Fungal Genetics and Biology 49 (2012) 426–432

Contents lists available at SciVerse ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Deletion of the *Ustilago maydis* ortholog of the *Aspergillus* sporulation regulator *medA* affects mating and virulence through pheromone response

Nadia Chacko [a], Scott Gold [a,b,*]

[a] Department of Plant Pathology, University of Georgia, Athens, GA 30605, USA
[b] USDA, ARS, Russell Research Center, Toxicology & Mycotoxin Research Unit, Athens, GA 30605, USA

ARTICLE INFO

Article history:
Received 13 October 2011
Accepted 11 April 2012
Available online 19 April 2012

Keywords:
Ustilago maydis
medA
Pheromone response
Virulence
DelsGate
Sporulation

ABSTRACT

Mating of compatible haploid cells of *Ustilago maydis* is essential for infection and disease development in the host. For mating and subsequent filamentous growth and pathogenicity, the transcription factor, *prf1* is necessary. Prf1 is in turn regulated by the cAMP and MAPK pathways and other regulators like *rop1* and *hap1*. Here we describe the identification of another putative Prf1 regulator, *med1*, the ortholog of the *Aspergillus nidulans* medusa (*medA*) transcription factor and show that it is required for mating and full virulence in *U. maydis*. *med1* deletion mutants show both pre- and post-mating defects and are unresponsive to external pheromone. The expression of *prf1* is down-regulated in Δ*med1* compared to the wild type, suggesting that *med1* is upstream of *prf1*. Additionally, indicative of a role in secondary metabolism regulation, deletion of the *med1* gene de-represses the production of glycolipids in *U. maydis*.

Published by Elsevier Inc.

1. Introduction

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

Ustilago maydis has a tetrapolar mating system made up of the biallelic *a* locus and multiallelic *b* locus. At the *a* locus are the genes *mfa* and *pra* that encode mating pheromone and receptor, respectively, that are required for the recognition of and response to opposite mating type cells. The *b* locus encodes homeodomain proteins *bE* and *bW* that are the subunits of a non-self recognition heterodimeric transcription factor that regulates filamentation, dikaryon maintenance and pathogenicity. The expression of *a* and *b* mating type genes is regulated by the transcription factor Prf1. The expression of genes at the *a* mating type locus occurs only if Prf1 is phosphorylated by the cyclic-AMP dependent protein kinase (PKA) while the expression of genes from the *b* locus requires phosphorylation of Prf1 via PKA and the pheromone responsive MAP kinase. The expression of mating genes is also dependent on the regulation of *prf1* by two other factors, *rop1* and *hap2* and MAPKs Ubc3/Kpp2 and Crk1 (Brefort et al., 2005; Garrido et al., 2004; Hartmann et al., 1999; Mayorga and Gold, 1999; Mendoza-Mendoza et al., 2009). Morphogenesis that follows mating and the subsequent development of the fungus in the host leads to sporulation and is regulated by several other transcription factors like *rum1*, *sql1*, *hgl1* and *ust1* (Durrenberger et al., 2001; Garcia-Pedrajas et al., 2010; Loubradou et al., 2001; Quadbeck-Seeger et al., 2000). Rum1 is required for teliospore development; Sql1 for filamentation and Hgl1 regulates both filamentation and teliospore maturation (Durrenberger et al., 2001; Loubradou et al., 2001; Quadbeck-Seeger et al., 2000).

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

The APSES domain transcription factor encoding gene, *ust1*, is a major regulator of morphogenesis and virulence. The haploid *ust1*

* Corresponding author at: USDA, ARS, Russell Research Center, Toxicology & Mycotoxin Research Unit, 950 College Station Road, Athens, GA 30605, USA. Fax: +1 706 546 3116.
E-mail addresses: sgold@uga.edu, scott.gold@ars.usda.gov (S. Gold).

1087-1845/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.fgb.2012.04.002

mutant shows a dramatic phenotype of *in vitro* filamentation and production of teliospore-like structures (Garcia-Pedrajas et al., 2010). Ust1 and other fungal specific transcription factors with the characteristic APSES domain are known to be regulators of morphogenetic changes like yeast–hypha transition and sporulation (Borneman et al., 2002; Ohara and Tsuge, 2004; Pan and Heitman, 2000; Stoldt et al., 1997; Wu and Miller, 1997). *ust1* is the only APSES domain encoding gene in the *U. maydis* genome and is an ortholog of the *A. nidulans* APSES domain transcription factor *stuA*.

