A MADS-Box Homologue in *Ustilago maydis* Regulates the Expression of Pheromone-Inducible Genes but Is Nonessential

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ABSTRACT

Mating and pathogenic development in the smut fungus *Ustilago maydis* are controlled by a pheromone/receptor system and two homeodomain proteins, bEp and bWp, which form heterodimers in nonallelic combinations. We describe the isolation of a gene, *umc1*, encoding a MADS-box protein, which displays significant similarity to the *Saccharomyces cerevisiae* MCM1 gene. *umc1* complemented the viability defect of yeast *mcm1* mutants. In *U. maydis*, *umc1* deletion mutants were viable and pathogenic development was unaffected. Nevertheless, the basal expression levels of several pheromone-inducible genes were significantly reduced leading to an attenuated mating reaction. In contrast to *S. cerevisiae*, where Mcm1p plays a crucial role in the cell-type specific expression of α - and α -specific genes, the *U. maydis umc1* gene appears to have only a modulatory effect on the expression of mating type-specific genes.

THE initial step in sexual development of the phytopathogenic fungus *Ustilago maydis* involves recognition and fusion of haploid yeast-like cells. This event is controlled by the biallelic *a* mating type locus. The *a* locus consists of large dissimilar DNA regions, 4.5 kilobases (kb) for *a1* and 8 kb for *a2*, that occupy the same chromosomal location (FROELIGER and LEONG 1991; BÖLKER *et al.* 1992). In *a1*, this region contains single copies of a lipopeptide pheromone precursor (*mfa1*) and a receptor gene (*pra1*). In *a2*, we also find a pheromone (*mfa2*) and a receptor gene (*pra2*) plus two additional genes of unknown function (*lga2* and *rga2*) (BÖLKER *et al.* 1992; URBAN *et al.* 1996a). Binding of pheromones induces formation of mating hyphae and triggers cell fusion (SPELLIG *et al.* 1994). After cell fusion, the resulting dikaryon undergoes a morphological transition and assumes hyphal growth. For this transition to occur on artificial media, autocrine stimulation of the pheromone signaling pathway is required together with an active *b* gene complex (BANUETT and HERSKOWITZ 1989; SPELLIG *et al.* 1994) while for filamentous growth in planta the activation of the pheromone cascade is not required (BANUETT and HERSKOWITZ, 1996). The *b* gene products regulate pathogenic development through combinatorial interactions. The *b* locus exists in multiple alleles and encodes a pair of homeodomain proteins, termed bWp and bEp (GILLISEN *et al.* 1992). bWp and bEp have been shown to form an active heterodimeric complex only if they are

derived from different alleles (KÄMPER *et al.* 1995), so far no function has been ascribed to the monomeric protein species. Generation of such a heterodimeric complex in haploid cells is sufficient to trigger pathogenic development (BÖLKER *et al.* 1995). Therefore, it was inferred that these heterodimers control development by acting as transcriptional activators or repressors (see BANUETT 1995).

As in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (MARSH *et al.* 1991), the pheromone receptors of *U. maydis* belong to the family of membrane-bound receptors that are coupled to heterotrimeric G proteins and transmit the pheromone signal by a MAP kinase cascade to transcription factors that regulate a number of pheromone-inducible genes. In *U. maydis*, two components of this cascade, the G α subunit of a heterotrimeric G protein and the pheromone response factor Prf1p, have been identified (HARTMANN *et al.* 1996; REGENFELDER *et al.* 1997). Prf1p is an HMG-domain protein that binds to upstream regulatory elements of pheromone-inducible genes, among which are genes of the *a* locus as well as the *b* genes (HARTMANN *et al.* 1996). Prf1p mediates pheromone induction of all these genes and controls the basal expression levels of the pheromone precursor genes. Since *prf1* mutants generated in the haploid solopathogenic strain CL13 (BÖLKER *et al.* 1995) are nonpathogenic and this phenotype can be suppressed by constitutive expression of the *b* genes, it was inferred that Prf1p must also play a role in regulating the basal expression level of these genes (HARTMANN *et al.* 1996).

Until now, neither for the bEp or bWp monomers nor for the bEp/bWp heterodimer have targets been

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identified, presumably because of the low affinity of these proteins for DNA (T. ROMEIS and J. KÄMPER, unpublished data). This raised the possibility that efficient DNA binding may require the presence of additional proteins. Precedent for this exists in *S. cerevisiae* where MAT α 1p as well as MAT α 2p bind DNA in a ternary complex with the MADS-box protein Mcm1p (BENDER and SPRAGUE 1987; VERSHON and JOHNSON 1993). Specifically, it has been demonstrated that, in α cells, Mcm1p together with α 1 activates the α -specific genes, and together with α 2 represses the α -specific genes. In α cells, however, where α 2 is absent, Mcm1p alone activates the α -specific genes (BENDER and SPRAGUE 1987). In diploid cells, the α 1/ α 2 heterodimer represses the haploid-specific genes and this does not involve Mcm1p (GOUTTE and JOHNSON 1988). To establish whether determination of cell type in *U. maydis* also requires an additional factor that may not be cell-type specific we have searched for genes encoding MADS-box proteins.

The family of MADS-box proteins includes, in addition to Mcm1p, several plant and mammalian transcription factors that share a conserved DNA binding and dimerization domain but are otherwise unrelated (SHORE and SHARROCKS 1995). Characteristic for these regulatory proteins is that they interact with different accessory proteins.

In this article we describe the isolation of a gene whose product shows significant sequence similarity to yeast Mcm1p. This gene, *umc1*, is nonessential but modulates the expression of several genes in the *a* mating type locus.

