THE ROLE OF CALCINEURIN AND PROTEIN PHOSPHATASE 2A IN MORPHOLOGY, MATING, PATHOGENICITY AND CELL VIABILITY IN *USTILAGO MAYDIS*

by

JOHN DUICK EGAN

(Under the direction of Scott E. Gold)

ABSTRACT

Ustilago maydis is a dimorphic basidiomycete and the causal agent of corn smut disease. It serves as a genetic model for understanding dimorphism, pathogenicity, and mating response in filamentous fungi. Previous studies have elucidated signal transduction pathways involving cAMP-dependent protein kinase A (PKA) and mitogen-activated protein (MAP) kinases as being important for normal cellular function in *U. maydis*. Although most of the target substrates of these pathways are unidentified, it appears that the phosphorylation states of these target substrates determine mating, filamentous growth and pathogenicity. The purpose of this study was to determine the roles of two protein phosphatases that could be involved in the PKA and MAPK pathways in *U. maydis*; a reverse genetics approach was taken to identify the catalytic subunits of calcineurin (*ucn1*) and protein phosphatase 2A (*upa2*). Strains that are mutant in *ucn1* have a multiple budding phenotype with their cell cluster size inversely dependent on the inoculum density. Mating between *ucn1* mutants and wild-type strains was reduced, and mating between two *ucn1* mutants was severely reduced. This reduction in mating can be attributed to *ucn1* mutants' inability to upregulate *mfa1* during mating induction. Through pathogenicity assays, calcineurin was identified as a critical virulence factor within maize plants.

A synergistic phenotype was observed in the *uac1 ucn1* double mutant; both filamentous and multiple budding. The *ucn1* multiple budding mutant phenotype was determined to be epistatic to the filamentous solopathogenic SG200 phenotype. Cyclosporine A was successful in generating the u*cn1* mutant phenotype in wild-type strains and a distinct pattern of bud emergence was observed during calcineurin inhibition.

A gene disruption in the *U. maydis* PP2A (*upa2*) catalytic subunit was not obtained, even after multiple attempts. Consistent with this result, experiments with okadaic acid, a potent inhibitor of PP2A, stopped the growth of *U. maydis* cells at 2nM concentration, suggesting the probable lethality of a PP2A null mutation. Protein phosphorylation patterns between *U. maydis* wild-type and mutant strains could not be determined using a hexahistidine-tagged Prf1 transcription factor, or through western blotting of total protein lysates using anti-phosphoserine/threonine antibodies.

INDEX WORDS: *Ustilago maydis*, Calcineurin, CN, Protein Phosphatase 2B, PP2B, Protein Phosphatase 2A, PP2A, Protein Kinase A, PKA, cAMP, MAP Kinase, MAPK, Signal Transduction, Basidomycete, Fungal Genetics

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

INTRODUCTION

Ustilago maydis is a dimorphic basidiomycete and the causal agent of corn smut disease (Christensen, 1963). It serves as a genetic model for understanding dimorphism, pathogenicity, and mating response in filamentous fungi. The long-term goal of this project is to elucidate pathways involving morphogenesis and pathogenicity in order to provide possible strategies for the control of fungal diseases of plants. To accomplish this goal, an understanding of the genetic basis for pathogenicity is essential. While trying to decipher the link between *U. maydis* morphology and pathogenicity, two pathways have been discovered: cAMP dependent protein kinase (PKA) and the mitogen-activated protein kinase (MAPK), which are necessary for completion of its life cycle.

In the haploid stage, *U. maydis* is a saprophytic budding yeast, that undergoes a dimorphic switch to become an infectious, filamentous dikaryon. To generate the filamentous dikaryon, two compatible haploid yeast strains differing at two genetic loci (termed *a* and *b*) must mate (Holliday, 1961, 1974; Banuett, 1995; Kahmann et al., 1995; Kronstad and Staben, 1997).

The two alleles of the *a* locus (*a*1 and *a*2) are idiomorphs that encode pheromones (Mfa1/2) and pheromone receptors (Pra1/2) (Froeliger and Leong, 1991; Bolker et al., 1992; Bakkeren and Kronstad, 1994). In a compatible mating reaction, the pheromone (Mfa) interacts with the receptor (Pra) of the opposite mating type and induces increased pheromone production and conjugation tube formation followed, ultimately by a fusion between the strains (Bolker et al., 1992; Trueheart and Herskowitz, 1992; Banuett, 1995; Urban et al., 1996). The *b* locus has at least 25 allele specificities (Puhalla, 1970; Schulz et al., 1990), and all nonself

combinations are able to promote pathogenicity and sexual development. The *b* locus encodes two homeodomain proteins, bEast (bE) and bWest (bW), which form heterodimers when derived from different *b* alleles (Gillissen et al., 1992a, b; Kamper et al., 1995). The bE/bW heterodimer is the master regulator of pathogenicity and is necessary to establish a stable dikaryon (Kahmann et al., 1999).

Mating occurs on the surface of maize stigmas, and the dikaryotic infectious hypha grows to form a slightly swollen appressorium over epidermal cell-wall junctions of the host plant (Snetselaar, 1993; Snetselaar and Mims, 1993, 1994). The dikaryotic hypha in the initial phase of infection grows intracellularly (breaching the host cell-wall but not plasma membrane), then later intercellulary in the ovaries, and leaves eventually forming galls (Snetselaar, 1993; Snetselaar and Mims, 1993, 1994).

Gall formation in the plant presumably results from chemical signals between *U. maydis* and maize (Banuett and Herskowitz, 1996). The dikaryotic hypha becomes fragmented in gall tissue and undergo morphological changes to develop into teliospores (Banuett and Herskowitz, 1996). During teliospore formation, karyogamy takes place (induced by putative plant signals) (Snetselaar and Mims, 1994). Meiosis eventually occurs within mature teliospores to produce haploid basidiospores (Banuett and Herskowitz, 1996).

The budding yeast, *Saccharomyces cerevisiae*, provides a model for signal transduction in *U. maydis*. In yeast cells, there are multiple pathways, which each respond to distinct extracellular signals transmitted to produce specific cellular responses. Signaling in yeast cells involves multiple pathways, redundant components, crosstalk between pathways, components used in more than one pathway, or even one signal producing a multitude of

responses (Banuett, 1998). The budding yeast paradigm has become a valuable tool in understanding the highly conserved signal pathways found in filamentous fungi such as: *U. maydis*, *Magnaporthe grisea*, *Candida albicans*, *Schizophyllum commune*, and *Coprinus cinereus* (Banuett, 1998). As described below, antagonistic roles for the cAMP and MAPK pathway components produce different morphological phenotypes, which have been determined through mutational analysis of *U. maydis* (Durrenberger et al., 1998; Kahmann et al., 1999).

The first pathway determined to be a morphological determinant was the cAMP pathway (Gold et al., 1994). Based on genetic evidence, signaling through a G-protein alpha subunit (Gpa3) by an unknown ligand and receptor complex, activates the adenylate cyclase protein Uac1 (Regenfelder et al., 1997). Presumably, Uac1 converts ATP to cAMP (Barrett et al., 1993; Gold et al., 1994), which acts as a second messenger by binding to the regulatory subunit (Ubc1) of protein kinase A (PKA). This binding induces a conformational change in the subunits and a subsequent disassociation of the Ubc1 dimer from the catalytic subunits of PKA (Adr1) (Gold et al., 1994; Gold et al., 1997; Durrenberger et al., 1998). Once released, the primary PKA catalytic subunit, Adr1, can phosphorylate target protein(s) that are directly or indirectly responsible for budding growth (Gold et al., 1994; Gold et al., 1997; Durrenberger et al., 1998). In a *uac1* null mutant, cAMP is not produced (confirmed recently, A. Martinez personal communication); consequently, the Ubc1 dimer stays bound to the Adr1 subunits resulting in a loss of PKA activity. This loss of PKA activity (in a *uac1* or *adr1* mutant) prevents the phosphorylation of target proteins required for budding growth; thus a constitutive filamentous phenotype appears in the haploid stage (Gold et al., 1994; Durrenberger et al., 1998).

Using changes in haploid morphology, a forward genetic screen identified genes that affect growth form and also mating and pathogenicity in *U. maydis*. Mutagenesis of a constitutively filamentous haploid mutant strain (*uac1*-) allowed the isolation of suppressor mutants (termed *ubc* for *Ustilago* bypass of cyclase) that no longer require the production of cAMP to grow in the budding morphology (Barrett et al., 1993; Gold et al., 1994; Gold et al., 1997; Durrenberger et al., 1998). Using this forward genetic screen, five *ubc* genes have been cloned thus far.

As discussed above, *ubc1* encodes the regulatory subunit of PKA. In Ubc1 mutants, Adr1 is unregulated and an increase in PKA activity occurs independent of cellular cAMP levels (Gold et al., 1994; Gold et al., 1997). *U. maydis* strains with high levels of PKA activity produce lateral buds and have a multiple budding phenotype (Gold et al., 1994). An important connection exists between haploid cellular morphology and pathogenicity. In filamentous and multiple budding haploid strains where levels of PKA activity are affected (*ubc1*, *adr1* and *uac1* mutant strains), pathogenicity and mating are severely compromised (Barrett et al., 1993; Gold et al., 1994; Gold et al., 1997; Durrenberger et al., 1998).

The remaining *ubc* class of suppressor mutants are members of a pheromone signaling MAP kinase pathway (Mayorga and Gold, 1998, 1999; Andrews et al., 2000; Mayorga and Gold, 2001). In *U. maydis*, mating pheromones interact with receptors, presumably on the cell surface, and a signal is sent to a MAPK module by an undefined means. However, genetic evidence suggests that a Ras-like protein could be downstream of the receptor (J.W. Kronstad, personal communication). The MAPK module is activated and a presumed phosphorylation cascade occurs. The exact sequence of phosphorylation events remains undefined in *U.*

maydis, however, based on the *S. cerevisiae* paradigm, phosphorylation should occur from MAPKKK (Ubc4) to MAPKK (Fuz7/Ubc5) to MAPK (Ubc3/Kpp2) (In *S. cerevisiae* this occurs Ste11p, Ste7p, Fus3p) (Banuett, 1998).

Mutations in the genes involved in the pheromone response pathway have distinct phenotypes. A mutation in the *fuz7/ubc5* gene (MAPKK homolog) has a normal budding phenotype, however, mating, conjugation tube formation, and maintenance of filamentous growth in culture are all attenuated (Banuett and Herskowitz, 1994; Mayorga and Gold, 1999).

Disruption mutants in other genes of the MAPK module have somewhat similar phenotypes. In *ubc3/kpp2* (MAPK) mutant strains, normal cellular morphology is present, but reductions in pheromone response and pathogenicity occur (Mayorga and Gold, 1999; Muller et al., 1999; Andrews et al., 2000). The *fuz7/ubc5*, and *ubc3/kpp2*, genes are also shown to be virulence factors not absolutely required for disease development (Banuett and Herskowitz, 1994; Mayorga and Gold, 1999; Muller et al., 1999; Andrews et al., 2000). In contrast, the *ubc2* gene is critical for pathogenicity (Mayorga and Gold, 2001). The *ubc2* gene encodes a product with multiple recognizable protein-protein interaction motifs (Mayorga and Gold, 2001). Partial phenotypic complementation of a temperature sensitive *ubc2* allele by multiple copies of *ubc4* provides further evidence of a possible protein-protein interaction (Mayorga and Gold, 1998).

Crosstalk between the pheromone MAPK and cAMP pathways likely impinges on the HMG (high motility group) Prf1 transcription factor (Hartmann et al., 1996; Kahmann and Basse, 1997; Hartmann et al., 1999; Kahmann et al., 1999; Muller et al., 1999). Prf1 has phosphorylation consensus sites for MAPK and PKA, which indicate possible phosphorylation

by Adr1 and/or Ubc3/Kpp2 (Muller et al., 1999). Strains with mutations in MAP kinase phosphorylation consensus sites are attenuated in their ability to fuse with wild-type strains but can still stimulate filamentous growth in the pheromone tester strain CL13 (*a1bE1bW2*) (Muller et al., 1999). These results indicate that elimination of putative MAP kinase sites is insufficient in destroying Prf1 activity completely.

Calcineurin, a Regulator of Morphogenesis and Pathogenicity:

As demonstrated above, the phosphorylation state of PKA targets plays an important role in morphogenesis and pathogenicity. Therefore, we hypothesize that a protein phosphatase would also play an important role in morphogenesis and pathogenicity. Two protein phosphatases are potential candidates for acting as the antagonistic protein phosphatase to PKA, calcineurin and PP2A.

The calcineurin (CN) holoenzyme is comprised of three polypeptides: the catalytic (A) and regulatory (B) subunits, and calmodulin that binds reversibly (in the presence of Ca^{2+}) to the (A) subunit and forms a functional trimeric protein phosphatase (Kincaid, 1993; Shenolikar, 1994). CN has been studied extensively in the budding yeast *S. cerevisiae* and the fission yeast *Schizosacchromyces pombe*. In these yeasts, CN has multifunctional roles in post translational modification of proteins (Stark, 1996), while in filamentous fungi CN has been shown to have a variable effect on cell viability, virulence, and cell and colony morphology.

Two genes, *CNA1* and *CNA2* (also called *CMP1* and *CMP2*), encode budding yeast CN catalytic subunits (Cyert et al., 1991; Liu et al., 1991). The CN holoenzyme is required for promoting adaptation of haploid yeast cells to pheromone response *in vivo* (Cyert et al., 1991; Cyert and Thorner, 1992). During mating, pheromones induce the rise of cytosolic Ca^{2+} to

activate CN, which is involved in further downstream mitotic cellular signals (Withee et al., 1997). Genetic data show that the MAPK (Mpk1) and Ca^{2+} signaling through CN act redundantly through parallel pathways to regulate cellular processes important for growth and morphogenesis (Nakamura et al., 1996, 1997; Withee et al., 1997).

CN is involved in ion regulation in yeast cells through the activation of ion pumps and transcription factors. In *S. cerevisiae*, VMA (vacuolar membrane ATPase) genes are involved in intracellular ionic homeostasis and vacuolar biogenesis (Tanida et al., 1995). CN plays a role in conjunction with VMAs to regulate ion homeostasis (Cunningham and Fink, 1994; Tanida et al., 1995; Cunningham and Fink, 1996; Withee et al., 1998).

CN, through the Crz1p/Tcn1p transcription factor, activates the *ENA1/PMR2A* gene which encodes the primary sodium-pumping ATPase (Mendoza et al., 1994; Danielsson et al., 1996). In contrast, *ENA1/PMR2A* transcription is down regulated by an increase in PKA levels, which is an example of the antagonist role that PKA plays relative to CN in ion tolerance (Hirata et al., 1996; Mendoza et al., 1996).

In *S. cerevisiae*, two proteins, FKS1p and FKS2p of the glucan synthase complex, control cell wall 1,3-β-D-Glucan synthesis. During mating, elevated Ca^{2+} levels or increased temperatures, *FKS2* expression is controlled by CN and the *RHO1-PKC1*-regulated signaling pathway (Zhao et al., 1998). In trying to understand the mechanisms of CN induced transcription, a 24-bp region of the *FKS2* promoter named CDRE (calcineurin-dependent response element) was discovered as sufficient to confer calcineurin-dependent transcriptional induction (Stathopoulos and Cyert, 1997). Crz1p/Tcn1p (calcineurin-responsive zinc finger protein) is a calcineurin dependent transcription factor that, in conjunction with CDRE, regulates

several genes: *PMC1*, *PMR1*, *PMR2A* and *FKS2*, whose high levels of expression confer high tolerance to Ca^{2+}, Mn^{2+}, Na^{+} and cell wall damage, respectively (Matheos et al., 1997; Stathopoulos and Cyert, 1997). Other such mechanisms of calcineurin-dependent gene expression remain to be discovered in budding yeast.

In *S. pombe*, CN (*ppb1*$^{+}$) gene expression is controlled by the *ste11* transcription factor (Plochockazulinska et al., 1995). *S. pombe ste11* activation of CN occurs during mating pheromone response, sporulation and nitrogen starvation, which indicates the intricate role that CN plays in the cell cycle (Plochockazulinska et al., 1995). A *ppb1* null mutant is sterile and displays noticeable growth defects at low temperatures, such as the morphological phenotypes of multi-septate and branched cells at 22°C. When wild-type colonies are compared to null mutant colonies at 22°C, cell polarity is impaired and cytokinesis is delayed in mutant cells. In *ppb1* null mutants, frequency of branched cells increased 20-fold when the temperature is decreased from 33°C to 22°C; also, binuclear cells in *ppb1* null cells displayed mostly the X-shaped post anaphase microtubules that are characteristic of cells just prior to cytokinesis, indicating that cells prematurely arrest prior to cytokinesis (Yoshida et al., 1994).

CN plays a pivotal role in virulence and normal cell growth in other filamentous fungi. *Cryptococcus neoformans* is a heterothallic basidiomycete and a pathogen of immunocompromised humans. Cyclosporine A (CsA) and FK506 are anti-microbial drugs that inhibit signal transduction in *C. neoformans*, by forming a complex with FKBP12 (FK binding protein in *C. neoformans*) which in turn bind and inhibits CN *in vitro* at 37°C, but not at 24°C (Odom et al., 1997). CN (*CNA1*) mutants in *C. neoformans* are attenuated in growth under *in vitro* conditions that mimic a normal host environment such as growth at 37°C, 5% CO_2, or

alkaline pH, and are no longer pathogenic in an animal model of cryptococcal meningitis (Odom et al., 1997). These results strongly suggest that CN is required for *C. neoformans* virulence in humans.

CN is an essential gene in *Aspergillus nidulans*, and a null mutant (*cnaA*$^{-}$*)* produces cells arrested in early mitotic division, possibly at G1 (Rasmussen et al., 1994). Heterozygous diploid strains that produce haploid conidia lacking the *cnaA* gene undergo a maximum of two nuclear divisions. This indicates that cytoplasmic CN levels from the conidiophore are high enough for initial nuclear cell division (Rasmussen et al., 1994). In *Neurospora crassa*, growth of the hyphal tip is altered by the addition of a CN inhibitor. When FK506 is added to the medium, *N. crassa* hyphal tips branch extensively and expand more slowly. This phenotype is also observed through antisense RNA expression of the CN catalytic subunit *cna-1* gene (Prokisch et al., 1997). A mutation in the regulatory subunit of *N. crassa*, *cnb-1*, is lethal which further demonstrates that CN is required for normal vegetative growth (Kothe and Free, 1998).

Protein Phosphatase 2A a Regulator of Morphogenesis and Pathogenicity:

Protein phosphatase 2A (PP2A) is another heterotrimeric protein complex comprised of one catalytic subunit (C) and two regulatory subunits (A) and (B) (Shenolikar, 1994; Stark, 1996). In *S. cerevisiae*, PP2A has been implicated in actin cytoskeleton localization, cell cycle regulation and glycogen metabolism (Clotet et al., 1995; Lin and Arndt, 1995; Minshull et al., 1996; Evans and Stark, 1997). In *S. pombe*, PP2A is also involved in cell cycle regulation in addition to cell morphogenesis and cell wall synthesis (Lundin et al., 1991; Yanagida et al., 1992; Kinoshita et al., 1993; Kinoshita et al., 1996). In *N. crassa*, PP2A is involved in the

regulation of hyphal growth, macroconidiation, and other developmental processes (Yatzkan and Yarden, 1995; Yatzkan et al., 1998; Yatzkan and Yarden, 1999).

S. cerevisiae has two genes that code for the PP2A catalytic subunits, *PPH21* and *PPH22*; a null mutation in one gene produces no phenotype, but a double deletion displays a severe slow growth phenotype (Ronne et al., 1991; Lin and Arndt, 1995; Evans and Stark, 1997). However, a double *PPH21/22* deletion in conjunction with a *PPH3* deletion is lethal (Ronne et al., 1991). This suggests that the non-essential Pph3p has a residual overlapping function with PP2A, but both sequence and biochemical properties of the Pph3p protein are distinct from those of PP2A catalytic subunits (Hoffmann et al., 1994). In *S. pombe*, catalytic subunits of PP2A are also encoded by two genes, *ppa1* and *ppa2* (Lundin et al., 1991; Kinoshita et al., 1993). A single mutation, in either *ppa1* or *ppa2* causes a small-cell phenotype, suggesting premature entry into mitosis, while a disruption of both genes results in lethality (Kinoshita et al., 1993). The small-cell phenotype possibly indicates that PP2A is a negative regulator of the G2-M transition in the cell cycle (discussed in more detail below) (Kinoshita et al., 1993).

Through analysis of a *S. cerevisiae* temperature sensitive mutant (*pph21*^{Ts}), PP2A was found to be required for proper organization of the actin cytoskeleton, which affects bud morphology and chitin localization (Lin and Arndt, 1995). Cell wall defects in PP2A mutant strains are partially rescued through the addition of 1M sorbitol to the growth medium; which was hypothesized to be the result of activating the PKC cell integrity pathway in response to high osmotic stress (Evans and Stark, 1997). However, genetic evidence suggests that PP2A regulates cell wall integrity either downstream of known PKC1-MAPK components or through

a different pathway altogether; because high copy expression of PKC1 or MAPK components failed to rescue the PP2A mutant cell wall growth defect (Evans and Stark, 1997).

PP2A can have a dual role in the regulation of certain cellular processes. This is evident in the negative regulation of MPF (maturation promoting factor) necessary for the G2-M transition (Minshull et al., 1996), and glycogen metabolism in budding yeast (Clotet et al., 1995). In MPF regulation, PP2A regulates the G2-M transition by inhibiting one protein (Mih1) while simultaneously activating the protein kinase Swe1 (Minshull et al., 1996). In glycogen metabolism, PP2A also presents a dual role by repressing glycogen phosphorylase while also activating glycogen synthase; this combination results in an increase in glycogen synthesis and a repression in glycogen breakdown (Clotet et al., 1995).

Very little has been done to explore the role of PP2A in filamentous fungi. Inactivation of *pph-1*, the catalytic subunit PP2A of *N. crassa*, is lethal (Yatzkan and Yarden, 1995). A commonly used inhibitor of PP2A, okadaic acid, had no effect on *N. crassa in vivo*, however, two PP2A inhibitors, cantharidin and calyculin A, perturbed hyphal morphology in a temperature dependent manner. Inactivation of the regulatory subunit (*rgb-1*)of *N. crassa* by repeat-induced point (RIP) mutation resulted in progeny that grew slowly with abnormal hyphal morphology. RIP *rgb-1* strains were also female sterile and produce abundant amounts of arthroconidia (Yatzkan and Yarden, 1999). These data suggest that PP2A is involved in regulating the budding subroutine and macroconidiation process.

Fascinatingly, a possible substrate for PP2A is PKA. The catalytic subunit of PKA has been shown to autophosphorylate on the threonine residue at amino acid 197 (Thr-197), which is essential for its optimal kinase activity (Steinberg et al., 1993; Cauthron et al., 1998). Liauw

and Steinberg (1996) have suggested that PP2A (or a closely related enzyme) can dephosphorylate Thr-197 on the catalytic subunit of PKA, thereby reducing its optimal activity. Although, in *N. crassa* it appears that an increase in PKA activity is morphologically dissimilar (mislocalization of septa, and perturbed hyphal growth polarity) to loss of PP2A activity phenotypes; this finding may not be the case in other organisms (Yatzkan et al., 1998).

Protein Phosphorylation in *Ustilago Maydis*

Protein kinase signal transduction pathways transmit signals by the phosphorylation of target proteins that affect downstream cellular functions. In *U. maydis*, the MAP kinase and PKA pathways impinge on one known transcription factor, Prf1, that acts as a pathogenicity determinant (Hartmann et al., 1996; Hartmann et al., 1999; Muller et al., 1999). Other unknown common and unshared target proteins almost certainly exist between these two signaling pathways. If CN acts antagonistically to PKA in regulating a morphological determinant, we hypothesized that CN mutants would have a multiple budding morphology similar to a *ubc1* mutant. This multiple budding morphology would result from PKA phosphorylation on target substrates in the absence of the antagonistic protein phosphatase. Since genes that are involved in regulating morphology are also determinants in mating and pathogenicity, we hypothesized that CN mutants would also be compromised in these functions as well. If PP2A acts antagonistically to PKA, then physiological phenotypes would be like those described above for CN. If, however, PP2A is involved in the regulation of PKA by desphosphorylating the catalytic subunit and thereby inactivating it, a mutation in PP2A would have the phenotype of a constitutively active PKA, which is that of a *ubc1* mutant (multiple budding).

The goals of the project detailed here were to understand the roles of two protein phosphatases, CN and PP2A, in morphogenesis, mating, and pathogenicity in *U. maydis*. The method by which we aimed to analyze the involvement of these protein phosphatases in the above processes was via the cloning and subsequent disruption of their catalytic subunits. To further elucidate phosphorylation patterns that control cellular processes, an attempt was made to visualize phosphorylated target proteins and to determine commonalities and differences between wild-type and various characterized mutant strains affected in morphogenesis, mating, and pathogenicity. This investigation was intended to allow an initial assessment of the degree of interrelatedness of the pathways, particularly the role of protein phosphatases, CN and PP2A, in counteracting the phosphorylation by the MAPK and/or PKA pathways.

PROJECT OBJECTIVES

The following objectives were designed to answer questions regarding the role of CN and PP2A in *U. maydis* and their relation to the PKA and MAP kinase pathways.

Primary Objective 1: Molecular analysis of the *U. maydis* calcineurin (*ucn1*) catalytic subunit.

Specific Aim A: Clone, sequence and characterize the *ucn1* gene.

Specific Aim B: Disrupt the *ucn1* gene and characterize the mutant for its effect in dimorphism, pathogenicity, and mating.

Specific Aim C: Determine epistatic/synergistic relationships between PKA (*uac1*, *adr1* and *ubc1*) and *ucn1* mutants.

Primary Objective 2: Molecular analysis of the *U. maydis* Protein Phosphatase 2A (*upa2*) catalytic subunit.

Specific Aim D: Clone, sequence and characterize the *upa2* gene.

Specific Aim E: Disrupt the *upa2* gene and characterize the mutant for its effect in dimorphism, pathogenicity, and mating.

Primary Objective 3: Compare protein phosphorylation patterns of protein kinase and protein phosphatase mutants with wild-type strains.

Specific Aim G: Extract and quantify protein from wild-type and mutant strains.

Specific Aim H: Determine differences in phosphorylation patterns between protein kinase mutants, protein phosphatase mutants, and wild-type strains, through western blotting with anti-phospho-(serine, threonine, tyrosine) antibodies.

Specific Aim I: Construct a six histidine tag Prf1 protein and determine if functional *in vivo*.

Specific Aim J: Determine hexahistidine tagged Prf1 phosphorylation differences between protein kinase mutants, protein phosphatase mutants, and wild-type strains, through western blotting with anti-phospho-(serine, threonine, tyrosine) antibodies.

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CHAPTER 2

THE ROLE OF CALCINEURIN AND PROTEIN PHOSPHATASE 2A IN MORPHOLOGY, MATING, PATHOGENICITY AND CELL VIABILITY IN *USTILAGO MAYDIS*

ABSTRACT

Ustilago maydis is a dimorphic basidiomycete and the causal agent of corn smut disease. It serves as a genetic model for understanding dimorphism, pathogenicity, and mating response in filamentous fungi. We are interested in identifying genes required for these processes. Previous studies indicate the importance of regulating cAMP dependent Protein Kinase A (PKA) for normal cellular function in *U. maydis*. Although most of the target substrates of PKA are unidentified, it appears that the phosphorylation states of PKA substrates determine filamentous growth and pathogenicity. The purpose of this study was to determine the roles of two protein phosphatases that potentially act antagonistically to PKA. We have taken a reverse genetics approach to identify the catalytic subunits of calcineurin (CN, protein phosphatase 2B or PP2B) and protein phosphatase 2A (PP2A) in *U. maydis*. A mutation in the CN catalytic subunit, *ucn1*, causes a dramatic multiple budding phenotype with cell cluster size inversely dependent on the inoculum density. Mating between a *ucn1* mutant and a wild-type strain is slightly attenuated. However, mating between two *ucn1* mutants is severely reduced. The pathogenicity of *ucn1* mutant strains is minimal when compared to wild-type strains, but this is not solely due to the mating defect as indicated by the reduced pathogenicity of an SG200 *ucn1* mutant constructed in a solopathogenic haploid strain. Through genetic and inhibitor analysis, strains with nonfunctional *ucn1* and *uac1* were shown to generate a synergistic phenotype, which is both filamentous and multiple budding. Additionally, the solopathogenic haploid filamentous strain, SG200, has a multiple budding phenotype when disrupted in *ucn1*. A gene disruption in the *U. maydis* PP2A (*upa2*) catalytic subunit was not obtained, even after multiple attempts. Consistent with this result, experiments with okadaic

acid, a potent inhibitor of PP2A, stopped the growth of *U. maydis* cells at 2nM concentration, suggesting the probable lethality of a PP2A null mutation.

INTRODUCTION

U. maydis, the causal agent of corn smut disease, is a heterothallic basidiomycete (Christensen, 1963). In the haploid stage, *U. maydis* exists as a saprophytic budding yeast that undergoes a dimorphic switch to become an infectious filamentous dikaryon. To generate this filamentous dikaryon, two compatible haploid yeast strains differing at two genetic loci (termed *a* and *b*) must mate (Holliday, 1961, 1974; Banuett, 1995; Kahmann et al., 1995; Kronstad and Staben, 1997). The two alleles of the *a* locus (*a*1 and *a*2) are idiomorphs that encode pheromones (Mfa1/2) and pheromone receptors (Pra1/2) (Froeliger and Leong, 1991; Bolker et al., 1992; Bakkeren and Kronstad, 1994). In a compatible mating reaction, the pheromone (Mfa) interacts with the receptor (Pra) of the opposite strain and induces increased pheromone production and conjugation tube formation; followed ultimately by a fusion between the strains (Bolker et al., 1992; Trueheart and Herskowitz, 1992; Banuett, 1995; Urban et al., 1996). In contrast, the *b* locus has at least 25 allele specificities (Puhalla, 1970; Schulz et al., 1990), and all nonself combinations are able to promote pathogenicity and sexual development. The *b* locus codes for two homeodomain proteins, bEast (bE) and bWest (bW), which form heterodimers when derived from different alleles (Gillissen et al., 1992; Kamper et al., 1995). The bE/bW heterodimer is the master regulator of pathogenicity and is necessary to establish a stable dikaryon (Kahmann et al., 1999).