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

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

2. Materials and methods

2.1. Fungal and bacterial strains and growth conditions

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

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

2.2. Nucleic acid manipulations

The *med1* deletion construct was made using the DelsGate method (Garcia-Pedrajas et al., 2008). To produce the deletion construct the following primers were employed: um03588-1 (**TAGGGATAACAGGGTAATTGTACTGTGGCTGTACTGTGCTGT**); um03588-2 (**GGGGACAAGTTTGTACAAAAAAGCAGGCTAAAGAGCGTTGAGTGAGAAAGCGAGA**); um03588-3 (**GGGGACCACTTTGTACAAGAAAGCTGGGTATCTCGTGTAATCGTGTCCAGCCTT**); um03588-4 (**ATTACCCTGTTATCCCTAGCTGCATCCATAACCAGCAACGAA**). The *med1* ORF of strains *a1b1*, *a2b2* and SG200 were replaced completely, from 149 bp upstream of the start codon to 63 bp downstream of the stop codon, with the DelsGate deletion construct plasmid containing a carboxin resistance marker (Table 1). *U. maydis* transformation was carried out as previously described (Barrett et al., 1993). Gene deletion was analyzed and confirmed using PCR and Southern hybridization, respectively. The DNA from all mutant strains and the wild type were digested with *Bgl*I (New England Biolabs, Ipswich, MA, USA) and separated on a 0.8% agarose gel. The DNA was transferred to a Hybond XL nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using 0.4 M NaOH as the transfer solution. The 1231 bp 5′ flank ending 150 bp upstream of the start of the open reading frame of the *med1* gene was used as the probe. The probe was prepared by PCR amplification with the primers *med1*-probe-P1 (TGTACTGTGGCTGTACTGTGCTGT) and *med1*-probe-P2 (AGAGCGTTGAGTGAGAAAGCGAGA), purification of the PCR product and labeling using the DIG-High Prime labeling and detection kit (Roche, Indianapolis, IN, USA). Hybridization and subsequent development of the blot was done according to the manufacturer's instructions.

Table 1
U. maydis strains.

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

2.3. Complementation of Δmed1 mutants and curing of complemented stains

The Δ*med1* mutants were complemented using a genomic DNA library previously constructed in the plasmid pJW42 (Barrett et al., 1993). The cosmid containing the open reading frame and flanks of the *med1* gene was identified by PCR from pools of DNA from the library. Transformed Δ*med1* strains were selected on YEPS agar plates supplemented with 150 μg/ml of hygromycin B (Roche, Indianapolis, IN, USA). Complemented Δ*med1* strains were cured of the cosmid by three successive transfers in PDB medium without hygromycin B. The cured strains were identified by plating on PDA without 150 μg/ml hygromycin B followed by screening for sensitivity to hygromycin B.

2.4. Plate mating assays

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

2.5. Pheromone stimulation

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

2.6. Virulence assays

Maize seedlings of variety Golden Bantam (Athens Seed Co., Watkinsville, GA, USA) were grown in potting soil and injected

after 7 days with cell suspension mixtures of 10^6 cells per ml for each strain. Solo pathogenic strains were inoculated at 10^6 cells per ml. Plants were grown in a Conviron E15 growth chamber (Mannitoba, Canada) with daily cycles of 16 h light at 28 °C followed by 8 h dark at 20 °C. Three replications of 20 plants each were used for each cross. The symptoms were scored at 7, 10 and 14 days post inoculation (dpi) based on the disease scale of 0, no symptom; 1, chlorosis/anthocyanin production; 2, leaf galls; 3, small stem galls; 4, large stem galls and 5, plant death (Gold et al., 1997). The results were analyzed using the non-parametric statistical test (Shah and Madden, 2004).

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

2.7. Real time PCR

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

For glycolipid expression studies, the wild type and Δ*med1* mutant strains were grown in PDB for 24 h at 30 °C for tissue collection and RNA was extracted. RNA was also extracted from wild type and mutant strains first grown in nitrogen starvation medium containing 1.7 g/l of yeast nitrogen base and 5% glucose and supplemented with 0.2% ammonium sulfate for 15 h at 30 °C with constant shaking at 200 rpm, until they reached logarithmic phase and transferred to the medium without ammonium sulfate. The cells were further cultured for 12 h under similar conditions and harvested. cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) using oligo dT primers according to the manufacturer's instructions. qPCR was conducted using the SYBR-GREEN Supermix kit (Bio-Rad, Hercules, CA, USA) on a Cepheid Smartcycler I (Cepheid, Sunnyvale, CA, USA). All real time PCR primers used in the expression studies were designed using the Integrated DNA Technology (IDT) website. The sequences of gene specific primers for mating associated genes are as follows: um02713 (*prf1*) (AACGCATCAAGATGAGCTTGGCAC, ATGCGAAGGTGTCAAAGGATTGCG), um02382 (*mfa1*) (ATGCTTTCGATCTTCGCTCAGACCAC, AACAACACAGCTGGAGTAGCCGAT) and um02383 (*pra1*) (TCTTCGCAATGTTTGGCCTTGGTC, TCGAATGTGTCGTGAGACCGGAAA. Primers for study of expression of genes required for glycolipid biosynthesis were as follows: um06458 (*rua1*) (GCTCTTCGGTCTCTGCTTTG, CTTGTCCGTGATTGTGAAGC) and um06463 (*cyp1*) (AGCACCTTCTGAGCATAGTTG, CACCGTTTTCAACCTGCAAG). The reference gene used was cyclophilin um03726 (*cpr1*) amplified using primers, ACGCCGATTCACTTCGTC and AACGACGATCCCTCGTAACCGAAA). The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