MATERIALS AND METHODS

Strains, plasmids and plasmid constructions: The *Escherichia coli* K12 strain DH5 α was used as a host for plasmid amplification and the *E. coli* B derivative BL21::DE3 for overproduction of the Umc1₈₃₋₁₈₀ protein. The *U. maydis* strains FB1 (*a1 b1*), FB2 (*a2 b2*), FB6a (*a2 b1*) and FBD11 (*a1 a2 b1 b2*) have been described previously (BANUETT and HERSKOWITZ 1989). The *S. cerevisiae* strains 136 (SL2-2B): MAT α , *mcm1::LEU2 GAL1p::mcm1-98* and 137 (SL2-2A): MAT α , *mcm1::LEU2 GAL1p::mcm1-98* (PRIMIG *et al.* 1991) were used for the complementation analysis. Plasmids pSL1180 and pTZ18R (Pharmacia), pSP72 (Promega) pSMUT (BÖLKER *et al.* 1995) pYep-ubiquitin-StuI (ECKERT *et al.* 1987, 1989) and pCBX122 (KEON *et al.* 1991) were used for cloning and pET15b was used for protein expression. The plasmid pTZumc1 contains a genomic 4.8-kb *Hind*III fragment encompassing the open reading frame (ORF) of *umc1* and flanking sequences. The vectors pSLUmc1₈₃₋₁₈₀/pSLUmc1₁₋₄₅₄ were constructed by amplification of the MADS-box/the ORF of *umc1* using the primers MB41-44 and pTZumc1 as template. The resulting PCR products were cleaved with *Nde*I and *Bam*HI and cloned in the respective sites of pSL1180. The same fragments were cloned in the respective sites of pYep-ubiquitin-StuI generating pYumc1₈₃₋₁₈₀ and pYumc1₁₋₄₅₄, respectively. p3'*umc1* was constructed by cloning a 1.9-kb *Xma*I/*Eco*RI fragment of pCBX122 containing the carboxin resistance cassette and a 1.0-kb *Msc*I/*Bgl*II fragment of pTZumc1 containing the 3' region of *umc1* in pSP72 linearized with

*Xma*I and *Bgl*II. A 1.2-kb *Xba*I/*Ngo*MI fragment of pTZumc1 containing the 5' region of *umc1* was cloned into p3'*umc1* linearized with *Xba*I/*Xma*I to generate p Δ umc1. The *Nde*I/*Bam*HI fragment of pSLUmc1₈₃₋₁₈₀ was cloned into the respective sites of pET15b to generate pET83-180. The DNA oligonucleotides CA1 (GATCCTTTTCCTAATTAGGTAAG)/CA2 (GATCCTTACCTAATTAGGAAAG) and CA3 (GATCCTTTCAT AATTAGGTAAG)/CA4 (GATCCTTACCTAATTATGAAAG) were hybridized and ligated into the *Bam*HI site of pTZ18R to generate pMCE and pMCEmut, respectively.

PCR procedures: A fragment of *umc1* was isolated from genomic *U. maydis* DNA using the following primers: MB31 (guesser): ACGTTCACGAAGCGTAAGCATGGTATCATG AAAAAGGC, MB36: GACGGATCC(AG)TTNA(AG)(AG)CA NG(CT)(CT)TG(AGT)AT. For the amplification of the MADS-box of *umc1* we used MB41: TGGCCATATGGGTA AGCGCTCC and MB42: GCCGGATCCCTAATCGGGAGC ATTCAAGCA and for the amplification of the ORF MB43: CGGTACATATGTCGACCTTATCCGCCGGC and MB44: GC CGGATCCCTAGACGACGATGACCCGATG.

The PCR reaction was performed using Boehringer Taq polymerase for 30 cycles. Annealing temperature for the isolation of *umc1* was 57° and genomic DNA of strain FBD11 was used as template. The MADS-box and the ORF of *umc1* were amplified from pTZumc1 at an annealing temperature of 50°, respectively. In both reactions extension was carried out at 72° for 1 min and strand separation was at 94° for 1 min.

Retention analysis: For retention analysis a His-tagged version of the Umc1p MADS-box, Umc1₈₃₋₁₈₀His6p, was overproduced, purified as described by Invitrogen and buffered for storage in 500 mM NaCl, 25 mM Tris HCl pH 7.5, 0.1 mg/ml BSA, 2 mM EDTA, 1 mM DTT, 5 mM MgCl₂ and 10% glycerol. DNA fragments were end labeled with ³²P using T4 polynucleotide kinase (NEB). As probes, the 122 base pair (bp) *Kpn*I/*Sac*I fragments from pTZMCE and pTZMCEmut were used. DNA binding reactions were performed in 25 mM Tris HCl pH 7.5, 3 mM DTT, 2 mM EDTA, 0.1 mg/ml BSA, 100 mM NaCl, 10 mM MgCl₂ and competitor DNA as indicated for 30 min at 25°. Protein-DNA complexes were separated on a 4.2% polyacrylamide gel.

Growth conditions for *U. maydis*: Strains were grown at 28° in YEPS (TSUKUDA *et al.* 1988) or in CM medium (HOLLIDAY 1974). The mating reaction was observed by co-spotting strains on charcoal-containing plates (HOLLIDAY 1974; BANUETT and HERSKOWITZ 1989). The formation of mating structures was assayed by growing cells in CM to a maximal OD₆₀₀ of 0.8, centrifugation and resuspension of the cells in H₂O. Next, 1- μ l droplets of strains to be assayed were spotted on 2% H₂O-agar ~0.5–1 mm apart. After drying, the spots were covered with 5 μ l liquid paraffin. The formation of mating structures was observed after 12 hours at room temperature (SNETSelaar *et al.* 1996). The pathogenicity test has been described (GILLISSEN *et al.* 1992). Overnight cultures of strains to be tested were resuspended in water at a concentration of ~4 $\times$ 10⁷ cells/ml and injected into young corn seedlings. For individual strains, at least 20 plants were infected and anthocyanin biosynthesis and tumor development were scored for a period of 7–21 days after inoculation.

