An ste20 Homologue in Ustilago maydis Plays a Role in Mating and Pathogenicity

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The mitogen-activated protein kinase (MAPK) pathways are conserved from fungi to humans and have been shown to play important roles in mating and filamentous growth for both Saccharomyces cerevisiae and dimorphic fungi and in infectivity for pathogenic fungi. STE20 encodes a protein kinase of the p21-activated protein kinase family that regulates more than one of these cascades in yeasts. We hypothesized that an Ste20p homologue would play a similar role in the dimorphic plant pathogen Ustilago maydis. The full-length copy of the U. maydis gene was obtained from a genomic library; it lacked introns and was predicted to encode a protein of 826 amino acids, whose sequence confirmed its identity as the first Ste20p homologue to be isolated from a plant pathogen. The predicted protein contained both an N-terminal regulatory Cdc42-Rac interactive binding domain and a C-terminal catalytic kinase domain. Disruption of the gene smu1 resulted in a delayed mating response in a mating-type-specific manner and also in a severe reduction in disease production on maize. Unlike the Ustilago bypass of cyclase (ube) mutations previously identified in genes in the pheromone-responsive MAPK cascade, mutation of smu1 does not by itself act as an extragenic suppressor of the filamentous phenotype of a uac1 mutant. Thus, the direct connection of Smu1p to MAPK cascade function has yet to be established. Even so, Smu1, though not absolutely required for mating, is necessary for wild-type mating and pathogenicity.

Signal transduction pathways are important for a variety of features of fungal development. For example, many human fungal pathogens exhibit some form of dimorphism, and a common element in these and other fungi is a conserved mitogen-activated protein kinase (MAPK) pathway. This MAPK pathway plays an important role in a multitude of processes for the saprophytic fungi (e.g., Saccharomyces cerevisiae and Schizosaccharomyces pombe), in human pathogens, such as Candida albicans and Cryptococcus neoformans, and even in plant pathogens, such as Ustilago maydis. Each pathway consists of a cascade involving phosphorylation of and by three protein kinases, a MAPK kinase (MAPKK), a MAP kinase (MAPK), and finally, the MAPK. This protein, in turn, phosphorylates one or more target transcription factors, among other substrates. Some of the best characterized of these cascades are in S. cerevisiae, where they are known to regulate such cellular processes as mating, cell integrity, spore formation, growth under conditions of high osmolarity, and pseudohyphal or filamentous growth (for a review, see reference 22). Upstream of the three-kinase module, additional components such as a MAPKK kinase and GTP-binding proteins (e.g., Cdc42, Rho/Rac, and Ras) (3) are also found.

U. maydis is a pathogen of maize, for which cell fusion and subsequent pathogenic development are controlled by two separate mating loci, a and b (5). The a locus encodes the pheromone and a seven-transmembrane protein pheromone receptor (5, 12), and these constitute the cell recognition system. Genetic and biochemical data indicate that the interactions between the U. maydis pheromones and receptors are similar to the events in the S. cerevisiae paradigm (4). Heterozygosity at this locus is required for the characteristic mating reaction observed on charcoal agar but not for production of dikaryotic hyphae in planta (5). The b locus is required for control of pathogenic development (12). The pheromone-responsive MAPK pathway is involved in the activation of both the a and b loci.

In addition to the a locus, several U. maydis genes orthologous to those in the pheromone response pathway of S. cerevisiae have been described. Of the four G protein subunits so far identified, gpa3 is required for the pheromone response (29). Mutants lacking this gene display an elongated morphology reminiscent of adenylate cyclase (uac1) mutants (10). Both the elongated morphology and sterility phenotypes of the gpa3 mutant were remedied by addition of exogenous cyclic AMP (cAMP), which was interpreted to show that cAMP signaling is a prerequisite for pheromone response (16) and shows that gpa3 is not required for transmission of the pheromone signal to a MAPK cascade. Additional components of the MAPK cascade have been identified in U. maydis (5, 11, 25–27). Besides the protein kinase components, the high mobility group protein encoded by prf1 appears to play the role of the downstream transcription factor and is required for expression of the genes at the a and b loci (12). Differential phosphorylation of Prf1 by the pheromone-responsive MAPK and the cAMP-dependent protein kinase regulates the activity of this transcription factor toward its various targets (14).
In addition to the triggering of filamentous growth via the action of the a and b loci, we have shown that a CAMP signal transduction pathway is critical for the maintenance of budding growth of the U. maydis wild-type haploid (10). Mutants in the uac1 gene encoding adenylyl cyclase are converted to a constitutive filamentous, but nonpathogenic, phenotype (6, 10). Several genes playing roles in dimorphism, in addition to uac1, have been identified by complementation of the mutations that suppress the filamentous phenotype of this mutant (10, 25). These suppressor mutations are called ubc (Ustilago bypass of cyclase), and so far five genes that influence the morphogenetic response of U. maydis to CAMP have been defined. The first of these, ubc1, encodes the regulatory subunit of protein kinase A (PKA) and is required for gal112 formation (8, 10, 11). Of the remaining genes, ubc3, ubc4, and ubc5, each encodes components of the MAPK cascade, while the predicted Ubc2 protein shares homology to the yeast Ste50p, specifically in the sterile alpha motif domain, used in binding to and preventing autoinhibition by Ste11p. Moreover, the Ubc2 protein contains a putative Ras association domain, and two-hybrid analyses have demonstrated an interaction between Ubc2 and a Ras1-like putative Ras association domain, used in binding to and preventing autoinhibition by Ste11p. Therefore, we sought the genes for such homologues so as to characterize them in terms of DNA sequence and possible functions in this plant pathogenic fungus.