3. Results

3.1. Identification of med1

In *A. nidulans*, transcription factors *medA* and *stuA* are critical regulators of the pathway leading to asexual sporulation (conidiation) (Clutterbuck, 1969; Martinelli, 1979; Miller et al., 1993). Deletion of the ortholog of *stuA* in *U. maydis* (*ust1*) generated a filamentous strain that produced pigmented teliospore-like structures in culture, suggesting its possible role in regulation of sporulation (Garcia-Pedrajas et al., 2010). In the transcriptome analysis of *ust1* compared to the wild type, a gene (um03588) with high similarity to the ortholog of *A. nidulans* transcription factor *medA* was identified by virtue of the fact that it was down-regulated 4.5-fold in the *ust1* deletion mutant. A BLASTp search of *A. nidulans medA* against the *U. maydis* genome database also identified the same gene with an expected value of 8e−61 and 57% identity. Bioinformatic analysis of the protein sequence of this gene (designated *med1*), like the *A. nidulans* protein did not have identifiable functional domains other than a DNA binding region. These data suggest that *U. maydis med1* may be a positively regulated direct or indirect target of Ust1 and its sequence similarity to *A. nidulans medA* suggests a role in development and sporulation.

3.2. Δmed1 secretes excess quantities of glycolipids in culture

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

3.3. med1 is required for in vitro mating and filamentation

To test the Δ*med1* mutants for defects in mating or post-mating filamentation, deletion mutants were spotted on YPD charcoal plates (Fig. 2). On mating wild type compatible haploids, typical dikaryotic hyphae that appeared as white fuzzy growth were produced (Fig. 2A). However, when compatible haploid *med1* deletion strains were co-spotted, fuzzy growth was not observed (Fig. 2A). The complemented Δ*med1* mutants on the other hand behaved like wild type. Complemented strains cured of the *med1* containing cosmid reverted to the Δ*med1* phenotype and were unable to produce filamentous growth indicative of successful mating. Absence of filamentation was also observed when mutants were co-spotted with a compatible wild type mating partner, suggesting a defect in fusion ability in Δ*med1* strains. The solopathogenic haploid strain SG200, which has the active, *bE/bW* heterodimer required for filamentation, is capable of filamentation on YPD charcoal plates (Fig. 2B). The *med1* deletion mutant in the SG200 background, however, failed to filament on these plates (Fig. 2B) indicating that *med1* is critical for post-mating filamentation. Taken together, these data indicate that deletion of *med1* results in loss of filamentous growth in mating reactions and in solopathogenic strains and

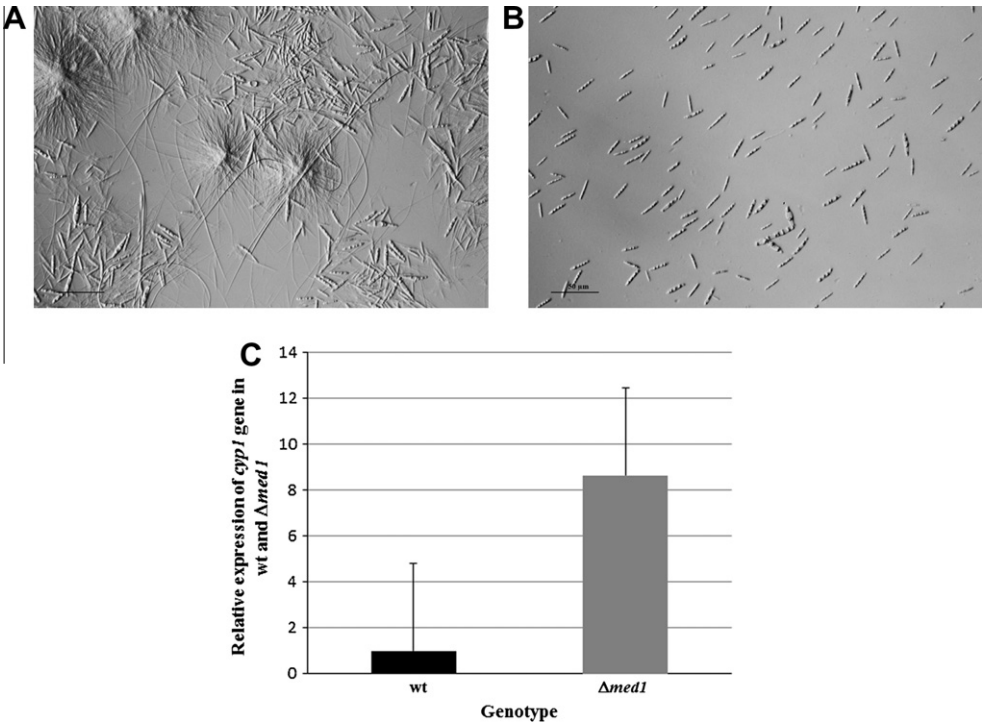

Fig. 1. Over-production of secreted glycolipids by Δ*med1*. (A) Δ*med1* cells in potato dextrose broth (PDB) culture 48 hours post inoculation (hpi) showed the presence of large quantities of secreted glycolipids that appeared as elongated and star shaped crystals that were not visible in 48 hpi cultures of wild type in PDB (B). (C) The expression of the *cyp1* gene was up-regulated in Δ*med1* compared to the wild type in glycolipid inducing low nitrogen medium conditions. The values are an average of three biological replicates with two technical replicates each. The *cpr1* gene was used as control and the expression of *cyp1* in Δ*med1* is relative to *cyp1* expression in wild type when it is arbitrarily fixed at 1.