In *U. maydis*, two pathways are known to play antagonistic roles in producing different morphological phenotypes: the cAMP pathway and the pheromone response MAP kinase

pathway (Durrenberger et al., 1998; Kahmann et al., 1999; Mayorga and Gold, 1998, 1999; Andrews et al., 2000; Mayorga and Gold, 2001). Based on genetic evidence and interactions in similar well-characterized pathways from other organisms, signaling through a G-protein alpha subunit (Gpa3) (Regenfelder et al., 1997) activates the adenylate cyclase protein Uac1 which converts ATP to cAMP (Barrett et al., 1993; Gold et al., 1994a). Cyclic AMP acts as a second messenger by binding to Ubc1, the regulatory subunit of protein kinase A (PKA). This binding induces a conformational change in the subunits and a subsequent disassociation of the Ubc1 dimer from the catalytic subunits of PKA (Adr1) (Gold et al., 1994a; Gold et al., 1997; Durrenberger et al., 1998). (Note that this statement is speculated based on the paradigm; since no evidence exists in U. maydis, this claim is based on phenotypes, not biochemistry.) Upon release, PKA phosphorylates target proteins that are directly or indirectly responsible for budding growth (Gold et al., 1994a; Gold et al., 1997; Durrenberger et al., 1998). Haploid strains with a mutation in *uac1*, or *adr1* result in a loss of PKA activity, which produces a filamentous phenotype (Gold et al., 1994a; Regenfelder et al., 1997; Durrenberger et al., 1998). In *ubc1* mutants, Adr1 is presumed to be unregulated, causing an increase in PKA activity independent of cellular cAMP levels; this, in turn, results in lateral buds and a multiple budding phenotype (Gold et al., 1994a). An important connection exists between haploid cellular morphology and pathogenicity. In filamentous and multiple budding haploid strains where levels of PKA activity are affected (*ubc1*, *adr1* and *uac1* mutant strains), pathogenicity and mating are severely compromised (Barrett et al., 1993; Gold et al., 1994a; Gold et al., 1997; Durrenberger et al., 1998). With this in mind, the phosphorylation state of PKA substrates are believed to be determinants of morphology and pathogenicity. Therefore, our

objective for the work reported here was to determine the exact roles of two protein phosphatases that potentially act antagonistically to PKA by dephosphorylating PKA substrates: calcineurin (CN) which has been shown in yeast and mammalian systems to act antagonistically to PKA (Kincaid, 1993; Shenolikar, 1994), and PP2A which acts antagonistically to PKA in glycogen metabolism in *S. cerevisiae* (Clotet et al., 1995).

The calcineurin (CN) holoenzyme is comprised of three polypeptides: the catalytic (A) and regulatory (B) subunits, and calmodulin that binds reversibly (in the presence of Ca^{2+}) to the (A) subunit and forms a functional heterotrimeric protein phosphatase (Kincaid, 1993; Shenolikar, 1994). CN has been studied extensively in the budding yeast *S. cerevisiae* and the fission yeast *S. pombe*. In *S. cerevisiae* two genes *CNA1* and *CNA2* code for catalytic subunits of CN and a single gene, *CNB1*, encodes the regulatory subunit (Cyert et al., 1991; Liu et al., 1991; Cyert and Thorner, 1992). Yeast cells with mutations in either the regulatory subunit, or both catalytic subunits, are viable with no morphological phenotypes, however, mutant cells are unable to recover from cell cycle arrest after pheromone exposure (Cyert et al., 1991; Cyert and Thorner, 1992; Mizunuma et al., 1998; Mizunuma et al., 2001) and have increased ion sensitivity (Cyert et al., 1991; Cyert and Thorner, 1992; Parent et al., 1993; Eng et al., 1994; Chaudhuri et al., 1995; Garrett-Engele et al., 1995; Withee et al., 1998). In *S. cerevisiae*, it has been shown that PKA and CN act antagonistically to control at least one transcription factor which is involved in ion and cell wall synthesis regulation (Matheos et al., 1997; Stathopoulos and Cyert, 1997).

In filamentous fungi, CN has been demonstrated to have an effect on cell viability, virulence, and cell and colony morphology. In *Cryptococcus neoformans* CN (*CNA1*),

mutants are sensitive to conditions that mimic a normal host environment and are no longer pathogenic in an animal model of cryptococcal meningitis (Odom et al., 1997a). Evidence suggests that CN also plays an important role in appressorium development and virulence in the rice blast fungus *Magnaporthe grisea* (Viaud et al., 2002). CN is an essential gene in two other filamentous fungi *Aspergillus nidulans*, and *Neurospora crassa* (Rasmussen et al., 1994; Prokisch et al., 1997; Kothe and Free, 1998).

Protein phosphatase 2A is also heterotrimeric protein complex comprised of one catalytic subunit and has two regulatory subunits (A) and (B) (Shenolikar, 1994; Stark, 1996). In *S. cerevisiae*, PP2A has been implicated in the actin cytoskeleton localization, cell cycle regulation and glycogen metabolism (Clotet et al., 1995; Lin and Arndt, 1995; Minshull et al., 1996; Evans and Stark, 1997). PP2A is also involved in *S. pombe* cell cycle regulation, in addition to cell morphogenesis and cell wall synthesis (Lundin et al., 1991; Yanagida et al., 1992; Kinoshita et al., 1993; Kinoshita et al., 1996). In the filamentous fungus, *N. crassa*, PP2A is an essential gene involved in the regulation of hyphal growth, macroconidiation, and other developmental processes (Yatzkan and Yarden, 1995; Yatzkan et al., 1998; Yatzkan and Yarden, 1999). Interestingly, a possible substrate for PP2A is PKA. The catalytic subunit of PKA has been shown to autophosphorylate on threonine residue (Thr-197), which is essential for its optimal activity (Steinberg et al., 1993; Cauthron et al., 1998). Liauw and Steinberg (1996) have suggested that PP2A (or a closely related enzyme) can dephosphorylate Thr-197 on the catalytic subunit of PKA, thereby reducing its optimal activity.

If CN acts antagonistically to PKA in regulating a morphological determinant, we hypothesized that CN mutants would have a multiple budding morphology similar to a *ubc1*

mutant. This multiple budding morphology would result from PKA phosphorylation on target substrates in the absence of CN dephosphorylation. Since genes that are involved in regulating morphology are also determinants of mating and pathogenicity, we hypothesized that CN mutants would also be compromised in these functions as well. If PP2A were to act antagonistically to PKA by dephosphorylating PKA substrates, physiological phenotypes would be like those described above for CN. Alternatively, if PP2A is involved in the direct regulation of PKA by dephosphorylating the catalytic subunit, thereby inactivating it, a mutation in PP2A would generate cells with constitutively active PKA; this, in turn, would yield a multiple budding phenotype similar to the *ubc1* mutant.

Here we describe the importance of CN and PP2A in *U. maydis*. CN mutants exhibit a multiple budding phenotype similar to *ubc1*, except that *ucn1* mutants can form much larger clusters. Mating and pathogenicity are compromised in *ucn1* mutants. Epistasis analysis through the use of double mutants and Cyclosporine A indicates that CN is required for filamentation in a SG200 solopathogenic haploid, but not in an adenylate cyclase mutant. Cyclosporine A was also used to determine how the multiple budding phenotype is formed in a *ucn1* mutant. A gene disruption in *upa2* was never obtained, although non-homologous integrated transformants were generated. Consistent with this finding, *U. maydis* is sensitive to okadaic acid, a PP2A inhibitor, at 2nM concentrations, suggesting that *upa2* is an essential gene.

MATERIALS AND METHODS

Fungal and bacterial strains and culture conditions

All *U. maydis* strains in this study (Table 1) were maintained on potato dextrose agar (PDA, Difco) supplemented to 2% agar (2PDA) and grown at 30°C. Transformants were grown on double complete medium with 1 M sorbitol (DCM-S) and 300µg/ml hygromycin B (Boehringer Mannheim) (Barrett *et al*., 1993), except that instead of 2% yeast extract, 0.5% yeast extract was used. Resistant progeny were selected on PDA amended with 150µg/ml hygromycin B. Potato dextrose broth (PDB, Difco) was used for all fungal liquid cultures, which were grown at 250 rpm at 30°C. *Escherichia coli* strains DH5α (Bethesda Research Laboratories) and DH10B cells (Gibco BRL, Gaithersburg, MD) were used for DNA manipulations (Ausubel *et al*., 2002). *E.coli* was grown in and on Luria Bertani (LB)-medium amended with appropriate antibiotics.

Nucleic acid manipulations

PCR amplifications, as well as cloning procedures, were carried out with standard techniques (Ausubel et al., 1987; Sambrook et al., 1989). Degenerate primers OSG06 (VCGDMHGQY) and OSG12 (TFVDMFN) were used for the amplification of *U. maydis* genomic DNA from strain 1/2. Reactions contained: 50 mM KCl, 10 mM Tris pH8.3, 2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 10 µM of each dNTP, 50pmol of each primer, and 2 units of *Taq* polymerase (Boehringer Mannheim). Reactions were performed in an Amplitron II Thermocycler (Barnstead/Thermodyne) with the following reaction times: 4 min denaturing 94°C, followed by 40 cycles of 94°C 1 min, 55°C for 1min, and 72°C for 2 min with an 8 min final 72°C dwell at the end. The polymerase chain reaction (PCR) product was cloned into

pCRAmpSK$^+$ (Stratagene) and sequenced from the vector primer sites (T7 and M13-48 rev). All sequencing was performed on the ABI Prism 310 Genetic Analyzer (PE Applied Biosystems) using either the ABI Prism Big Dye or the dRhodamine Terminator Cycle Sequencing Reaction kits (PE Applied Biosystems) according to the manufacture's directions. MacDNAsis PRO v3.5 (Hitachi Software Engineering) was used for DNA and protein sequence analysis. MacDNAsis Higgins alignment was used for all amino acid comparisons. Primers OSG06 and OSG12 were used to screen a *U. maydis* cosmid genomic library (Barrett *et al.*, 1993) by PCR (conditions same as above) to identify the full-length cosmid clone, cosucn1.

For *upa2* gene isolation degenerate primers, OSG06 and OSG10 were used for amplification of *upa2* from *U. maydis* genomic DNA (strain 1/2). PCR reaction times and cloning procedures were the same as above. The 450 bp PCR product was sequenced (from vector primer sites as above), and specific primers JDE201 (GGCAACTCACCTG ATACC) and JDE202 (GTCCAGAGTGTCGATAGACG) were made to screen the *U. maydis* cosmid library to retrieve cosupa2. The pUPA2 plasmid was made by subcloning a ~6 kb *BamH*I fragment containing the *upa2* gene from Cosupa2 into the *Bam*HI site of pCM619 (Kojic and Holloman, 2000).

To create an *ucn1* disruption construct, the following procedure was employed. Using sequence specific primers JDE116 (ACGGATCTCTCTTGCTCACC) and JDE113 (GTGGAAGAAGCCGTTCAGTT), a 2431bp PCR product was amplified from cosucn1 and cloned into pCR2.1 (TA cloning kit Invitrogen, La Jolla, Ca) and designated pP2B2400. The *Bgl*II site was destroyed in an empty pCR2.1 vector and designated pCR2.1B. Both pCR2.1B

and pP2B2400 were cut with *Not*I and *Bam*HI. The ~2431 bp insert from pP2B2400 was inserted into pCR2.1B and the resulting plasmid was designated pP2B2400B. A 2.7kb (*Bam*HI *Bgl*II) hygromycin cassette (from pIC19RHL) was inserted into a *Bgl*II site located in the catalytic core region of *ucn1*, and the resulting plasmid was designated pP2B::HYG. To complement a *ucn1* mutant strain, plasmid pUCN1 was made by subcloning a ~5 kb *Bam*HI fragment from cosucn1 into the *Bam*HI site of pCM619 (Carboxin resistant marker).

A *upa2* deletion construct was made by amplifying a 2323 bp fragment using primers JDE213 (ACAAGAAGGAAAGCCGTC) and JDE216 (TGCATCTCATCCCTC TTG) and cloning the fragment into pCR2.1 to produce pUPA2-2.3kb. The cloned fragment was then shuttled from pUPA2-2.3 to pCR2.1B as above to produce plasmid pUPA2-2.3B. Two *Nru*I sites within the catalytic core of the *upa2* gene were cut and *Bgl*II linkers (Promega) were ligated onto the resulting blunt ends. A 2.7kb (*Bam*HI *Bgl*II) hygromycin cassette (from pIC19RHL) replaced a 492 bp *Bgl*II fragment located in the catalytic core, and the resulting plasmid was designated pUPA2::HYG. All fungal transformations were performed according to Tsukuda *et al*. (1988).

To confirm *ucn1* and *uac1* gene disruptions, Southern blotting was performed using the non-radioactive DIG/Genius system (Boehringer Mannheim). For *ucn1* disruption verification, genomic DNA was isolated from *U. maydis* strains, digested with *Xba*I and *Hind*III, and blotted to a positively charged nylon membrane (Boehringer Mannheim). The blot was probed with a *ucn1* 2431 bp PCR fragment (previously described above). For *uac1* gene disruption verification, genomic DNA was isolated from *U. maydis* strains and digested with *BamH*I, and blotted as above. A 2019 bp *uac1* fragment was amplified from pFUZ60C (Barrett et al.,

1993) using primers JDE048 (GGTCAACATGCTTCCTCGC) and JDE049 (TCGAATTGAATGAC ACATC) to generate a *uac1* probe.

For northern analysis, RNA was isolated from 100ml *U. maydis* cultures using Triazol (Amerscham Pharmacia) reagent and following the manufacture's protocol. For RNA manipulation, standard molecular techniques were used (Ausubel et al., 1987; Sambrook et al., 1989). Radioactive probes were made using Prime-a-Gene Labeling System (Promega) and ^{32}P-dCTP (Amerscham Pharmacia). For *ucn1* northern blots, probes were made by labeling the previously mentioned 2431 bp *ucn1* PCR fragment. For a loading control, a ~2 kb fragment that detects the iron-sulphur succinate dehydrogenase message (*cbx*) was amplified and labeled as above (Keon et al., 1991). For *mfa1* northern analysis, the entire *mfa1* gene was amplified using primers JDE050 (ATGCTTTCGATCTTCGCTCA) and JDE051 (CTAGGCAACAACACAGCT GG) and labeled as above. All hybridizations were done with Church buffer (5mM EDTA, 0.25M Na_2HPO_4, 1% hydrolyzed casein, 0.17% H_3PO_4, and 7% SDS) and Zeta-Probe membranes (BioRad). All blots were pre-hybridized for 4 hrs and hybridized overnight at 68°C. The first set of washes were conducted at room temperature (RT) in 2 X SSC and 0.05% SDS for 40 min with the solution being changed three times. The second set of washes was at 50°C in 0.1 X SSC and 0.1% SDS for 40 min and the solution being changed twice. For signal detection, the Storm phosphoimager and ImageQuant software (Molecular Dynamics) were used.

To determine *mfa1* expression in *ucn1* mutant strains, the growing of cultures for RNA isolation was as follows. For each sample, 1ml of an overnight PDB culture was inoculated on four 2PDA plates containing 1% charcoal; for mating reactions, an extra 1ml of its respective

partner was used. Plates were dried in a transfer hood, then covered and wrapped with parafilm to be incubated for 24 h at 30°C. After incubation, fungal cultures were scraped from plates using a sterile bent glass rod and washed 2X 25ml with sterile distilled water before extracting the RNA with Triazol reagent. For a loading control a 1315 bp *upa2* fragment was amplified from pUPA2 with primers JDE212 (CACCGTTCGATTTGACCA) and JDE208 (GGTGCCGAAAAGATGGTA) and labeled as previously described.

Growth conditions for *ucn1* multiple budding cluster assays

A single *ucn1* mutant colony from a 2PDA plate that had grown for 3 days at 30^0C was used to inoculate 100ml PDB to produce an initial culture. After 24 h at 30^0C, three different flasks containing 100ml of PDB were inoculated with 1000µl, 100µl, and 10µl, respectively. For strains grown in PDB filtrate, a 500ml overnight culture of strain 6/1 was filter sterilized through a 0.22µm filter (Whatman). Filtrate (100ml) was measured into sterile flasks, and inoculated as described for the fresh media flasks.

For three-dimensional analysis of multiple budding cell clusters, a 100ml (inoculated with 10µl of strain 6/1) overnight culture was allowed to settle to the bottom of the flask. The colony clusters were removed and placed in a 1.5ml microcentrifuge tube. Molten 1% agarose (cooled to 60°C) was added to the tube and poured onto a chilled (at 4°C) drop suspension glass slide. Observations were made under a dissecting scope and photographed. Individual clusters were chosen and cut out in agarose blocks for ease in manipulating different planes of view.

Pathogenicity assay

Truckers Favorite (Imperial Garden Seed, Athens Seed Co.) seedlings were grown in sterilized potting soil and, at 7 days, were inoculated according to procedures previously described (Gold et al., 1997; Mayorga and Gold, 1999) with the following modifications: all *ucn1* mutant strains were at cell suspensions of 10^7 cells ml^{-1}, and each cell cluster was considered one point of inoculation, therefore having a count of 1 (e.g., Fig 7A panel A has a count of ~17, panel C has a count ~10). Disease symptom data were collected on 7, 10 and 14 days after inoculation. Disease ratings were as previously described (Gold et al., 1997). Briefly, ratings 0-5 were: 0, no disease; 1, anthocyanin or chlorosis; 2, leaf galls; 3, small stem galls; 4, large stem galls; 5, plant death due to disease (Table 2). Experiments to analyze pathogenicity were carried out a minimum of three times.

Plate mating and drop mating assays

For plate mating reactions, charcoal plates were made as previously described by Holliday (1974), except that 0.5% yeast extract was used instead of 2%. Five microliters from an overnight PDB culture was spotted on charcoal plates and dried in the transfer hood; the respective mating partner was then spotted in the same manner. After the plates were completely dried, they were wrapped in parafilm and incubated for 24 h at 30°C. Confrontational drop mating assays were conducted as described in Snetselaar et al. (1996) with modifications described in Mayorga and Gold (1999).

Microscopy

Photographs of fungal cell morphology were taken using a FujiFilm digital camera FinePix S1PRO on an Olympus BH-2 microscope with differential interference optics as previously

described (Mayorga and Gold, 1998). For fluorescent microscopy, 2mls of a fungal culture was centrifuged for 30 sec in a 1.5ml microcentrifuge tube. The sample was washed once in phosphate buffered saline (PBS) and resuspended in 1 ml of DEP fixing solution (20% DMSO, 50% EtOH in PBS). After 30 min of incubation at RT, the sample was centrifuged at 14 k rpm for 2 min and resuspended in 1µg/ml 4',6-diamindino-2-phenylindole in PBS (DAPI; Sigma). Samples were gently vortexed for 10 min, before being centrifuged at 13k x g for 2 min. They were then washed twice in PBS before viewing with a Zeiss Axioplan Universal microscope (Carl Zeiss) and photographed with a Zeiss MC100 microscope digital camera system. All confocal imaging was done with the assistance of Dr. John Shields at the University of Georgia Center for Ultrastructure Research.

Cyclosporine A and Okadaic acid procedures

Cyclosporine A (CsA) (Sigma) and FK506 (Alexis) were both diluted in 95% EtOH. For all CsA experiments listed below, 95% EtOH was used as a negative control. For time course studies, an overnight 100 ml PDB culture of wild-type strain 1/2 was set to G_0 according to Ruiz-Herrera *et al*. (1995). CsA was added to 5ml PDB cell culture of 10^5 cells per ml to reach a final concentration of 10µg/ml. For each time interval, 1ml of cells was removed, centrifuged at 5,000 x g for 5 min and resuspended in 10µl of DH_2O for observation. Samples were observed using a haemocytometer. For non-time course experiments, 50µl of an overnight 5ml PDB cell culture was added to PDB + 10µg/ml CsA. Cell cultures were grown for 24 h at 30°C before being photographed.

Okadaic acid sodium salt (OA) was purchased from Sigma and diluted in DH_2O. A 5ml overnight culture of wild-type strain 1/2 was counted on a haemocytometer and diluted to

10^5 cells per ml with PDB. Appropriate amounts of OA were added to each culture and grown under normal conditions. Cell counts were made at 12 and 28 h using a haemocytometer. For photography of OA cultures, 1ml of cells was centrifuged at 5,000 x g for 5 min and resuspended in 10µl of DH_2O.

RESULTS

Cloning and analysis of *ucn1* and *upa2*

Degenerate primers OSG06 and OSG12 were designed according to known CN catalytic subunit genes, based on the conserved sequence of the catalytic core and the regulatory subunit-binding domain (Fig. 1) (Means, 1994). A PCR fragment (794bp) was amplified from wild-type (wt) strain 1/2 genomic DNA, cloned, and sequenced. NCBI BLAST results indicate the PCR fragment's homology to other known CN catalytic subunit genes. Using a PCR-based sib selection strategy (Banuett and Herskowitz, 1994), the full-length cosmid clone, cosucn1, was recovered from a genomic cosmid library (Barrett et al., 1993). The *ucn1* gene was sequenced by primer walking to reveal a single open reading frame of 1836 nucleotides coding for a polypeptide of 626 amino acids (Appendix 1). Comparisons with database entries demonstrated striking similarities to other fungal CN catalytic subunits (Fig. 1). We designated the corresponding *U. maydis* CN catalytic subunit *ucn1*.

The catalytic subunit of PP2A was cloned in a similar manner, except in the initial step, the degenerate reverse primer was OSG10 (instead of OSG12) based on a conserved sequence found in many protein phosphatases (Fig. 2). The *upa2* gene consisted of 1038 nucleotides separated into two open reading frames by a 120 bp intron spanning between

nucleotides 478 to 597 (Appendix 2). The *upa2* gene encoded a putative 306 amino acid polypeptide with strong similarities to other PP2A catalytic subunits (Fig. 2).

ucn1 mutant analysis

To study the function of *ucn1*, we generated a mutant allele. This was accomplished by inserting a hygromycin resistance cassette into nine amino acids upstream from the conserved phosphatase activation site RGNHE (Fig. 1 and 3). The allele *ucn1*::hyg was introduced into the wild-type strain 1/2 by gene replacement, and verified though Southern and northern blots (Fig. 4 and 5), to produce the *ucn1* mutant strain 6/1 (see Table 1). Morphology of the 6/1 strain resembled a *ubc1* mutant phenotype; liquid grown cells had a cell separation defect (multiple budding). When comparing colony morphology, wild-type colonies appear flat while *ucn1* mutants appear more convoluted (Compare Fig. 6B to D). Complemented calcineurin mutants carrying the plasmid pUNC1 (pCM619 + *ucn1* gene) have wild-type budding patterns and colony morphology (Compare Fig. 6E to F).

Unlike wild-type or *ubc1* mutants, *ucn1* mutants produce multiple-budding clusters of varying size in response to growth at different inoculum concentrations in liquid media (Fig. 7A-C). At lower inoculum concentrations, large multiple budding clusters form, which can be seen easily without magnification. The multiple budding clusters appear to be sphere-shaped and rarely get larger than 1mM in diameter (Fig. 7D-I).

When comparing colony morphology between *ucn1* and other *U. maydis* haploid mutants, unexpected differences are revealed. The multiple budding strain *ubc1* has a wet colony phenotype when compared to the wild-type strain (Compare Fig. 8A to D). However, the *ucn1* strain, which is also multiple budding, does not have a wet phenotype (Fig. 8B).

There were two basic differences between the colony morphologies of haploid filamentous strains: invasive growth and color. The *uac1* (adenylate cyclase) mutant had a whitish-gray filamentous phenotype that grew invasively into the agar (invasive growth was determined by the ability to remove the fungus from agar plates without tearing/damaging the agar surface), while the solopathogenic haploid strain, SG200 (*a1::mfa2 bE1bW2*) had a white filamentous phenotype, that did not exhibit invasive growth. The PKA catalytic subunit mutant, *adr1*, had a grayish filamentous phenotype, however, it does not grow invasively into the medium (Fig. 8E). The differences between the *uac1* and SG200 colony morphologies became more apparent when they were coupled with a mutation in *ucn1*. The SG200 *ucn1* double mutant had a colony phenotype that resembled the single *ucn1* mutant (Compare Fig. 8B to H). Strikingly different was the colony phenotype of the *uac1 ucn1* double mutant, which had a different phenotype than either the *ucn1* or *uac1* single mutants: convoluted and filamentous (Compare Fig. 8B, C, and G). The *uac1 ucn1* double mutant does not have the *uac1* mutant invasive growth phenotype (data not shown).

Morphologically, the *ucn1* mutant cells appeared the same as wild-type cells when comparing nuclei content. A DAPI staining technique revealed a single nucleus per cell in *ucn1* mutants (Fig. 9 and 10). Often there were more than 10 cells that originated from a single source (Fig. 10A, white arrow), while large cell clusters may have formed from one centrally located cell within the cluster (Fig 10B).

The role of *ucn1* in pheromone response

The *ucn1* mutant (6/1, *a1b1*) was crossed in maize plants to the wild-type strain 2/9 (*a2b2*) to produce teliospores and, ultimately, progeny. Progeny analysis indicated that the 43/48

hygromycin resistant strains had the multiple budding phenotype (data not shown). Through this cross, strain 6/9 was identified as a *ucn1* mutant in an *a*2*b*2 mating type background and used in further studies. To determine the effect of the *ucn1* mutation on cell fertility, charcoal plate mating and confrontational assays were used. Figure 11 presents plate mating assays that were carried out on charcoal mating medium (Holliday, 1974). In matings between compatible wild-type strains (1/2 X 2/9), very strong filamentous growth was observed 24 h after co-spotting. However, when one strain was disrupted in *ucn1* (1/2 X 6/9 or 2/9 X 6/1), an easily visible, but significantly reduced, reaction was observed. If both mating partners were disrupted for *ucn1* (6/1 X 6/9), a very weak mating reaction occurred after 24 h. These data indicate that *ucn1* mutants are blocked in efficient formation of filamentous dikaryons by either pre- or post-mating defects.

To distinguish pre- and post-mating points of blockage to dikaryon formation, a confrontational drop assay was used (Snetselaar et al., 1996). This method determines whether *ucn1* mutants produced and/or responded to mating pheromone. It should be noted that pheromone peptide is not directly quantified in this assay; morphological responses are thus simply interpreted as a result of pheromone production and/or response. Wild-type strains possessing opposite *a* mating-type specificity (*b* is irrelevant in this assay) responded to each other by producing copious filaments (Fig. 12A). As reported by Snetselaar *et al*. (1996), *a*2 strains (top strain in all images) responded more rapidly than did *a*1 strains (bottom strain in all images). Apparently, *ucn1* mutant strains secrete less pheromone than wild-type strains, as indicated by the reduction of mating hyphae produced by the wild-type *a*2*b*2 strain when paired with the *a*1*b*1 *ucn1* strain (compare Fig. 12A and B). A slight reaction appears when *a*1*b*1

wild-type strains are paired with *ucn1 a2b2* strains, however this reaction is less pronounced when compared to wild-type reactions (compare Fig. 12A and C). No visible response was seen from either partner when two *ucn1* mutant strains were paired (Fig 12D). However, due to the morphology (or unusual hydrophobic characteristics) of the *ucn1* phenotype, a uniform margin of cells could not be produced in this assay (Fig 12, thin cell groups of *ucn1* mutants when compared to wild-type), bringing into question the significance of these results.

Northern analysis was used to determine whether Ucn1 influences the expression of the mating pheromone gene *mfa1* (Fig. 13). The expression of *mfa1* is increased in a *ucn1* mutant (Compare Fig. 13 6/1 to 1/2). Interestingly, this coincides with what is seen in a *ubc1* mutant (Hartmann et al., 1999; Muller et al., 1999). To examine the expression of *mfa1* during mating, the wild-type tester strain 2/11 (*a2b1*) was paired with 1/2 wild-type (*a1b1*) and the mutant strain 6/1 *ucn1* (*a1b1*) (*b* is irrelevant in this assay). Opposite to what is seen at a basal level, the expression of *mfa1* appeared reduced in conjunction with a *ucn1* mutant during mating induction (Fig. 13, compare 6/1 X 2/11 to 1/2 X 2/11). This result could account for the mating reduction seen in the two previous mating experiments: confrontational assay and the charcoal mating. These data indicate that a mutation in *ucn1* affects *mfa1* expression at a basal level and during pheromone induction.

The role of *ucn1* in virulence on maize

To determine the effect of mutation of the *ucn1* gene on pathogenicity, maize plants were inoculated with various pair-wise combinations of wild-type and *ucn1* single mutant-compatible mating partners. Plant inoculations were performed at least three times with the typical results of one such test shown in Table 2. The *ucn1* gene acts as a critical virulence and pathogenicity

factor, as evident in finding that tumors were never formed and solopathogenic haploids were capable of inducing only the weakest of symptoms; occasional sparse localized chlorosis on only about 40-60% of inoculated plants (Table 2).

Fungal hyphae were observed when staining symptomatic plant tissue of compatible crosses (Fig. 14). However, because a filamentous dikaryon was never observed within the plant when two *ucn1* mutants were crossed (Fig. 14 C and D, blue fungal hyphae on the cell surface), it was questionable whether the loss of virulence was caused by a pre-mating defect rather than a post-mating defect. To overcome this obstacle, *ucn1* was disrupted in the SG200 solopathogenic haploid strain, which no longer requires mating to be pathogenic. SG200 *ucn1*$^{-}$ (strain 6/26 and 6/27) pathogenicity results were similar to 6/1 X 6/9 inoculations, and multiple budding clusters of cells could only be seen around inoculation sites (Table 2; Fig. 15E and F).

