct they were grown in culture and observed microscopically. All four replacement transformants showed wild type morphology. The expression of the tagged protein was tested by Western blotting using HRP conjugated anti-HA antibodies (Miltenyi Biotec, CA, USA). As a positive control we used a 85 KDa HA tagged protein and for a negative control the *U. maydis* 1/2 wild type strain was used. The 65 KDa Ust1 protein with the HA tag was expressed in all four transformants (Fig. 2)

Reference

Dutton, J.R., et al., 1997. StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. EMBO J. 16**,** 5710-5721.

Figures

The polylinker 1 : 0.6/5'T3/ *Sac*I.(*Bst*XI/ *Sac*II). *Not*I. *Eag*I. *Xba*I. *Spe*I. *Bam*HI. *Sma*I. *Pst*I. *Eco*RI

Polylinker 2: 2.89/ *Hind*III. *Cla*I. (*Hinc*II/ *Acc*I/ *Sal*I). *Xho*I. *Dra*II. *Kpn*I/T73'/

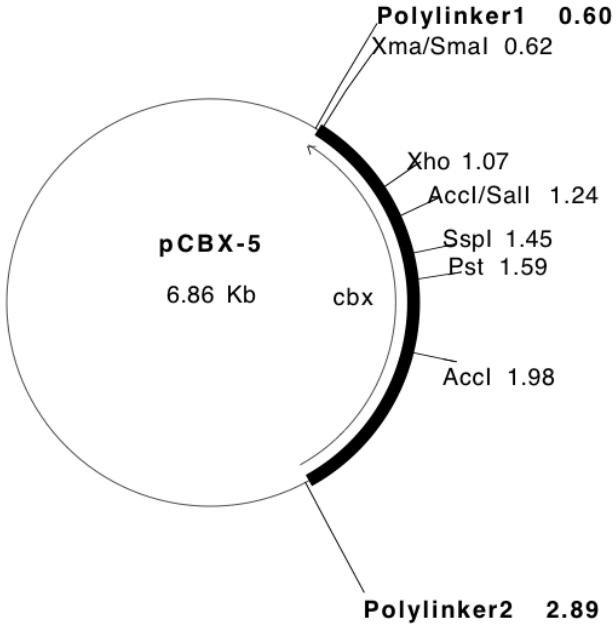


Figure B.1. Vector map of pCbx-5 vector**.**

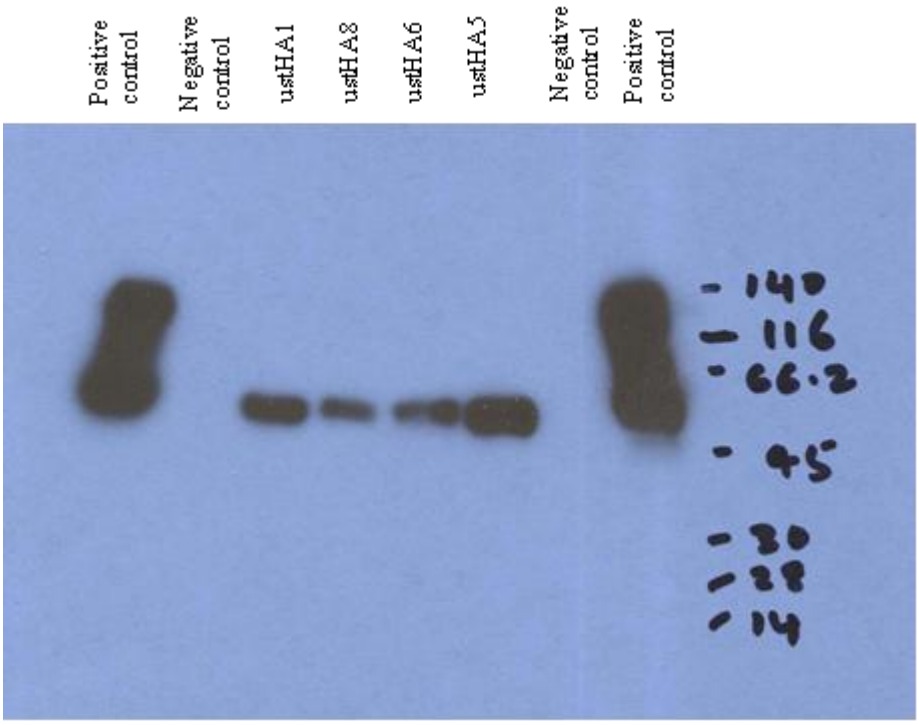


Figure B.2. Expression of HA tagged Ust1 protein