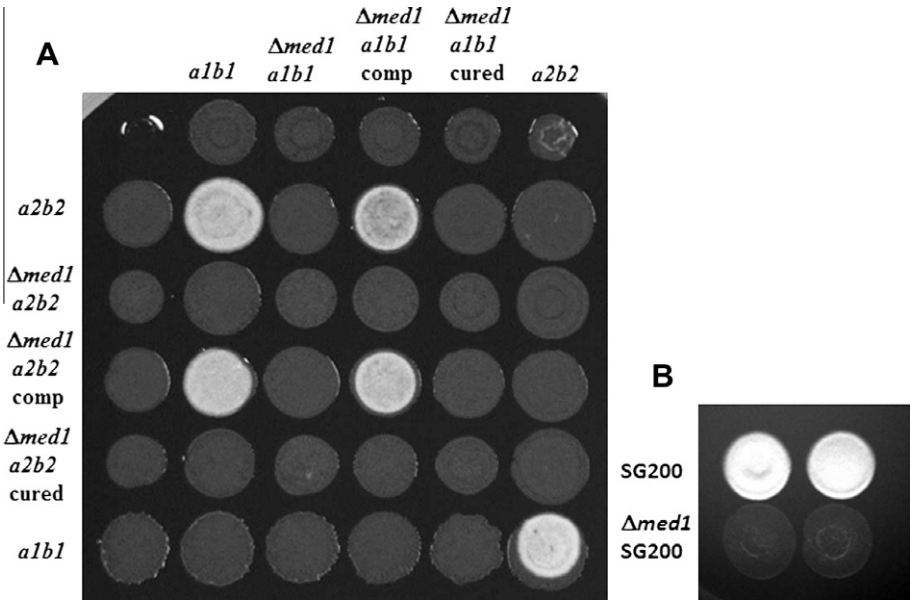

Fig. 2. Impairment of *in vitro* mating in Δ*med1*. (A) Plate mating assay with wild type, Δ*med1*, Δ*med1* complemented and cured strains. Positive mating reactions appear as white fuzzy growth. (B) The filamentation of SG200 strain on charcoal plates was affected when *med1* gene was deleted in this genetic background. The loss of filamentation was consistent in two independent Δ*med1* mutants in the SG200 background.

that transformation of Δ*med1* mutants with a cosmid carrying *med1* results in phenotypic complementation.

3.4. The Δmed1 mutant does not produce conjugation tubes in vitro

To investigate the requirement of *med1* for the production of conjugation tubes, the Δ*med1* mutant and wild type (2/9) in the *a2b2* background, grown in Complete Medium (CM), were treated with synthetic *a1* pheromone dissolved in water. After 8 h of incubation, Δ*med1* cells treated with pheromones did not produce conjugation tubes. The Δ*med1* cells appeared to have a multiple budding phenotype in the presence of artificial pheromones that is reminiscent of the *ubc1* mutant or a wild type in the presence of cAMP (Fig. 3A). Wild type cells after 8 h of treatment with

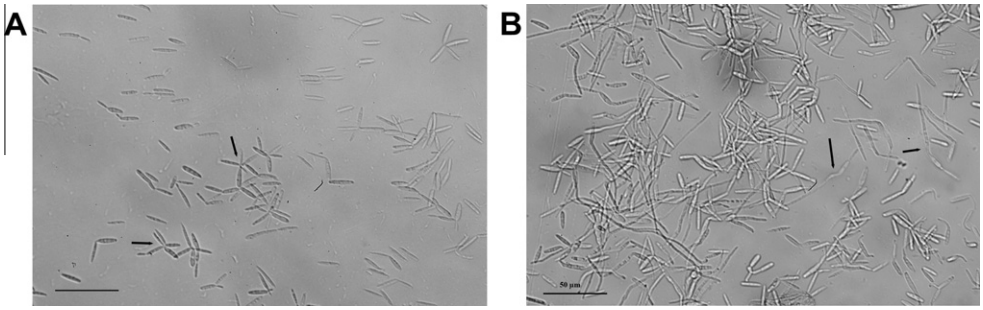

Fig. 3. The Δ*med1* mutant is unable to produce conjugation tubes in response to artificial pheromones. (A) The Δ*med1* mutant cells appeared to have multiple budding phenotype (arrows) and did not produce conjugation tubes in response to pheromone stimulation. (B) After 8 h of treatment with artificial *a1* pheromones, wild type cells produced conjugation tubes (arrows).