DNA and RNA procedures: Standard molecular techniques followed SAMBROOK *et al.* (1989). *U. maydis* DNA was prepared according to HOFFMAN and WINSTON (1987). RNA isolation was performed as described (TIMBERLAKE 1986). Transformation of *U. maydis* followed published procedures (SCHULZ *et al.* 1990), and transformation of *S. cerevisiae* was performed as described by SHERMAN *et al.* (1986). The following probes were used for northern analysis: *mfa1*, a 675-bp fragment of pUMa1 (URBAN *et al.* 1996); *pra2*, a 1.3-kb *Eco*RI

cDNA fragment (URBAN *et al.* 1996); *mfa2*, a 386-bp *PstI-SpeI* fragment of pUMa2 (URBAN *et al.* 1996); *prf1*, a 1.4-kb *SalI* fragment of pHA63 (A. HARTMANN, unpublished data); *lga2*, a 800-bp *HindIII* fragment of pUMa2 (URBAN *et al.* 1996); *bE1*, a 1.4-kb *NdeI/BamHI* fragment of pHA196 (A. HARTMANN, unpublished data) and *umc1*, a 300-bp *NdeI/BamHI* fragment of pSLUMc1₈₃₋₁₈₀. As loading control a 2.2-kb *EcoRI-EcoRV* fragment of pCBX122 was used that detects the constitutively expressed *cbx* gene. A STORM phosphorimager (Molecular Dynamics) was used for signal detection. The intensity of the signals was compared with respect to the *cbx* signals using ImageQuant.

Sequence analysis: Sequences were determined using the T7 polymerase dideoxy sequencing kit (Pharmacia).

Light microscopy: *U. maydis* was microscopically analyzed using a Zeiss Axiophot with differential interference contrast optics.

RESULTS

Cloning of the *umc1* gene: MADS-box domains consist of 80 amino acids that display a high degree of sequence conservation (TREISMAN and AMMERER 1992). Based on short stretches of nearly invariant residues we designed primers for PCR amplification. With the combination of the primers MB31 and MB36 (Figure 1A), a DNA fragment of 183 bp could be amplified from genomic *U. maydis* DNA. Sequencing revealed significant similarity to the known MADS-box sequences (not shown). This fragment was used to isolate cosmid clones on which an ORF coding for a protein of 454 amino acids could be identified (Figure 1A). The respective gene was designated *umc1* (*Ustilago* MCM1 homologue). Within the MADS-box domain, Umc1p displays the highest degree of amino acid identity (64%) to Mcm1p of *S. cerevisiae* and the human serum response factor SRFp (61% identity). The transcription of *umc1* was not detectable by Northern analysis in either haploid or dikaryotic cells, and irrespective of pheromone stimulation (data not shown). Nevertheless, from a cDNA library (SCHAUWECKER *et al.* 1995), one full-length and two truncated clones could be isolated (pUMCc1-3). The 5' ends of the respective cDNAs were at position -263, +122 and +486 of the *umc1* sequence while the polyA tail in pUMCc1 was found at position +1863 (see Figure 1A). Sequencing of the full-length cDNA clone pUMCc1 revealed no introns. The first translational start codon is likely to be used, although it is embedded in a sequence context (Figure 1A) that does not perfectly fit the fungal consensus (BALLANCE 1991). The next possible start codon, however, is located within the highly conserved MADS box domain. With the exception of this domain, Umc1p does not share significant similarity with other known MADS-box proteins. Interestingly, Umc1p contains, between amino acids 220 and 230, an additional short DNA binding motif, the A·T-hook (REEVES and NISSEN 1990), which is characteristic for the family of mammalian HMG-I(Y) proteins (Figure 1C). In addition, Umc1p

contains a region rich in acidic amino acids at the N terminus and two glutamine/histidine tracks near the C terminus (see Figure 1A).

***umc1* can complement the viability defect of *S. cerevisiae* *mcm1* mutants:** To assess the function of *umc1*, we tried to complement a *mcm1* mutant of *S. cerevisiae* by introducing the *U. maydis* *umc1* gene. To this end *umc1* was fused to the yeast *CUP1* promoter (HOTTIGER *et al.* 1994) on plasmids pYumc1₈₃₋₁₈₀ and pYumc1₁₋₄₅₄ (see MATERIALS AND METHODS). In these constructs, the coding region of *umc1* containing only the MADS-box (amino acids 83–180) or the entire ORF (amino acids 1–454), respectively, were expressed as N-terminal ubiquitin fusion proteins, which are posttranslationally processed to yield the indicated protein domains of Umc1p. Since MCM1 is an essential gene in *S. cerevisiae*, we used strains 136 and 137 in which the chromosomal copy of MCM1 is disrupted by insertion of *LEU2* but which contain the region of MCM1 coding for the MADS-box domain (amino acids 1–98) fused to the *GAL1* promoter (PRIMIG *et al.* 1991). These strains are viable only on galactose-containing media. Both strains were transformed with plasmids pYumc1₁₋₄₅₄ and pYumc1₈₃₋₁₈₀, respectively. Plasmid pYep-Ubiquitin-StuI served as control. Transformants were selected on galactose-containing media. Individual transformants were then transferred to media containing either glucose or galactose as carbon source (Figure 2). Whereas on galactose-containing plates all transformants were viable, on glucose-containing plates only transformants harboring either plasmid pYumc1₁₋₄₅₄ or pYumc1₈₃₋₁₈₀ formed single colonies, while transformants harboring pYep-Ubiquitin-StuI were unable to do so (Figure 2). This illustrates that Umc1p can complement the viability defect of *mcm1* mutants of *S. cerevisiae* and that the complementing activity resides within the MADS-box domain of Umc1p.