This study describes the isolation and characterization of the gene for one such Ste20p homologue and an examination of its role in the biology of U. maydis. Its predicted structural similarity to Ste20p from S. cerevisiae and S. pombe (7) as well as in C. albicans (18) and C. neoformans (35). We hypothesized that an Ste20p homologue would play a similar role in the dimorphic plant pathogen U. maydis. Therefore, we sought the genes for such homologues so as to characterize them in terms of DNA sequence and possible functions in this plant pathogenic fungal.

MATERIALS AND METHODS

Fungal strains, isolation of genomic DNA, and microscopy. U. maydis wild-type strain 521 (a1b1) and its near-isogenic counterpart 2/9 (a2b2) (11) were used, as were the additional tester wild-type strains 2/14 (a2b2) and 2/1 (a2b1). Disruption of the smu1 gene in strain 521 yielded carboxin-resistant strain 10/18, while strain 10/17 was a carboxin-resistant a2b2 segregant recovered after infection of maize plants with a cross between strains 10/18 and 2/9. Strain 1/52 contains both the uac1 mutation eliminating production of adenylyl cyclase and yielding constitutive filamentous growth (10) and a temperature-sensitive ubc2 (27) allele. Thus, this strain continues to filament at 18°C, whereas, when shifted to 30°C, it produces the multiple-budding phenotype consistent with ubc mutations (26, 27). To test if smu1 could function as a ubc gene, strain 14/24 was generated by disrupting smu1 in the 1/52 background strain. This step was done rather than making the disruption directly in a ubc1 background strain because filamentous strains are notoriously refractive to transformation (25). S. Gold and M. Perlin, unpublished observations). Cells were inoculated on potato dextrose agar (PDA) plates, and the sporidia were harvested after 5 days for DNA isolation. The procedure used to isolate total genomic DNA was that developed by Luo and Perlin (23). Images of fungal cell morphology were taken with an Olympus BH-2 microscope with differential interference optics as previously described (25). For fluorescence microscopy, preparations were visualized with a Zeiss Axioscop Universal microscope (Carl Zeiss, Microscope Division), and digital images were compiled by using the Photoshop 5.5 (Adobe Systems) software.

Primer design for degenerate PCR. Amino acid sequences from six Ste20p homologues found in the GenBank database were aligned by the Clustal W multiple sequence alignment tool (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). The sequences were used from those of S. cerevisiae (Ste20p; accession no. NP_011856), C. albicans (serine/threonine-protein kinase Ste20p homologue; accession no. Q92212), S. pombe (serine/threonine protein kinase paki1b-hsk1; accession no.CAA22347), Dictyostelium discouleum (sererin kinase; accession no. AAC24522), and Homo sapiens (PAK 1B and protein kinase AIIA1142; accession no. AAC24716 and BAA86456, respectively).

Based on these comparisons, two conserved regions were identified: AIKQMNL and TPYWMAPE. The regions were chosen because they would yield relatively less degeneracy when used to design primers. The following primers were used: STE 20-5’#1, 5’-GCNATHAARCARATGAA, and STE 20-3’#1, 5’-TCNGNNGCCATCCARTA, where N is A, C, G, or T; R is A or G; and H is A, C, or T. Additional primers for PCR and sequencing were designed with the Primer3 program (S. Rosen and H. J. Skaltsky, 1997 [http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi]).

The primers were obtained from Sigma-Genosys (The Woodlands, Tex., and MWG Biotech (High Point, N.C.).

PCR. PCR was run on a PTC-100 thermal controller (MJ Research Inc., San Francisco, Calif.) by an initial denaturing temperature of 94°C for 4 min, followed by 35 cycles of the following program: 94°C for 1 min, annealing temperature (ranging from 58°C to 62°C) for 1 min, and 72°C for 2 min. This program was followed by a final extension at 72°C for 5 min. Gradient PCR was run on a Mastercyclus gradient cycler (Eppendorf Scientific Inc., Westburg, N.Y.). For colony PCR, the template was replaced by a small portion of the cells from a colony that was picked with a sterile pipet tip.

Nucleic acid manipulations. PCR products were separated by electrophoresis on an 0.8% low-melting agarose gel (agarose II; AMRESCO, Solon, Ohio) and purified by using a Wizard PCR Prep DNA purification system kit (Promega, Madison, Wis.). Plasmid DNA was purified with a Wizard miniprep kit (Promega), while cosmid DNA was extracted with the same miniprep kit for plasmid, with extra caution to minimize mechanical shearing. PCR products were either sequenced directly or cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, Calif.). PCR products, cloned DNA fragments, and cosmid DNAs were sequenced by using the Big Dye terminator kit and a 310 Genetic Analyzer (ABI/Perkin-Elmer, Foster City Calif.), following the manufacturer’s protocol. Primers designed for additional PCR and sequencing included mds_ste5in (5’-GGCGCATCGCAACATTGTCAAC-3’) and mds_ste3in (5’-TCGGCGATGGGA TAAACGCCGCTGTT-3’).

The cosmid library was screened by a sib selection PCR procedure which identified a pool of 48 cosmid clones in which the smu1 gene was present. Cosmid DNAs from the 48 colonies in this pool were harvested, digested, and subjected to Southern blotting. A 600-bp PCR product produced by the primer pair of mds_ste5in (5’-GCCGATCGCAACATTGTCAAC-3’) and mds_ste3in (5’-TCGGCGATGGGA TAAACGCCGCTGTT-3’).