The effect of cyclosporine A on *U. maydis*

Two specific inhibitors of CN are FK506 and cyclosporine A (CsA) (Breuder et al., 1994; Cardenas et al., 1994; Cardenas et al., 1995; Dolinski et al., 1997). FK506 had no visible effect on *U. maydis* at 50µg/ml (data not shown), which is fifty times the drug minimum inhibitory concentration on *C. neoformans* at 37°C (Odom et al., 1997a; Odom et al., 1997b). However, CsA affected wild-type strains at 10µg/ml concentrations. Although this concentration of CsA is ten-fold greater than what is seen to cause inhibition of CN in *C. neoformans* at 37 °C (Odom et al., 1997a; Odom et al., 1997b), the phenotype caused by CsA on wild-type strains is identical to a *ucn1* phenotype (Compare Fig. 16A, B, and C). As we predicted, the addition of CsA to *ucn1* mutant strains had no additional effect on morphology (Fig. 16C and D), unless the *ucn1* mutant strain was previously complimented with

a plasmid containing the full-length *ucn1* gene (Fig. 16E and F). These data coupled with existing data on CsA's mode of action lead us to conclude that CsA is able to inhibit CN in *U. maydis* cells.

For epistasis analysis, attempts were made to disrupt *ucn1* in other haploid mutant strains to no avail, except for the solopathogenic haploid strain SG200. The morphology of a SG200 *ucn1* mutant appears to be the same as a *ucn1* mutant in another wild-type haploid strain (Compare Fig. 8B and 8H). The phenotype of the SG200 *ucn1*$^{-}$ mutant strain is indistinguishable to that seen when SG200 is grown in the presence of CsA (Fig. 17F). A similar result (multiple budding phenotype) is seen when CsA is added to the diploid strain D132 (Fig. 17H), and the multiple budding mutant strain 1/68 (*ubc1*-) (Fig. 17B). A significant finding was that CsA seemed to have no effect on the *adr1* mutant strain 6/55 (Fig. 17 C and D).

Additionally, one of our hypotheses was that the filamentous *uac1* mutant phenotype would be epistatic to a *ucn1* phenotype. To test this hypothesis, a *uac1 ucn1* double mutant was created. Since a *uac1* mutant is unable to mate with a *ucn1* mutant (data not shown), it was necessary to complement one of the mutant strains prior to plant inoculations. To produce the *uac1 ucn1* double mutant, strain 2/27 (*uac1* mutant with the complementing plasmid pUAC1) was crossed to strain 6/1 (*ucn1* mutant) within maize plants; progeny were isolated from teliospores that conferred drug resistance to both phleomycin (linked to *uac1*) and hygromycin (linked to *ucn1*). Southern analysis indicated that of 74 progeny scored, only one was disrupted for both genes and the strain was designated 6/25 (Table 1; Fig. 4A and 4B). The phenotype of strain 6/25 appears to be a combination of the two single mutant phenotypes

(multiple budding and filamentous) (Fig 18D). The phenotype of strain 6/25 is similar to strain 1/9 (*uac1* mutant) grown in the presence of CsA (18C). An additional phenotype is seen when strain 1/9 is grown in the presence of CsA, with the possible breakdown of an extracellular matrix (Fig. 18A-C) that holds the filamentous cells together in liquid cultures.

To understand the formation of the *ucn1* mutant phenotype, a time course experiment was designed to determine bud site selection during CN inhibition. After setting cell cultures to G_0 (Ruiz-Herrera et al., 1995) and adding CsA, observations were made of growing cultures over 10 h. In wild-type growth conditions, mother cells form a single daughter bud at one of the poles, that when mature, is released via cell separation (Jacobs et al., 1993, 1994). The mother cells appear to form center septations; cell buds form from both ends of the mother cell, indicating that the first possible cell division was within the mother cell (Fig. 19C arrows). After 8 h, a definite pattern was seen where buds continued to form from the mother cell and subsequent daughter cells in a predictable manner. Fascinatingly, the mother cell was seen to have 2 or 3 long buds forming from its center septation at 10 h (Fig. 19D arrows).

Because CsA induced a *ucn1* mutant phenotype, an attempt was made to use CsA to prevent the infection of wild-type *U. maydis* strains in maize plants. Even at 5-fold of the minimum inhibitory concentrations (50µg/ml) with two-hour incubation periods, CsA had no effect on reducing pathogenicity of wild-type strains on maize plants (data not shown).

The effect of okadaic acid on *U. maydis*

A *upa2* deletion construct was made that removed 249 bp of the catalytic core (Fig. 2 and 20; Appendix 2). Five separate transformation attempts were made to disrupt *upa2* in the wild-type strain 1/2. Unfortunately, all 95 hygromycin resistant transformants were determined to be

ectopic integrations (data not shown). Because *U. maydis* responded to CsA, we thought a PP2A inhibitor might provide insight into the phenotype of a *upa2* deficiency. Okadaic acid (OA), a potent inhibitor of PP2A (Bialojan and Takai, 1988; Haystead et al., 1989; Cohen et al., 1990), was used on wild-type strain 1/2 in a cell growth assay to determine the effect of PP2A inhibition. At very low OA concentrations (2nM), *U. maydis* cells appeared swollen (Fig. 21B). As the concentration of OA increased, cells became more spherical in shape (Fig. 21C through E), while at the highest concentration tested, 12nM OA, cells appeared to be lysing (Fig. 21F). Cell counts taken at 12h and 28h revealed that at 2nM and 4nM OA, cells went through only two divisions (starting concentration of cells $1x10^5$ to final concentration of cells $4x10^5$), while at 8nM OA, cells completed only one division (final concentration of cells $2x10^5$) (Fig. 22). These data suggests that loss of PP2A activity results in lethality in *U. maydis*.

DISCUSSION

One of the goals of our laboratory is to identify fungal genes that are morphology and pathogenicity determinants. The haploid filamentous strain *uac1*$^-$ has been the leverage point of our forward genetic screen to find suppressor mutants in signal transduction pathways (Barrett et al., 1993; Gold et al., 1994b; Gold et al., 1997; Gold et al., 2000). In this screen, our lab has cloned and characterized five filamentous suppressor genes *ubc1-5* (Mayorga and Gold, 1998). Mutations in *ubc* genes have reduced the virulence of *U. maydis* within maize plants (Banuett and Herskowitz, 1994; Mayorga and Gold, 1999; Muller et al., 1999; Andrews et al., 2000; Mayorga and Gold, 2001). For this reason, haploid morphology is an instrumental marker in finding pathogenicity determinants. Because we believed that a *uac1* mutant

phenotype would be epistatic to a loss of protein phosphatase activity on target substrates, a reverse genetics approach was taken to identify the *U. maydis* catalytic subunits of PP2A and CN.

Both catalytic subunits had a high degree of homology compared to other protein phosphatase catalytic subunits (Fig. 1 and 2). In trying to understand the roles of these genes, an attempt was made to generate mutant alleles in both *ucn1* and *upa2*. Unfortunately, since cell growth appears to be halted in the presence of okadaic acid, a *upa2* null mutant is probably lethal (Fig. 21 and 22). Northern analysis was employed to determine if the regulation of *upa2* fluctuates during mating. Transcript levels of *upa2* appeared unchanged during mating (Compare Fig. 13 *upa2* to agarose gel control). Because the cells were not synchronized in the northern assay, it is difficult to determine what factor that played in understanding the expression of *upa2* during mating. Wild-type *U. maydis* cells become morphologically distorted when grown in the presence of okadaic acid. In *S. pombe* and *S. cerevisiae*, PP2A is believed to play a role in cytoskeleton organization and cell wall integrity (Lin and Arndt, 1995; Kinoshita et al., 1996; Evans and Stark, 1997). The swollen cell morphology seen after the addition of okadaic acid suggests that PP2A may have the same role in *U. maydis*. After the addition of okadaic acid, *U. maydis* cells appear to halt growth after two divisions (Fig. 22). These data suggest that *upa2* likely is an essential gene for cell viability.

Unlike PP2A, inhibition of CN activity induces a unique multiple budding cell cluster phenotype (Fig. 6C and D). We predicted this phenotype based on where we thought CN would play a role in the cAMP pathway. A *ubc1* mutant has a multiple budding phenotype because PKA activity is unregulated and target substrates are presumed to be hyper-

phosphorylated (Fig. 17A and 8D). We hypothesized, in the case of the CN mutant, that PKA target substrates were being phosphorylated, but CN was not able to dephosphorylate the target substrates, causing a *ubc1*-like phenotype (multiple budding and reduced pathogenicity). Our data support our hypothesis; the *ucn1* mutant strains have a multiple budding phenotype and are reduced in pathogenicity. However, our data indicate that the *ucn1* mutant phenotype is not exactly like a *ubc1* mutant phenotype (Compare Fig.17A to 6C, and 8D to 6D). Interestingly, even with the addition of exogenous cAMP, the *ucn1* mutant phenotype seems to be predominant (Fig. 16C and H). These results indicate that CN likely interacts both with PKA target substrates, as well as other protein kinases.

Phenotypic analysis was consistent with the interpretation that CN is inhibited by CsA (Fig. 16). This finding became a useful tool in understanding the effects of CN inhibition in other haploid mutant backgrounds and in determining CN's influence in bud site selection (Fig. 16, 17, 18, and 19). Figure 23 illustrates what occurs during bud site selection when CN is inhibited. Interestingly, a pattern can be discerned regarding where bud sites will be placed off the mother cell. The most fascinating aspect of this discovery is the consistency at which this pattern occurs. What appears to happen during CN inhibition is that the mother cell goes through nuclear division and makes a septation horizontally in the center of the cell. This compartmentalization explains how at 4 h there are consistently two identical daughter cells at the polar ends of the mother cell (Fig. 23A and 19B). The next set of buds occurs again at the polar ends of the mother cell, indicating that there is a cell separation defect (Fig 23B and 19C). The mother cell continues to divide at the polar ends until its fourth division, which occurs at the septation point; long central buds (Fig. 23B, bud site 4 and 19D) can be observed only after 8

h. The daughter cells divide at their polar ends in what seems to be a similar pattern to that of the mother cell. Unfortunately, due to the number, size, and structure of these clusters, following their budding pattern past 10 h is futile.

Large multiple budding cluster formations are an interesting phenomenon that cannot be fully explained yet (Fig.7). An obvious assumption about the formation of these clusters was that with an abundance of nutrition, large clusters of cells would form. However, when growing *ucn1* mutants in filtrate media extract, large clusters still formed when we expected to see small clusters of cells (data not shown). This result indicates that nutrition is not the essential requirement for large cell cluster formation. It also suggests that a compound secreted by cells is not inhibitory to large cell cluster formation. We report here the occurrence of these clusters, but are unable at this time to explain the cause behind their variable formation dependent on inoculum concentration.

We hypothesized that a *uac1* mutant phenotype would be epistatic to a *ucn1* mutant phenotype, but this was not the case. The *uac1 ucn1* mutant presents a phenotype that appears to be additive of the *ucn1* and *uac1* mutant phenotypes (Fig. 18D). Strain 6/25 appears not only filamentous, but also multiple budding (branching) when compared to strain 1/9 (*uac1*) (Compare Fig. 18A to D). This phenotype has a fuzzy donut-shaped appearance when grown on 2PDA, and lacks the invasive filamentous phenotype associated with the *uac1* mutant (Fig. 8 and data not shown). These data indicate that CN is required for filamentous invasive growth in a *uac1* haploid mutant. The question still remains whether or not CN is antagonistic to PKA and target substrates required for normal haploid budding growth.

However, because the a*dr1* mutation is epistatic to *ucn1* mutant phenotype (based on CsA data Fig 17C and D), a possible antagonistic interaction exists between PKA and CN in *U. maydis* morphogenesis (Fig. 24). In an *adr1* mutant, target proteins required for budding growth are not phosphorylated and filamentous phenotype is seen, even if CN is inhibited by CsA. These data suggests that CN acts antagonistically to PKA in U. maydis morphogenesis.

The solopathogenic haploid strain SG200 reveals the role of CN in forming the filamentous dikaryon that is essential for pathogenicity in maize plants. SG200 *ucn1* mutants exhibit the CN mutant phenotype of multiple budding cell clusters (Fig. 8 and 17F). Interestingly, unlike the *uac1 ucn1* mutant, the SG200 *ucn1* mutant is not a synergistic phenotype, which indicates a large difference between the various phenotypes termed "haploid filamentous" (Fig. 8C, E, and F). Unexpectedly, the diploid D132 exhibited the CN phenotype when grown in the presence of CsA (Fig. 17H). Because D132 and SG200 had the same phenotype when grown in CsA, their phenotypes were more closely related than they were to the *uac1* filamentous haploid phenotype (Compare Fig. 17F and H to Fig.18). This realization seems logical since D132 and SG200 can behave as pathogens within maize, whereas *uac1* haploid mutant cannot. Also, SG200 (and presumably D132) had an activated cAMP pathway required for bE/bW heterodimer activation, which is involved in the upregulation of genes that induce filamentous dikaryotic growth. In a *uac1* haploid mutant, cAMP is not produce (Martinez and Gold, unpublished data), which is a presumed requirement for PKA activity.

Figure 25 explains the possible role that CN has in the pheromone response pathway. Based on the phenotype of the SG200 *ucn1* mutant, CN appears required for filamentation and

pathogenicity on maize. At what specific point in the signal transduction pathway that CN plays a role has yet to be determined. CN also seems to be required for the proper regulation of both basal and mating induced pheromone gene (*mfa)* expression (Fig 13). An interesting point regarding *mfa1* regulation is that the *ucn1* mutant mimics the *ubc1* mutant's effect on pheromone gene expression (Hartmann et al., 1999; Muller et al., 1999). This result suggests that there is co-regulation by PKA and CN of the signaling pathway leading to pheromone gene regulation. Because an over abundance of PKA activity in a *ubc1* mutant has the same effect on *mfa1* expression as does a loss of CN activity, we are able to conclude that CN and PKA may modify the same target substrate.

The reverse genetics approach we have taken to identify candidate genes with an impact on morphology, mating, and pathogenicity has been successful, leading to two major findings. PP2A is essential for cell viability in *U. maydis*, although the exact cellular processes controlled by PP2A remains to be determined. A mutation in *ucn1* has a dramatic impact on cell morphogenesis, mating and pathogenicity. Therefore, PP2A and CN appear to be important potential targets for fungal control.

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Table 1. Strains of *Ustilago maydis*

Strain	Relevant Genotype	Source
1/2	*a1 b1*	Gold *et al.*, 1997
2/9	*a2 b2* (BX7A22, near isogenic to 1/2)	Gold *et al.*, 1997
1/9	*a1 b1 uac1*::*ble*[a]	Gold *et al.*, 1997
1/68	*a1 b1 ubc1::hyg*	Gold *et al.*, 1994
2/11	*a2 b1* (BX7A21, near isogenic to 1/2)	Gold *et al.*, 1997
2/21	D132 diploid (*a1a2 b1b2*)	Banuett and Herskowitz, 1994
6/1	*a1 b1 ucn1::hyg*	this study
6/9	*a2 b2 ucn1::hyg*	this study
6/25[b]	*a1 b1 uac1*::*ble ucn1::hyg*	this study
6/26	SG200 *ucn1::hyg*	this study
6/27	SG200 *ucn1::hyg*	this study
6/55	*a1 b1 adr1::ble*	Durrenberger *et al.*, 1998
7/20	SG200 (*a1::mfa2 bE1bW2*)	Muller *et al.*, 1999

[a] *uac1* gene disruption with phleomycin marker

[b] double mutant strain

Fig. 1. Alignment showing homology of Ucn1 to other calcineurin catalytic subunits from fungi and yeasts. Predicted translation products are in the following order from the top: *U. maydis*, Ucn1; *C. neoformans*, Cna1; *N. crassa*, Cna1; *A. nidulans*, CnaA; *S. cerevisiae*, Cna1p; *S. pombe*, Ppb1. OSG06 and OSG12, indicated by lines, are the amino acid sequences to which degenerate oligonucleotides were used to amplify the 794 bp *ucn1* fragment. OSG06 and OSG12 were based on the catalytic core and regulatory subunit-binding domain, respectfully. The asterisk indicates the insertion site of the hygromycin phosphotransferase cassette, 10 amino acids upstream of the RGNHE catalytic site. MacDNAsis PRO v3.5 (Hitachi Software Engineering) Higgins alignment function was used for protein sequence alignment.

```
                    1
U.ma   Ucn1         MTTPAQVDRQ Q--RLVKAIG QITNKPA--P PEID------ ---------- 30
C.ne   Cna1         MASPAT---- ---QTANAIA AINNRSNLVI PEID------ ---------- 27
N.cr   Cna1         M--------- ---------- ---------- ---------- ---------- 1
A.ni   CnaA         M--------- ---------- ---------- ---------- ---------- 1
S.ce   Cna1p        MSSDAIRNTE QINAAIKIIE NKTERPQSST TPIDSKASTV AAANSTATET 50
S.po   Ppb1         MTSGPH-NLE D--PTVRAIR QKNQ-----A PSHD------ ---------- 26

U.ma   Ucn1         ---FTIHTTD DGAQVSTQER VIKDVQAPAF QKPTDEQFFS AKDPSKPDIA 77
C.ne   Cna1         ---FTQHQLE NGEIVSTTER VIKDVQAPAM YVPTDDQFWS KVDKTKPDIA 74
N.cr   Cna1         ---------E DGSQVSTMER VCKDVQAPAM FKPSDEQFFE DETHTKPDIQ 42
A.ni   CnaA         ---------E DGTQVSTLER VVKEVQAPAL NKPSDDQFWD PEEPTKPNLQ 42
S.ce   Cna1p        SRDLTQYTLD DGRVVSTNRR IMNKVPAITS HVPTDEELFQ PNGIPRHE-- 98
S.po   Ppb1         ---FTIFVQE DGSSVSTLDR VVKNVQAPAT YIPTDVEFFD INEPDKPDLH 76

                                                                OSG06
U.ma   Ucn1         FLKNHFYREG RLTDDQARFI LTRATEILRQ EPNLLEVDAP ITVCGDMHGQ 127
C.ne   Cna1         FLKNHFYREG RLTEEQALYI LEKGGELLRS EPNLLEVDAP ITVCGDIHGQ 134
N.cr   Cna1         FLKQHFYREG RLTEEQALWI IREGTKLLRA EPNLLEMDAP ITVCGDVHGQ 92
A.ni   CnaA         FLKQHFYREG RLTEDQALWI IQAGTQILKS EPNLLEMDAP ITVCGDVHGQ 92
S.ce   Cna1p        FLRDHFKREG KLSAAQAARI VTLATELFSK EPNLISVPAP ITVCGDIHGQ 148
S.po   Ppb1         FLRNHFIREG RLSEEQTLYI IKKATEILKS EDNLIEVDAP VTVCGDIHGQ 136

                    ▶                                           *
U.ma   Ucn1         YYDLMKLFEV GGNPADTRYL FLGDYVDRGY FSIECVLYLW ALKIWYPDTL 177
C.ne   Cna1         YYDLMKLFEV GGNPADTRYL FLGDYVDRGY FSIECVLYLW SLKMWYPDTL 184
N.cr   Cna1         YYDLMKLFEV GGDPAETRYL FLGDYVDRGY FSIECVLYLW ALKIHYPKTL 142
A.ni   CnaA         YYDLMKLFEV GGDPAETRYL FLGDYVDRGY FSIECVLYLW ALKIWYPNTL 142
S.ce   Cna1p        YFDLLKLFEV GGDPATTSYL FLGDYVDRGS FSFECLIYLY SLKLNFNDHF 198
S.po   Ppb1         YYDLMEIFEV GGNPANTQYL FLGDYVDRGY FSIECLLYLW ALKIWYPKTL 186

U.ma   Ucn1         FLLRGNHECR HLTDYFTFKL ECKHKYSEEI YDLCMESFCT LPLAAVMNKQ 227
C.ne   Cna1         FLLRGNHECR HLTDYFTFKL ECKHKYSETV YNACMESFCN LPLAAVMNKQ 234
N.cr   Cna1         WLLRGNHECR HLTDYFTFKL ECKHKYSEAI YEACMESFCC LPLAAVMNKQ 192
A.ni   CnaA         WLLRGNHECR HLTDYFTFKL ECKHKYSERI YEACIESFCA LPLAAVMNKQ 192
S.ce   Cna1p        WLLRGNHECK HLTSYFTFKN EMLHKYNLDI YEKCCESFNN LPLAALMNGQ 248
S.po   Ppb1         WLLRGNHECA HLTDYFTFKL ECTHKYNIKV YEACLQSFNA LPLAAIMNKQ 236

U.ma   Ucn1         FLCIHGGLSP ELQTLDDLRS IDRFREPPTH GLMCDILWAD PLEDFGS--- 274
C.ne   Cna1         FLCIHGGLSP ELHTLDDLRS INRFREPPTQ GLMCDILWAD PLEDFGS--- 277
N.cr   Cna1         FLCIHGGLSP ELHTLDDIRN IDRFREPPTQ GLMCDILWAD PLEDFGQ--- 239
A.ni   CnaA         FLCIHGGLSP ELHTLEDIKS IDRFREPPTH GLMCDILWAD PLEDFGQ--- 239
S.ce   Cna1p        YLCVHGGISP ELNSLQDINN LNRFREIPSH GLMCDLLWAD PIEEYDEVLD 298
S.po   Ppb1         FLCVHGGLSP ELHTLNDIRM INRFCEPPTH GLMCDLLWSD PLEDFGS--- 287

U.ma   Ucn1         ---------- ---------- -EKTNEEFIH NHVRGCSYFF TYNAACQFLE 303
C.ne   Cna1         ---------- ---------- -EKTNENFLH NHVRGCSYFF TYNAACQFLE 306
N.cr   Cna1         ---------- ---------- -EKTTDFFVH NHVRGCSYFF SYSAACHFLE 268
A.ni   CnaA         ---------- ---------- -EKTGDYFIH NSVRGCSYFF SYPAACAFLE 268
S.ce   Cna1p        KDLTEEDIVN SKTMVPHHGK MAPSRDMFVP NSVRGCSYAF TYRAACHFLQ 348
S.po   Ppb1         ---------- ---------- -EKSNKHFIH NNVRGCSYFY SYQAVCTFLE 316
```

```
U.ma   Ucn1        RNQLLSIIRA HEAQDAGYRM YRKTKTTGFP SVMTIFSAPN YLDVYNNKAA 353
C.ne   Cna1        RNNLLSIIRA HEAQDAGYRM YRKTKTTGFP SVMTIFSAPN YLDVYSNKAA 356
N.cr   Cna1        KNNLLSIIRA HEAQDAGYRM YRKTRTTGFP SVMTIFSAPN YLDVYNNKAA 318
A.ni   CnaA        KNNLLSVIRA HEAQDAGYRM YRKTRTTGFP SVMTIFSAPN YLDVYNNKAA 318
S.ce   Cna1p       ETGLLSIIRA HEAQDAGYRM YKNTKTLGFP SLLTLFSAPN YLDTYNNKAA 398
S.po   Ppb1        NNNLLSVIRA HEAQDVGYRM YRKTKTTGFP SLMTIFSAPN YLDVYNNKAA 366

                                                  OSG12
                                                  <----
U.ma   Ucn1        VLKYENNVMN IRQFNCTPHP YWLPNFMDVF TWSLPFVGEK ITDMLIAILN 403
C.ne   Cna1        VLKYESNVMN IRQFNCTPHP YWLPNFMDVF TWSLPFVGEK ITDMLIAILN 396
N.cr   Cna1        VLKYENNVMN IRQFNCTPHP YWLPNFMDVF TWSLPFVGEK ITDMLIAILS 368
A.ni   CnaA        VLKYENNVMN IRQFNCTPHP YWLPNFMDVF TWSLPFVGEK ITDIVIAILN 368
S.ce   Cna1p       ILKYENNVMN IRQFNMTPHP YWLPDFMDVF TWSLPFVGEK VTEMLVAILN 448
S.po   Ppb1        VLKYENNVMN IRQFNCSPHP YWLPNFMDVF TWSLPFVGEK VSEMLISMLN 316

U.ma   Ucn1        VCSKEELEEE EEEDEIPTTP TSA------- AEE------- --------ET 431
C.ne   Cna1        CCTKEELEEE DEEFPL-NAP EPT------- DAE------- --------SA 425
N.cr   Cna1        TCSEEELRED SATT-SPGSA SPALPSAANQ DPD------- --------SI 402
A.ni   CnaA        TCSKEELEDE TPSTISPAEP SPPMPMDTV- DTE------- --------ST 402
S.ce   Cna1p       ICTEDELEND TPVIEELVGT DKKLPQAGKS EATPQPATSA SPKHASILDD 498
S.po   Ppb1        ICSKEELYET DLKESAPTQH KQPAPSENEN KADQE----- -------IDI 354

U.ma   Ucn1        AERRTLIKNK ILAVGRMSRV FALLREEAER VSELKS-SQT AKLPYGSLVL 480
C.ne   Cna1        AERRQIIKNK ILAVGRMSRV FSLLREESER VSELKSISGS NALPAGMLAS 475
N.cr   Cna1        EFKRRAIKNK ILAIGRLSRV FQVLREESER VTELKTVSGG -RLPAGTLML 451
A.ni   CnaA        EFKRRAIKNK ILAIGRLSRV FQVLREESER VTELKTAAGG -RLPAGTLML 451
S.ce   Cna1p       EHRRKALRNK ILAVAKVSRM YSVLREETNK VQFLKDHNSG V-LPRGALSN 547
S.po   Ppb1        EARRQIIKNK IMAIGRISRV FSVLREERES VSELKNVSGT QRLPAGTLML 404

U.ma   Ucn1        SSEAAKDAIA NFDDARKVDI ENERLPPDLI DADEAGPASP AEGARVSSPA 530
C.ne   Cna1        GAEGIKEAIQ GFEDARKSDI ENERLPPDII DPDEDKPASP SASPIMPATP 525
N.cr   Cna1        GAEGIKNAIS SFEDARKVDL QNERLPP--- ---------- --------SH 480
A.ni   CnaA        GAEGIKQAIT NFEDARKVDL QNERLPP--- ---------- --------SH 480
S.ce   Cna1p       GVKGLDEALS TFERARKHDL INEKLPPSLD ELKNENKKY- ---------- 586
S.po   Ppb1        GAEGIKNAIN SFDDARKLDI QNERLPP--- ---------- ---------- 431

U.ma   Ucn1        ---------- ---------- FEDMASPGSP ASPATPSSPI AGGH-----R 555
C.ne   Cna1        EEIPSEIPYD SPITGTPRTP ISSAIASGSP GSPGTPTSPS IGGPPLTAWR 575
N.cr   Cna1        DEV------- ---------- ---------- ---------- ---------- 483
A.ni   CnaA        DEV------- ---------- ---------- ---------- ---------- 483
S.ce   Cna1p       --------YE K--------- ---------- ---------- -------VWQ 592
S.po   Ppb1        ---------- ---------- ---------- ---------- ---------- 431

U.ma   Ucn1        RGHSRTSSLG TTMSSPSNRR RSLESTVSMI REALEGTDAA DDKHLEKLAN 605
C.ne   Cna1        PGHGRRTSLG TTKTSPSTRR RSLENTMHLI RDVVGGKDAQ GDGQLERLAE 625
N.cr   Cna1        ---------- -VKMQDEERA QALERATR-- -------EAD NDKKLQTLSR 513
A.ni   CnaA        ---------- -DRRSEEERR IALDRAQH-- -------EAD NDTGLATVAR 513
S.ce   Cna1p       KVHEH----- ---------- ---------- ---------- ---------- 597
S.po   Ppb1        ---------- -----SNSRR RSTD--LKAF EEVMNSSE-- DDTSIDHLVE 462
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U.ma   Ucn1          DITSPVSPKG TDPPAQARFA NK..... ..... ..... 627
C.ne   Cna1          VISSP--TKG GQ-------- GE..... ..... ..... 637
N.cr   Cna1          RLS------- ---------- TS..... ..... ..... 518
A.ni   CnaA          RISMSVRRIR KIPST----- TR..... ..... ..... 530
S.ce   Cna1p         ---------- -----DAKND SK..... ..... ..... 604
S.po   Ppb1          RFADKKSSL- ---------- --..... ..... ..... 471
```