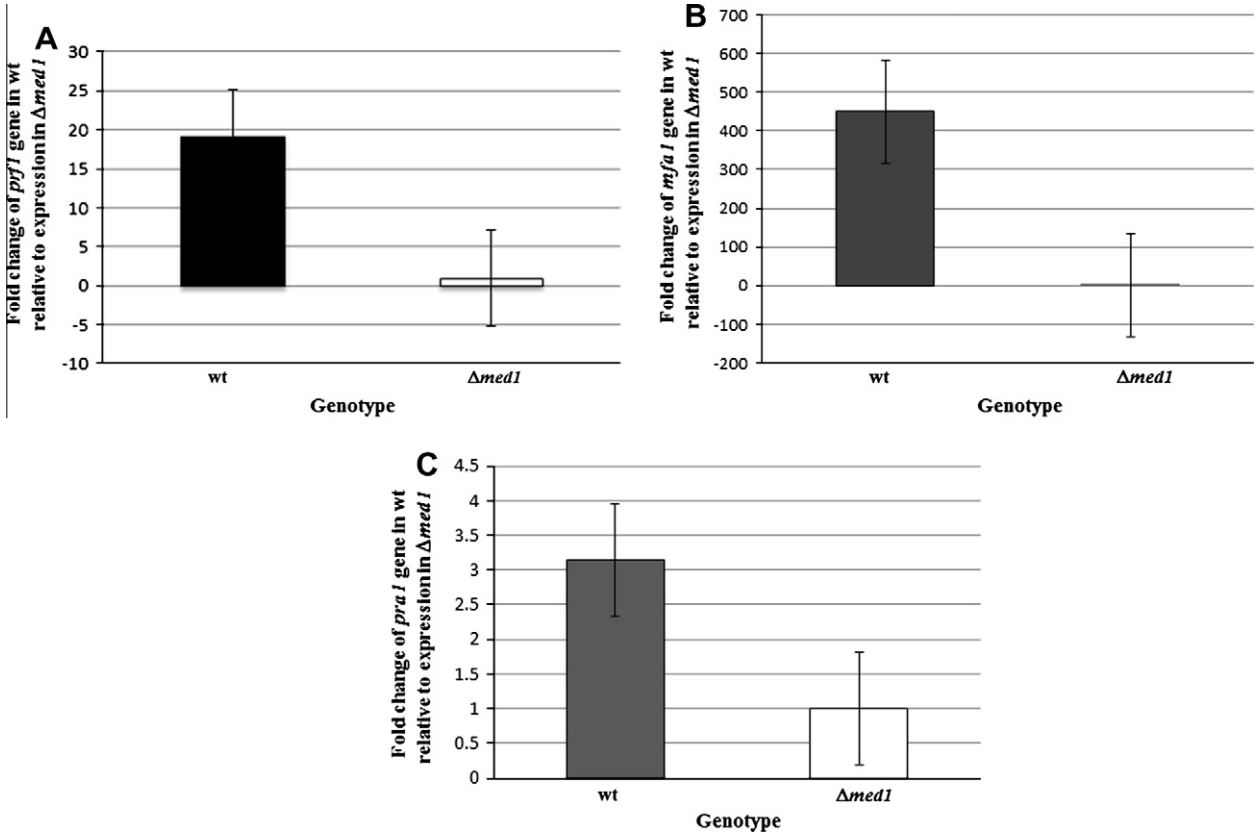

Fig. 4. Reduced expression of pheromone responsive genes in the Δ*med1* mutant. The expression levels of genes encoding (A) the transcription factor *prf1*, (B) the *a1* pheromone *mfa1*, and (C) the pheromone receptor *pra1* were all lower in Δ*med1* compared to the wild type. Relative transcript levels were calculated based on qRT-PCR methodology. Transcript levels were normalized to control gene *cpr1*(um03726). Indicated values correspond to average of three biological replicates and error bars represent the standard deviation.

pheromones produced visible conjugation tubes (Fig. 3B). These data indicate that *med1* plays a critical role in transmission of pheromone signaling.

3.5. Δmed1 mutants are reduced in expression of prf1, pra1 and mfa1

The inability of the Δ*med1* mutant to produce functional conjugation tubes suggests potential reduction or absence in production of pheromones and/or pheromone receptors. To investigate the reduction in expression of pheromone and receptor genes and their regulator *prf1*, in the Δ*med1* mutant, RT-PCR experiments were conducted using RNA extracted from the compatible cells co-spotted on YPD charcoal plates. Control RNA was extracted from wild type cells similarly co-spotted on YPD charcoal plates. The average Ct values from three biological replications each with two technical replications, normalized against the reference gene *cpr1* (um03726) showed a relative down-regulation in expression of *mfa1*, *pra1* and *prf1* genes in the *med1* mutant when compared to the wild type strain (Fig. 4). The 19-fold reduction in the expression of *prf1*, in the *med1* mutant suggests that *med1* functions upstream of *prf1* in the pheromone response pathway.