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Restriction enzymes were purchased from New England Biolabs, Inc. (Beverly, Mass.), or American Allied Biochemical, Inc. (Aurora, Colo.). Digested DNAs were electrophoresed in 0.8% agarose and transferred in alkali to a nylon membrane (NYTRAN PLUS; Schleicher & Schuell, Keene, N.H.), according to the procedure of Sambrook et al. (31). Hybridization and washes were at 65°C.

Production of cDNA and RACE. First, total RNA was isolated (23). Poly[A]+ RNA was purified from total RNA with an Oligotex kit (QIAGEN, Chatsworth, Calif.), following the manufacturer’s protocol. Then a RETROscript kit (Ambion, Austin, Tex.) was used to make single-stranded cDNA for PCR. A Marathon cDNA amplification kit was used to get the double-stranded cDNA from the amplified cDNA. The use of RACE, we followed the protocol from the user manual of the Marathon cDNA kit. The program employed for amplification was as follows: 94°C for 1 min,
followed by 5 cycles of 94°C for 30 s and 72°C for 4 min and then 5 cycles of 94°C for 30 s and 70°C for 4 min; the last 22 cycles were at 94ºC for 20 s and 68ºC for 4 min.

Phylogenetic analysis. The amino acid sequence of the Ste20p homologue obtained in this study was compared with others in the GenBank database by BLASTX (basic local alignment search tool) (1) software available through the National Center for Biotechnology Information at the National Library of Medicine (www.ncbi.nlm.nih.gov). The sequences were also aligned by Clustal W (www.ebi.ac.uk/clustalw). Alignments were then adjusted as necessary, after assessment by eye, and gaps were added to facilitate the alignments. The alignment results were analyzed by the neighbor-joining method of Saitou and Nei (30), and a tree was produced by using PAUP4b10 (34). In order to screen for similar genes in U. maydis, when the first draft of the publicly available U. maydis genome project was released, BLAST searches (1, 2) were also conducted on this database (http://www-genome.wi.mit.edu/annotation/fungi/ustilago_maydis/index.html).

Design of disruption and overexpression constructs for the smu1 gene. The disruption vector was designed to use a PCR product containing only the Ste20p gene and a selectable marker inserted within the gene to disrupt its function. Primers mds_ste_big3 (5’-CCGGATGAGATCGAAGTGAGT-3’) and mds_ste_Send (5’-ACGTCGGCTTCAACTCGGACA-3’) were used with U. maydis genomic DNA to yield a 1.57-kb PCR product that encoded the most conserved part at the 3’ end of the gene, including the functional catalytic domain. The PCR product was purified and cloned into the vector pBAD4.1-TOPO (Invitrogen). This was digested with HindIII and PstI. The linear vector or gene was purified and ligated with a HindIII/NotI fragment (about 2.5 kb) of plasmid pCR2.1_Cbx (27) containing the carboxin resistance gene for selection in U. maydis. A PCR product containing the smu1 gene with the carboxin resistance cassette was produced and purified. Linear DNA was used since it has a higher recombination rate than the circular plasmid (9). Also, by providing only the smu1 disruption without plasmid sequences flanking the CbxR, we greatly reduced ectopic recombination. Strains 521 and 1/52 were used as recipient strains for such transformations and targeted disruptions. Carboxin was used at a concentration of 3 μg/ml to select U. maydis transformants.

For overexpression, the genomic region encoding smu1 was amplified by PCR and cloned, and along with the U. maydis gapd promoter (15), into plasmid pHyg101 (25). The correct construct was confirmed by restriction mapping, PCR, and sequencing.

Plate-mating and confrontation (drop-mating) assays. Plate-mating assays were used to examine the effects of the smu1 mutations on the filamentous phenotype associated with the mating reaction. Strains were grown overnight in yeast extract-peptone-sucrose (12) at 30°C and then, after equal volumes of paired cell types were mixed in approximately equal number, 20-μl drops were spotted onto complete medium or yeast extract-peptone-dextrose (YPD) or double-complete medium containing 1% charcoal at room temperature (13). Drop-mating assays were carried as described by Snetselaar et al. (33) with previously noted modifications (26). This assay allowed indirect observation of both production and response to pheromone signals in mutant and wild-type cells, including production of mating filaments. Northern blot analyses (31) of participant strains in these plate-mating assays were used to characterize gene expression of the mfa (pheromone) genes.

Plant infection studies. Trucker’s Favorite (Imperial Garden Seed; Athens, Georgia) seedlings were grown and inoculated, and disease ratings were recorded as previously described (11). Inocula of 10⁶ cells ml⁻¹ for each of the paired strains were quantified with a hemocytometer. Experiments to analyze pathogenicity were conducted a minimum of three times, with 20 plants used for each treatment.

RESULTS

A U. maydis gene encoding an Ste20p-like protein is detected by PCR amplification with degenerate primers. Two blocks of conserved amino acids were found in the C-terminal portion of the Ste20p-like proteins analyzed. These conserved portions of the proteins were chosen to design two degenerate primers. For PCR, a gradient of annealing temperatures was used, varying from 46°C to 60°C at intervals of 4°C. All PCRs showed one clear, strong band of approximately 450 bp at all temperatures in the gradient in addition to fainter bands in some cases. The band was purified and cloned into vector pCR2.1-TOPO. This gene fragment was sequenced. A BLASTX (1, 2) search showed high similarity to Ste20p in S. cerevisiae and to other Ste20p homologues (data not shown). Analysis by 3’ RACE yielded the 3’ end of the gene. A subsequent comparison of the sequence obtained by PCR of the corresponding genomic region detected no introns in this region.