Fig. 2. Alignment showing homology of Upa2 to other PP2A catalytic subunits from other organisms. Predicted translation products are in the following order from the top: *U. maydis*, Upa2 (*U.m*); *Arabidopsis thaliana*, P2A1 (*A.th*); *N. crassa*, PPh1 (*N.cr*); *S. cerevisiae*, Pph21p (*S.ce*); *S. pombe*, Ppa2 (*S.po*); *S. cerevisiae*, Pph22p (*S.ce*), *Homo sapiens* PP2Aα-isoform, PP2Aα (*H.s*). OSG06 and OSG10, indicated by lines, are the amino acid sequences, where degenerate oligonucleotides were used to amplify a 450 bp *upa2* fragment. The sequence between the two asterisks indicates where the deletion was made and a hygromycin cassette was inserted to construct p*UPA2*::*hyg*. Parameters for protein alignment was the same as in figure 1.

```
                    1
U.ma   Upa2         MVD------- ---------- ---------- ---------- ---------- 3
A.th   P2a1         MPSNG----- ---------- ---------- ---------- ---------- 5
N.cr   Pph1         MDTNMEDV-- ---------- ---------- ---------- GRVPAELTSS 18
S.ce   Pph21p       MDTDLDVPMQ DAVTEQLTPT VSEDMDLNN- -------NSS DNNAEEFSVD 42
S.po   Ppa2         MSVSGK---- ---------- ---------- ---------- ---------- 6
S.ce   Pph22p       MDMEIDDPMH GSDEDQLSPT LDEDMNSDDG KNNTKARSND EDTDEELEDF 50
H.sa   PP2Aα        MDEKV----- ---------- ---------- ---------- ---------- 5

U.ma   Upa2         ---------- ---------- ----ITEQDA WIAHLSECKQ LSENDIKRLC 29
A.th   P2a1         ---------- ---------- ------DLDR QIEQLMECKP LSEADVRTLC 29
N.cr   Pph1         NFEP------ ---------- --TTIPTLDG WIESLMNCKQ LAESDVQRLC 50
S.ce   Pph21p       DLKPGSSGIA DHKSSKPLEL NNTNINQLDQ WIEHLSKCEP LSEDDVARLC 92
S.po   Ppa2         ---------- ---------- ----IGEVDR WIEQLSRCEP LSEEDVIQMC 32
S.ce   Pph22p       NFKPGSSGIA DHKSSKPLKL TNTNINQLDQ WIEHLSKCEP LSEDDVARLC 100
H.sa   PP2Aα        -F-------- ---------- ----TKELDQ WIEQLNECKQ LSESQVKSLC 32

                       *                 OSG06
U.ma   Upa2         DKAREILLGE SNVQPVRCPV TVCGDIHGQF HDLSELFRIG GNSPDTNYLF 79
A.th   P2a1         DQARAILVEE YNVQPVKCPV TVCGDIHGQF YDLIELFRIG GNAPDTNYLF 79
N.cr   Pph1         EKAREVLQDE SNVQPVKCPV TVCGDIHGQF HDLMELFKIG GSCPDTNYLF 100
S.ce   Pph21p       KMAVDVLQFE ENVKPINVPV TICGDVHGQF HDLLELFKIG GPCPDTNYLF 142
S.po   Ppa2         DLAKEVLSVE SNVQSVRCPV TVCGDIHGQF HDLMELFNIG GPSPDTNYLF 82
S.ce   Pph22p       KMAVDVLQFE ENVKPINVPV TICGDVHGQF HDLLELFKIG GPCPDTNYLF 150
H.sa   PP2Aα        EKAKEILTKE SNVQEVRCPV TVCGDVHGQF HDLMELFRIG GKSPDTNYLF 82

U.ma   Upa2         MGDYVDRGYY SVETVTLLVA LKVRYRDRVT ILRGNHESRQ ITQVYGFYDE 129
A.th   P2a1         MGDYVDRGYY SVETVSLLVA LKVRYRDRLT ILRGNHESRQ ITQVYGFYDE 129
N.cr   Pph1         MGDYVDRGYY SVETVTLLVA LKIRYPNRIT ILRGNHESRQ ITQVYGFYDE 150
S.ce   Pph21p       MGDYVDRGYY SVETVSYLVA MKVRYPHRIT ILRGNHESRQ ITQVYGFYDE 192
S.po   Ppa2         MGDYVDRGYH SVETVSLLIA FKIRYPQRIT ILRGNHESRQ ITQVYGFYDE 132
S.ce   Pph22p       MGDYVDRGYY SVETVSYLVA MKVRYPHRIT ILRGNHESRQ ITQVYGFYDE 200
H.sa   PP2Aα        MGDYVDRGYY SVETVTLLVA LKVRYRERIT ILRGNHESRQ ITQVYGFYDE 132
                                                     *
U.ma   Upa2         CLRKYGNANV WKYFTDLFDY LPLTALIDDQ IFCLHGGLSP SIDTLDHIRS 179
A.th   P2a1         CLRKYGNANV WKYFTDLFDY LPLTALIESQ VFCLHGGLSP SLDTLDNIRS 179
N.cr   Pph1         CLRKYGNANV WKYFTDLFDY LPLTALIDNQ IFCLHGGLSP SIDTLDNIRA 200
S.ce   Pph21p       CLRKYGSANV WKMFTDLFDY FPITALVDNK IFCLHGGLSP MIETIDQVRE 242
S.po   Ppa2         CLRKYGNANV WQYFTDLFDY LPLTALIEDR IFCLHGGLSP SIDTLDHVRI 182
S.ce   Pph22p       CLRKYGSANV WKMFTDLFDY FPVTALVDNK IFCLHGGLSP MIETIDQVRD 250
H.sa   PP2Aα        CLRKYGNANV WKYFTDLFDY LPLTALVDGQ IFCLHGGLSP SIDTLDHIRA 182

                                  OSG10
U.ma   Upa2         IDRIQEVPHE GPMCDLLWSD PDDRCGWGIS PRGAGYTFGQ DISEAFNHNN 229
A.th   P2a1         LDRIQEVPHE GPMCDLLWSD PDDRCGWGIS PRGAGYTFGQ DIAAQFNHNN 229
N.cr   Pph1         LDRIQEVPHE GPMCDLLWSD PDDRCGWGIS PRGAGYTFGQ DISEAFNHNN 250
S.ce   Pph21p       LNRIQEVPHE GPMCDLLWSD PDDRGGWGIS PRGAGFTFGQ DVSEQFNHTN 292
S.po   Ppa2         LDRVQEVPHE GPICDLLWSD PDDRPGWGIS PRGAGYTFGP DIAEAFNHNN 232
S.ce   Pph22p       LNRIQEVPHE GPMCDLLWSD PDDRGGWGIS PRGAGFTFGQ DISEQFNHTN 300
H.sa   PP2Aα        LDRLQEVPHE GPMCDLLWSD PDDRGGWGIS PRGAGYTFGQ DISETFNHAN 232

U.ma   Upa2         GLTLVARAHQ LVMDGFNWSQ ERNVVTIFSA PNYCYRCGNQ AAIMEIDENL 279
A.th   P2a1         GLSLISRAHQ LVMEGFNWCQ DKNVVTVFSA PNYCYRCGNM AAILEIGENM 279
```

```
N.cr   Pph1          GLTLIARAHQ LVMEGYNWSQ DRNVVTIFSA PNYCYRCGNQ AAIMEIDEHL 300
S.ce   Pph21p        DLSLIARAHQ LVMEGYAWSH QQNVVTIFSA PNYCYRCGNQ AAIMEVDEN- 341
S.po   Ppa2          GLDLIARAHQ LVMEGYNWTT NHNVVTIFSA PNYCYRCGNQ AAIMGIDDHI 282
S.ce   Pph22p        DLSLIARAHQ LVMEGYSWSH QQNVVTIFSA PNYCYRCGNQ AAIMEVDEN- 349
H.sa   PP2Aα         GLTLVSRAHQ LVMEGYNWCH DRNVVTIFSA PNYCYRCGNQ AAIMELDDTL 282

U.ma   Upa2          KYT-FLQFDP APRAGEPLVS RRVPDYFL.. .......... .......... 306
A.th   P2a1          EQN-FLQFDP APRQVEPDTT RKTPDYFL.. .......... .......... 306
N.cr   Pph1          KYTLYVLFPP ---------- --------.. .......... .......... 310
S.ce   Pph21p        HNRQFLQYDP SVRPGEPSVS RKTPDYFL.. .......... .......... 369
S.po   Ppa2          NYA-FIQYDT APRKEELHVT RRTPDYFL.. .......... .......... 309
S.ce   Pph22p        HNRQFLQYDP SVRPGEPTVT RKTPDYFL.. .......... .......... 377
H.sa   PP2Aα         KYS-FLQFDP APRRGEPHVT RRTPDYFL.. .......... .......... 309
```

Fig. 3. *ucn1* disruption construct strategy. A *Bgl*II (B)-*Bam*HI(Bm) hygromycin phosphotransferase cassette from pIC19RHL was inserted into a *Bgl*II (B) site in the center of the catalytic core of *ucn1*. Restriction enzyme digests of *U. maydis ucn1* disrupted strains with *Xba*I (X) and *Hind*III (H) were predicted to produce two bands (1294 bp, 745 bp) while the wild-type *ucn1* gene should produce a single larger band (2039 bp). Bent arrows indicate the direction of transcription.

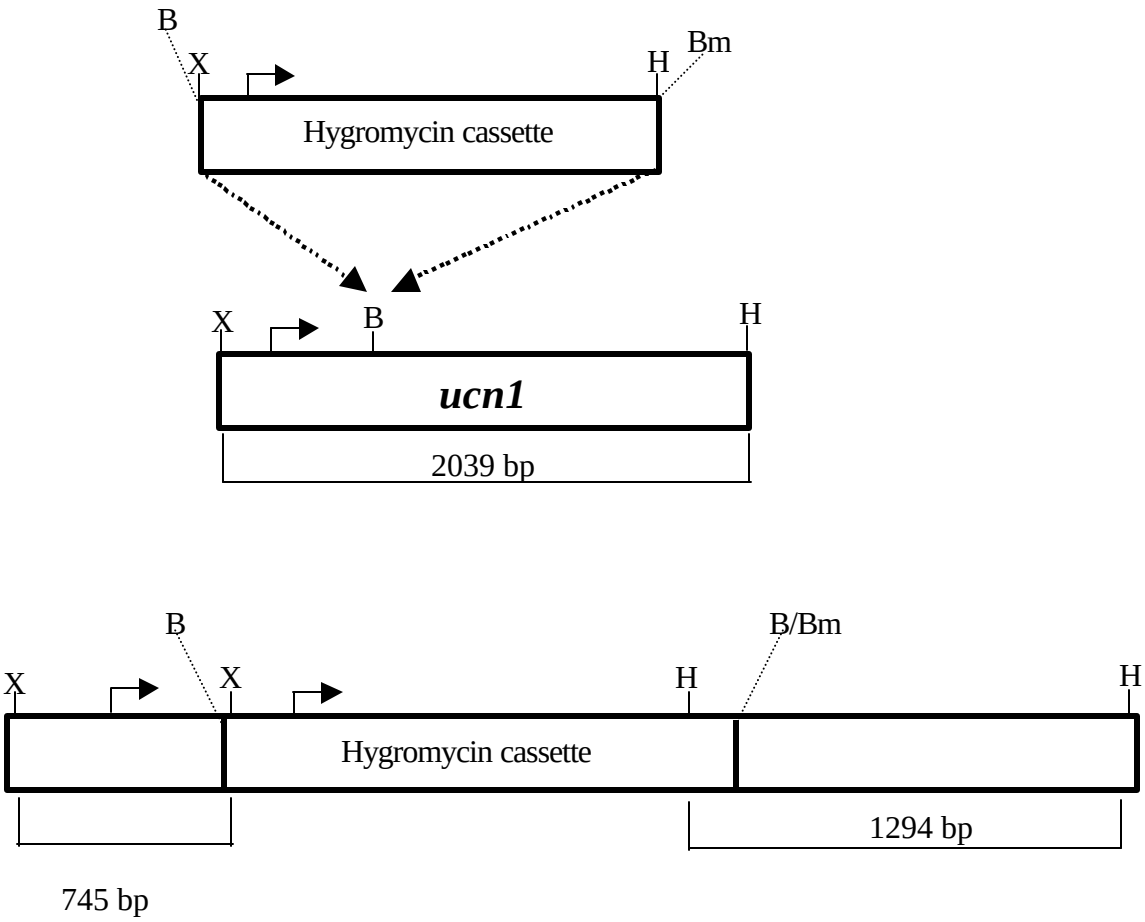

B
X
H
Bm
Hygromycin cassette
X
B
H
ucn1
2039 bp
B
B/Bm
X
X
H
H
Hygromycin cassette
1294 bp
745 bp

Fig. 4A. Southern blot analysis of *ucn1*$^{-}$ strains. Genomic DNA was extracted from the *U. maydis* tranformants, digested with *Xba*I and *Hind*III (fragments were resolved through 0.8% agarose) and transferred to a positively charged nylon membrane. A DIG labeled *unc1* probe was hybridized to the membrane according to the manufacturer's instructions. Lanes are as follows:

(1) 1/2 (wild-type)

(2) SG200 (solopathogenic haploid)

(3) 6/1 (*ucn1*$^{-}$) a1b1

(4) 6/9 (*ucn1*$^{-}$) a2b2

(5) 6/25 (*uac1*$^{-}$ *ucn1*$^{-}$)

(6) 6/26 (SG200 *ucn1*$^{-}$)

(7) 6/27 (SG200 *ucn1*$^{-}$)

Fig. 4B. Southern blot analysis of the double *ucn1*$^{-}$ *uac1*$^{-}$ mutant. Genomic DNA was extracted from *U. maydis* strains and cut with *Bam*HI and subjected to the same procedures as in Fig. 4A. The DIG probe used for this membrane was *uac1*. Lanes are as follows:

(1) 1/2 (wild-type)

(2) 1/9 (*uac1*$^{-}$)

(3) 6/1 (*ucn1*$^{-}$)

(4) 6/25 (*uac1*$^{-}$ *ucn1*$^{-}$)

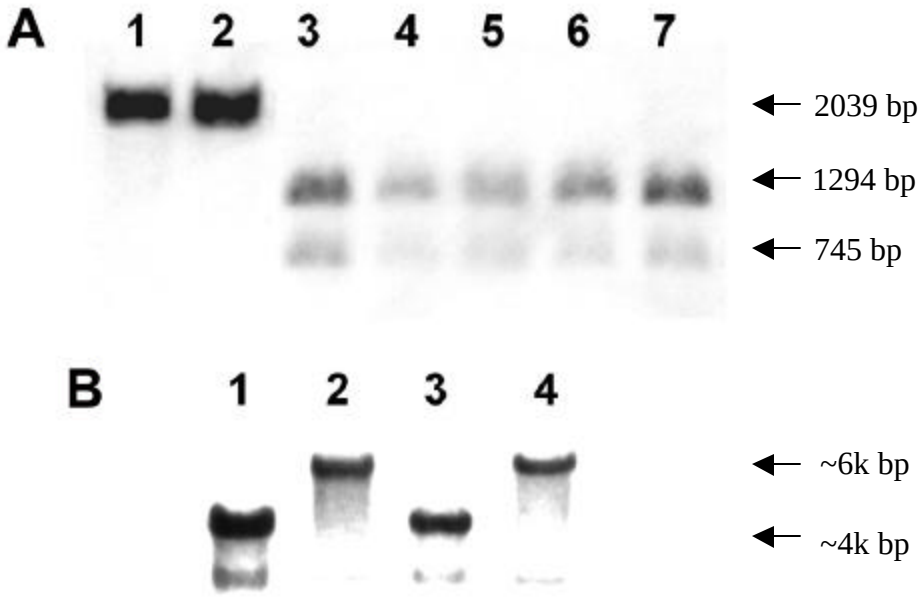

A
1
2
3
4
5
6
7
2039 bp
1294 bp
745 bp
B
1
2
3
4
~6k bp
~4k bp

Fig. 5. Northern analysis of 1/2 (wild-type) and strain 6/1 (*ucn1*$^{-}$). Strains were grown in PDB for 30 h. RNA was isolated and subjected to northern analysis after loading approximately 15µg total RNA per lane. Lane 1 is the 1/2 wild-type strain, lane 2 is the 6/1 *ucn1*disrupted strain. A second hybridization with the carboxin (succinate dehydrogenase) gene probe and the transferred EtBr agarose gel are shown as loading controls.

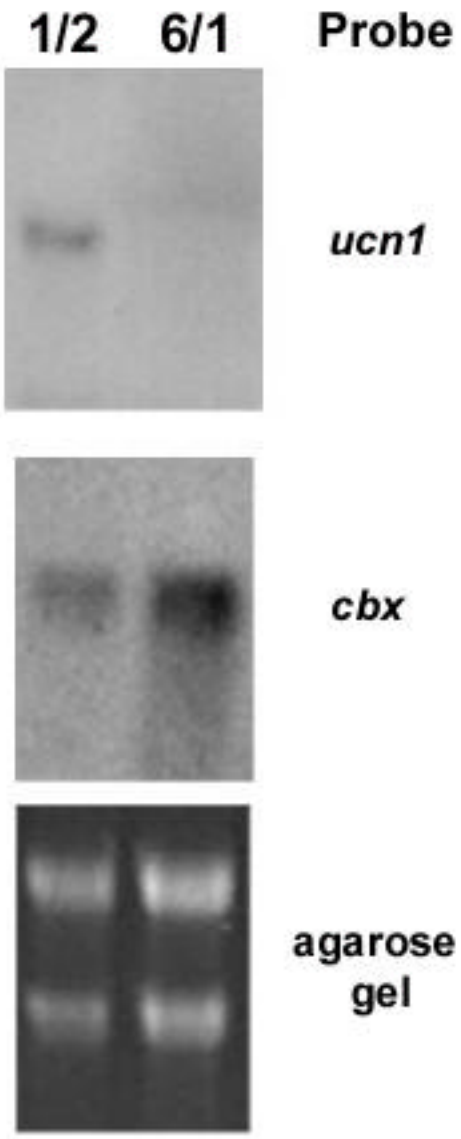

1/2
6/1
Probe
ucn1
cbx
agarose
gel

Fig. 6. Cellular and colony morphology of *U. maydis ucn1* mutants. Micrographs A, C and E are cell morphology of various *U. maydis* strains grown overnight in PDB. B, D and F are colony morphology of various *U. maydis* strains grown on 2PDA for 4 days at 30^0C. In left panels bar equals 50µm, and in the right panels bar equals 1mm. Strains are as follows:

(A, B) 1/2 (wild-type)

(C, D) 6/1 (*ucn1*$^-$)

(E, F) 6/64 (*ucn1*$^-$ + pUCN1)

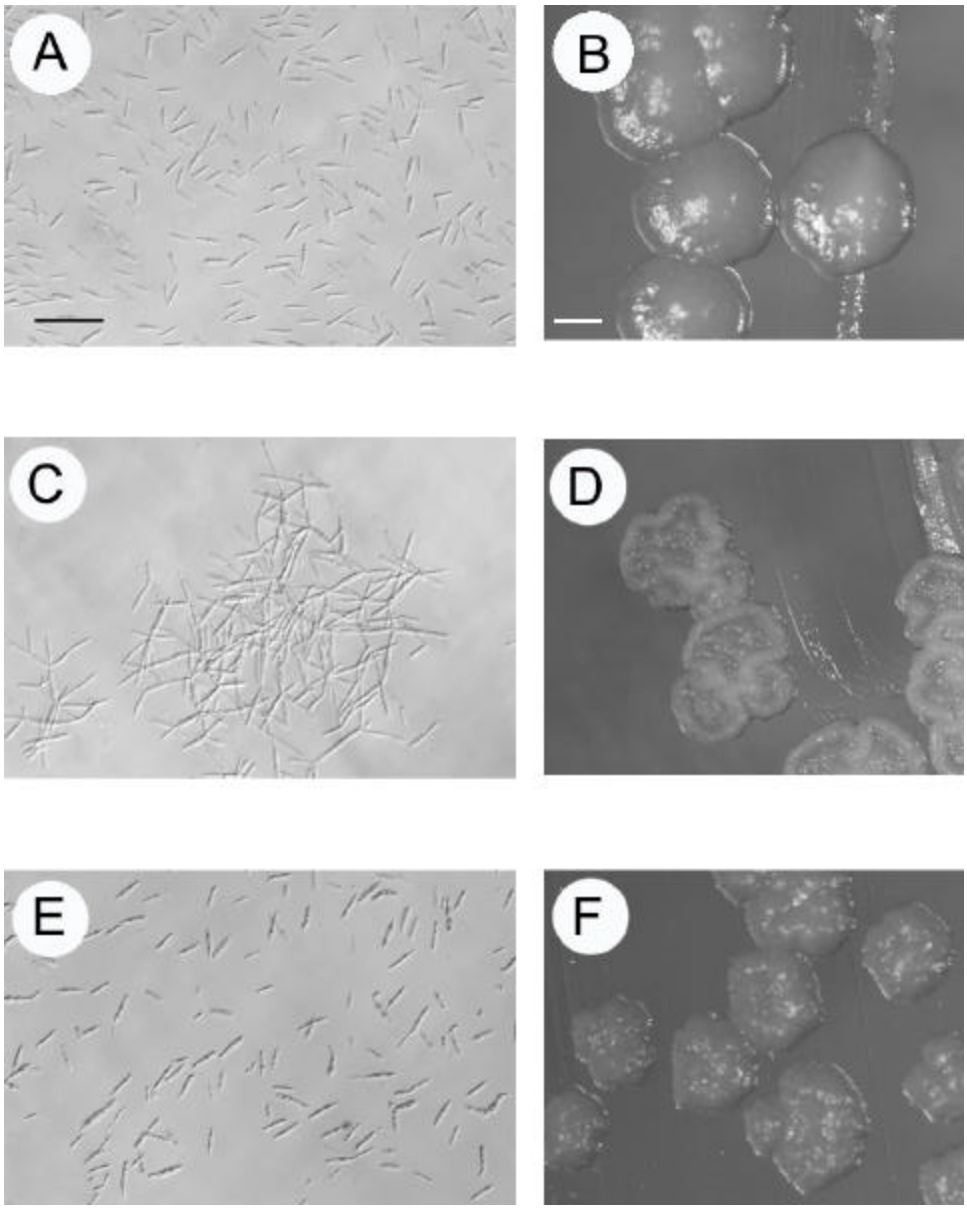

A
B
C
D
E
F

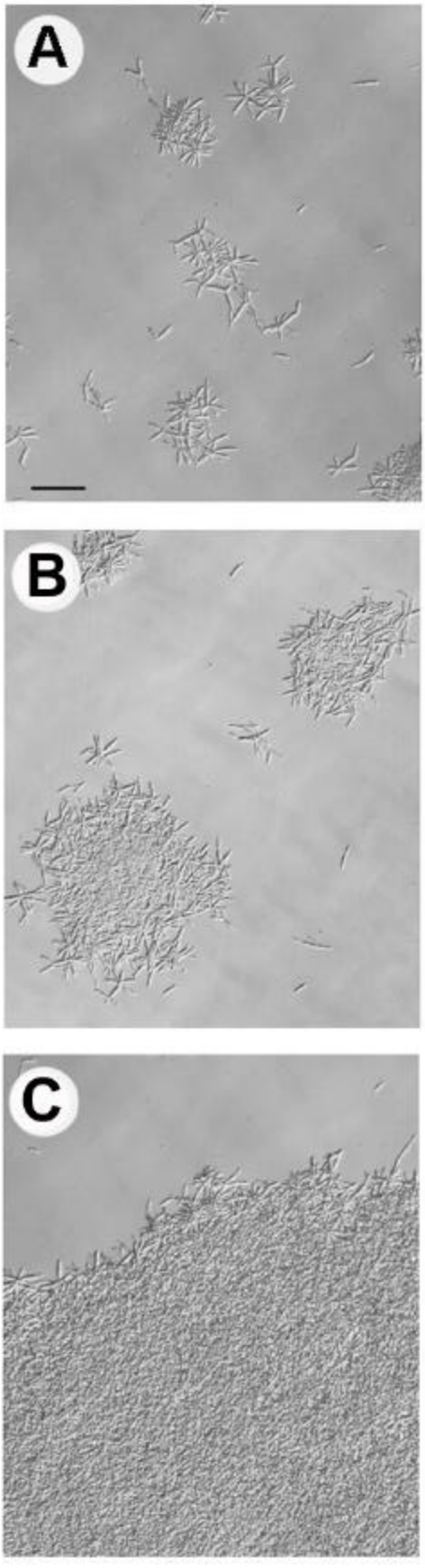
Fig. 7. The effect of inoculum concentration on large multiple-budding cluster formation in *ucn1* mutant strains. Micrographs are of the *ucn1* mutant strain grown for 30 h in PDB using different amounts of initial inoculum from an overnight culture grown in 100ml of PDB. Bar equals 50µm.

(A) 1000µl

(B) 100µl

(C) 10µl

(D-I) A liquid broth culture of *ucn1*$^{-}$ was grown overnight in 100ml PDB to produce an initial culture. From the initial culture, 10µl was used to inoculate 100ml PDB and grown under the same conditions as described above. The multiple-budding clusters were pipetted into molten 1% agarose and solidified for viewing under the dissecting scope. White arrow indicates the cluster that is seen in panels H, I and J from different planes of view. The black arrow indicates the cluster seen in panels K and L from different planes of view. Five clusters were observed for each experiment, and the experiment was replicated four times. Bar equals 1mM.


A
B
C

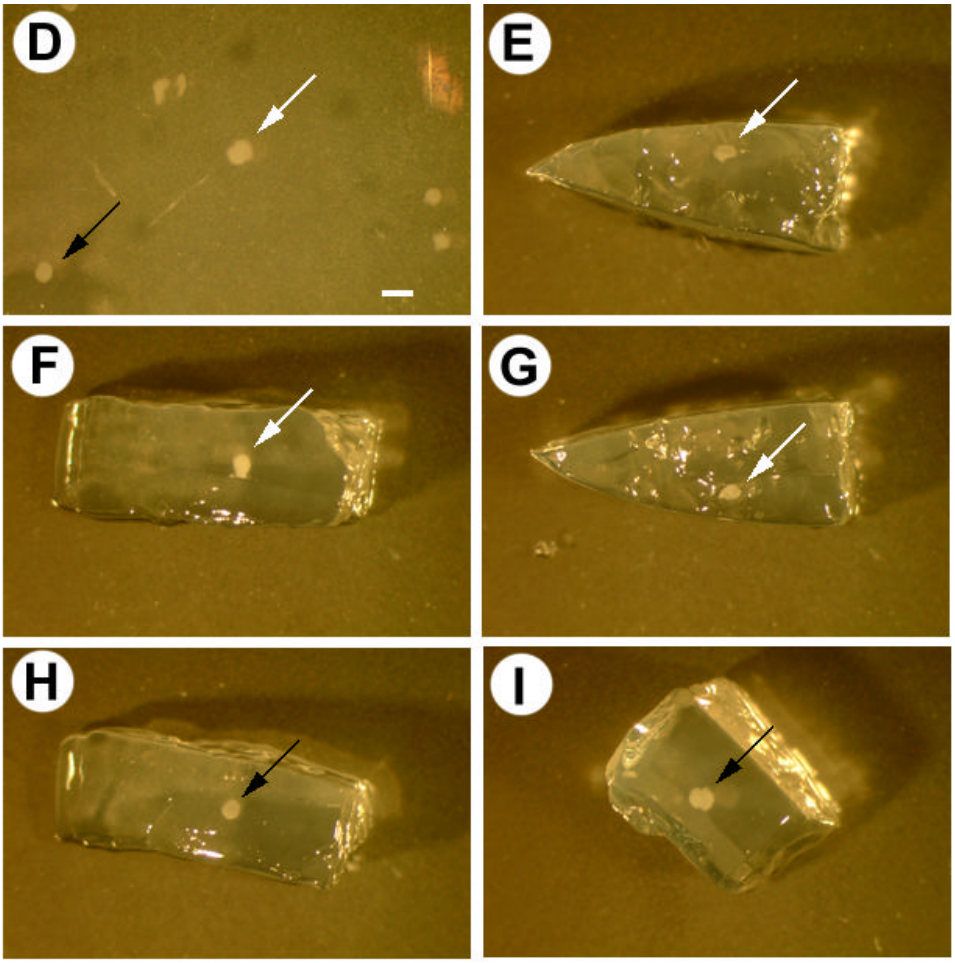

D
E
F
G
H
I

Fig. 8. Epistasis anaylsis of *ucn1* mutants by colony comparison. Strains were streaked onto 2PDA medium and photographed after incubation at 30^0C for 4 days. Bar equals 1mm. Colonies are as follows:

(A) 1/2 (wild-type)

(B) 6/1 (*ucn1*$^-$)

(C) 1/9 (*uac1*$^-$)

(D) 1/68 (*ubc1*$^-$)

(E) 6/55 (*adr1*$^-$)

(F) SG200 (solo pathogenic haploid)

(G) 6/25 (*uac1*$^-$ *ucn1*$^-$)

(H) 6/26 (SG200 *ucn1*$^-$)