3.6. Δmed1 mutants are significantly reduced in virulence

To assess the effect of deletion of the *med1* gene on pathogenicity of *U. maydis*, various pair-wise combinations of compatible mutant and wild type cells were injected into 7 day old maize plants. The disease index measured on 7, 10 and 14 days after inoculation

Table 2
med1 is required for full virulence of mated haploids.

Sl. No.	Cross	Disease index		
		7 days	10 days	14 days
(A) Virulence assay of Δmed1-1(a1b1) and Δmed1-4(a2b2) mutant dikaryons				
1.	Δ*med1*-1 × Δ*med1*-4	0.92[a]	1.40[a]	1.82[a]
2.	Δ*med1*-1 × *2/9*	1.02[a]	1.50[ab]	2.08[ab]
3.	*1/2* × Δ*med1*-4	1.20[a]	2.07[b]	2.61[b]
4.	*1/2* × *2/9*	1.92[b]	3.03[c]	4.00[c]
(B) Virulence assay of Δmed1-2 (a1b1) and Δmed1-5 (a2b2) mutant dikaryons				
1.	Δ*med1*-2 × Δ*med1*-5	1.05[a]	1.30[a]	1.38[a]
2.	Δ*med1*-2 × *2/9*	1.47[b]	1.72[ab]	2.02[ab]
3.	*1/2* × Δ*med1*-5	1.68[b]	2.08[b]	2.47[b]
4.	*1/2* × *2/9*	2.28[c]	2.85[c]	3.40[c]

Panel A: $N = 60$ plants in three replications of 20 plants each. Disease index is calculated as the average disease rating [(Σ disease rating for each plant)/total number of plants]. Statistical analysis was performed using a non-parametric test of ordinal data in designed factorial experiments. Treatments with different letters at specific time points indicate a statistically significant difference at the 95% confidence interval. Panel B: $N = 60$ plants in three replicates of 20 plants each. For statistical analysis refer to Table 2A legend.

Table 3
med1 is required for full virulence of solopathogenic haploids.

Sl. No.	Strain	Disease index		
		7 days	10 days	14 days
1.	Δ*med1* SG200	0.51[a]	0.58[a]	0.48[a]
2.	SG200	1.45[b]	1.55[b]	1.63[b]

$N = 60$ plants in three replications of 20 plants each. For statistical analysis refer to Table 2A legend.

from three separate replications was recorded. The mutant was found to be significantly reduced in virulence compared to wild type and that wild type–mutant combinations showed intermediate virulence indicative of a mating defect (Table 2A and B). The plants infected with Δ*med1* mutants displayed symptoms including chlorosis and leaf, stem and basal galls but the symptoms were reduced and progressed at a slower rate than wild type. Moreover, unlike wild type-infected plants, plant death was not observed when inoculated with *med1* mutants. The Δ*med1* mutants in the solopathogenic SG200 background also showed statistically significant reduction in virulence compared to those inoculated with wild type SG200 (Table 3). The plants inoculated with Δ*med1* SG200 generally did not show advanced symptoms like stem and basal galls. Despite the reduction in virulence of the Δ*med1* mutant, it produced galls when injected into ears of adult cv Tom Thumb maize plants. The teliospores produced developed normally and were able to germinate and produce sporidia (data not shown). These data indicate that *med1* is an important virulence factor that when deleted negatively affects disease development in both a pre- and post-mating manner.

4. Discussion

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

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

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

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

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

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

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

The mutation in *ubc1*, the gene encoding the regulatory subunit of PKA, or addition of a high external concentration of cAMP to the wild type haploid, triggers a multiple budding phenotype (Gold et al., 1994). Δ*med1* in response to pheromone stimulation resembles a weak recapitulation of this cAMP induced phenotype. The transfer of pheromone signal requires the normal functioning of the cAMP pathway and high cAMP can stimulate the expression of pheromone gene *mfa1* indicating a crosstalk between cAMP and pheromone pathways (Kruger et al., 1998). This high expression of *mfa1* in response to cAMP does not however induce conjugation tube formation. A constitutively high cAMP level is, on the other hand, known to cause diminished response to pheromone and in turn to reduce conjugation tube formation in the wild type (Muller et al., 2004). Therefore in Δ*med1*, activation of the cAMP pathway may yield multiple budding and repress conjugation tube formation. However, complete loss of conjugation tube formation occurs in mutants in which Pra1, the pheromone receptor, or the pheromone responsive MAP kinase pathway are inactivated (Muller et al., 2003). Thus the complete loss of conjugation tube formation in Δ*med1* also makes it plausible for the gene to be a positive regulator of the pheromone response pathway. Therefore *med1* may function as a point of convergence of the cAMP and MAPK pathways with respect to pheromone related morphogenesis.