Umc1p can bind specifically to the MCM1 consensus site: Since Umc1p can restore the viability defect of *mcm1* mutants in yeast it was likely that the two respective proteins have similar DNA-binding properties. To test this hypothesis, the MADS-box domain of Umc1p (amino acids 83–180) was overproduced in *E. coli* after cloning into the expression vector pET15b. This generates a MADS-box domain carrying an N-terminal His6 epitope that facilitates the purification of the overproduced protein by affinity chromatography (see MATERIALS AND METHODS). To analyze the binding of Umc1₈₃₋₁₈₀His6p to DNA, we used double-stranded oligonucleotides, MCE and MCEmut, that contain either a consensus Mcm1p binding sequence (PASSMORE *et al.* 1989) or a mutant version of the binding site in which a single nucleotide critical for binding is altered (see MATERIALS AND METHODS). Both DNA fragments were endlabeled with ³²P and incubated with increasing amounts of purified Umc1₈₃₋₁₈₀ protein. The analysis of complexes was

A

GTGCGGTGCA	AGTCGCACAG	ATGGGCAGGA	CTGTAGATCG	GGCATCGGC	TGCATCAGTT	TCCGATGCTC	CCCCAAGCGG	-842															
CATATCGTGT	TTGTGGCTTA	AGTTACATTTC	TTGACGCGCG	CGCCGAGTGT	GCAACAGTAG	CTTGGGCATT	TCGGGTGTAAT	-762															
GGACITTTACT	TGGCATTTGA	ATAGATATTC	AAAGATATGT	ATAGTTTGTA	AGTACACAAAT	TCCATCCCGA	TTGATGATTC	-682															
ATGCGAGCTG	TAATCCAAAT	TCAATCCGCG	TAAATCATAT	GGTTGTATCA	GCTCAGCCAA	AGCTAAGGGG	GCAGATCGTT	-602															
TCAAAAACCTT	GAAATTTGGC	AAGCGCCAACT	CTGCAAGTCT	GACAGCCTCA	GGTGAACACG	GGCGGTACG	ATGTCAGAGT	-522															
CCCAGCCACA	AGTACGAGC	GTCACACAGA	GTGGCAAAACA	CGCAAGAGC	ACAGCGAGAA	GGCGAACAGC	CAGCTTTGTT	-442															
TTGTTTCGGT	CAGTACGGTG	AGCGAGAACG	AGAAGGGAGG	CCAAGGCGGA	GGCGGTGGGA	GTTTGGCTTCC	AGCGTGTAGT	-362															
CTCTCGACTT	GCCGAAACGA	AGTTACCGCT	TCCAGAGAGC	AAACAGTTGC	CAGTCAACCGT	CAGTGTGTGGT	TCACACACACA	-282															
CACGCTCTCC	TCTGACTTGG	ATCTTAGGAC	TGGCAGCCAG	CAAGACAGGT	CTCTCTTCA	GACCTTAGCC	GACACTGGCT	-202															
TGCTTCGTTT	CGTCGACCTC	TCTACCGCTG	CCCTCGTATT	GTCGTACCAC	CTTCTATATC	ATATCTACAC	GCTGCCGCTC	-122															
AGACATAATT	ACTATACCTG	TATCTCGCTG	TTTCCATCG	GCTCCACACC	TTTGGCGATA	TACTCGGAGC	CGCTTTTGA	-42															
TCCAATCTAC	CCTAATAACA	ACCACGAGCC	CACCTGCACA	GA	ATG	TCG	ACC	TTA	TCC	GCC	GGC	ATT	GTT	GCG	30								
GTG	CGT	CAA	GCA	GGT	TCC	TCT	AGT	AAC	AGC	CCA	GTC	TAT	GAC	ACA	CCC	TTT	GCC	ACC	GTT	CAA	CTT	96	
V	R	Q	A	G	S	S	N	S	N	S	T	F	F	F	G	T	V	Q	L				
GCT	TCT	TCC	TCA	AAA	GAC	CTC	TGC	GCC	AAT	TTC	GAC	CTC	ACT	GCT	GGC	TTT	GCC	GCT	TCT	GCC	GAC		162
A	S	S	K	D	L	S	A	N	F	D	L	L	L	A	G	F	A	A	S	A	D		
TAT	GAG	AAT	CAA	GAG	GAC	GAA	GAC	GAG	GAA	GAC	GAC	GAT	GAT	GGC	GAT	TCC	GAA	GAT	GGC			228	
Y	E	N	Q	E	D	E	D	E	D	E	D	D	D	D	D	D	S	E	D	D			
AAA	GGC	AAA	GGC	AAA	AAG	GGT	AAG	CGT	GCG	CGC	TCC	GAA	GCC	AAA	GGC	GAG	CGC	GAT	CTC	AAG	AGT	294	
K	G	K	G	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K		
GGT	CGT	CGC	AAG	ATC	AAG	ATC	GAG	TTT	ATC	GAA	GAC	GAT	GCT	CGC	CGA	CAC	ATC	ACG	TTC	AGC	AAG	360	
G	R	R	K	I	K	I	E	F	I	E	D	D	A	R	R	H	I	T	F	S	K		
CGC	AAG	GCG	GGT	ATC	ATG	AAA	AAG	GCA	TAC	GAG	CTG	GCC	ACC	TTG	ACG	GGA	ACT	CAG	GTG	CTC	TTG	426	
R	K	A	G	I	M	K	K	A	A	Y	E	L	A	T	L	T	G	T	Q	V	L	L	
CTC	GTT	GTC	TCG	CAG	ACG	GGT	CTG	GTG	TAT	ACA	TTT	ACC	ACG	CCC	AAG	TTG	CAA	GCG	CTC	GTG	ACG	492	
L	V	V	S	Q	T	G	L	V	Y	T	T	T	T	P	K	L	Q	A	L	V	T		
CAA	GCC	GAA	GGA	CGC	AAC	CTG	ATT	CAG	GCT	TGC	TTG	AAT	GCT	CCC	GAT	CCA	CCC	AAC	GGT	GGC	GGA	558	
Q	A	E	G	R	N	L	I	Q	A	C	L	N	A	P	D	P	P	N	G	G	G		
GAG	AGC	GGA	GGC	GAG	CAC	TGC	GGC	GAC	GAG	CCA	AGT	GAG	GCA	GAC	GAT	GCC	TTT	TCT	CCA	TCC		624	
E	S	G	G	E	D	H	S	G	D	E	P	S	E	A	D	D	A	F	S	P	S		
GAA	TCA	CAG	GCT	AGC	TCC	AAC	AAG	CGT	GCC	GTG	CAA	CCT	CGC	AAA	CGT	CCT	CGC	ACC	GCA	TCC	ACG	690	
E	S	Q	A	S	S	N	K	R	A	V	Q	P	R	K	R	P	R	T	A	S	T		
AGC	AAG	TCG	TCG	AGC	GCG	TCC	ACT	TCC	GTC	GCC	GGT	GCT	GCG	CCT	GGC	ATC	GGT	GGT	GGC	GAA	GAG	756	
S	K	S	S	S	A	S	T	S	V	A	G	A	A	P	G	I	G	G	G	E	E		
GAT	GCT	ACC	TCT	GGC	TCG	CAG	CTA	GCA	ACA	GTC	AAA	CGC	GGT	CGG	GGT	CGA	CCT	CGT	AAG	AAT	ACT	822	
D	A	T	S	G	T	L	A	T	V	K	R	G	R	G	R	P	R	K	N	T			
GCC	GGA	AAC	GCA	CCA	GCC	TCG	AGC	TCG	AAC	ACG	TCC	AAC	GAC	GCG	CAT	CCC	ATG	CCT	CCG	CCA	CCT	888	
A	G	N	A	P	A	S	S	S	N	T	S	N	D	A	H	P	M	P	P	P	P		
GCG	ATG	CAT	CAG	AGC	ATC	TCC	GAC	GAT	GCC	GTA	CAT	CAG	CAT	CAT	CAG	CAC	CAA	CAT	CAT	CAG	CAC	954	
A	M	H	Q	S	F	S	D	D	A	V	H	Q	H	H	Q	H	Q	H	Q	H			
CAA	CAT	CGA	CAG	CAG	CAA	CAG	CAA	CAG	TAC	ACA	CAC	GTC	CCT	TCT	GCT	GAT	TTC	CCC	AAC		1020		
Q	H	R	Q	Q	Q	Q	Q	Q	Q	Q	Y	T	H	V	P	S	A	D	F	P	N		
TAC	ACC	GCT	GCT	GGC	TTT	TAT	GGC	GAC	GTC	CCC	AAT	TCG	GCA	GCC	AAC	CCC	GAT	CTC	GGT	TTT	GGC	1086	
Y	T	A	A	G	F	Y	G	A	D	V	P	N	S	A	A	N	P	D	L	G	F	G	
CAA	GCG	GGC	GGA	CTG	TCA	ATT	GAC	AAT	CAA	GCT	GTT	TGG	GCC	CAG	ACC	ATG	TCT	CCC	AAC	CCC	AAC	1152	
Q	A	G	G	L	S	I	D	N	Q	A	V	W	A	Q	T	M	S	P	N	P	N		
CAG	ATG	TTT	GAT	CCT	TCC	AAC	CAC	CAT	CAA	CAT	CAT	CAA	CAG	CAA	CAG	CAG	CCG	GGG	CAG	CAG	CAA	1218	
Q	M	F	D	P	S	N	H	H	Q	H	H	Q	Q	Q	Q	Q	P	G	Q	Q	Q		
TCA	TCA	CAG	CAG	CAA	TCG	CAG	CAG	CAG	CAG	CAG	CAG	CAG	TCC	TCA	GTA	CAG	TTT	GGA	TAC	GCA	ACC	1284	
S	S	Q	Q	Q	S	Q	Q	Q	Q	Q	Q	Q	Q	S	S	V	Q	F	G	Y	A	T	
CAG	CAT	TTT	GGT	CAC	TTT	AAT	CCA	CAG	CAG	TTG	GCC	GCC	ATG	CAA	CCG	CAG	GAG	ATT	CAG	GCT	CCC	1440	
Q	H	F	G	H	F	N	P	Q	Q	L	A	A	M	Q	P	Q	E	I	Q	A	P		
AGA	CCC	GCT	TGG	TAG		GATGTTTCAGG	ATCGAAAATT	GGATTCTGTT	ATACCCCCCT	CCATTGCGCG	AGCTTGTGAT										1515		
R	P	A	W	*																			
GTCTCTTTGC	CGGTCTATCGG	GTCATCGTCT	AGCTTCTACT	CGTTTCTGTA	TTGCACACAT	AAATCTTATC	ACGCTCGTTC	1595															
CACCAACCGCA	CGATATGCAC	AGAACAAGC	GCGGCGCATG	CATCTGCAAC	TGCGCGCATC	AAAGTGGTCT	ATCTATGGGT	1675															
TTGCGTACGT	ATCAGCCAA	GAATGCGGCC	TCGTGAGATT	GGACGCCAAA	CTCGTCCCTG	GCCATCTGGC	GTACTACTTTG	1755															