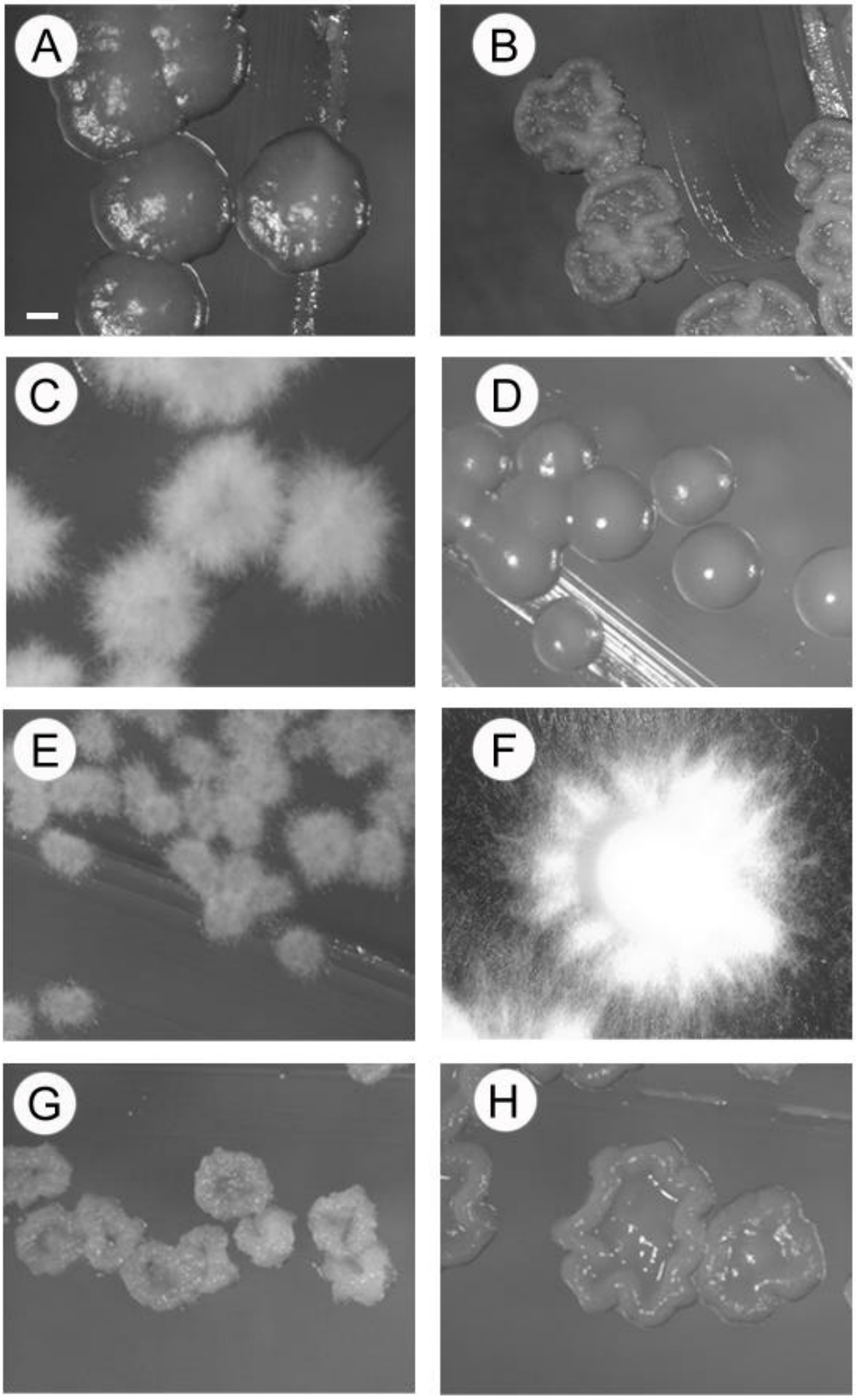

A
B
C
D
E
F
G
H

Fig. 9. Nuclear staining of wild-type and *ucn1*$^{-}$ mutant strains. Micrographs of wild-type cells compared to *ucn1*$^{-}$ mutant cells using DIC (A, C) and DAPI fluorescence stain (B, D). Bar equals 50μm. Images are as follows:

(A, B) 1/2 (wild-type)

(C, D) 6/1 (*ucn1*$^{-}$)

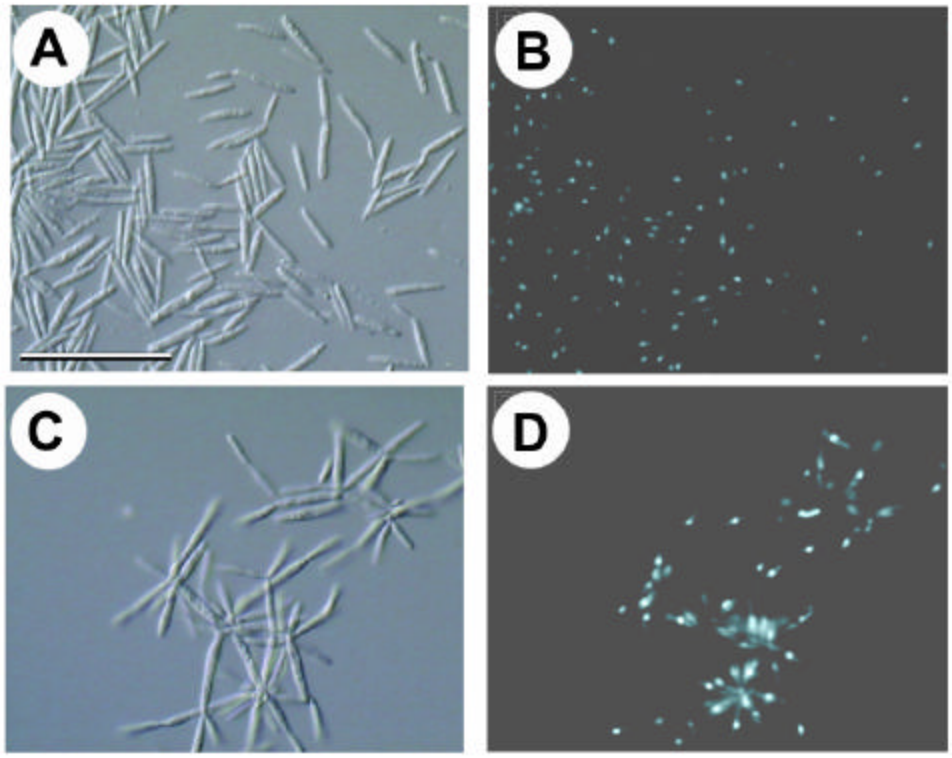

A
B
C
D

Fig. 10. Confocal sections of the *ucn1*$^{-}$ mutant. Confocal imaging of DAPI stained strain 6/1 (*ucn1*$^{-}$). Arrow indicates a single polar cell point where more than 10 buds emerged. Bar equals 50μm.

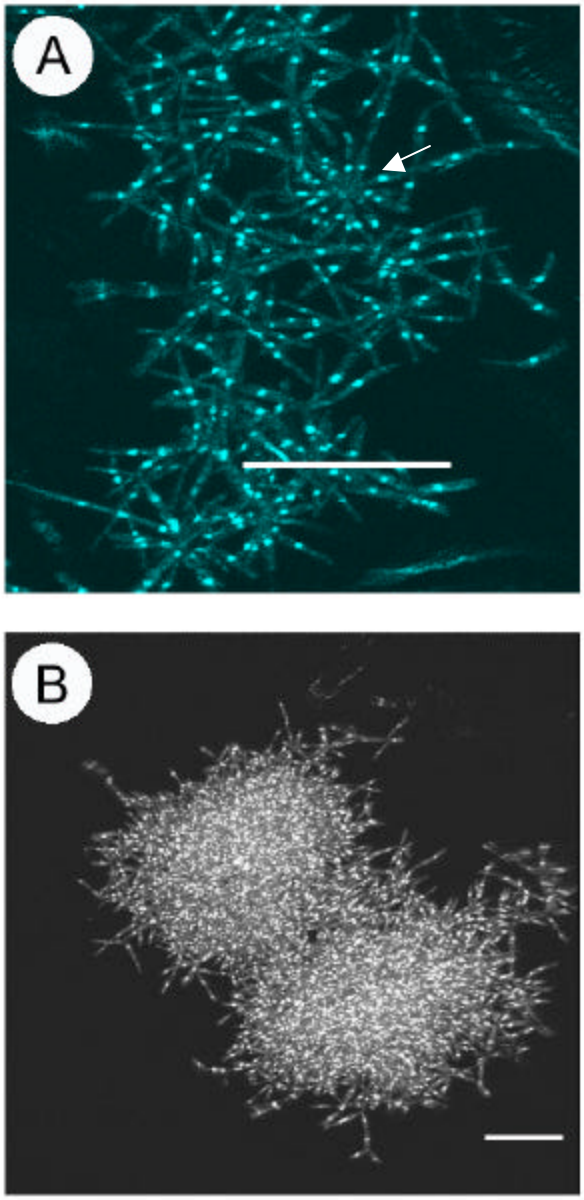

A
B

Fig. 11. Effect of the *ucn1* mutation on mating. Five µl spots of overnight cell cultures were placed on charcoal-containing medium. Rows were first placed and dried in a transfer hood, and then columns were spotted. Mating reactions were incubated at 30°C and photographed 24 hours post inoculation.

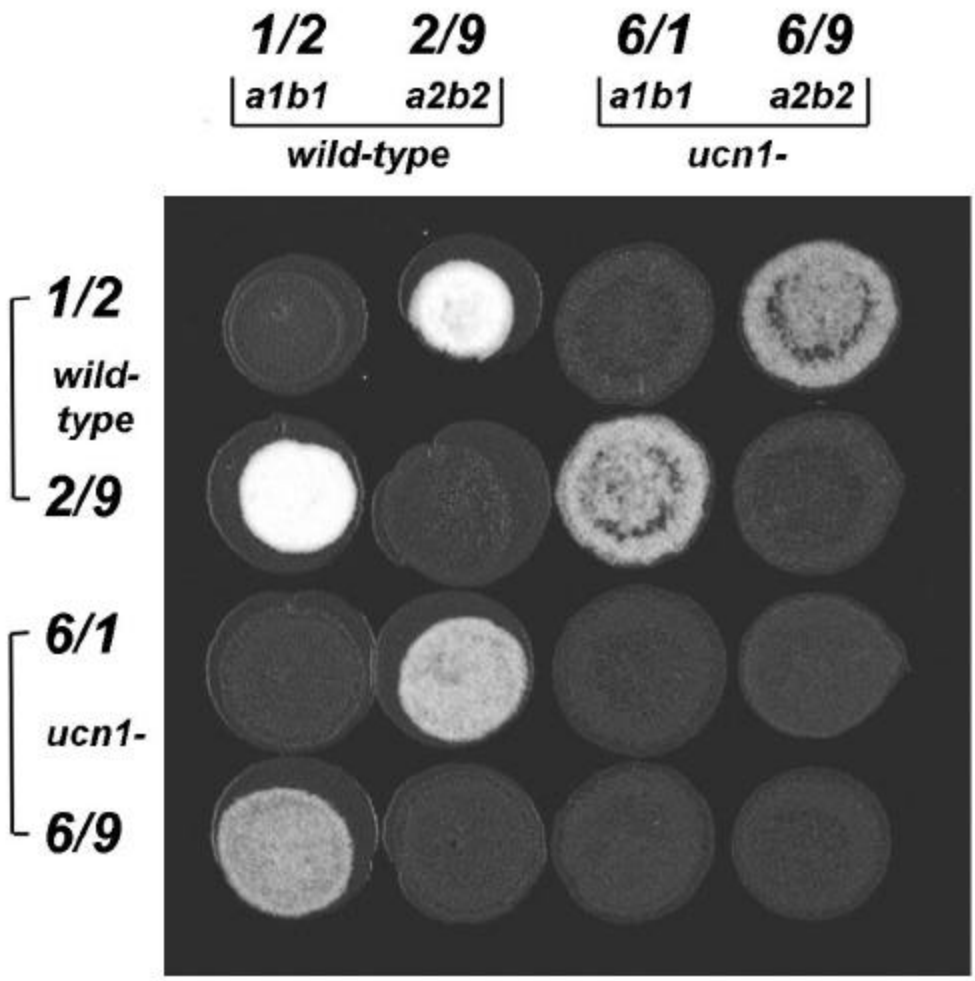

1/2
2/9
6/1
6/9
a1b1
a2b2
a1b1
a2b2
wild-type
ucn1-
1/2
wild-type
2/9
6/1
ucn1-
6/9

Fig. 12. Confrontational drop mating assay for pheromone response in *ucn1* mutant strains. (A-D) Drops of appropriate strains were spotted in close proximity on microscope slides covered with water agar and observed after 24 h (Snetselaar et al., 1996). In all cases *a2b2* strain is at the top and the *a1b1* strain is at the bottom of the image. Bar equals 50µm.

(A) 2/9, (wild-type) top; 1/2, (wild-type) bottom

(B) 2/9, top; 6/1, (*ucn1*$^{-}$) bottom

(C) 6/9, (*ucn1*$^{-}$) top; 1/2, bottom

(D) 6/9, top; 6/1, bottom

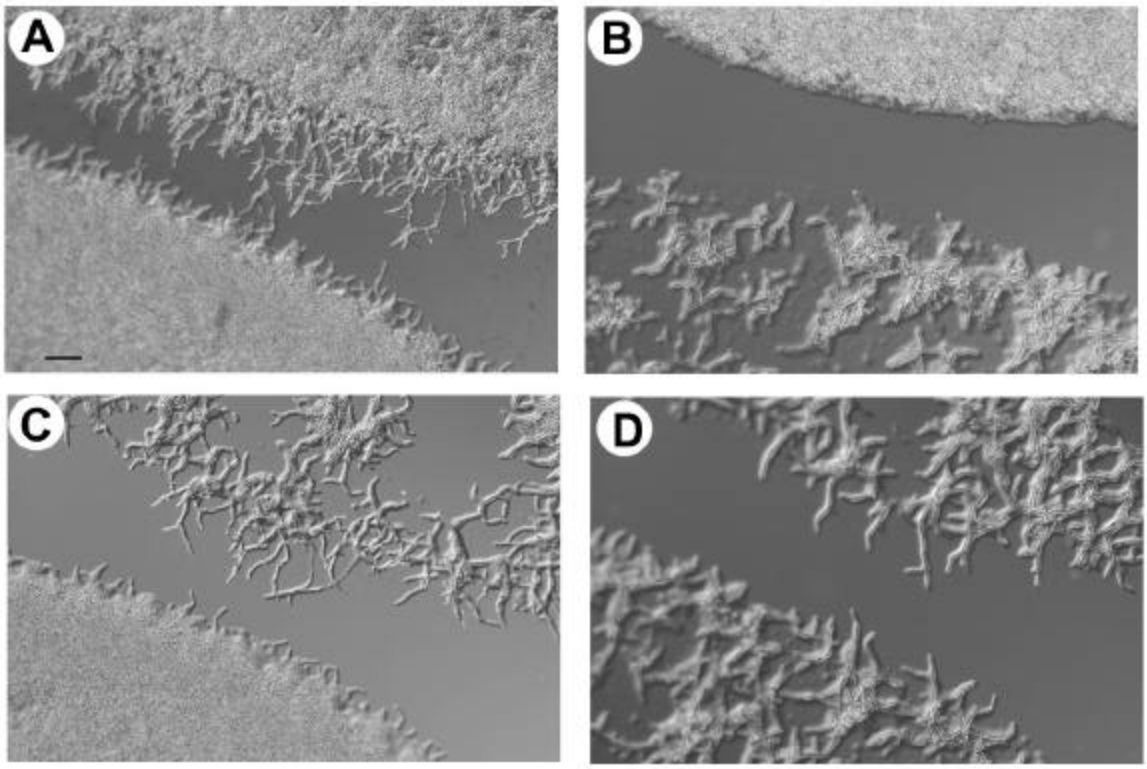

A
B
C
D

Fig. 13. Role of Ucn1 in pheromone induction. RNA was isolated from mating reactions and subjected to northern analysis. The filter was hybridized with a probe for *mfa1*. The same filter was then stripped and hybridized with a probe for *upa2* and the agarose gel is shown for a loading control. Lanes are as follows: 1/2 (wild-type) *a1b1*; 6/1 (*ucn1*$^{-}$) *a1b1*; 1/2 X 2/11 (wild-type) *a2b1*; 6/1 X 2/11.

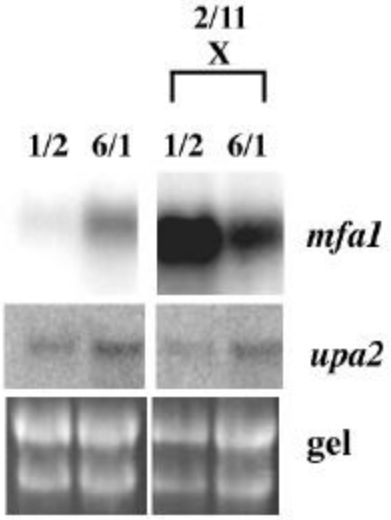

2/11
X
1/2
6/1
1/2
6/1
mfa1
upa2
gel

Table 2. Pathogenicity of *ucn1* mutants[a]

Treat-ment[c]	Dikaryon	No. of Plants	Disease Rating[b] 0	1	2	3	4	Death 5	Disease[d] Index
1	+/+	20			4	2		12	3.70
2	+/*ucn1-*	20		3	5	1		11	3.55
3	*ucn1-*/+	20		2	5	2		11	3.65
4	*ucn1-*/*ucn1-*	20	8	12					0.6
5	*7/20*[e] +	20		3	1	12	3	1	2.9
6	*6/26 ucn1-*	20	12	8					0.4
7	*6/27 ucn1-*	20	13	7					0.35

[a] Results of one of three tests. All results were similar

[b] Recorded 14 days post inoculation.

[c] Treatments were inoculations of 10^6 cells per ml for all wild-type strains (+) and 10^7 colony forming units (cell clusters) per ml for all mutant strains (*ucn1-*). Paired strains are as follows (see Table 1): 1 = (1/2 x 2/9), 2 = (1/2 x 6/9), 3 = (6/1 x 2/9), 4 = (6/1 x 6/9), 5 = SG200 (solopathogenic haploid), 6 = 6/26 (*ucn1* SG200), 7 = 6/27 (*ucn1-* SG200)

[d] Disease index is calculated as disease ratings divided by number of plants. Disease ratings are described from 0-5 as: 0, no disease; 1, anthocyanin or chlorosis; 2, leaf galls; 3, small stem galls; 4, large stem galls; 5, plant death due to disease.

[e] Note that treatments 5-7 were with solopathogenic haploids that should be able to cause disease without needing a mating partner

Fig. 14. *ucn1* mutants within *Zea maize* plants. Photographs are of *in planta* observation of fungal hyphae by crossing strains. Plants were harvested at 14 days post inoculation and leaf sections were subjected to anailine blue staining. Arrows indicate blue fungal hyphae. Bar equals 50μm. Panels are as follows:

(A) 1/2 X 2/9 (both wild-type)

(B) 6/1 (*ucn1*$^{-}$) X 2/9

(C-D) 6/1 X 6/9 (*ucn1*$^{-}$)

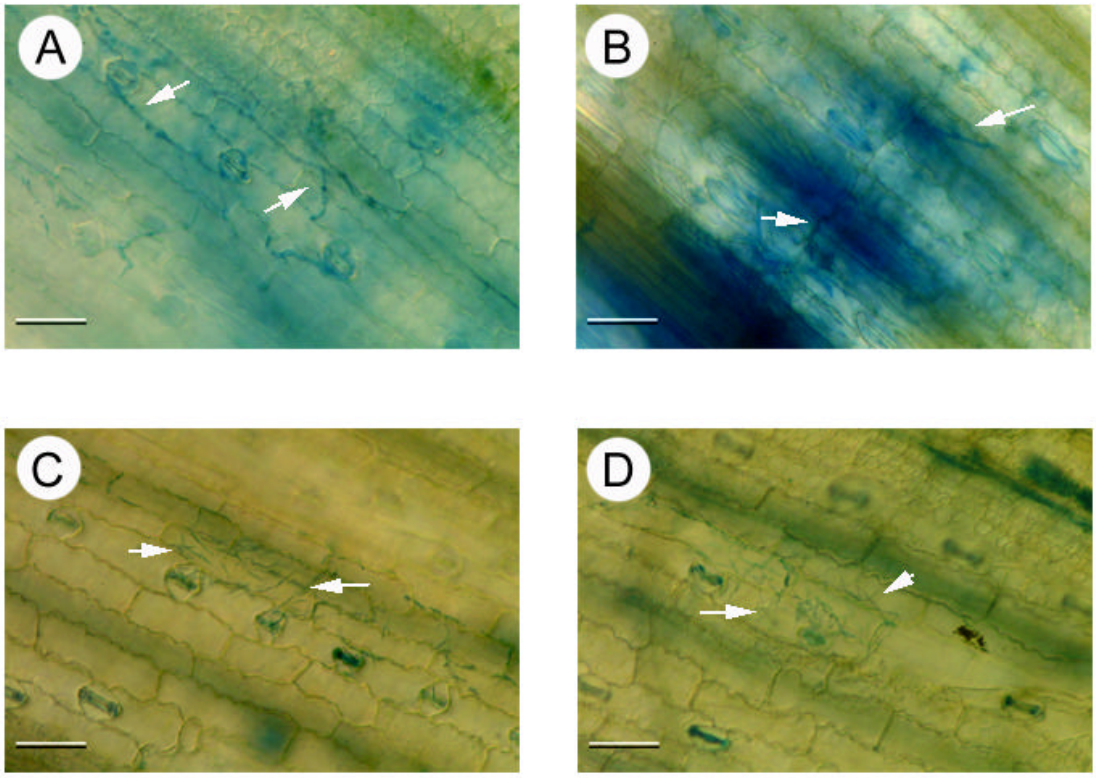

A
B
C
D

Fig. 15. Pathogenicity of solopathogenic SG200 and SG200 *ucn1*⁻ strains on *Zea maize* plants. Plants were harvested at 14 days post-inoculation and were photographed for symptoms (left panels), then subjected to anailine blue staining for detection of *in planta* fungal growth (right panels). Bar equals 50μm.

(A, B) 1/2 X 2/9, (both wild-type)

(C, D) SG200, (solopathogenic haploid)

(E, F) 6/26, (SG200 *ucn1*-)

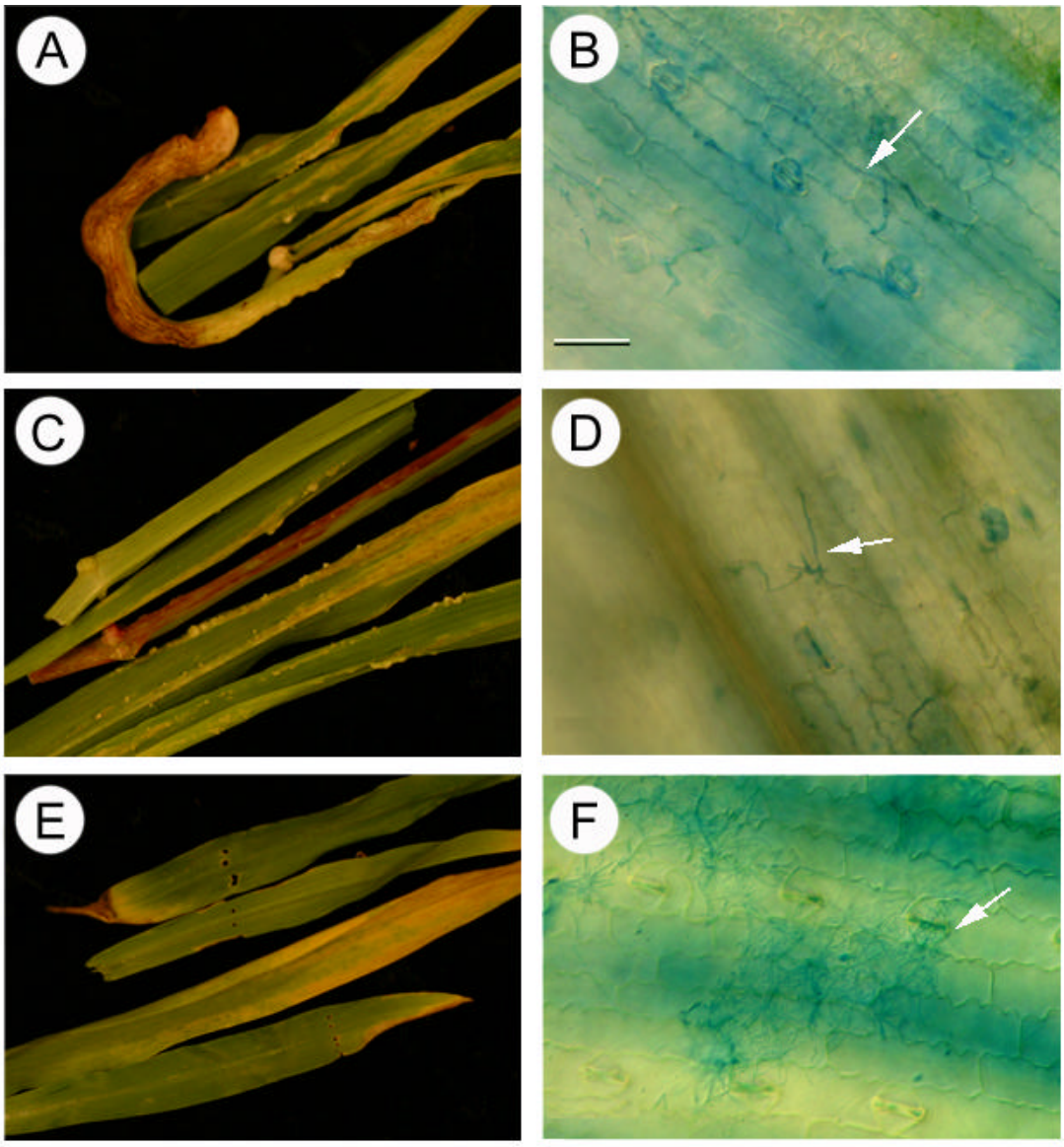

A
B
C
D
E
F

Fig. 16. Morphology of wild-type and *ucn1* mutant strains *U. maydis* strains grown in Cyclosporine A or cAMP. Micrographs are of strains grown overnight in PDB. Where noted strains were grown with 10µg/ml CsA or 25mM cAMP. Bar equals 50µm. Strains are as follows:

(A) 1/2 (wild-type)

(B) 1/2 + CsA

(C) 6/1 (*ucn1*$^{-}$)

(D) 6/1 + CsA

(E) 6/64 (*ucn1*$^{-}$ + p*UCN1*)

(F) 6/64 + CsA

(G) 1/2 + cAMP

(H) 6/1 + cAMP

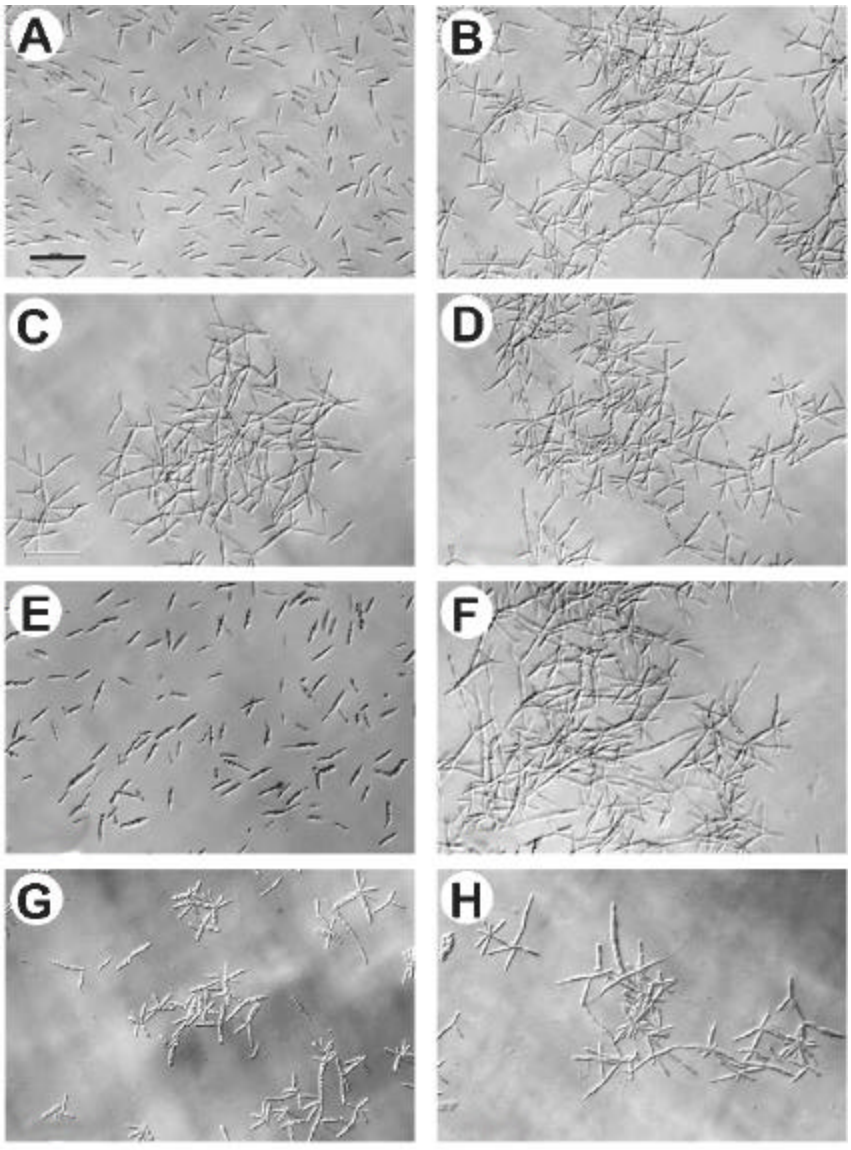

A
B
C
D
E
F
G
H

Fig. 17. Morphology of cAMP pathways mutants and pathogenic *U. maydis* strains grown in Cyclosporine A. Micrographs on the left side are of strains grown overnight in PDB, while the right side micrographs are of the same strains grown with the addition of Cyclosporine A (CsA) at 10µg/ml. Bar equals 50µm. Strains are as follows:

(A) 1/68 (*ubc1*$^{-}$)

(B) 1/68 + CsA

(C) 6/55 (*adr1*$^{-}$)

(D) 6/55 + CsA

(E) SG200 (solo pathogenic haploid)

(F) SG200 + CsA

(G) D132 (diploid)

(H) D132 + CsA

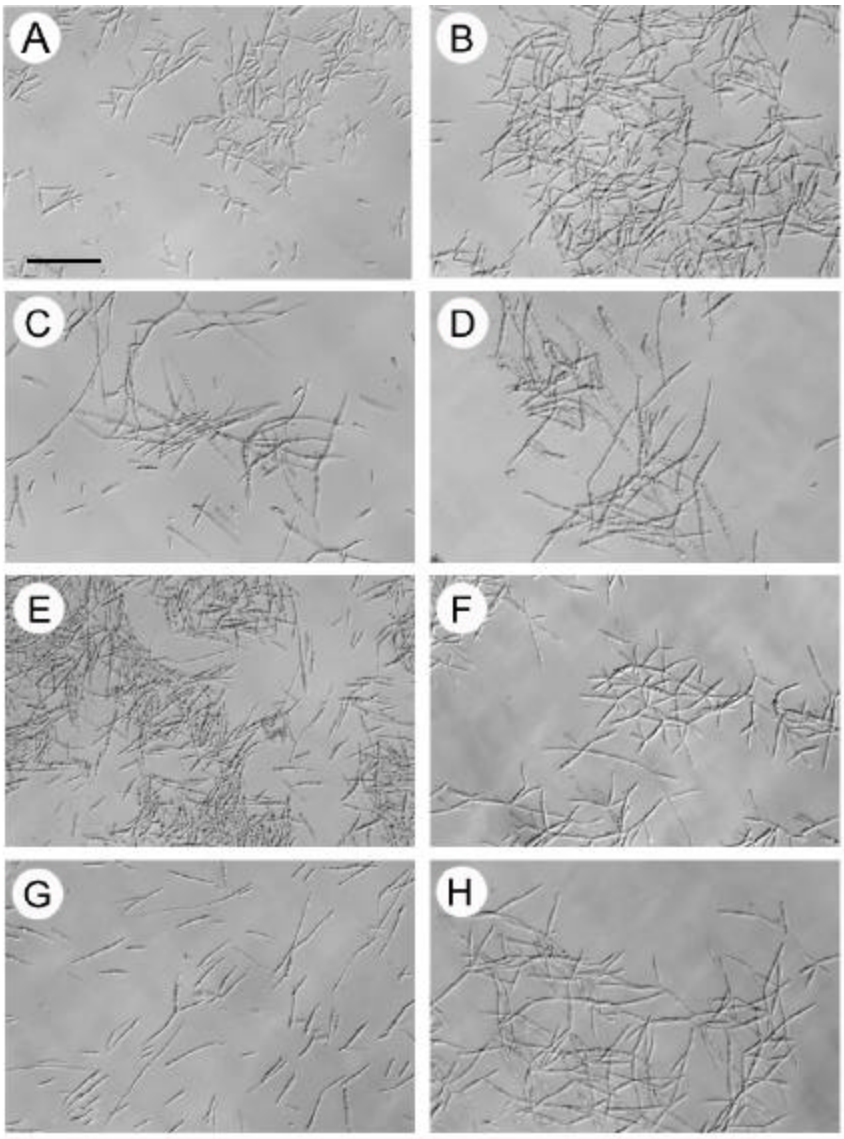

A
B
C
D
E
F
G
H

Fig. 18. The effect of cyclosporine A on the adenylate cyclase mutant *uac1*$^{-}$. Micrographs are of strain 1/9 (*uac1*$^{-}$) grown in PDB or with the addition of cyclosporine A, 10µg/ml. Micrographs B and C are from the same culture at the same time, indicating a possible breakdown of an extracellular matrix. Bar equal 50µm.