The activity of the *prf1* gene is required for mating, both *in vitro* and *in planta* (Hartmann et al., 1999). When the direct regulator of *prf1*, *hap2*, is deleted, the mutant loses the ability to mate and is non-pathogenic (Mendoza-Mendoza et al., 2009). Hap2 binds to a

CCAAT box in the promoter of the *prf1* gene and regulates its expression. A similar regulatory role was shown for the HMG domain protein Rop1. The *rop1* gene is required for pheromone induced gene expression and plate mating reactions but not for pathogenicity, indicating that *rop1* mutants can mate *in planta* (Brefort et al., 2005). This result can be explained by another factor influencing the regulatory role of Rop1 on *prf1 in planta*. However, the Δ*rop1* mutant shows reduced filamentation when co-spotted with its corresponding compatible wild type strain indicating a reduced mating reaction even though it is incapable of forming conjugation tubes in response to pheromones (Brefort et al., 2005). The Δ*med1* mutant on the other hand is incapable of forming conjugation tubes and visible mating *in vitro*. Since *prf1* is required for cell fusion and filamentous growth (Hartmann et al., 1999) the phenotype of Δ*med1* taken together with that of Δ*rop1* suggests that the *med1* gene is a regulator of *prf1 in vitro*. The loss of *med1* also reduces the virulence of *U. maydis*, another characteristic regulated by *prf1*. The reduction of virulence in Δ*med1* could indicate at least a partial regulatory role of *med1* over *prf1 in planta*.

Acknowledgments

We thank Dr. David Andrews for technical assistance with complementation experiments. This work was supported by the United States Department of Agriculture National Research Initiative Competitive Grant No. 20033531913361 to S.E.G.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2012.04.002.

References

Adams, T.H., Wieser, J.K., Yu, J.H., 1998. Asexual sporulation in *Aspergillus nidulans*. Microbiol. Mol. Biol. Rev. 62, 35–54.

Aguirre, J., 1993. Spatial and temporal controls of the *Aspergillus brlA* developmental regulatory gene. Mol. Microbiol. 8, 211–218.

Banuett, F., Herskowitz, I., 1996. Discrete developmental stages during teliospore formation in the corn smut fungus, *Ustilago maydis*. Development 122, 2965–2976.

Barrett, K.J., Gold, S.E., Kronstad, J.W., 1993. Identification and complementation of a mutation to constitutive filamentous growth in *Ustilago maydis*. Mol. Plant Microbe Interact. 6, 274–283.

Boothroyd, B., Thorn, J.A., Haskins, R.H., 1956. Biochemistry of the Ustilaginales. Characterization of extracellular glycolipids produced by *Ustilago* sp. Can. J. Biochem. Physiol. 34, 10–14.

Borneman, A.R., Hynes, M.J., Andrianopoulos, A., 2002. A basic helix-loop-helix protein with similarity to the fungal morphological regulators, Phd1p, Efg1p and StuA, controls conidiation but not dimorphic growth in *Penicillium marneffei*. Mol. Microbiol. 44, 621–631.

Boylan, M.T., Mirabito, P.M., Willett, C.E., Zimmerman, C.R., Timberlake, W.E., 1987. Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans*. Mol. Cell. Biol. 7, 3113–3118.

Brefort, T., Muller, P., Kahmann, R., 2005. The high-mobility-group domain transcription factor Rop1 is a direct regulator of *prf1* in *Ustilago maydis*. Eukaryot. Cell 4, 379–391.

Busby, T.M., Miller, K.Y., Miller, B.L., 1996. Suppression and enhancement of the *Aspergillus nidulans* medusa mutation by altered dosage of the bristle and stunted genes. Genetics 143, 155–163.

Clutterbuck, A.J., 1969. A mutational analysis of conidial development in *Aspergillus nidulans*. Genetics 63, 317–327.

Durrenberger, F., Laidlaw, R.D., Kronstad, J.W., 2001. The *hgl1* gene is required for dimorphism and teliospore formation in the fungal pathogen *Ustilago maydis*. Mol. Microbiol. 41, 337–348.

Garcia-Pedrajas, M.D., Baeza-Montanez, L., Gold, S.E., 2010. Regulation of *Ustilago maydis* dimorphism, sporulation, and pathogenic development by a transcription factor with a highly conserved APSES domain. Mol. Plant Microbe Interact. 23, 211–222.

Garcia-Pedrajas, M.D., Nadal, M., Kapa, L.B., Perlin, M.H., Andrews, D.L., Gold, S.E., 2008. DelsGate, a robust and rapid gene deletion construction method. Fungal Genet. Biol. 45, 379–388.

Garrido, E., Voss, U., Muller, P., Castillo-Lluva, S., Kahmann, R., Perez-Martin, J., 2004. The induction of sexual development and virulence in the smut fungus *Ustilago maydis* depends on Crk1, a novel MAPK protein. Genes Dev. 18, 3117–3130.

Gold, S., Duncan, G., Barrett, K., Kronstad, J., 1994. CAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*. Genes Dev. 8, 2805–2816.