B

UMC1	aa	95-139	DLKTGRRKIKIEFIEDDARRHITFSKRKAGIMKKAYELATLTGTQ
MCM1	aa	13- 57	GQQKERRKIEIKFKENKTRRHVTFSKRKHGIMKKAFELSVLTGTQ
SRF	aa	138-182	KKTRGRVKIKMEFIDNKLRRYTFFSKRKTGIMKKAYELSTLTGTQ
ARG80	aa	75-119	TSTVTRRKQPIRYIENKTRRHVTFSKRRHGIMKKAYELSVLTGAN
MAP1	aa	15- 59	EKAYTGSSQGSYLEDQRONTFTKRKAGIFKKANELALLTGSE
UMC1	aa	140-184	VLLLVVSQTGLVYTFSTTPKLOALVTQAEGRNLIQACLNAPDPENG
MCM1	aa	58-102	VLLLVVSETGLVYTFSTPKFPIVTOQEGRNLIQACLNAPDDEEE
SRF	aa	183-227	VLLLVASETGHVYTFATRLQPMITSETGKALIQTCNLNSPDSPPR
ARG80	aa	120-164	ILLILANSGLVYTFSTTPKLPVVREDEGKSLIRACINASDTEA
MAP1	aa	60-104	VMVLVSETGLVHTFSTPKLENVVKSPEGOKLITESLINATTDQN