(A) 1/9 (*uac1*$^{-}$)

(B) 1/9 + CsA

(C) 1/9 + CsA

(D) 6/25 (*uac1*$^{-}$ *ucn1*$^{-}$)

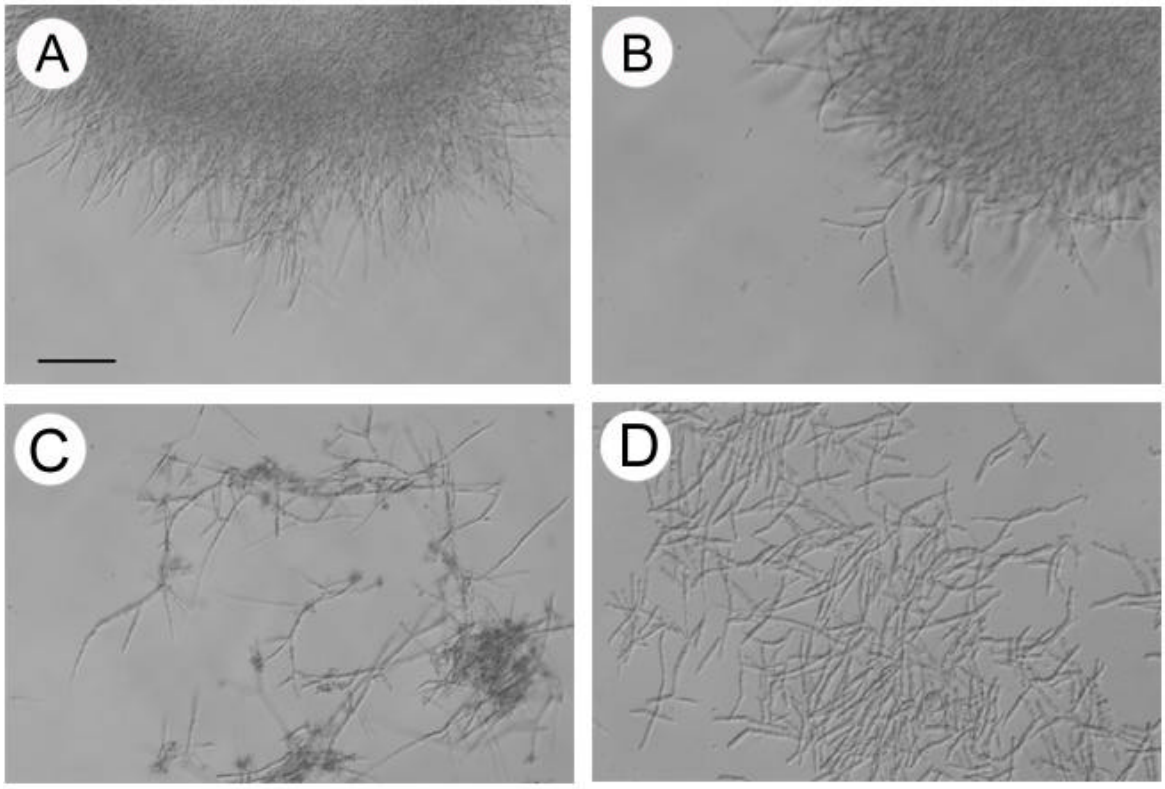

A
B
C
D

Fig. 19. Time course of cell division after the addition of cyclosporine A. Micrographs are a time course of wild-type cells (1/2) after the addition of 10µg/ml CsA. A 100ml culture of wild-type 1/2 was grown in PDB overnight. The culture was set to G_0, Cyclosporinee A was added and photographs were taken at the given time intervals. Experiment was replicated three times with similar results. Arrows indicate the center septation of the mother cell. Bar equals 50µm.

(A) 0 h

(B) 4 h

(C) 8h

(D) 10h

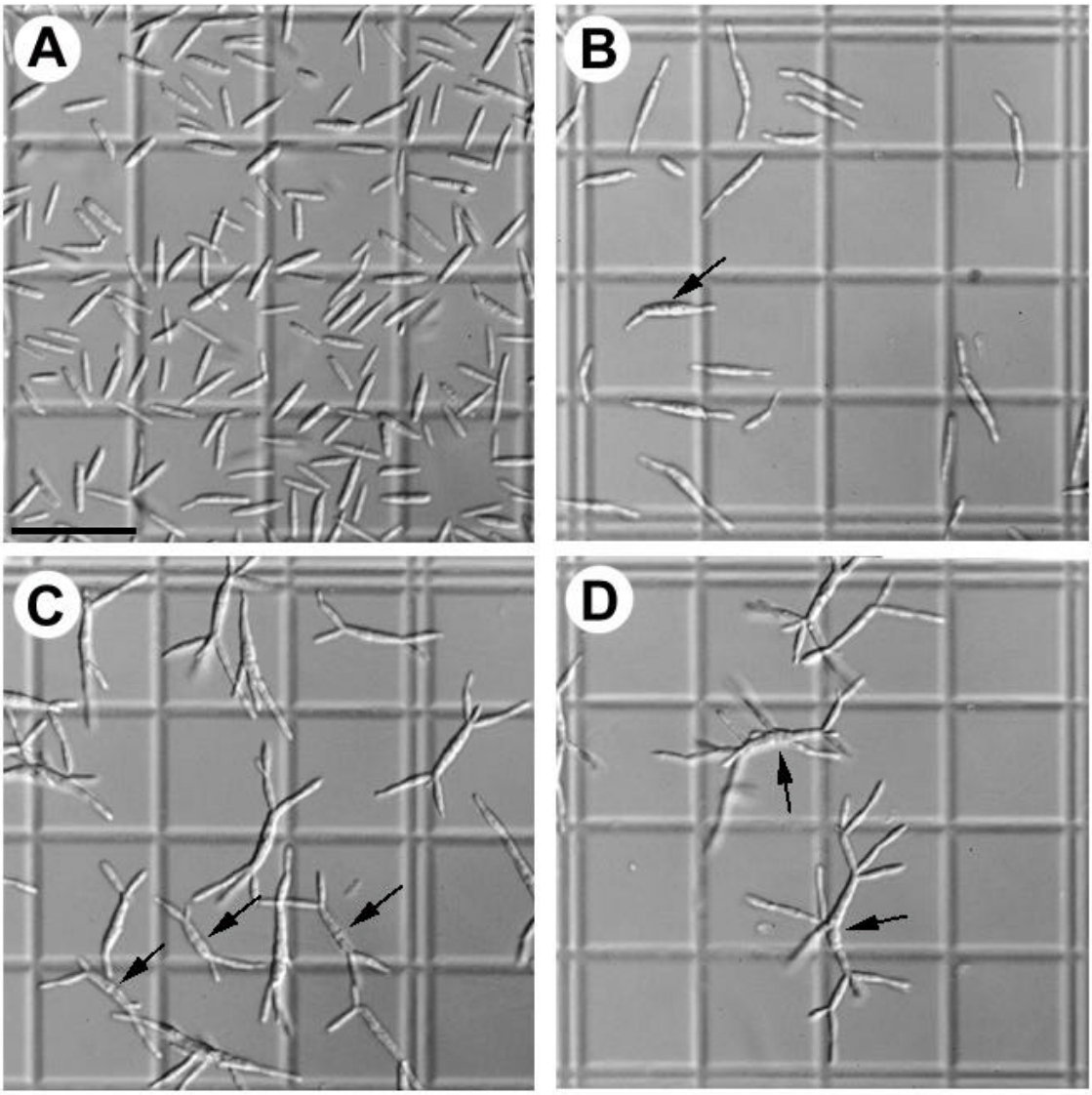

A
B
C
D

Fig. 20. *upa2* disruption construct strategy. *Bgl*II linkers were ligated to digested *Nru*I sites located within the *upa2* gene (N/B). A *Bgl*II (B) digestion excised a 574 bp fragment from the center of *upa2* (N/B-B). A 2.7 kbp *Bgl*II (B) -*Bam*HI (Bm) hygromycin cassette from pIC19RHL was ligated in the opposite orientation into the resulting *Bgl*II sites of *upa2*. *Bam*HI restriction enzyme digests of wild-type *upa2* gene produces a ~4k bp band (dashed line is not to scale), while *upa2* disrupted transformants were predicted to produce a ~6.2k bp band. Bent arrows indicate the direction of transcription.

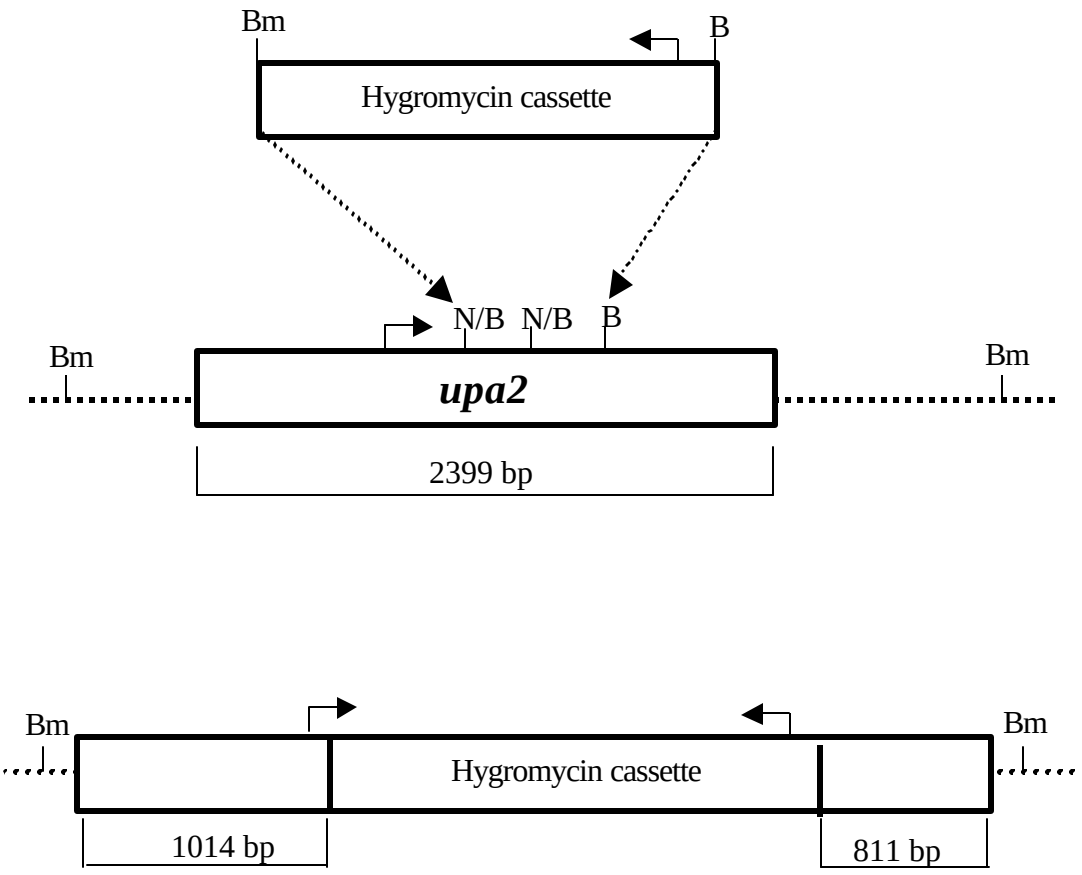

Bm
B
Hygromycin cassette
N/B
N/B
B
Bm
upa2
Bm
2399 bp
Bm
Hygromycin cassette
Bm
1014 bp
811 bp

Fig. 21. The effect of okadaic acid on the growth of *U. maydis*. Wild-type strain 1/2 grown 30 h in PDB with different amounts of okadaic acid (OA).

(A) 0nM

(B) 2nM

(C) 3nM

(D) 4nM

(E) 8nM

(F) 12nM

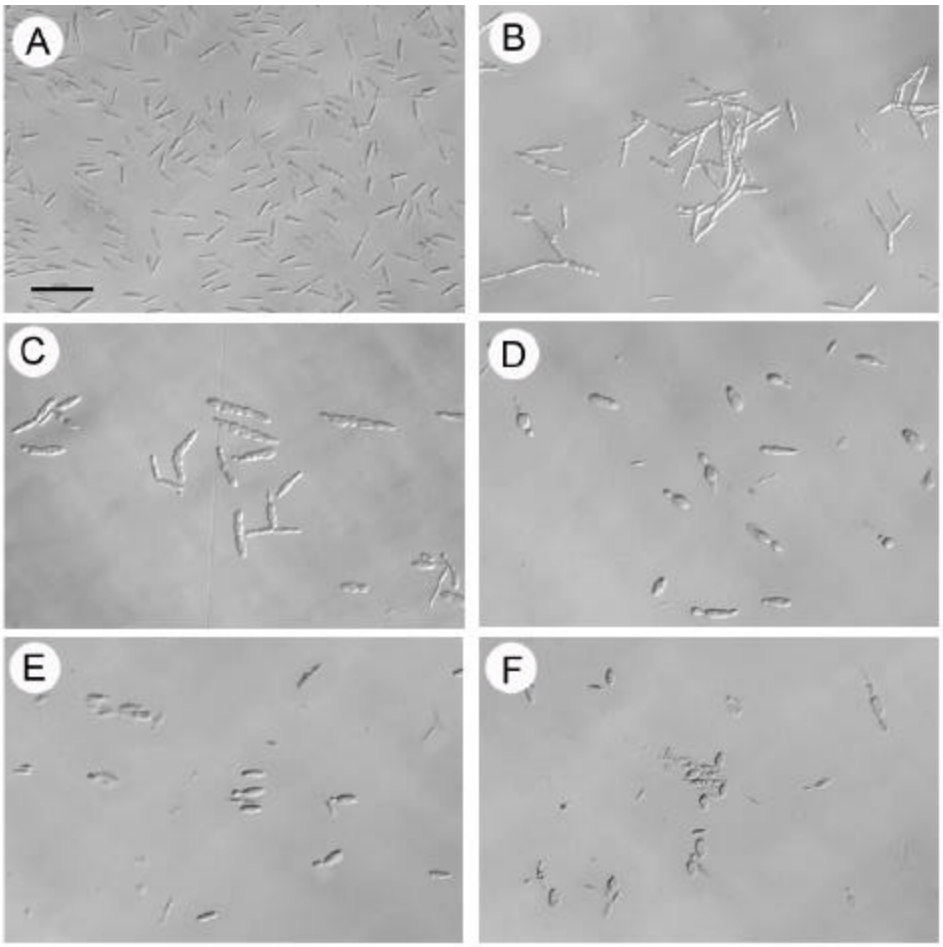

A
B
C
D
E
F

Fig. 22. Okadaic acid inhibits growth in *U. maydis*. The wild-type strain (1/2) was grown in PDB with different amounts of okadaic acid. All cell cultures were started with 10^5 cells/ml and cell counts were taken on 12 h and 28 h. Values are the mean of three separate replications

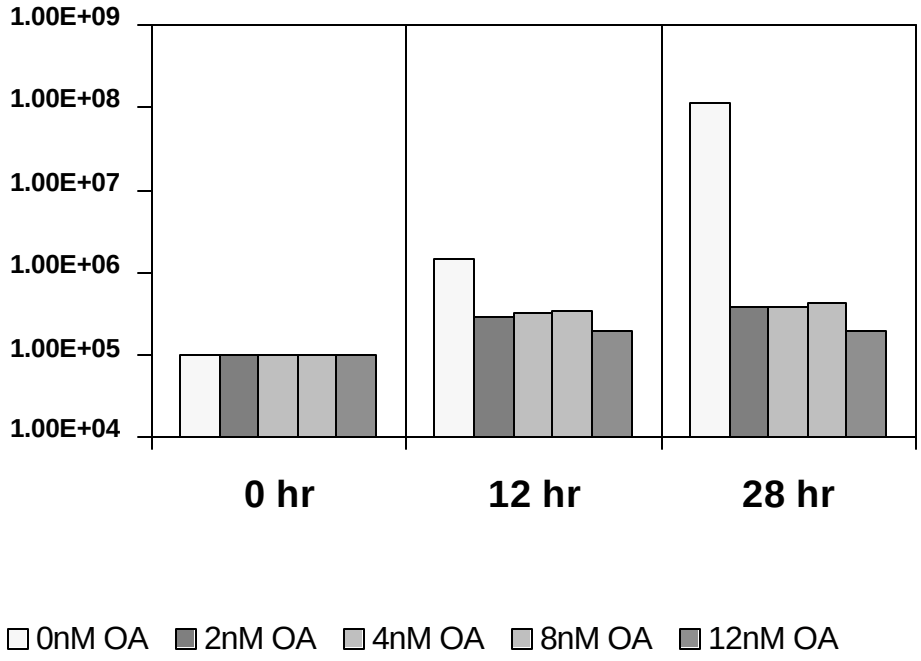

1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
0 hr
12 hr
28 hr
0nM OA
2nM OA
4nM OA
8nM OA
12nM OA

Fig. 23. Bud site selection during CN inhibition in *U. maydis*. The model is based on the data from figure 19. The mother cell (MC) goes through multiple rounds of division to produce various buds. At four hours, the mother cell underwent one nuclear division and separated into two compartmentalized cells. The next cell division was at the polar ends of the mother cell giving rise to two daughter cells (buds 1). At eight hours, the mother cell has divided two more times (buds 2 and 3), and the daughter cells have budded off the end distal to the mother cell (buds 2b). At 10 hours, two to three long buds emerge from the center septation of the mother cell (bud 4).

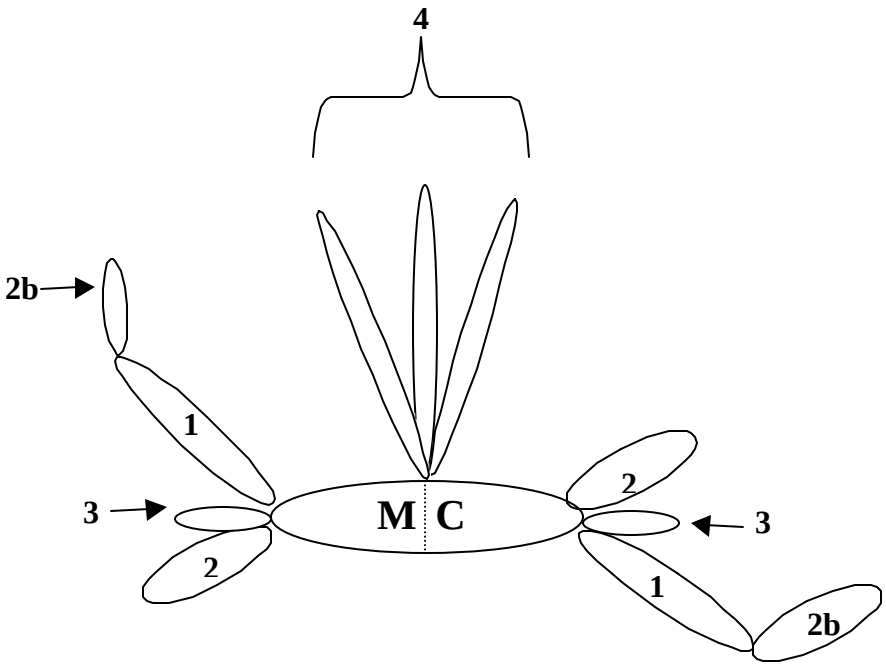

4
2b
1
3
2
M C
2
3
1
2b

Fig. 24. Proposed model for the hypothetical role of CN in the *U. maydis* cAMP pathway. Environmental signals activate a cell surface receptor that transmits a signal through a trimeric G-protein (Gpa3 being important for signal transmission). It is presumed that Gpa3 activates Uac1, which converts ATP to the secondary signal molecule cAMP. Cyclic AMP binds to the regulatory subunit of PKA (Ubc1), inducing a conformational change causing the release of the catalytic subunit of PKA (Adr1). Adr1 is then able to phosphorylate target proteins responsible for haploid budding morphology. In a *uac1* mutant, invasive filamentous growth is seen in the haploid state. However, in the *uac1 ucn1* double mutant, invasive growth is diminished, which indicates that CN is required for invasive growth in a *uac1* mutant.

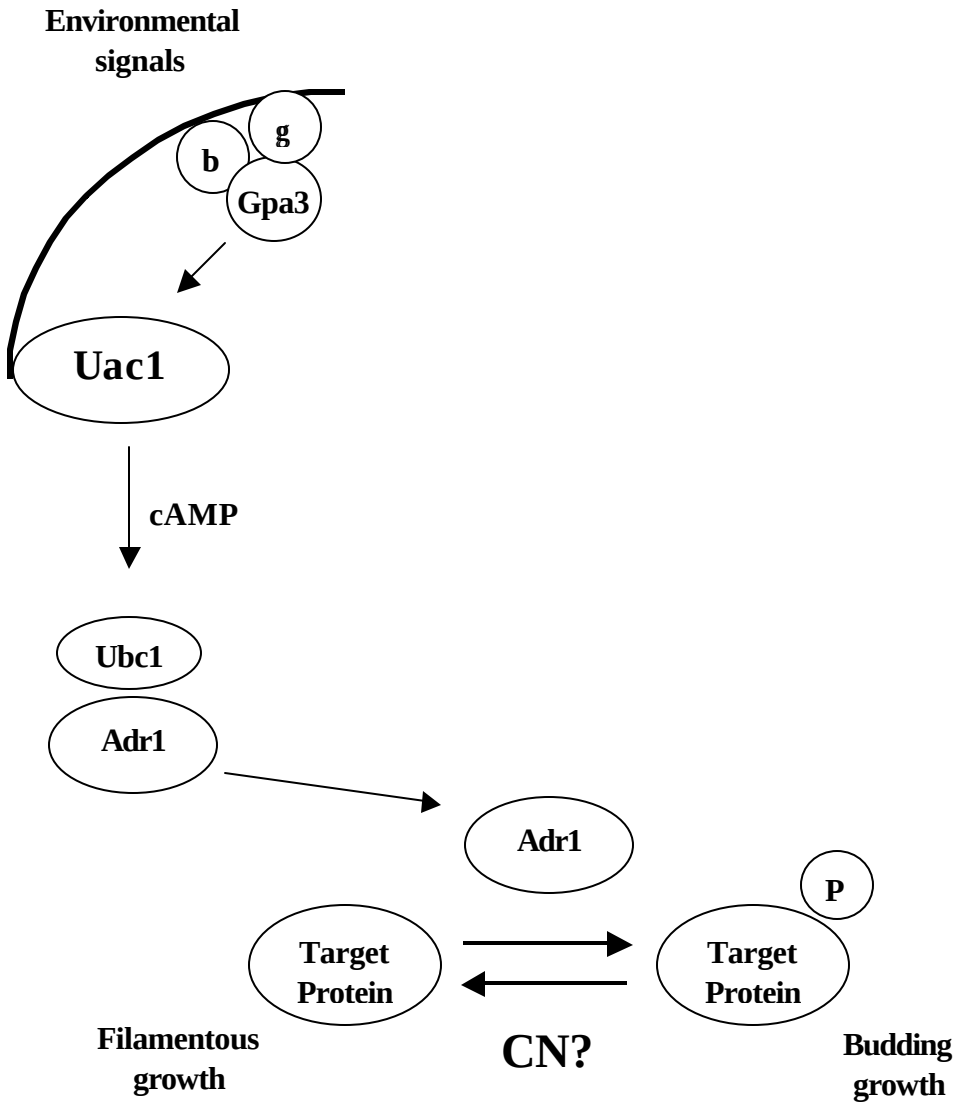
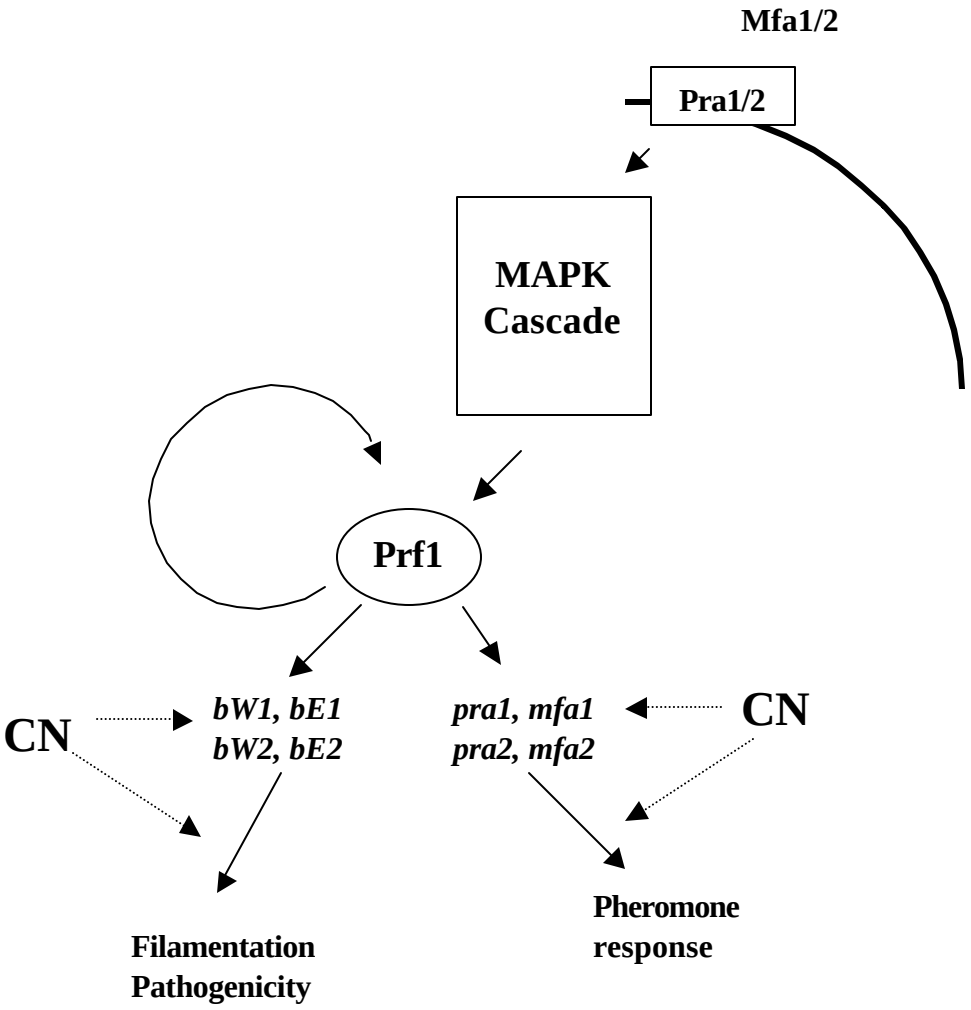

Environmental signals
g
b
Gpa3
Uac1
cAMP
Ubc1
Adr1
Adr1
P
Target Protein
Target Protein
Filamentous growth
CN?
Budding growth

Fig. 25. Proposed model for CN's involvement in the pheromone response pathway in *U. maydis*. During mating, Mfa1/2 interacts with its respective receptor Pra1/2. This interaction activates a MAP kinase module, which transmits a signal to Prf1. Prf1 is auto induced, and activates genes involved in the formation of the bE/bW heterodimer and pheromone stimulation. CN has a role in regulating directly or indirectly filamentation, pathogenicity and pheromone stimulation.