Cosmid library provides the full-length sequence of smu1. Only approximately 200 bp of additional sequence information was provided by 5’ RACE, so a cosmid library was screened to recover the entire genomic copy of the gene and flanking material (accession no. AF299352). The predicted amino acid sequence for the protein was found to be closest to the S. pombe PAK (accession no. CAA22347; identity, 46%; positives, 361 of 594 [59%]; gaps, 33 of 594 [5%]) and was also closely related to S. cerevisiae Ste20p (accession no. NP_011856; identity, 46%; positives, 331 of 563 [58%]; gaps, 65 of 563 [11%]) and to C. albicans Cst20 (accession no. AAB68613; identity, 52%; positives, 242 of 342 [70%]; gaps, 14 of 342 [4%]). The U. maydis protein contained at the C terminus the conserved kinase domain and what appears to be a Gβ binding region: RLTPLIKAAR. The underlined residues fit the consensus of the gene encoding for Smu1p in Ustilago maydis 1.164 [scaffold 13]. The next three highest matches (scores 364, 210, and 180) were to regions that primarily bore similarity in the encoded kinase domains to those of Ste20a from C. neoformans (accession no. AAN75173; Don3 (accession no. AAM73879), a U. maydis germlinal-center PAK-like kinase (36); and a serine kinase from D. discoideum (accession no. AAC24522). When the regulatory region (CRIB) from the S. cerevisiae Ste20p was used in the search, the only significant matches were to the region containing the smu1 gene (highest match, Ustilago maydis 1.164 [scaffold 13]) and to that containing the gene for the U. maydis Cla4 homologue (Ustilago maydis 1.83 [scaffold 4]).

Computer analyses of the 1,000-bp region upstream of the putative start codon for the gene encoding the U. maydis Ste20 homologue did not reveal a likely TATA box or similar transcriptional start signals. It appears that transcription begins at a point between 550 and 350 bp upstream of the predicted ATG start codon. This conclusion is based on the success or failure of reverse transcription-PCR using a primer at the upstream 350 and 550 positions, respectively, together with a primer from the coding region. The corresponding reactions with genomic DNA yielded the expected products (data not shown).

Disruption of smu1 attenuates mating and slows filamentous growth in response to low ammonium. A disruption vector was constructed in which a carboxin resistance cassette was inserted into the 3’ end of the gene where the functional catalytic domain is normally encoded. The entire construct was amplified as a 3.6-kb PCR product and then purified and used
to transform *U. maydis* to disrupt the Ste20p homologue. In this way an a1b1 strain with a disruption was initially obtained and an a2b2 strain was later obtained through backcrossing and recovery of carboxin-resistant progeny from the plants. Confirmation of the gene disruptions in all cases was made by Southern analyses and PCR (data not shown). Disruption strains did not display any obvious differences from the wild type in colonial morphology on PDA or YPD. Nor were there any obvious differences in growth relative to that of the wild type at either 18°C or 30°C.

In order to examine whether Smu1p might play a role in filamentation induced by environmental stimuli, *smu1* disruption strains were compared with their wild-type haploid progenitors grown either on low ammonium (SLAD; 50 μM ammonium) (32) or in acid pH (pH 3.5). All strains tested produced hyphae on SLAD (Fig. 1) or in acid pH (data not shown). However, the ability of the disruption strains to produce such hyphae on SLAD was reduced relative to that of the wild type, although not eliminated. In contrast, such filamentation was completely absent in a strain with a disruption of the *ump2* gene for an ammonium permease (Fig. 1E) (32). As with the wild-type progenitor strains, filamentation due to growth on low ammonium could be reversed in the *smu1* disruption strains by the addition of exogenous cAMP (10 to 25 mM; data not shown). In addition, overexpression of *smu1* in the disruption backgrounds yielded strains that produced filamentation at or above that seen with the corresponding wild-type strains on SLAD (Fig. 1F and G).

Segregants of opposite mating types were obtained in the background strains 521 (a1b1) and 2/9 (a2b2). Such segregants were used in plate-mating assays to assess their efficiencies of mating. As seen from the results shown in Fig. 2, disruption of the gene encoding the Ste20p homologue produced a dramatic delay and overall reduction in mating. Thus, the gene was named *smu1* (Ste20p affecting mating in *Ustilago*). The observed differences were most pronounced when the plate mating was done on YPD-charcoal media. On double-complete medium containing charcoal, such differences were less noticeable. On either medium, mating between wild-type strains produced the aerial hyphae characteristic of the dikaryon on charcoal agar. In contrast, when mating was between the wild type and an a2b2 strain with an *smu1* disruption (e.g., strain 10/17), mating was substantially reduced and did not reach the level observed for the wild type, even after 48 h. As seen in Fig. 2,
the mating reaction between the wild type and an \(a1b1\) strain bearing the \(smu1\) disruption was delayed by about 6 h. During the entire 48-h period, there was no visible reaction between strains that each had the \(smu1\) disruption. Thus, disruption of \(smu1\) appears to attenuate the mating reaction. This phenotype was returned to the wild type when disruption strains were transformed with the cosmid bearing the wild-type copy of \(smu1\).