C

UMC1	(<i>U.maydis</i>)	aa	211-237	DATSGSQLATVKGRRGRPRKNTAGNAP
HMG-Y	(<i>H.sapiens</i>)	aa	12- 38	LASKQEKDGTGTEKRGRGRPRKQPPKEPS
HMG-Y	(<i>M.musculus</i>)	aa	12- 38	LASKQEKDGTGTEKRGRGRPRKQPPKEPS
HMG-I	(<i>H.sapiens</i>)	aa	12- 38	LASKQEKDGTGTEKRGRGRPRKQPPVSPG
AT1	(<i>O.sativa</i>)	aa	297-321	VPPTAAAVLGVKRGRRPRKKDKPLQS

FIGURE 1.—Sequence of *umc1* and alignments with related proteins. (A) The complete nucleotide sequence (GenBank accession no. AF020538) and derived amino acid sequence of *umc1* are given. The locations of primers MB31 and MB36 used to amplify a part of the gene are indicated. (B) MADS-box domains of Umc1p, Mcm1p (PASSMORE *et al.* 1988), SRFp (TREIMANN

FIGURE 2.—*umc1* complements the viability defect of *mcm1* mutants. *S. cerevisiae* strains 136 (top row) and 137 (bottom row) were transformed with plasmids indicated. Single colonies were streaked on medium containing galactose or glucose as carbon source. *umc1* is expressed constitutively under the conditions used. Expression of the endogenous *mcm1-98* gene is under control of a galactose-inducible promoter (*GAL1p::mcm1-98*). Strains 136 and 137 do not grow because the medium lacks tryptophan. In all transformants, the vector carries the *TRP1* gene (ECKERT *et al.* 1987) and complements this defect. On glucose-containing plates, where the endogenous *mcm1-98* gene is not expressed, only transformants expressing *Umcl*₈₃₋₁₈₀p or *Umcl*₁₋₄₅₄p are viable.

carried out on nondenaturing polyacrylamide gels (Figure 3). *Umcl*₈₃₋₁₈₀p was able to form specific complexes with MCE but was unable to bind to the MCEmut oligonucleotide (Figure 3). This illustrates that the MADS-box domains of *Mcm1*p and *Umcl*p can recognize the same sequences.

Phenotype of *umc1* deletion strains: To assess the role of *umc1* in *U. maydis* we have generated null mutants in haploid wild-type strains FB1 (*a1 b1*), FB2 (*a2 b2*) and FB6a (*a2 b1*) by gene replacement. For this purpose we constructed plasmid p Δ *umc1* in which the region encoding amino acid 6–360 of *umc1* was deleted and replaced with a carboxin resistance cassette. In all three strains gene replacement occurred in ~25% of all transformants and was verified by Southern analysis (see Figure 4 and not shown). This illustrates that *umc1* is a nonessential gene. In terms of cell morphology and growth characteristics, strains FB1 Δ *umc1*, FB2 Δ *umc1* and FB6a Δ *umc1* were indistinguishable from the corresponding wild-type cells (not shown).

Next we have analyzed the mating reaction of Δ *umc1* strains and have compared it to that of compatible wild-type cells. Upon mixing of two compatible Δ *umc1* strains on PD charcoal plates, we consistently observed an attenuation in the appearance of dikaryotic filaments in the mutant combination compared to the wild-type combination (Figure 5A). Since this assay does not distinguish between defects in cell fusion and defects in hyphal development, we next assayed for mating tube formation in response to pheromone stimulation in a confrontation assay (SNETSEL-AAR *et al.* 1996). Between spots of FB1 and FB2 strains vigorous growth of mating tubes directed toward each other was observed after 12 hours (Figure 5B, top). In the confrontation of compatible Δ *umc1* strains, mating hyphae could not be observed (Figure 5B, bottom), not even after prolonged incubation (not shown). This suggests that Δ *umc1* strains are unable to sense each other over a certain distance, an effect that could be caused by a decrease in pheromone

and AMMERER 1992), Arg80p (DUBOIS *et al.* 1987) and Map1p (NIELSEN *et al.* 1996) were aligned to maximize similarity indicated by shaded bars. (C) A·T hooks and flanking sequences of *Umcl*p, human HMG-Yp (KARLSON *et al.* 1989), mouse HMG-Yp (JOHNSON *et al.* 1988), human HMG-Ip (JOHNSON *et al.* 1989) and *O. sativa* AT1p (GenBank accession no. 1362172) were aligned.

FIGURE 3.—The MADS-box domain of Umc1p binds to the Mcm1p consensus binding element (MCE). DNA fragments containing either the MCE or the MCEmut sequence, respectively, were 32 P-end-labeled and incubated with different amounts of purified Umc1₁₈₃₋₁₈₀His6 protein. Complexes were analyzed on a 4.2% nondenaturing polyacrylamide gel. In lanes 1 and 9 no protein was added, in lanes 2 to 6 protein concentrations were 4, 8, 40, 100 and 300 ng, respectively. In lanes 7 and 8 the protein concentrations were 300 and 40 ng, respectively.

production or a decrease in membrane-bound pheromone receptor molecules.

When assayed for pathogenicity by injecting compatible $\Delta umc1$ combinations (FB1 $\Delta umc1$ $\times$ FB2 $\Delta umc1$) or compatible wild-type combinations (FB1 $\times$ FB2) into young corn plants, tumor development was not signifi-

cantly different. For the FB1 $\Delta umc1$ $\times$ FB2 $\Delta umc1$ combination, 97% of infected plants produced tumors (121 plants inoculated) while the wild-type combination produced tumors in 98% of all infected plants (109 plants inoculated). These results indicate that, despite the attenuated formation of dikaryotic hyphae on plates, pathogenic development of $\Delta umc1$ strains is unaffected under the infection conditions used.