Mfa1/2
Pra1/2
MAPK
Cascade
Prf1
CN
bW1, bE1
bW2, bE2
pra1, mfa1
pra2, mfa2
CN
Filamentation
Pathogenicity
Pheromone
response

CHAPTER 3

A COMPARATIVE STUDY OF PHOSPHORYLATION PATTERNS IN SIGNAL TRANSDUCTION MUTANTS OF *USTILAGO MAYDIS*

ABSTRACT

A two-pronged approach was taken to analyze protein phosphorylation in *Ustilago maydis*. The first approach focused on a specific target protein pheromone response factor 1 (Prf1), which is an important pathogenicity determinant. A five-tier approach was taken to tag, isolate, and determine the proteins that phosphorylate or dephosphorylate the transcription factor Prf1: (1) create a *prf1*-hexahistidine plasmid, (2) determine its function in a *prf1* deletion strain, (3) extract, quantify, and analyze the tagged Prf1 protein by using HIS-binding columns, (4) determine its phosphorylation state by anti-phospohserine and anti-phosphothreonine antibodies, and (5) establish the phosphorylation state of the tagged Prf1 protein in different signal transduction mutant backgrounds in *U. maydis*. Experimental analysis was halted with the inability to isolate the tagged Prf1 protein.

The second approach was to extract total soluble proteins from wild-type and mutant strains for analysis using anti-phospohserine and anti-phosphothreonine antibodies. However, due to background binding of the secondary antibody, protein phosphorylation patterns between these strains still remain undetermined.

INTRODUCTION

Signal transduction cascades impinge on a variety of protein targets. The goal of this study was to determine the target proteins that are phosphorylated by protein kinases and dephosphorylated by protein phosphatases in *U. maydis*. Muller et al. analyzed the putative MAP kinase docking and recognition sites in Prf1 by substituting alanine residues in place of serine residues (Muller et al., 1999). This change yielded mutant strains that were attenuated in mating with wild-type strains, but behaved differently than a Prf1 null mutant in two respects:

they were able to fuse with wild-type tester strains, and they stimulated filamentous growth in the pheromone tester strain CL13 (*a1bE1bW2*) (Muller et al., 1999). This study showed that the elimination of putative MAP kinase sites did not destroy the activity of Prf1 completely, but did affect its function during mating. This finding suggests two possibilities: (1) Prf1 can function without being phosphorylated, and/or (2) another protein kinase is able to phosphorylate and activate Prf1 during mating on an unknown amino acid residue. In support of possibility (2), Prf1 appears to have five putative cAMP-dependent kinase sites (Kahmann et al., 1999). In *U. maydis*, it has been well documented that cross-talk occurs between the MAP kinase cascade and the PKA pathway to impinge on at least one target protein Prf1 (Kahmann et al., 1999; Gold et al., 2000).

In this study, we attempted to elucidate the protein phosphorylation targets in *U. maydis* through a two-pronged approach. In the first approach, the Prf1 protein was modified with a hexa-histidine epitope tag on the carboxy terminus. An attempt was made to purify the tagged Prf1 protein from *U. maydis* cell extracts for further analysis. In the second approach, specific antibodies that recognize phosphorylated serine and threonine residues were used to probe total soluble protein extracts from different mutant strains of *U. maydis*, in an attempt to discover common and different target substrates of signal transduction mutants.

MATERIALS AND METHODS

Construction of *prf1* hexa-histidine epitope tag

Oligonucleotides were designed based on the known *prf1* gene sequence (NCBI accession #U40753) (Hartmann et al., 1996). Template genomic DNA was used from strain FB1 *a1b1* to amplify a 4219bp fragment by PCR (4min denaturing 94°C followed by 40 cycles of 94°C

1min, 55°C for 1min, and 72°C for 5min with a 7min final 72°C dwell) using the 5' upstream primer JEPR01 5' ttt taa aag cct cct tac ggg 3' and the 3' downstream primer JEPR05 5' ttt aaa tca atg gtg atg gtg atg gtg gat gca gtg ctg agg aga tg 3' that conforms to the following amino acid sequence (Dra1 sites are underlined):

```
      S   P   Q   H   C   I   H   H   H   H   H   H   *
5' CATCT CCT CAG CAC TGC ATC CAC CAT CAC CAT CAC CAT TGA TTT AAA 3'
3' GTAGA GGA GTC GTG ACG TAG GTG GTA GTG GTA GTG GTA ACT AAA TTT 5'
```

Two PCR fragments, from independent PCR reactions, were cloned into a pCR2.1 TOPO cloning kit and the resulting plasmids were designated pTOPO5 and pTOPO20, both containing the *PRF6HIS* gene. For verification, pTOPO5 and pTOPO20 were end sequenced using primer JEPR04 5' caa ctc caa cat cgt cca ac 3' (~250bp upstream of 6-His integration), to determine if the hexahistidine integration was successful (Perkin Elmer, ABI310 and Big Dye sequencing terminator mix). Both plasmids contained *PRF6HIS*, pTOPO5, which was used to subclone the *PRF6HIS* gene into a *U. maydis* transformation vector. The *PRF6HIS* gene was cut out of pTOPO5 with *Dra*1 and blunt end cloned into a (*Sac*1) *Eco*RI CR1 site in pCM642 (G418 resistance) and pCM619 (Carboxin resistance), with both vectors containing a *U. maydis* autonomously replicating sequence (ARS) (Kojic and Holloman, 2000). The resulting plasmids were verified using restriction enzyme digest and designated p642PRF6HIS and p619PRF6HIS, respectively.

Transformation of *U. maydis* strain 6/10 (*prf1*DHYG)

Protoplasts of *U. maydis* strain Ha99 (6/10) *prf1*Δ*HYG* and wild-type (1/2) were generated according to Tsukuda *et al*. (1988). Approximately 1 ìg of plasmid DNA (Qiagen Midiprep)

was used in each transformation reaction. The concentration of plasmid DNA was estimated using agarose gel electrophoresis and Lambda DNA digested with *Hind* III for comparison.

Genomic DNA (GDNA) extractions from *U. maydis* were accomplished following Elder *et al*. (1983) with slight modifications. First, 3mls of an overnight culture of cells was pelleted in an eppendorf microcentrifuge tube, washed once in DH_2O, and resuspended in 500µl lysis buffer. 0.3g of glass beads was added along with 500µl of phenol:chloroform:isoamyl alcohol (PCI) (25:24:1). The sample was vortexed at high speed for 8 min then centrifuged at 13k x g for 10 min. The aqueous phase was transferred to a microcentrifuge tube and 1 ml of 95% EtOH was added. The sample was incubated at -80°C for 30 min to precipitate the GDNA then centrifuged at 13k x g for 10min. The GDNA pellet was washed twice in 70% EtOH and dried in a speed vac for 10 min with heat. The GDNA pellet was resuspended in 50µl of TE (10mM Tris, 1mM EDTA pH8.0).

pPRF6HIS in vivo functional analysis

For plate matings, charcoal plates were made as previously described by Holliday (1974) except for the following two changes: 0.5% yeast extract was used instead of 2%, and plates contained 4µg/ml final concentration of carboxin. Carboxin resistant strains were grown overnight at 250rpm, 30°C in 5ml PDB with carboxin at 2µg/ml. 5µl spots of strain 6/72 (wild-type strain 2/9 *a2b2* with carboxin resistant plasmid pGR3) were placed on charcoal plates and allowed to dry in the transfer hood. Experimental strains were added to the charcoal plate, which was then allowed to dry; the plate was wrapped in parafilm and incubated inverted at 30°C for 24hr.

Protein isolation technique.

Strains were grown in 100ml of PDB at 30°C with vigorous shaking until reaching the OD_{600} ~0.6 (10^7 cells/ml). Cultures were centrifuged in 50ml conical tubes at 6000 X g for 6 min at 4°C; the supernatant was discarded and the pellet was resuspended on ice with 20mls of PBS (20mM Sodium Phosphate (pH 7.5), 150mM NaCl) and centrifuged as before. Cells were resuspended in 1ml PBS-EPPI (EDTA, Protease, Phosphatase Inhibitors: 20mM EDTA, 50mM NaF, 40mM Beta-glycerolphosphate, 10mM HEPES, Aprotinin 2µg/ml, Leupeptin 2µg/ml, PMSF 10µg/ml), and kept on ice unless otherwise stated. The sample was divided (~750µl) into two screw-cap microcentrifuge tubes, which contained a cap-full of glass beads. Samples were then placed into a bead-beater (Glenn Mills Inc. Retsch) and agitated at full speed for seven 5 min intervals with a 2 min on-ice resting period in between agitations. Samples were visually assessed for breakage under a microscope at 200X magnification, centrifuged at 30,000 X g for 30 min to remove insoluble proteins and lipids, and quantified according to the Bradford assay (Bio-Rad).

Slight modifications were made for *PRF6HIS* experiments. Strains were grown overnight in 100ml PDB (2µg/ml carboxin) at 30°C. For each experimental strain, 1 ml of the tester strain 6/72 was spotted on four charcoal plates (as described above) with the addition of 1 ml from each experimental strain. Strains were grown for 24 hr at 30°C, and cells were harvested by adding 2 ml of DH_2O to each plate and collecting cells with a bent glass rod into 50 ml conical tubes. Cultures were then processed as stated above except that EDTA was left out of all buffers and solutions. For His*Bind column (Novogen) applications, 3000µg of total soluble protein was used for each column, and the manufacture's protocol was followed.

Polyacrylamide gel electrophoresis and western blotting techniques

For SDS-PAGE separation of total soluble proteins, 8-16% gradient gels were used (ISC bioexpress). For each strain used, 33µg of total protein extract was placed in a microcentrifuge tube. All samples were brought up to the same volume with the addition of PBS-EPPI, and dried in a speed-vac for 27 min with heat. Samples were resuspended in 1X SDS phosphate running buffer (Ausubel et al., 1987), and incubated at 100°C for 5 min. Samples were centrifuged for 15 sec and loaded onto SDS-polyacrylamide gel and electrophoresed in 1 X SDS running buffer (Ausubel et al., 1987) for 90 min at 150V.

All SDS-polyacrylamide gels were blotted onto PVDF membranes overnight at 150mA in transfer buffer (20% methanol, 0.3% Tris, 1.44% Glycine) and post stained with Coomassie brilliant blue to determine protein transfer efficiency. India ink staining was performed according to Ausubel et al., 1987, to visually assess proteins transferred to PVDF membranes.

For western blotting using anti-phosphoserine and anti-phosphothreonine antibodies, Zymed methods for western blotting were followed (http://www.zymed.com/ methods/blotting.html). For all experiments using the Anti-His (C-term) antibody, the manufacture's protocol was followed for western blotting (Invitrogen). For an anti-body control, 100ng of a hexahistidine tagged protein was used (Bingli Gao, unpublished data).

RESULTS AND DISCUSSION

Prf1 hexa-histidine epitope tag

Prf1 was chosen for analysis because it is a putative target substrate of the MAP kinase and cAMP pathways. It is also a very important transcription factor that regulates the *bE-bW* heterodimer essential for pathogenicity in *U. maydis*. An approach was taken to determine the phosphorylation state of Prf1 during its pivotal role in the fungus during mating. The created tagged construct included the upstream activation sequence (UAS) to assure that Prf1 expression was under the control of its own promoter to mimic what happens naturally in mating reactions. One difference between wild-type expression of *prf1* and the tagged expression is that the tagged gene is on an autonomously replicating vector that produces 25 additional copies of the *prf1* (Tsukuda, 1988). However, it was previously shown that the constitutive expression of *prf1* did not interfere with normal development of *U. maydis* nor produced any obvious phenotype regarding morphology, mating, and pathogenicity (Hartmann et al., 1999).

For protein phosphorylation anaylsis of Prf1, a hexahistidine tag was incorporated on the carboxy terminus and verified through sequencing (Fig. 26A and data not shown). Transformation of *prf1* mutant strain 6/10, revealed that pPRF6HIS is able to complement during mating and that the hexahistidine tag appeared to not disrupt the function of Prf1 *in vivo* (Fig. 26B). This result was consistent with a previously published result that showed a *prf1-E1* allele (*prf1* gene with an amino terminus hexahistidine tag and a carboxy terminus triple-myc tag) did not disrupt the function of Prf1 during mating (Muller et al., 1999). However, unlike the construct in Muller et. al., where the *prf1-E1* allele was integrated into the *prf1* locus, the plasmid pPRF6HIS was created for the ease of shutting the construct into mutant strains for

analysis. An attempt was made to isolate Prf1 via His*Bind columns (Novogen), however a predicted 90 kDa band was never seen in polyacrylamide gels (Coomasie stained) or western blots using Anti-His(C-term) antibody (Invitrogen) (Fig. 26C). A smaller band (<60 kDa) was seen but dismissed as an artifact because it was seen in the empty vector control lanes (Fig. 26C). These Prf1 results suggest four possible reasons for why the tagged Prf1 protein is not seen: (1) expression of *prf* and subsequent protein yield is too low to detect, (2) Prf1 is an unstable protein, (3) Prf1 is post-translationally modified on the carboxy terminus under normal conditions, (4) the hexahistidine epitope tag is being cleaved during processing.

Protein phosphorylation patterns of *U. maydis* mutants

Total soluble protein extracts were resolved through polyacrylamide gels and blotted onto PVDF membranes (Fig 27A-D). Presumably because of differences in the fungal cell wall, concentrations of the total soluble protein varied greatly between the strains. The filamentous strain *uac1* (1/9) yielded protein concentrations of 0.1µg/µl, while other mutant strains such as *fuz7/ubc5* (2/37) yielded ten times the protein concentrations, 1µg/µl. To overcome these differences, all samples were brought up to an equal volume, and then concentrated by drying the sample down in a speed vac. This procedure seemed to have very little, if any, effect on the protein samples (data not shown). A negative control probing with the secondary goat anti-mouse horseradish peroxidase conjugated antibody revealed that most of the bands that were seen in the experimental blots are background (Fig. 27B-D). Several bands were seen in the anti-phosphoserine/threonine western blots, however they were not always reproducible and were at the same molecular weight as some weaker bands seen in the negative control blot (black arrows Fig 27B-D). Although the use of anti-phosphoserine/threonine antibodies

appeared as a relatively safe alternative to using radioactivity to distinguish phosphorylation patterns in *U. maydis*, they were ineffective under these experimental conditions.

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Table 3. Strains of *Ustilago maydis*

Strain	Relevant Genotype	Source
1/2	*a1 b1*	Gold *et al.*, 1997
1/9	*a1 b1 uac1*::*ble*[a]	Gold *et al.*, 1997
1/68	*a1 b1 ubc1::hyg*	Gold *et al.*, 1994
2/9	*a2 b2*	Gold *et al.*, 1997
2/37	*a1 b1 fuz7::hyg*	Banuett and Herskowitz 1994 I.
2/58	*a1 b1 ubc3/kpp2::nat*	Muller *et al.*, 1999
5/10	*a1 b1 ubc2::cbx*	Mayorga and Gold 1998
6/1	*a1 b1 ucn1::hyg*	This study
6/10	*a1 b1 prf1*::*hyg*	Hartman *et al.*, 1996
6/30	*a1 b1 prf1*::*hyg*, pCM619	This study
6/31	*a1 b1 prf1*::*hyg*, p619PRF1HIS	This study
6/34	*a1 b1* (strain 1/2), p619PRF1HIS	This study
6/35	*a1 b1* (strain 1/2), pCM619	This study
6/55	*a1 b1 adr1*::*ble*	Durrenberger *et al.*, 1998
6/72	*a2 b2* (2/9), + pGR3 cbx^R	This study

[a] *uac1* gene disruption with phelomycin resistance marker

Fig. 26. Strategy for incorporating a six histidine tag on Prf1 for protein isolation and phosphorylation analysis in *U. maydis* mutant strains.

A. Prf1-6HIS construct strategy. Oligonucleotides were constructed based on the known *prf1* sequence (NCBI accession # U40753) to amplify the entire 5' promoter region and incorporate a six histidine tag on the 3' end.

B. Complimentation of *prf1* mutant *in vivo* with pPRF16HIS.

5µl spots of overnight cell cultures were placed on charcoal-containing medium. The row of wild-type tester strain 6/72 (*a2b2* strain + pGR3) was first placed and dried, then strains above the row were either spotted alone (top row) or on top of the wild-type pGR3 strain (bottom row). Mating reactions were photographed 24 hours post inoculation.

C. Extraction of Prf1-6HIS using His-Bind columns. Strains were grown 24 h at 30^0C in PDB with 2µg/ml carboxin and co-inoculated on CM plates with charcoal. Plates were incubated at 30^0C for 24 h and total soluble protein was isolated. 33 µg of soluble protein was loaded per lane and separated by SDS-PAGE and blotted onto a PVDF membrane. Lanes were as follows: 1-3 total protein extracts; 4-6 eluted His-Bind column (Novagen) protein samples. The left membrane was stained for total protein with India ink. The right membrane was incubated with a primary mouse anti-His (C-term) (Invitrogen) monoclonal antibody, followed by a secondary goat anti-mouse horseradish peroxidase conjugated antibody and detected with the BCPI/NBT substrate/chromogen solution.

The experiment was repeated four times with similar results. Lanes were as follows:

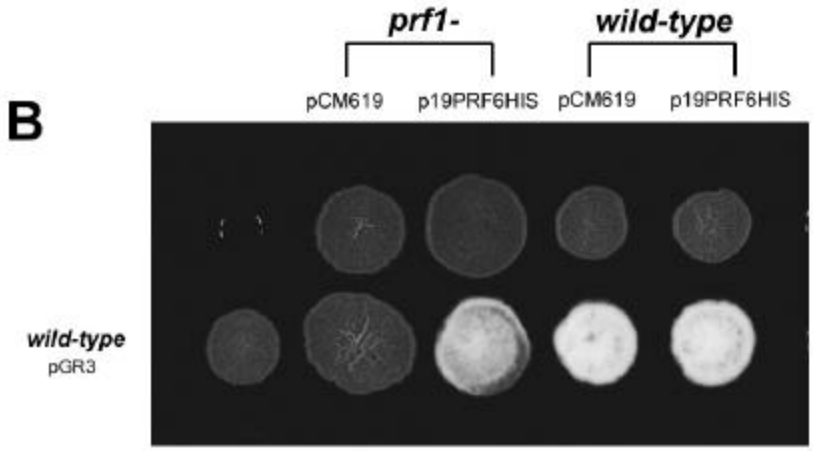
(1, 4) 6/35 (*a1b1* pCM619) wild-type strain crossed to 6/72 (*a2b2* pGR3)

(2, 5) 6/30 (*a1b1 prf1*$^{-}$ pCM619) crossed to 6/72 (*a2b2* pGR3)

(3, 6) 6/31 (*a1b1 prf1*$^{-}$ p619PRF1HIS) crossed to 6/72 (*a2b2* pGR3)

(mw) molecular weight marker, the small arrow indicates the 148 kDa phosphorylase B protein and the large arrow indicates the 60 kDa glutamic dehydrogenase protein (MultiMark multi-colored standard, Invitrogen)


A
DraI
uas
ATG
prf1
TGA
HHHHHHTGA


B
prf1-
wild-type
pCM619
p19PRF6HIS
pCM619
p19PRF6HIS
wild-type
pGR3

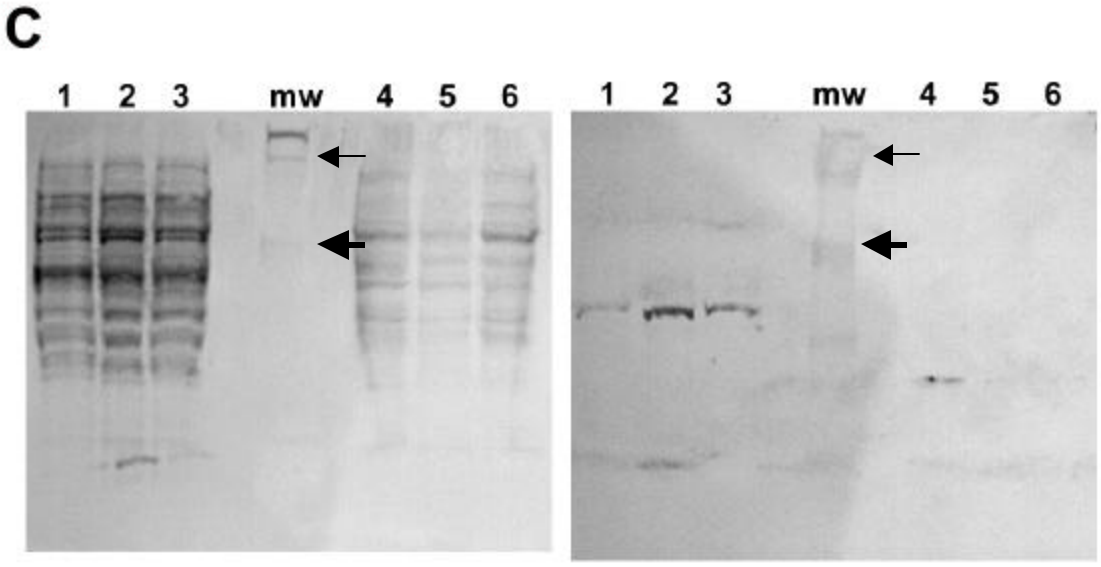

C
1 2 3 mw 4 5 6
1 2 3 mw 4 5 6

Fig. 27. Detection of protein phosphorylation patterns in various *U. maydis* strains using anti-phosphoserine and anti-phosphothreonine antibodies.

A. Detection of *U. maydis* total soluble protein by India ink staining for loading control. Total soluble protein was extracted from various *U. maydis* strains. As determined by Bradford assay, 33µg of protein extract was loaded per lane and separated by SDS-PAGE. The polyacrylamide gel was blotted onto a PVDF membrane and stained with India ink to confirm efficient transfer of proteins. Lanes are as follows:

(mw) molecular weight marker

(1) 1/2, wild-type

(2) 1/9, *uac1*$^{-}$

(3) 1/68, *ubc1*$^{-}$

(4) 6/55, *adr1*$^{-}$

(5) 6/1, *ucn1*$^{-}$

(6) 2/37, *fuz7*$^{-}$/*ubc5*$^{-}$

(7) 2/58, *ubc3/kpp2*$^{-}$

(8) 5/10, *ubc2*$^{-}$

(9) 6/10, *prf1*$^{-}$

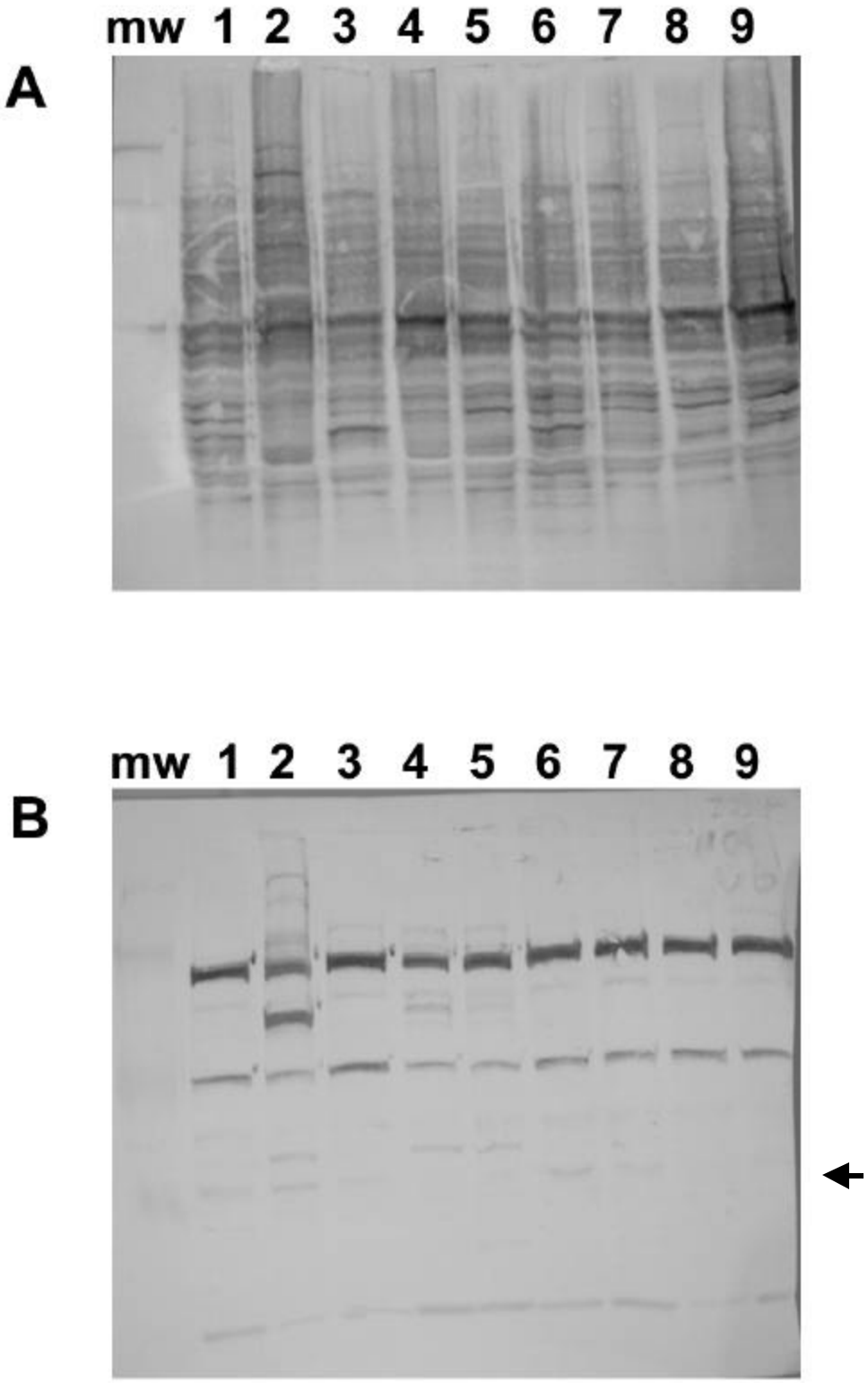
B. Detection of non-specific hybridization with secondary antibody.

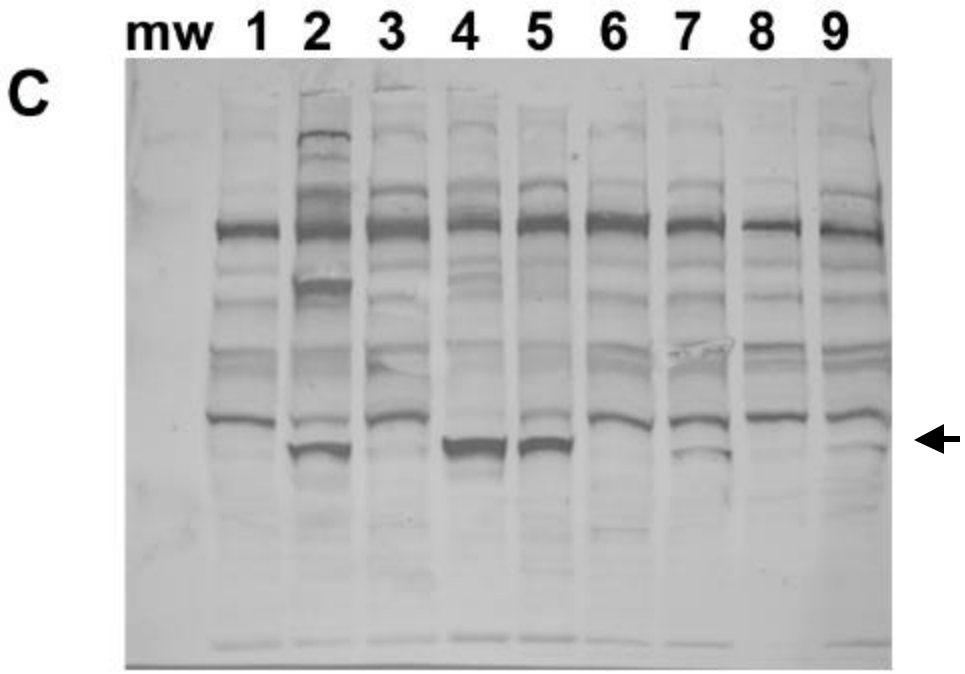
Detection of *U. maydis* total soluble protein extract with goat anti-rabbit horseradish peroxidase. PVDF membrane blot is same as above except that instead of India ink staining the blot was subjected to goat anti-rabbit horseradish peroxidase antibody which reacts with the BCPI/NBT substrate/chromogen solution to produce dark bands indicating background binding. Lanes are the same as in A.

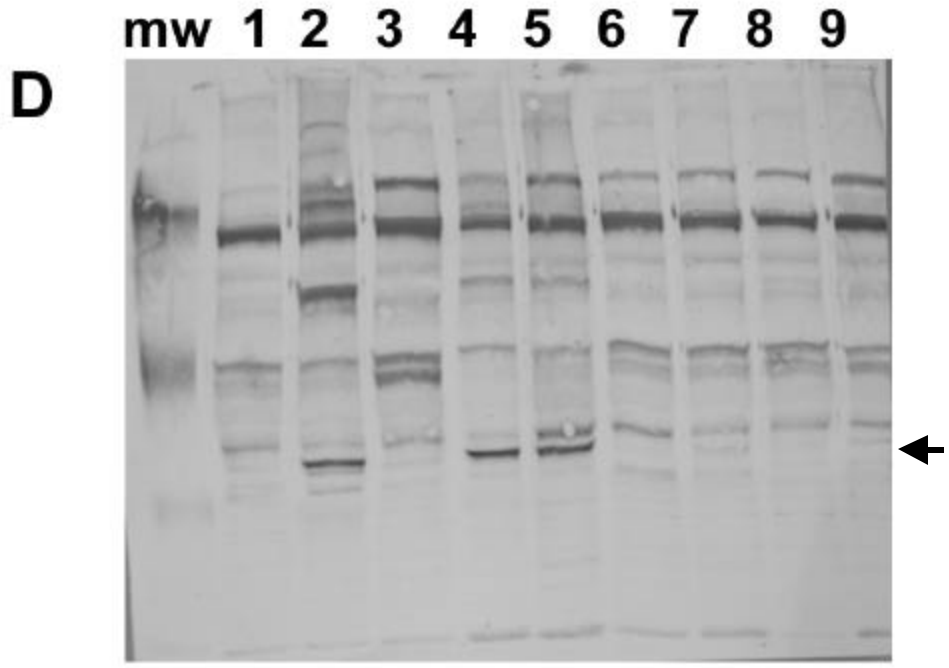
C. Detection of phosphorylated serine residues in total *U. maydis* protein extracts.