Gold, S.E., Brogdon, S.M., Mayorga, M.E., Kronstad, J.W., 1997. The *Ustilago maydis* regulatory subunit of a cAMP-dependent protein kinase is required for gall formation in maize. Plant Cell 9, 1585–1594.

Hartmann, H.A., Kruger, J., Lottspeich, F., Kahmann, R., 1999. Environmental signals controlling sexual development of the corn smut fungus *Ustilago maydis* through the transcriptional regulator Prf1. Plant Cell 11, 1293–1306.

Holliday, R., 1974. *Ustilago maydis*. In: King, R.C. (Ed.), Handbook of Genetics, pp. 574–595.

Kruger, J., Loubradou, G., Regenfelder, E., Hartmann, A., Kahmann, R., 1998. Crosstalk between cAMP and pheromone signalling pathways in *Ustilago maydis*. Mol. Gen. Genet. 260, 193–198.

Loubradou, G., Brachmann, A., Feldbrugge, M., Kahmann, R., 2001. A homologue of the transcriptional repressor Ssn6p antagonizes cAMP signalling in *Ustilago maydis*. Mol. Microbiol. 40, 719–730.

Martinelli, S.D., 1979. Phenotypes of double conidiation mutants of *Aspergillus nidulans*. J. Gen. Microbiol. 114, 277–287.

Mayorga, M.E., Gold, S.E., 1999. A MAP kinase encoded by the *ubc3* gene of *Ustilago maydis* is required for filamentous growth and full virulence. Mol. Microbiol. 34, 485–497.

Mendoza-Mendoza, A., Eskova, A., Weise, C., Czajkowski, R., Kahmann, R., 2009. Hap2 regulates the pheromone response transcription factor *prf1* in *Ustilago maydis*. Mol. Microbiol. 72, 683–698.

Miller, K.Y., Wu, J.G., Miller, B.L., 1993. The *medA* gene of *Aspergillus nidulans*. J. Cell. Biochem. 17C, 144.

Mirabito, P.M., Adams, T.H., Timberlake, W.E., 1989. Interactions of three sequentially expressed genes control temporal and spatial specificity in *Aspergillus* development. Cell 57, 859–868.

Muller, P., Aichinger, C., Feldbrugge, M., Kahmann, R., 1999. The MAP kinase Kpp 2 regulates mating and pathogenic development in *Ustilago maydis*. Mol. Microbiol. 34, 1007–1017.

Muller, P., Leibbrandt, A., Teunissen, H., Cubasch, S., Aichinger, C., Kahmann, R., 2004. The G{beta}-subunit-encoding gene *bpp 1* controls cyclic-AMP signaling in *Ustilago maydis*. Eukaryot. Cell 3, 806–814.

Muller, P., Weinzierl, G., Brachmann, A., Feldbrugge, M., Kahmann, R., 2003. Mating and pathogenic development of the smut fungus *Ustilago maydis* are regulated by one mitogen-activated protein kinase cascade. Eukaryot. Cell 2, 1187–1199.

Ohara, T., Inoue, I., Namiki, F., Kunoh, H., Tsuge, T., 2004. REN1 is required for development of microconidia and macroconidia, but not of chlamydospores, in the plant pathogenic fungus *Fusarium oxysporum*. Genetics 166, 113–124.

Ohara, T., Tsuge, T., 2004. FoSTUA, encoding a basic helix-loop-helix protein, differentially regulates development of three kinds of asexual spores, macroconidia, microconidia, and chlamydospores, in the fungal plant pathogen *Fusarium oxysporum*. Eukaryot. Cell 3, 1412–1422.

Pan, X., Heitman, J., 2000. Sok2 regulates yeast pseudohyphal differentiation via a transcription factor cascade that regulates cell–cell adhesion. Mol. Cell. Biol. 20, 8364–8372.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, e45.

Quadbeck-Seeger, C., Wanner, G., Huber, S., Kahmann, R., Kämper, J., 2000. A protein with similarity to the human retinoblastoma binding protein 2 acts specifically as a repressor for genes regulated by the *b* mating type locus in *Ustilago maydis*. Mol. Microbiol. 38, 154–166.

Shah, D.A., Madden, L.V., 2004. Nonparametric analysis of ordinal data in designed factorial experiments. Phytopathology 94, 33–43.

Stoldt, V.R., Sonneborn, A., Leuker, C.E., Ernst, J.F., 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J. 16, 1982–1991.

Urban, M., Kahmann, R., Bolker, M., 1996. Identification of the pheromone response element in *Ustilago maydis*. Mol. Gen. Genet. 251, 31–37.

Weber, I., Gruber, C., Steinberg, G., 2003. A class-V myosin required for mating, hyphal growth, and pathogenicity in the dimorphic plant pathogen *Ustilago maydis*. Plant Cell 15, 2826–2842.

Wu, J., Miller, B., 1997. *Aspergillus* asexual reproduction and sexual reproduction are differentially affected by transcriptional and translational mechanisms regulating stunted gene expression. Mol. Cell. Biol. 17, 6191–6201.