In $\Delta umc1$ strains the expression pattern of pheromone-inducible genes is altered: The observed attenuation in morphological responses to pheromone stimulation in $\Delta umc1$ strains could result from altered expression levels of the pheromone and receptor genes. Since these genes (*mfa1*, *mfa2*, *pra2*) are themselves induced by pheromone, we have studied their expression pattern by Northern analysis in haploid $\Delta umc1$ and wild-type strains as well as in mixtures of compatible $\Delta umc1$ strains and mixtures of respective wild-type strains (Figure 6). In addition, the expression levels of the pheromone-inducible genes *prf1*, *lga2* and *bE1* were studied. All strains were grown on CM-charcoal plates and RNA was prepared after 48 hours incubation at 22°.

In haploid $\Delta umc1$ strains, the mRNA levels of the pheromone precursor genes *mfa1* (Figure 6, compare lanes 1 and 5) and *mfa2* (Figure 6, compare lanes 2 and 6) were reduced 15-fold and sixfold, respectively, compared to the wild-type strain. For the receptor gene *pra2*, expression is about threefold reduced in the FB2 $\Delta umc1$ strain in comparison to the respective wild-type strain (Figure 6, compare lanes 2 and 6). *prf1* and *lga2* mRNAs are clearly detectable in the haploid wild-type strains but are undetectable in the $\Delta umc1$ mutant

FIGURE 4.—Deletion of the *umc1* gene by homologous recombination. (A) The upper part shows a restriction map of the *umc1* locus, the *umc1* gene is indicated by the shaded box. The lower part shows the corresponding parts of the deletion construct p $\Delta umc1$ in which the *umc1* gene is largely replaced by a carboxin resistance cassette. (B) DNA of the strains indicated was cleaved with *HindIII* and *PstI* and subjected to Southern analysis using a 4.8-kb *HindIII* fragment spanning the *umc1* gene as a probe. Replacement of the endogenous *umc1* gene by the deletion construct results in the disappearance of the signal at 4.8 kb and generates two signals at 2.9 and 2.4 kb, respectively.

FIGURE 5.—Mating is attenuated in $\Delta umc1$ strains. The following strains were used either alone or in mating assays: FB1 (*a1 b1*), FB2 (*a2 b2*), FB1 Δ is FB1 $\Delta umc1$ and FB2 Δ is FB2 $\Delta umc1$. (A) Strains and strain combinations listed on top and below were spotted on charcoal plates. The photograph was taken after 24 hours incubation at room temperature. Dikaryotic filaments appear as white cover of the colonies (Fuz⁺ phenotype). (B) Strains indicated were analyzed by confrontation assay (SNETSelaar *et al.* 1996); cells were spotted ~1 mm apart on water agar, covered with liquid paraffin and photographed after 12 hours. During this period FB1 and FB2 cells (top) and not FB1 $\Delta umc1$ and FB2 $\Delta umc1$ cells (bottom) sensed each others presence and formed mating structures directed toward each other.

strains (Figure 6, compare lanes 1 and 2 to 5 and 6). *bE1* transcripts could not be detected in haploid strains, irrespective of whether they contained the $\Delta umc1$ mutation. These data illustrate that *umc1* has notable effects

FIGURE 6.—Expression of pheromone responsive genes in wild-type and $\Delta umc1$ strains. Strains and strain combinations indicated on top were grown on charcoal plates for 48 hours for RNA preparation. FB1 (*a1 b1*), FB2 (*a2 b2*), FB6a (*a2 b1*) and their respective $\Delta umc1$ derivatives were used. Two identical gels with 25 μ g of total RNA per lane were prepared and hybridized in succession with gene probes indicated on the right. *cbx* (KEON *et al.* 1991) serves as a loading control.

on basal transcription levels of the genes located in the *a* locus.

To analyze the effects of the $\Delta umc1$ mutation on pheromone-stimulated gene expression, we have used mixtures of cells that differ at the *a* locus and are either identical or differ at *b*. The presence of an active bEp/bWp heterodimer after cell fusion affects the expression pattern of some of the pheromone-inducible genes (URBAN *et al.* 1996b). In the cross of FB1 $\Delta umc1$ x FB6a- $\Delta umc1$, where strains carry different *a* but identical *b* alleles, the expression of all genes examined, with the exception of *lga2*, is significantly lower than in the FB1 x FB6a cross (Figure 6, compare lanes 3 and 7). The barely detectable level of *lga2* expression in the absence of an active b heterodimer did not allow to monitor a further decrease (Figure 6, compare lanes 3 and 7).

When wild-type strains that differ both in *a* and *b* are mixed and allowed to develop dikaryotic hyphae, the expression levels of the *mfa1*, *mfa2* and *pra2* genes are lower than under conditions of pheromone stimulation (URBAN *et al.* 1996b; Figure 6, compare lanes 3 and 4). For the FB1 $\Delta umc1$ x FB2 $\Delta umc1$ combination, however, we observe *mfa1*, *mfa2* and *pra2* levels comparable (or even higher for *mfa1*) than the pheromone-induced levels (Figure 6, compare 7 and 8). For *bE1*, *lga2* and *prf1* expression levels in FB1 x FB2 and FB1 $\Delta umc1$ x

FB2 $\Delta umc1$ crosses are comparable (Figure 6, compare lanes 4 and 8), although their expression in the pheromone-induced state is significantly lower than in the wild-type situation (Figure 6, compare lanes 3 and 7).

DISCUSSION

In this communication we describe the cloning of the *umc1* gene of *U. maydis*. Umclp displays significant similarity to the MADS-box protein Mcmlp of *S. cerevisiae*. Additional members of the MADS-box family have been identified in mammals, plants and *S. pombe* (reviewed by TREISMAN and AMMERER 1992). The *U. maydis umc1* gene is the first member of this family in filamentous fungi.