The \(smu1\) gene was also overexpressed from a multicopy plasmid. The construct was introduced into wild-type \(U.\ maydis\) strains of opposite mating type (i.e., strains 521 and 2/9) as well as \(smu1\) disruption strains of opposite mating type (strains 10/18 and 10/17). Hygromycin-resistant transformants were tested in both the plate-mating and confrontation assays. Effects of overexpression were subtle for either plate mating or performance in the confrontation assay (data not shown). In general, strains that overexpressed \(smu1\) mated less efficiently than their untransformed wild-type progenitor strains, with the greatest effect observed when both partners in the mating overexpressed the gene. For all other comparisons, overexpression in the \(a1\) background slightly improved mating, while overexpression in the \(a2\) background strains reduced efficiency relative to matings with the corresponding untransformed progenitor.

To visualize if the reduction in cell fusion could be ac-

FIG. 2. Mating is attenuated in strains with a disruption in \(smu1\). Colonies were examined after growth for 16, 24, 40, and 48 h at 25°C on mating medium containing activated charcoal (13). Coinoculation of the wild-type strains 521 (\(a1b1\)) and 2/9 (\(a2b2\)) produced a strong mating reaction, with the characteristic aerial hyphae. Coinoculation of two \(smu1\) mutants resulted in many fewer aerial hyphae (comparable to haploid strains alone), while the reaction with either mutant together with a wild-type strain of the opposite mating-type yielded a reduction in the fus reaction that was most obvious when the \(a2b2\) strain contained the lesion (gray arrow). Transformants of the mutants with the cosmid bearing the wild-type \(smu1\) gene produced strains whose mating efficiency was equivalent to that of the wild type (data not shown). Haploid strains fail to make aerial hyphae when inoculated alone onto mating medium.
counted for by altered pheromone response or production, we employed a confrontation or drop-mating assay (33). Figure 3 shows the results of this second assay for premating defects. It should be noted that while this assay has been used reliably to address the role of pheromone in *U. maydis* mating reactions (33), pheromone is not directly measured in these experiments, and morphological responses are, thus, simply interpreted to be a result of pheromone production and/or response. Wild-type strains possessing opposite mating-type specificity (b is irrelevant in this assay) responded to each other by the production of copious filaments (Fig. 3A). As reported by Snetselaar et al. (33), a2 strains (Fig. 3, bottom strain in all panels) respond more rapidly than do a1 strains (Fig. 3, top strain in all panels). Apparently *smu1::Cbx* strains secrete a highly reduced amount of pheromone compared to the wild type as indicated by the lack of mating hyphae produced by the wild-type a2 strain when paired with the a1 *smu1::Cbx* strain (compare Fig. 3A and B). However, sparse filaments were produced by the mutant strain when it was confronted with the wild type (Fig. 3B and C). The *smu1::Cbx* strains showed essentially no visible filamentation response when two *smu1* mutant strains were paired (Fig. 3D). This result likely means that mating between two *smu1::Cbx* strains can only occur if the colonies grow to bring mating partners to near-direct physical contact.

**Disruption of smu1 differentially influences mfa1 and mfa2 expression.** Northern blot analyses (Fig. 4) allowed us to examine some of the predictions made above from the confrontation assays. In unmated cells, expression of *mfa1* was only slightly reduced in the a1b1 *smu1* mutant (strain 10/18) relative to the wild-type a1b1 (strain 521). However, *mfa2* expression was substantially lower in the a2b2 *smu1* mutant (strain 10/17) than in the a2b2 wild type (strain 2/9). In mating reactions between strains with different a and b alleles, the expression of *mfa1* was similar regardless of the status of *smu1*, whereas

**FIG. 3.** Drop-mating assays for pheromone response in *smu1::Cbx* disruption strains. (A-D) Drops of appropriate strains were spotted in close proximity on microscope slides covered with water agar and observed after 24 h (33). In all cases, an a1b1 strain is at the top and an a2b2 strain is at the bottom of the image. (A) Top, wild-type (strain 521); bottom, wild-type strain 2/9. (B) Top, *smu1::Cbx* (strain 10/18); bottom, wild-type strain 2/9. (C) Top, wild type (strain 521); bottom, *smu1::Cbx* (strain 10/17). (D) Top, *smu1::Cbx* (strain 10/18); bottom, *smu1::Cbx* (strain 10/17). Size bar, 50 μm.

**FIG. 4.** Effect of *smu1* disruption on expression of *mfa* genes. Strains and strain combinations listed were grown on complete medium-charcoal plates for 48 h at room temperature. Total RNA was then extracted and subjected to Northern blot analysis with probes *mfa1*, *mfa2*, and *cbx* (carboxin resistance; succinate dehydrogenase) consecutively. In each case the blot was probed, stripped, and then reprobed with the next probe. Lanes 1 to 12 contained total RNA from individual strains or from matings of strains 521 (lane 1), 2/14 (lane 2), 2/9 (lane 3), 10/18 (lane 4), 10/17 (lane 5), 521 × 2/9 (lane 6), 521 × 10/17 (lane 7), 10/18 × 2/9 (lane 8), 10/18 × 10/17 (lane 9), 521 × 2/11 (lane 10), 10/18 × 2/11 (lane 11), and 2/14 × 10/17 (lane 12).
expression of mfa2 was much lower when the a2 allele was in the smu1 mutant background. The mfa1 expression was similar (and low) in all these reactions, presumably because after 48 h mating is advanced enough for the b-dependent repression of a to take place.