U. maydis total soluble protein was extracted from various strains and separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was first incubated with a primary rabbit anti-phosphoserine antibody, then incubated with a secondary goat anti-rabbit horseradish peroxidase conjugated antibody. Lanes are the same as in A.

D. Detection of phosphorylated threonine residues in total *U. maydis* protein extracts. *U. maydis* total soluble protein was extracted from different strains and subjected to the same procedures as above, except that the primary antibody is a rabbit anti-phosphothreonine antibody. Lanes are the same as in A.


mw 1 2 3 4 5 6 7 8 9
A
mw 1 2 3 4 5 6 7 8 9
B


C
mw 1 2 3 4 5 6 7 8 9


D
mw 1 2 3 4 5 6 7 8 9

CHAPTER 4

SUMMARY

Ustilago maydis is an ideal organism for studying host-pathogen interaction and determining the influence of fungal genes in morphology, mating and pathogenicity. Our laboratory studies have focused primarily on a forward genetics approach to discovering genes that are morphology determinants, because, in *U. maydis*, morphology has been successfully used as an indicator of how the fungus responds to mating and virulence within maize plants. The major goals of this project were: to determine the roles of (1) calcineurin and (2) PP2A in regard to morphogenesis, mating and pathogenicity in *U. maydis*, and (3) to compare protein phosphorylation patterns of protein kinase and protein phosphatase mutants with wild-type strains.

Our first goal was to analyze the role of the *U. maydis* calcineurin (*ucn1*) catalytic subunit in morphogenesis, mating, and pathogenicity. The *ucn1* gene was cloned, sequenced, and determined to be homologous to other CN catalytic subunits. To understand the functional role of *ucn1*, phenotypic analysis was done on *ucn1* null mutant strains. Consistent with our hypothesis, CN mutants exhibited a multiple budding phenotype, although different from what we expected. We hypothesized that a *ucn1* mutant would have the same multiple budding phenotype as a *ubc1* mutant. However, the phenotype of a *ucn1* mutant, has larger multiple budding cell clusters and lacks the "wet" *ubc1* mutant colony morphology seen on agar plates. Interestingly, the size of the *ucn1*$^{-}$ multiple budding cell clusters are inversely dependent on their inoculation density, and nutrient deficiency or *U. maydis* cell excretions appear not to be the cause of this phenomenon. Besides morphology, we also hypothesized that *ucn1* mutants would have a reduction of virulence within maize plants. Our data coincides with this

hypothesis; in addition, *ucn1* mutant infected maize plants were reduced in chlorosis when compared to *ubc1* mutant infected maize plants.

To determine epistatic relationships between *ucn1* and other previously characterized signal transduction pathway mutants in *U. maydis*, double mutant analysis and CN inhibitor assays were employed. The *uac1 ucn1* double mutant has an additive phenotype which appears to be both multiple budding and filamentous, and unlike the filamentous haploid *uac1* mutant, the double mutant lacks invasive filamentous growth.

The solopathogenic haploid strain SG200 (*a1::mfa2 bE1bW2*), has a noninvasive filamentous phenotype. Interestingly, the SG200 *ucn1* double mutant has a phenotype identical to the *ucn1* mutant, which suggests that the SG200 strain and the *uac1* mutant strain are utilizing different signal pathways to produce a filamentous phenotype.

The CN inhibitor, cyclosporine A (CsA), was successfully used to determine the effects of inhibiting CN in *U. maydis* strains. Surprisingly, wild-type strains followed a distinct budding pattern after the addition of CsA. All mutant strains assayed with CsA exhibited a *ucn1* mutant phenotype except the *adr1* mutant (catalytic subunit of PKA), which appeared to be unaffected. This phenotypic result suggests two possibilities: 1) the CsA did not enter the *adr1* mutant cells, or 2) a functional *adr1* catalytic subunit is necessary for the *ucn1* mutant phenotype. A double mutant (*adr1 ucn1*) haploid strain (which was not obtained) would have addressed these possibilities.

The diploid strain D132 produced a *ucn1* mutant phenotype when grown in the presence of CsA. This result brought two ideas to mind. First, in *ucn1* (*a1b1*) X *ucn1* (*a2b2*) mutant crosses, which have a non-filamentous phenotype on charcoal plates, these cells actually

mated and the resulting phenotype was a multiple budding diploid (*ucn1*$^{-}$ *ucn1*$^{-}$) strain. This hypothesis could have been tested by constructing a *ucn1*$^{-}$ *ucn1*$^{-}$ diploid, but obtaining another *ucn1* mutant with a different selective marker was not done.

The other idea is that since both the diploid strain D132 and the solopathogenic haploid strain SG200 exhibit the *ucn1* mutant phenotype in the presence of CsA, while the *uac1* mutant does not, the mechanisms that control filamentation in D132 and SG200 must be different than those in the *uac1* haploid mutant. To further emphasize the difference between these strains is the fact that the *uac1* haploid mutant is nonpathogenic, but SG200 and D132 are pathogenic in maize. Both SG200 and D132 have a functional cAMP pathway required for bE1/bW2 heterodimer activation, which is involved in the upregulation of genes that induce filamentous dikaryotic growth. However, in a *uac1* haploid mutant, cAMP is not made (A. Martinez, unpublished data), which suggests that a different pathway is involved in producing the filamentous phenotype.

Another interesting lesson learned from the SG200 *ucn1* double mutant was that signals involved in dikaryon formation appear to be compromised. In the case of a SG200 strain, it is presumed that filamentation occurs because Mfa2 (pheromone) interacts with Pra1 (receptor) to activate a MAPK module to activate the *bE1/bW2* heterodimer, which activates genes involved in filamentation. Our data indicate that *mfa* expression is altered in a *ucn1* mutant background, which may be the only reason why filamentation does not occur in SG200. However, this discovery cannot account for the loss of pathogenicity found in a SG200 *ucn1* mutant, because a previously described strain CL13 (*a1 bE1bW2*) has demonstrated that an activated pheromone pathway is not a prerequisite for pathogenicity.

The *ucn1* mutation appears to be a pathogenicity determinant, because the SG200 *ucn1* mutant is severely reduced in virulence, and we also know that this reduction in virulence is post mating. Because of these data, an attempt was made to use CsA to prevent wild-type *U. maydis* strains from infecting maize plants. However, CsA could not prevent infection in maize plants even with a two-hour incubation of the fungus with the inhibitor prior to inoculation within the maize plants. The reason for these results we cannot yet determine. To continue this investigation of how CN might be used in fungal control, future researchers could examine other analogs of CN inhibitors that might be potential fungicides. Because CN is essential for pathogenicity, it can be used as a new launching pad for investigating down stream genes needed to form a filamentous dikaryon. This experimentation could be accomplished be comparing the SG200 *ucn1* mutant strain to the SG200 strain through genomic tools such as microarrays or the Suppressive Subtraction Hybridization PCR (SSHP) Kit. These methods would identify genes regulated during filamentous growth and pathogenicity, and would also identify genes that are repressed or expressed under normal CN functioning.

The second goal of this study, to determine the role of PP2A in regard to morphogenesis, mating and pathogenicity in *U. maydis*, followed along the same lines of reasoning as our *ucn1* investigations. We were able to clone, sequence and characterize the *upa2* gene, however, disrupting the *upa2* gene was never accomplished. Through the use of the PP2A inhibitor, okadaic acid, we discovered that PP2A appears to be essential for cell viability. The morphology of *U. maydis* cells becomes less elongated and more spherical in the presence of okadaic acid, which indicates PP2A's role in normal cellular function. This finding is significant because it identifies PP2A as another potential target for fungal disease control.

Identification of PP2A inhibitors that are only toxic to fungi, not mammals or plants, would be necessary for PP2A to be a useful target for fungal control. Further observations of the effects of okadaic acid should include actin localization, nuclear division, and cell wall synthesis to illuminate the functional roles of PP2A in *U. maydis*.

For the third goal, to compare protein phosphorylation patterns of protein kinase and protein phosphatase mutants with wild-type strains, a two-prong approach was taken to understand phosphorylation patterns in *U. maydis* wild-type and mutant strains. The first approach was to hexahistidine tag the transcription factor Prf1 and isolate the protein from cell lysates to determine its phosphorylation state during mating. Unfortunately the protein was never recovered from cell lysates. The second approach was to visualize phosphorylated soluble proteins via western blotting and anti-phosphoserine/threonine antibodies. However the antibodies proved to be ineffective at identifying phosphorylation patterns in *U. maydis* strains. If this experiment were to be revised, radioactive ^{32}P might be utilized instead of anti-phosphoserine(threonine) antibodies to identify phosphorylation patterns between wild-type and mutant *U. maydis* strains.

Therefore, in summation, the catalytic subunit of CN (*ucn1*) was shown to be important in morphogenesis, mating, and pathogenicity. Although a deletion in the catalytic subunit of PP2A was not obtained in *U. maydis*, our data strongly suggest that PP2A is essential for cell viability. In our attempts to compare protein phosphorylation patterns between wild-type, protein kinase, and protein phosphatase mutants, we were

unable to discern patterns based on our experimental approach. While we made some advancement in the understanding of morphogenesis, mating, and pathogenicity, more research needs to be done to elucidate the role of CN and PP2A in the known signal transduction pathways.

APPENDICES

APPENDIX 1: *UCN1* GENE

UCN1

```
5'                                                                                -698
AACACATAAGCGAACACACACACACACACTCGTGACAACTCACGACTTCACGCTTGTGACTTGGG
                                                                                  -633
GACTTGCCAGCTCGACTTTCCTTTCGCTTGAGTCGTGAGTGAGTGAGACAAAGTCGTGAGCATTG
                                                                                  -568
TAGCCTCGTGCAAGTCCAAAATGCCATGCATCACTCTGACTGCTTGCTTCTGGTAACACCTTCCA
                                                                                  -503
GGACTCGTGACTCTCGTTTTCGTCGTACCGCCATCTGAACAACCCTAGCTCGCCCCGACACTCTC
                                                                                  -438
ATACTTCTGCGCAGCACGAATTTCATCAGCTCCTTACCGTACTGCAAGGCTTGCTCCATACCCCG
                                                                                  -373
TCGGCCCTACCTCATCAGGTCACCGCTCCTTCCGTGACCTATCTAGTGCTCCGTGGGCTATCTAG
                                                                                  -308
TGCTACGACTATCACGGATCTCTCTTGCTCACCATCTTTTACTGCTTTCTAGCGCGCGAAAAGGT
                                                                                  -243
AGGTCTCTTTCGCCAACCCGCCCTCGATTTCGGAGACATTGCCAGCTGTATGCTGGGTTCTACAG
                                                                                  -178
CACCTTTCTAGAGTGAGCATCGCGCTGACCCTTGACCCTTGCTCTATCTGATCTGCTCGTTTCTG
                                                                                  -113
TCCATTCTCGTTTTCTTTCTTGCCAACCTGCGTGCTTCGGCTCTCCCCCTACTCTCATCATATTT
                                                                                   -48
TCAAGGCTGGACACTCCTGGCTTTCCTCGAAACGCTGTCGTTAGGCAGCTTTCACACTCCCTTCC

                                                                                    12
CTTCCTTCTGAGCGCTAGGCGAGCGCCTGAATCGCTCTTCTCAGACA ATG ACT ACC CCT
                                                    M   T   T   P
                                                                                    60
GCG CAG GTC GAC AGG CAG CAG AGG CTT GTC AAG GCC ATC GGC CAA ATC
 A   Q   V   D   R   Q   Q   R   L   V   K   A   I   G   Q   I
                                                                                   108
ACC AAC AAG CCC GCA CCC CCC GAG ATT GAT TTC ACC ATT CAC ACC ACT
 T   N   K   P   A   P   P   E   I   D   F   T   I   H   T   T
                                                                                   156
GAT GAT GGA GCC CAG GTC AGT ACG CAG GAG CGT GTC ATC AAA GAT GTG
 D   D   G   A   Q   V   S   T   Q   E   R   V   I   K   D   V
                                                                                   204
CAG GCA CCT GCA TTC CAG AAG CCC ACC GAT GAA CAG TTC TTC AGC GCC
 Q   A   P   A   F   Q   K   P   T   D   E   Q   F   F   S   A
                                                                                   252
AAA GAC CCC TCC AAA CCA GAC ATT GCC TTC CTC AAG AAT CAT TTC TAC
 K   D   P   S   K   P   D   I   A   F   L   K   N   H   F   Y
                                                                                   300
CGT GAG GGT CGT CTG ACC GAT GAT CAG GCT CGC TTC ATT CTC ACA AGA
 R   E   G   R   L   T   D   D   Q   A   R   F   I   L   T   R
```

348
GCC ACC GAG ATT TTA CGT CAG GAA CCC AAT CTG CTC GAA GTC GAT GCA
 A   T   E   I   L   R   Q   E   P   N   L   L   E   V   D   A
396
CCC ATC ACC GTT TGC GGG GAC ATG CAT GGG CAG TAT TAT GAC CTC ATG
 P   I   T   V   C   G   D   M   H   G   Q   Y   Y   D   L   M
444
AAG CTC TTT GAA GTG GGT GGC AAC CCC GCC GAT ACC CGT TAT CTC TTC
 K   L   F   E   V   G   G   N   P   A   D   T   R   Y   L   F
492
TTG GGA GAC TAC GTC GAC CGC GGC TAC TTT TCG ATC GAG TGC GTC CTC
 L   G   D   Y   V   D   R   G   Y   F   S   I   E   C   V   L

*Bgl*II
540
TAC CTC TGG GCT TTG AAG ATC TGG TAT CCG GAT ACG CTC TTC CTC CTT
 Y   L   W   A   L   K   I   W   Y   P   D   T   L   F   L   L
588
CGT GGC AAT CAC GAA TGT CGC CAT CTG ACC GAC TAC TTC ACC TTC AAG
 R   G   N   H   E   C   R   H   L   T   D   Y   F   T   F   K
636
CTC GAG TGC AAG CAC AAG TAT TCC GAG GAA ATT TAC GAC CTT TGC ATG
 L   E   C   K   H   K   Y   S   E   E   I   Y   D   L   C   M
684
GAG TCG TTT TGT ACA CTG CCG CTT GCC GCT GTC ATG AAC AAG CAG TTC
 E   S   F   C   T   L   P   L   A   A   V   M   N   K   Q   F
732
CTC TGT ATC CAC GGC GGT CTC TCT CCC GAG CTC CAG ACC CTC GAC GAT
 L   C   I   H   G   G   L   S   P   E   L   Q   T   L   D   D
780
CTT CGC AGC ATC GAC CGC TTC CGC GAG CCG CCT ACA CAT GGT CTC ATG
 L   R   S   I   D   R   F   R   E   P   P   T   H   G   L   M
828
TGC GAC ATT CTC TGG GCT GAT CCA CTC GAG GAC TTT GGC TCT GAG AAG
 C   D   I   L   W   A   D   P   L   E   D   F   G   S   E   K
876
ACC AAC GAG GAG TTC ATC CAC AAC CAC GTC CGA GGT TGC TCT TAC TTC
 T   N   E   E   F   I   H   N   H   V   R   G   C   S   Y   F
924
TTC ACC TAC AAC GCT GCC TGT CAA TTC CTC GAG CGC AAT CAG CTA CTG
 F   T   Y   N   A   A   C   Q   F   L   E   R   N   Q   L   L
972
TCC ATC ATC CGA GCG CAC GAA GCC CAA GAC GCC GGA TAT CGC ATG TAC
 S   I   I   R   A   H   E   A   Q   D   A   G   Y   R   M   Y
1020
CGC AAA ACA AAG ACC ACA GGC TTT CCC TCG GTC ATG ACA ATC TTC TCA
 R   K   T   K   T   T   G   F   P   S   V   M   T   I   F   S
1068
GCA CCC AAC TAC CTC GAC GTC TAC AAC AAC AAG GCC GCC GTC CTC AAG
 A   P   N   Y   L   D   V   Y   N   N   K   A   A   V   L   K
1116
TAC GAA AAC AAC GTC ATG AAC ATT CGC CAA TTC AAC TGC ACC CCA CAT
 Y   E   N   N   V   M   N   I   R   Q   F   N   C   T   P   H

```
                                                                    1164
CCT TAC TGG CTA CCC AAC TTC ATG GAC GTC TTT ACA TGG AGT TTG CCA
 P   Y   W   L   P   N   F   M   D   V   F   T   W   S   L   P
                                                                    1212
TTC GTC GGT GAA AAG ATC ACC GAC ATG CTC ATT GCC ATT CTC AAC GTC
 F   V   G   E   K   I   T   D   M   L   I   A   I   L   N   V
                                                                    1260
TGC AGC AAA GAG GAG CTC GAG GAG GAG GAA GAG GAG GAC GAG ATC CCC
 C   S   K   E   E   L   E   E   E   E   E   E   D   E   I   P
                                                                    1308
ACC ACA CCC ACT TCT GCA GCT GAA GAA GAG ACC GCC GAG CGC AGA ACG
 T   T   P   T   S   A   A   E   E   E   T   A   E   R   R   T
                                                                    1356
CTC ATC AAG AAC AAG ATC CTC GCT GTG GGC CGT ATG TCT CGT GTC TTT
 L   I   K   N   K   I   L   A   V   G   R   M   S   R   V   F
                                                                    1404
GCC CTG TTG CGT GAG GAG GCC GAA CGC GTA TCA GAG CTC AAG TCA TCC
 A   L   L   R   E   E   A   E   R   V   S   E   L   K   S   S
                                                                    1452
CAG ACC GCC AAG CTG CCG TAC GGC TCG TTG GTG CTC AGC TCC GAG GCT
 Q   T   A   K   L   P   Y   G   S   L   V   L   S   S   E   A
                                                                    1500
GCC AAG GAC GCC ATT GCC AAT TTC GAC GAT GCT CGA AAG GTA GAC ATT
 A   K   D   A   I   A   N   F   D   D   A   R   K   V   D   I
                                                                    1548
GAG AAC GAG CGT CTG CCC CCC GAC TTG ATC GAC GCC GAC GAG GCT GGA
 E   N   E   R   L   P   P   D   L   I   D   A   D   E   A   G
                                                                    1596
CCT GCT TCA CCT GCC GAA GGA GCA CGT GTC TCG TCG CCC GCT TTC GAA
 P   A   S   P   A   E   G   A   R   V   S   S   P   A   F   E
                                                                    1644
GAC ATG GCT TCC CCG GGC AGC CCG GCA TCG CCT GCC ACG CCA AGC TCG
 D   M   A   S   P   G   S   P   A   S   P   A   T   P   S   S
                                                                    1692
CCC ATT GCT GGT GGT CAC CGC CGT GGA CAC TCG AGG ACA AGC AGT CTG
 P   I   A   G   G   H   R   R   G   H   S   R   T   S   S   L
                                                                    1740
GGA ACG ACC ATG TCG AGT CCC TCA AAC CGT CGC CGT TCG CTG GAG TCA
 G   T   T   M   S   S   P   S   N   R   R   R   S   L   E   S
                                                                    1788
ACC GTA AGC ATG ATT CGT GAG GCG CTC GAA GGT ACG GAT GCA GCA GAT
 T   V   S   M   I   R   E   A   L   E   G   T   D   A   A   D
                                                                    1836
GAC AAG CAC CTC GAG AAG CTT GCC AAT GAC ATC ACC TCG CCG GTC TCG
 D   K   H   L   E   K   L   A   N   D   I   T   S   P   V   S
                                                                    1884
CCA AAG GGT ACC GAT CCT CCA GCG CAA GCC CGC TTC GCC AAC AAG TGA
 P   K   G   T   D   P   P   A   Q   A   R   F   A   N   K   *
                                                                    1949
GAACAGACAATACCAAAACTTCAAGCGTACCGACGCATCCCAATCATCCCTTTCTTTTCACAATT
                                                                    2014
TCTGGTTTCAGCCGTTCTTTAGTTCCGTTTCAGGCTTGATCGCATCGTATTTTATAACCTCTTTG
                                                                    2062
CAAGCGTACTTGCATGATTACGGTGAACGTAAGAACAGGGTGTCGTCCGCTGAGTTTGAAACTGA
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2110
ACGGCTTCTTCCACTAAAATCGGTGAAGGCAGAAGAAAATAAACATGACAAAATGGCAGAAGTTG
2158
GGGCCAAAGGTCGAGGAGGAGCCACGCTAGCGAGTAGTCAAGCTCAGCGCGACAAACGCTCTCTC
2223
TGGTCAAAGTACTCGTCGAGGAGGTTGAAGAAGGCTTCCTTATCTTGTTGTGTGAAGCGCGAGAA
2288
CTCGATATGGGATGTAGTGCCTTGGCTGGTTGGATATTCGTGATATGCTTGTGTTGCTGGAGGGT
2353
AAGGTGGGGGAGGGTTATTGGTACCGTCTTCGTTCTGCATTCGAGCTGGAAGGGCTGGTGGTGCA
2384
ACTCCTGCTGCTGCTGAGCCAGAATGACGAC 3'

APPENDIX 2: *UPA2* GENE

UPA2

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5'                                                                    -1041
GATGCGATATGATTTGTGATTGGTGATTCTGAACTGGGCAAGGCATCTAACCTGTGAATTTCGAG
                                                                      -976
GGAATCCACATGTTAAGGCGCCATTTATACAGAGACAAGGTCGAAAGCGGTGATCGATACACGAT
                                                                      -911
CTGCACGGTGGTTTGAAAGGCTGCTATCTTGTACTGCTGCTAAACAAGAAGGAAAGCCGTCGCGC
                                                                      -846
TTAGACCAGCTTGATCAACTCACGACTCACGACTTATTCAACAGCCGTGTTCAAGTTTTCGGGTG
                                                                      -781
TTATGCTTCCAAGTAGTTTTTTTGGCGATTGGCTTGAATTTGGCCTGCTTTCCAAAAGTTGAAAA
                                                                      -716
TGGGTCAAATCTGAATTTGAATACAAAATTCCGTTATTTGAGTGGTTTAGCTAACTGACTCCGTG
                                                                      -651
ACGGGCTGGTATGCCGGCAGTGTGTGGTTCAACTCGCCACGACGTTTTCTGGCAGCCGCGTCGTG
                                                                      -586
CAGGCAGGTGGGCTATGCGTGCGAGTCGTGAGTCGAGACTGGCTCAGCAGCCGTCAAATTACGGA
                                                                      -521
ATTGCTGAGCGCGGTTTGGTGTGGCTTTGGCTCTTTTTCTTTGCTTTGGACTGAGTGACTGGCTG
                                                                      -456
GCTTGTGCCTCGTGCACGACTGGCTGAGCGTGTTAACTTGGGTTATCCTGAGGCTTGGCCTGCGT
                                                                      -391
CAGCTCCATGTTGGCTGCTCGTCAACACCGTTCGATTTGACCATCATCCTCTTGTTGCGGTTGTC
                                                                      -326
TCGATCATACCCTCAAAAGCCAGTCTGTCTTCTTCGATCTGCATTTCTCTCACCTCCAGCACGTC
                                                                      -261
ATACGCAGCACTTGTTCCAGCTTCTTCGAGCTCGAACCACTCATCTCTCGACGCATCCGCATAGA
                                                                      -196
TACGTTTCCGCATAGCCGTCTCCATCTTGGCATCGTGTACGGTTGTCTTTTCTCGCATTGCGTAC
                                                                      -131
CTTTGACCTGATCTCAGCTTCTGCACATATATACCCTCCAAGCACACGCACGCCGTTTTGCAAGG
                                                                      -66
AGTATCTCGTTGAAACGTCGCCTCGGTTTCTAGGCACATCCACCAAAGCTCTCTTGCTCTTATCA
                                                                      -1
TTATTTTTCATCGATCTTGACCAATCCTAGACTACTTTACAACCAAGCACACCATGGTCGACATC
                                                                      48
ATG GTC GAC ATC ACG GAG CAG GAT GCG TGG ATC GCG CAT CTC AGC GAG
 M   V   D   I   T   E   Q   D   A   W   I   A   H   L   S   E
                                                                      96
TGC AAA CAG CTC TCG GAA AAC GAT ATC AAG CGT CTC TGC GAC AAG GCT
 C   K   Q   L   S   E   N   D   I   K   R   L   C   D   K   A
                                                                     144
CGC GAA ATC TTG CTC GGC GAG TCC AAC GTC CAA CCT GTT CGC TGC CCC
 R   E   I   L   L   G   E   S   N   V   Q   P   V   R   C   P
                                                                     192
GTT ACT GTA TGC GGT GAC ATT CAC GGC CAA TTT CAC GAC TTG TCA GAG
 V   T   V   C   G   D   I   H   G   Q   F   H   D   L   S   E
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*Nru*I

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                                                                    240
CTC TTC CGC ATC GGT GGC AAC TCA CCT GAT ACC AAC TAC CTG TTT ATG
 L   F   R   I   G   G   N   S   P   D   T   N   Y   L   F   M
                                                                    288
GGC GAT TAC GTT GAC CGT GGT TAC TAC TCG GTC GAG ACC GTC ACG TTG
 G   D   Y   V   D   R   G   Y   Y   S   V   E   T   V   T   L
                                                                    336
CTC GTG GCA CTC AAG GTG CGC TAC CGT GAC CGC GTC ACC ATC TTG CGT
 L   V   A   L   K   V   R   Y   R   D   R   V   T   I   L   R

                        NruI                                        384
GGT AAC CAC GAA TCG CGA CAA ATT ACC CAG GTC TAC GGT TTC TAC GAC
 G   N   H   E   S   R   Q   I   T   Q   V   Y   G   F   Y   D
                                                                    432
GAA TGC CTA AGG AAA TAT GGC AAT GCG AAT GTG TGG AAG TAC TTT ACC
 E   C   L   R   K   Y   G   N   A   N   V   W   K   Y   F   T
                                                                    480
GAT CTT TTC GAT TAT CTT CCA TTG ACC GCC TTG ATT GAC GAC CAG GTA
 D   L   F   D   Y   L   P   L   T   A   L   I   D   D   Q
                                                                    528
AGT AGA AAC AAC AGC TTG CCC TGC CGG CTT GCT CTG CCG GCC TTG CCT
                                                                    576
CTT CCC GGC GGT TGC ACC TTG AAT TCA CGC TGA CCG ACT TGG CGC TCA

                        BglII                                       624
TTG TCT GTT TGC TTT AAT TAG ATC TTC TGT CTG CAC GGC GGT CTT TCA
                           I   F   C   L   H   G   G   L   S
                                                                    672
CCG TCT ATC GAC ACT CTG GAC CAC ATT CGA TCC ATC GAC CGT ATC CAA
 P   S   I   D   T   L   D   H   I   R   S   I   D   R   I   Q
                                                                    720
GAA GTG CCG CAC GAA GGT CCT ATG TGT GAC TTG CTG TGG TCT GAC CCA
 E   V   P   H   E   G   P   M   C   D   L   L   W   S   D   P
                                                                    768
GAT GAC CGA TGT GGA TGG GGC ATC TCG CCA CGA GGT GCG GGC TAC ACG
 D   D   R   C   G   W   G   I   S   P   R   G   A   G   Y   T
                                                                    816
TTT GGC CAA GAC ATT TCG GAA GCG TTC AAC CAC AAC AAT GGC CTG ACA
 F   G   Q   D   I   S   E   A   F   N   H   N   N   G   L   T
                                                                    864
CTG GTG GCA CGA GCA CAT CAA TTG GTC ATG GAC GGA TTC AAC TGG TCG
 L   V   A   R   A   H   Q   L   V   M   D   G   F   N   W   S
                                                                    912
CAA GAG CGT AAC GTT GTT ACC ATC TTT TCG GCA CCC AAC TAC TGC TAC
 Q   E   R   N   V   V   T   I   F   S   A   P   N   Y   C   Y
                                                                    960
CGA TGC GGC AAC CAG GCA GCC ATC ATG GAA ATC GAC GAG AAC CTC AAG
 R   C   G   N   Q   A   A   I   M   E   I   D   E   N   L   K
                                                                   1008
TAT ACC TTT TTG CAG TTT GAC CCA GCT CCG CGT GCC GGC GAG CCG TTG
 Y   T   F   L   Q   F   D   P   A   P   R   A   G   E   P   L
                                                                   1061
GTC TCG AGA CGC GTG CCG GAT TAC TTT TTG TAA  GATTAGTAGGATGTGTGAGA
 V   S   R   R   V   P   D   Y   F   L   *
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1126
GATTAGTAGGATGTGTGAATGGTGTTGCGAGCCAGTTGAAGACGAGGAAGCGTGTGAATTTGTGT
1191
GTCTGCGCACGGTGAAAATCACGACTGTGATTGTGTTTGTGTGCTGTTAGGTGCAGTGACAGTGC
1256
AAGGCGCTGGTGAGCGAAGCATGCACGGCAAAGTTGCTTCAAGGGAGGGAGTTTCGCCAGCTTTG
1321
ACCGTACGTTGTTCGCACATTCGTTCCACGTATCAGCAGTCTGCCGCATTGGGTGGAGAAGCGAG
1386
TGATGCTGAGCGCGGACGTGGGAGCGGGAGGAGGAAGCAGAGTTACAAGGGCAGGTGGGTCAGTG
1451
GAATGATATTGATTTCTTGTACAAGAGGGATGAGATGCATCTACAGGAAGGAAATTGTGAATCGA
1516
GAGCAAGTGTGAAGATTCACGAGTGGAGCCGAGTTAGTGCGATTTTGGTTTGTGAGCGTTTGCGT
1578
GTTTGGCCGCCGCTTTGGCCGCCGCTTTGGCCGCCCTGCGCGCCTTGTCCTTTGCGTACGTC 3’