The *U. maydis* Umclp protein can complement the lethal phenotype of yeast *mcm1* mutants. Although a large number of genes that are regulated by *MCM1* have been identified in yeast, the essential function of *MCM1* has not been ascribed to (a) specific gene(s) whose transcription is dependent on Mcmlp (CHRIST and TYE 1991). The N-terminal 98 amino acids of Mcmlp that contain the MADS-box as well as the corresponding domain between amino acids 83–180 of Umclp are sufficient to restore viability, indicating that this domain fulfills all essential functions. It is interesting to note that, in yeast, two additional genes encoding MADS-box proteins (*ARG80* and *RLM1*), which obviously cannot complement the Mcmlp function, exist. We take this as evidence that the *U. maydis* Umclp protein is functionally related to MCM1p. In line with this assertion is the observation that Umclp binds specifically to the consensus site that is recognized by Mcmlp. Since in most cases Mcmlp acts as a component of a ternary complex, we propose that the *U. maydis MCM1* homologue not only shares the same binding specificity as Mcmlp but also has the ability to recruit in yeast the same accessory proteins as Mcmlp.

Inspection of the Umclp amino acid sequence revealed a short domain that is highly similar to the DNA binding motif of nonhistone DNA binding proteins of the HMG-I(Y) family. This motif was termed the A·T hook because it recognizes A/T-rich DNA sequences (REEVES and NISSEN 1990). Whether this motif contributes to the DNA binding specificity of Umclp remains to be elucidated. At least for the complementation of yeast *mcm1* mutants it is not required, since the truncated version, which lacks this motif, could complement the *mcm1* defect.

Deletion of *umc1* in *U. maydis* did not affect viability and did not result in sterility of haploid cells. However, the morphological reaction of haploid cells to compatible mating partners was significantly attenuated when compared to wild-type cells. The expression of genes directly involved in cell recognition, *i.e.*, the pheromone and the receptor genes, was significantly reduced

both in the pheromone-induced and in the uninduced state. This could explain the absence of mating hyphae in the confrontation assay and the less vigorous development of the dikaryotic stage after cocultivation of compatible $\Delta umc1$ strains.

How can we reconcile the role of *umc1* in the expression of the pheromone-inducible genes with what is known about other regulators that affect the expression of these genes? Recently, we have demonstrated that the transcription factor Prflp plays a central role in the activation of pheromone-inducible genes that include the *a* and *b* mating type genes (HARTMANN *et al.* 1996). It is proposed that binding of pheromone to its cognate receptor results in the activation of Prflp by phosphorylation through a MAP kinase (HARTMANN *et al.* 1996). *prf1* gene expression is pheromone inducible and is in addition controlled by carbon source and cAMP (A. HARTMANN, unpublished results). Interestingly, in $\Delta umc1$ strains, the *prf1* expression level is below the detection level, both in pheromone-induced and -uninduced cells. This could indicate that the primary effect of Umclp is on the expression level of *prf1*. In $\Delta umc1$ strains the expression level of *prf1* is clearly decreased but may not be completely abolished. This may be sufficient for the observed activation of genes after pheromone stimulation, *i.e.*, under conditions where Prflp is presumed to be phosphorylated (HARTMANN *et al.* 1996). In crosses of strains differing in *a* and *b*, the absence of Umclp did not affect the expression of *prf1*, *bE1* and *lga2*. Expression of these genes is high also in the wild-type situation. However, the genes that are normally down-regulated after cell fusion, such as *mfa1*, *mfa2* and *pra2*, are not under this type of regulation in $\Delta umc1$ strains. Therefore, we are tempted to speculate that Umclp might be required for repression, but not for activation by the b heterodimer or a regulatory protein under b control. Also, with respect to Umclp, it is not clear whether the observed effects are direct or indirect. An inspection of the nucleotide sequences in the *a* and *b* loci as well as the *prf1* promoter revealed no significant matches to the Mcmlp consensus binding site, which might argue against a direct role of Umclp in the regulation of the genes examined. In addition, we have tested Umclp in combination with bEp, bWp and Prflp, respectively, in the yeast two-hybrid system but failed to gain evidence for an interaction (M. REICHMANN and J. KRÜGER, unpublished results).

Why are *umc1* mutants viable? The essential *MCM1* gene from yeast is part of a complex regulatory network that controls cell-type determining genes (KUO and GRAYHACK 1994), cell wall synthesizing enzymes (KUO and GRAYHACK 1994) as well as cell-cycle-dependent expression of certain genes (ALTHOEFER *et al.* 1995). Even if a similar network exists in *U. maydis* it is conceivable that the different functions of yeast Mcmlp are exerted

by distinct regulatory proteins. A search for additional MADS-box proteins in *U. maydis* failed (J. KRÜGER, unpublished results), but we cannot rule out that other members of this family remained undetected. The reduction in the transcriptional level of the mating type genes might serve as indication that *Umc1p* is a member of a group of genes with redundant function. The effect of the mutation could then be explained by the reduction in copy number. However, hybridization analysis with *umc1*, even under conditions of very low stringency, gave no evidence for the presence of a related gene in the genome of *U. maydis* (J. KRÜGER, unpublished results). Therefore, the essential function of *MCM1* in yeast might be a specific feature of this species, since, also in the ascomycetous yeast *S. pombe*, the *map1* gene, coding for a MADS-box protein is not essential (NIELSEN *et al.* 1996). Thus, the consequences of mutations in these genes might vary from loss of viability as in *S. cerevisiae*, sterility only for one mating type as in *S. pombe* to a modulation of mating response as in *U. maydis*. The attenuation in mating responses did not decrease pathogenicity, at least under the conditions used, where plants are infected with a high inoculum of cells. It is feasible, however, that under infection conditions of low inoculum, which may occur in nature, efficient pheromone recognition might be of crucial importance in the competition for mating partners.

In yeast at least three gene products are known to interact with *Mcm1p*: α -specific transcription is induced together with *MAT α 1p*, α -specific gene expression is repressed by a complex of *MAT α 2p* and *Mcm1p* and pheromone-induced transcription of α -specific genes results from interaction of *Mcm1p* with *Ste12p*; in addition, another uncharacterized factor, *Sffp*, interacts with *Mcm1p* during cell-cycle-dependent transcription of *SWI5* (LYDALL *et al.* 1991). Whether a similar abundance of accessory proteins also exists in *U. maydis* remains to be elucidated. The isolation of *umc1* may provide the tool for the isolation of such proteins and a better understanding of the complex scenario regulating pheromone response in this fungus.

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