The effect of b-dependent repression of mfa gene expression can be eliminated in matings in which the a alleles are different but the b alleles are the same. When the smu1 mutant strain was a1, a reduction in Northern signal was observed for mfa1 (Fig. 4) relative to that seen for the wild-type interaction. Smu1p, therefore, seems to affect induction of mfa1. The effect of the smu1 mutation on the induction of pheromones was more severe when the smu1 disruption mutant was in the a2 background; the signal was much lower with both mfa1 and mfa2 probes, and the mating reaction in general was greatly reduced. Thus, smu1 affects both basal and induced expression levels for mfa2 in the a2 background.

Effect of disruption of smu1 on the phenotype of cells also bearing the uac1 mutation. All other components of the MAPK pathway so far identified in U. maydis have behaved as ubc (Ustilago bypass of cyclase) mutations. Thus, we wanted to determine if disruption of smu1 would similarly mask the filamentous phenotype associated with the uac1 (adenylate cyclase) mutant. Approximately 13 carboxin-resistant transformants of strain 1/52 were obtained when transformation utilized the smu1 disruption construct. Of these, one was confirmed to contain the disruption of smu1 and at least one was shown to contain an ectopic integration of the disruption construct. These conclusions were drawn from PCR and Southern hybridization (data not shown). The strain with integration of the smu1 disruption at the homologous site also was shown to lack the wild-type copy of the gene. Growth of a transformant bearing the disruption was compared with that of a transformant with an ectopic integration of the construct (i.e., still containing the wild-type copy of smu1) and with that of the untransformed recipient, strain 1/52. When shifted to growth at 30°C, the cells had a multiple-budding phenotype in liquid culture, consistent with the ubc2 temperature-sensitive mutation in conjunction with the uac1 mutation (data not shown) (10, 27, 28). On solid medium at 30°C, colonies of the recipient and the ectopic transformants were indistinguishable, and all produced the frosty budding growth noted for strain 1/52 (27).

FIG. 5. The smu1 mutation appears to behave as a weak ubc mutation. Strain 1/52, with both a mutation in uac1 and a temperature-sensitive ubc2 mutation (left panels), was compared with its isogenic counterpart, strain 14/24, bearing a disruption of smu1 (center panels) on solid medium PDA. Comparison was made at both 30°C (top panels) and 18°C (bottom panels). The permissive and nonpermissive temperatures for the ubc2ts allele are 18 and 30°C, respectively. Thus, at 18°C this gene would be functional, and therefore the strain carrying the smu1 mutation should behave as a double uac1/smu1 strain. The images in the panels of the wild-type strain 1/2 are approximately two times the magnification of those in the other panels to emphasize the smooth, wet-like character of the colonies, similar to the character of the colonies seen for the 14/24 strain at 30°C (upper middle panel).

This result was possibly due to residual ubc2 activity at 30°C. Cells taken from these colonies appeared elongated and similar to short filaments. In contrast, colonies of the 14/24 triple mutant (uac1 ubc2 ts smu1) resembled wild-type colonies and did not display a filamentous character (Fig. 5, upper panels). Similarly, cells taken from these colonies were wild type in appearance. At 18°C, where the temperature-sensitive ubc2 allele is functional, all strains, unlike the wild type, were filamentous (in both liquid culture and on agar plates) (see Fig. 5). However, strain 14/24, the strain that additionally bore the smu1 disruption, had a colonial morphology distinct from the others. Rather than the strongly filamentous dense colonies of the 1/52 strain (Fig. 5, lower left panel) or an ectopic transformant (data not shown), the disruptant grew as less robust colonies that had a spiderweb-like character (Fig. 5, lower middle panel).

The smu1 gene is required for full virulence. In order to examine the role, if any, of the smu1 gene in the disease process, smu1 disruptant strains 10/17 and 10/18 were each mated either with wild-type tester strains or with each other and used to infect maize seedlings. Table 1 shows the results of three infection trials. An observed trend, though not borne out by statistical analyses, was reduced disease symptoms with only one smu1 disruption present and a greater reduction when the disruption was in the a2b2 background. When both strains contained the lesion, a Tukey’s test showed a significant difference compared to the other treatments. In such infections, the majority of symptoms were limited to those observed early
The smu1 gene appears to encode an Ste20p homologue of the PAK family. Comparison of the predicted amino acid sequence of Smu1p with other proteins in the database revealed its similarity with Ste20-like kinases. In particular, Smu1p contained the hallmark of proteins in one of the two subfamilies of kinases, the PAKs (p21Rac/Cdc42-activated kinases) (24). It contained a C-terminal Ste20-like kinase domain, as found in Ser/Thr and Tyr kinases, and a C-terminal Gβ binding site [SSLθFL(I/V)Xθφβ, where X is any residue; θ is A, I, L, S, or T; and β represents basic residues] that is solely present in members of the Ste20p/PAK family and that is conserved among Ste20 homologues from fungi to mammals (19, 21). It was also predicted to contain an N-terminal regulatory domain, with Cdc42/Rac binding regions. However, it did not appear to contain a pleckstrin binding domain found in the Cla4-like kinases (35; see below also). Its conservation with S. pombe PAK and S. cerevisiae Ste20p in phylogenetic analyses further supports these findings. Thus, the Smu1 protein would be expected to play a similar role in U. maydis as the proteins found in S. pombe and S. cerevisiae, including having a role in mating and possibly in filamentous growth.

The smu1 gene is required for typical strong mating reactions but not for filamentation of haploids normally induced by low ammonium or acid. There was no observed effect of disruption of smu1 on the normal response of haploid cells to growth in acid pH. Thus, the smu1 gene product would not appear to be required for this process. Interestingly, smu1 disruption strains produced less filamentation on low ammonium compared to wild-type strains. When the smu1 gene was reintroduced into the disruption strains and overexpressed from a plasmid (Fig. 1F and G), the degree of filamentation on SLAD equaled or exceeded that of the wild-type strains under the same conditions.

To examine another filamentous cell type for U. maydis, the effects of smu1 disruption on mating were examined. Compatible wild-type haploid strains, when mixed and plated onto medium containing 1% charcoal, produce a characteristic "fuz" reaction, where colonies are covered by aerial hyphae. This results from the production of a filamentous cell type after cell fusion of the U. maydis strains carrying different specificities at both a and b mating-type loci. Control of this reaction is governed, at least in part, by activation of the pherome-responsive MAPK cascade, several of whose components appear to fill the corresponding roles in the mating pathway in S. cerevisiae. Ste20p, the MAPKKK kinase, is known to regulate this pathway in S. cerevisiae, as well as the parallel pathway governing production of pseudohyphae. Thus, we reasoned that an Ste20-like protein in U. maydis would, in interacting with such pathways, play an important role in mating and, possibly, in subsequent downstream events.

In fact, disruption of smu1 affected mating as assessed by the plate-mating assay, in a gene dosage-dependent manner. Matings between compatible strains that each possessed the mutation were comparable to the haploid negative controls, i.e., no aerial hyphae were observed for up to 72 h postinoculation. Interestingly, when only one strain of the mating pair bore the disruption, the effect was more pronounced if the a2b2 strain carried the mutation. This result might be due to a2 strains responding morphogenetically to basal levels of a1 pherome produced by their compatible partner, whereas a1 strains are able only to respond morphogenetically to induced levels of pherome (see below). This finding would also be consistent with the commonly observed, more rapid response to pherome by a2 strains in confrontation assays (33).

The smu1 gene is required for normal response to pherome. Reduced filamentation in mating reactions with smu1::Cbx strains was observed, indicating a possible role for Smu1p in pherome response. A drop-mating assay demonstrated that smu1::Cbx strains are compromised for response to pherome signals. However, unlike mutants at ubc3/kpp2 (26, 28), which provoke no filaments in wild-type partners, and the ubc2 and fiz2/ube5 mutants that are unable to filament in response to pherome but still induce typical morphogenesis in their mating partner (27), smu1 mutants are able to filament in response to pherome produced by the wild type but induce no response in their wild-type partner. One possible explanation for this is that smu1 mutants are able to produce basal levels of pherome but are unable to be induced to produce pherome to a higher level in response to opposite a

### TABLE 1. Pathogenicity of smu1 mutant dikaryons

| Treatment | Dikaryon | Total no. plants tested | Disease rating by number of affected plants | Disease index |
|-----------|---------|-------------------------|---------------------------------------------|---------------|
| 1 (+/+ )  | 60      | 1 1 1 5 13 13 27        | 3.95 A                                      |
| 2 KO/ KO  | 60      | 1 1 4 5 14 15           | 3.60 A                                      |
| 3 +/KO    | 60      | 2 1 10 25 13 9          | 3.25 A                                      |
| 4 KO/ KO  | 60      | 6 9 5 17 19 4           | 2.02 B                                      |

*Table shows combined data from three identical experiments of 20 plants each reported for 10 days postinoculation.

b Treatment consisted of the inoculation of 10⁶ cells ml⁻¹ for each of the paired strains as follows: treatment 1, strain 521 × strain 2/9; treatment 2, strain 10/18 × strain 2/9; treatment 3, strain 521 × strain 10/17; and treatment 4, strain 10/18 × strain 10/17.

c KO, disruption mutation.

d The disease rating is measured on a scale of 0 to 5 points based on the severity of symptoms as follows: 0 points, no disease symptoms; 1 point (C), chlorosis; 1 point (A), anthocyanin production; 2 points, small leaf galls; 3 points, small galls on stems; 4 points, large stem galls; and 5 points, plant death.

The disease index is calculated as the sum of disease ratings divided by the number of plants. By analysis of variance with SAS of the disease indexes from the three independent repetitions of the experiment, it was found that there was a significant difference between treatments (P = 0.0016) but not between experiments (P = 0.3092). According to Tukey’s test, treatments 1 to 3 did not significantly differ (A) while treatment 4 differed (B) from the others at the 95% confidence level.

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pheromone. This possibility was examined by expression analysis for the mfa (pheromone) genes under conditions for mating. Sufficient pheromone must be produced by the wild-type partner to trigger a morphogenetic response in the smu1 mutants (compare Fig. 3B and C to D). Thus, this finding raises the interesting prospect that smu1 may be required for the induction of increased pheromone production but not for morphogenesis, suggesting an opposite role to the ubc2 gene.

The results from Northern blotting suggest a possible basis for the effect of the smu1 disruption on mating efficiency. Proper expression of smu1 is required for normal pheromone gene expression. The effect of the smu1 mutation on induction of pheromones was more severe when the mutant was a2; the signal was much lower with both mfa1 and mfa2 probes, consistent with the mating reaction also being greatly reduced. Thus, smu1 affects both the basal and induced expression levels of mfa2 in the a2 background. However, with signals in the a2b2 smu1 mutant alone compared with those in the cross a2b2 smu1 mutant × a1b2 wild type, we see that mfa2 in the mutant background can still undergo some level of induction. This result could explain why the smu1 mutants appear to be able to “catch up” and were able to produce some disease symptoms in maize, including large stem galls. This explanation also fits nicely with the mating reactions. There was much greater delay in the fuz reaction between an smu1 mutant a2b2 and a wild-type a1b1 than with an smu1 mutant a1b1 and a wild-type a2b2, although the latter was also delayed in comparison with the wild-type reaction. It is interesting that the smu1 mutation appears to have more effect on the strain bearing the a2 allele than on that with the a1 allele; a2 strains also normally tend to respond more rapidly in confrontation assays, forming mating hyphae before a1 strains do. Thus, these results suggest differential regulation of mfa alleles by smu1. One intriguing prospect is that the other PAK kinase identified in U. maydis, Cla4 (M. Mahlert, L. Leveleki, B. Sandrock, and M. Bölker, Abstr. 22nd Fungal Genetics Conference, abstr. 91, 2003), plays the primary role for mfa1 induction in a1 strains.

Another protein that signals through the MAPK cascade and regulates mfa1 pheromone gene expression is Ras2. This protein is an ortholog of the ras family of small GTP-binding proteins. Lee and Kronstad (20) observed that ras2 mutants were defective in budding growth, pathogenicity, and mating. In particular, such mutants did not even produce basal levels of mfa1 transcript. Thus, like smu1 mutants, they displayed greatly reduced aerial hyphae in the plate-mating assay, and they produced less pheromone.

**The smu1 gene is required for full virulence.** Other components of the MAPK cascade in U. maydis have been found to be important or required for virulence of the pathogen on maize (26, 27). As seen for mating on charcoal agar, the effect of disruption of smu1 on virulence was observed in a dosage-dependent manner. Inoculations where one copy of smu1 was disrupted produced a trend of lower disease index than inoculations with wild types. Moreover, a greater reduction in disease was observed when the mutation was in the a2b2 background, a result that correlated with those observed in plate-mating assays. A statistically significant decline in virulence was found when the smu1 gene was disrupted in both strains used for inoculation. Thus, the smu1 gene appears to be required for full virulence of this pathogen on maize. Reduced virulence seems likely attributable to reduced mating efficiency but may also involve additional unidentified defects.

The Smu1 protein, unlike other members of the MAPK pathway in U. maydis, may only act as a weak ubc for filamentous uac1 mutants. The uac1 gene of U. maydis encodes adenylate cyclase, and its disruption leads to constitutive filamentous growth of haploid cells (10). Genes whose mutation reverses this phenotype have been termed ubc genes (10, 25). Such genes include that encoding the regulatory subunit of the cAMP-dependent PKA pathway (ubcl1) (10), as well as all central components of the MAPK pathway so far identified (ubc2, ubc3, ubc4, and ubc5) (26, 27). Since smu1 is predicted to encode an Ste20p-like PAK kinase, we expected that this protein would participate in the MAPK pathway and, as such, that its disruption would also lead to a ubc mutant when present with a uac1 mutation. However, in this study, we found that disruption of smu1 by itself did not mask the phenotype of a uac1 mutant. For a ubc2 temperature-sensitive allele in a uac1 mutant, malfunction at 30°C led to a nearly complete reversal of the filamentous phenotype (Fig. 5, upper left panel). In contrast, the smu1 disruption did not have this effect at 18°C, where ubc2 was still functional. Instead, the only observable phenotype associated with this additional mutation at permissive temperature was on the density of the filamentous morphology of colonies on agar (Fig. 5, lower center panel). Thus, the smu1 mutation may interact with the uac1 mutation but not in a manner consistent with its behavior as a ubc gene. On the other hand, at 30°C the uac1 ubc2 mutation showed slight fuzzy growth due probably to remnant activity of ubc2 at this temperature. The addition of the smu1 mutation reverted colonies to a budding wild-type wet morphology. Therefore, smu1 mutation appears to behave as a weak ubc mutation.

**Conclusions.** A PAK-like kinase had previously been identified in U. maydis (36). The Don3 germinal-center kinase was found to be involved in initiation of the secondary septum required for proper cell separation (36). In addition to germinal-center kinases, other types of PAK-like kinases have been found in a variety of organisms. Two different classes of PAK kinases are found, those that contain both a C-terminal kinase catalytic domain and an N-terminal regulatory domain (CRIB) (17) and those that contain, in addition, an amino terminal pleckstrin homology domain. This latter domain is found in the Cla4-like kinases and is used to facilitate binding to membrane phosphoinositides (35). In fungi, the group of kinases containing the pleckstrin homology domain includes the Ste20α and Ste20a from serotypes A and D of C. neoformans (35), Cla4 from S. cerevisiae, S. pombe (7), and C. albicans (18), and a recently identified Cla4 from U. maydis (Mahlert et al., Abstr. 22nd Fungal Genetics Conference). Such proteins have unique roles in cytokinesis, and Cla4 plays a role in the switch from apical to isotropic growth for S. cerevisiae (35). Moreover, in addition to morphological defects associated with its disruption, Cla4 in C. albicans is completely required for virulence, and mutants lacking this kinase cannot undergo the dimorphic switch in vivo (18). In U. maydis, the Cla4 homolog regulates a parallel Cdc42-containing pathway that affects polarized growth and septum formation (Mahlert et al., Abstr. 22nd Fungal Genetics Conference).

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