Role of Hsl7 in Morphology and Pathogenicity and Its Interaction with Other Signaling Components in the Plant Pathogen *Ustilago maydis* \(^*\)\(^†\)

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The phytopathogenic fungus *Ustilago maydis* undergoes a dimorphic transition in response to mating pheromone, host, and environmental cues. On a solid medium deficient in ammonium (SLAD [0.17% yeast nitrogen base without ammonium sulfate or amino acids, 2% dextrose, 50 μM ammonium sulfate]), *U. maydis* produces a filamentous colony morphology, while in liquid SLAD, the cells do not form filaments. The p21-activated protein kinases (PAKs) play a substantial role in regulating the dimorphic transition in fungi. The PAK-like Ste20 homologue Smu1 is required for a normal response to pheromone, via upregulation of pheromone expression, and virulence, and its disruption affects both processes. Our experiments suggest that Smu1 also regulates cell length and the filamentous response on solid SLAD medium. Yeast two-hybrid analysis suggested an Hsl7 homologue as a potential interacting partner of Smu1, and a unique open reading frame for such an arginine methyltransferase was detected in the *U. maydis* genome sequence. Hsl7 regulates cell length and the filamentous response to solid *U. maydis* in a fashion opposite to that of Smu1, but neither overexpression nor disruption of hsl7 attenuates virulence. Simultaneous disruption of hsl7 and overexpression of smu1 lead to a hyperfilamentous response on solid SLAD. Moreover, only this double mutant strain forms filaments in liquid SLAD. The double mutant strain was also significantly reduced in virulence. A similar filamentous response in both solid and liquid SLAD was observed in strains lacking another PAK-like protein kinase involved in cytokinesis and polar growth, Cla4. Our data suggest that Hsl7 may regulate cell cycle progression, while both Smu1 and Cla4 appear to be involved in the filamentous response in *U. maydis*.

p21-activated protein kinases (PAKs) comprise a large, highly conserved family of serine/threonine protein kinases. They are involved in a myriad of cellular functions, including cell cycle regulation, cytokinetic organization, mating responses, cell polarity, morphogenesis, and separation, and responses to environmental conditions (7, 21, 22, 31, 41). Homologues of the PAK family are known to activate the mitogen-activated protein kinase (MAPK) pathway (17, 30). This pathway is conserved from fungi to humans and plays a role in several fungal processes, including mating, cell morphology, and filamentous growth in response to environmental stimuli. A serial cascade of phosphorylation events, triggered by and targeting three protein kinases, a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK, phosphorylates one or more downstream targets, some of which are transcription factors. In *Saccharomyces cerevisiae*, both mating and invasive growth/pseudohyphal differentiation depend on multiple elements of the MAPK cascade (41). In *Schizosaccharomyces pombe*, the MAPK pathway is involved in the mating response and subsequent meiosis and sporulation. Several components are shared by the two subsets of this pathway, including the PAK, the MAPKKK, and the MAPKK (17, 35, 36). Discrimination between various inputs and the eventual desired outputs is dependent on a series of scaffolding and adaptor proteins. In addition, upstream activators and coactivators differentiate between the eventual outputs through spatial regulation of components of this pathway. Upstream of the PAKs, and subsequently the MAPK pathway, is a series of conserved Rho/Rac-like GTP-binding proteins (GTPases) which bind and regulate the localization and activity of the PAKs (17, 35).

One member of the Rho/Rac family of GTPases is Cdc42. Cdc42 plays a role in regulating cell proliferation, polarity, and differentiation in many eukaryotes. In *S. cerevisiae*, Cdc42 is required for cell polarity, cytokinesis, invasive and pseudohyphal development, and mating of haploid cells (15, 30). Cdc42 specificity and activity are regulated by cycling between GTP (active) and GDP (inactive) states via two additional groups of proteins, guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). One such GEF is Cdc42, which localizes Cdc42 to the bud neck, allowing for proper bud formation, cytokinetic reorganization, and cytokinesis. A multiprotein complex comprised of several proteins, including Cdc42 and Scd1 (Cdc42 homologue), plays similar roles in *S. pombe* (11).

Of potential downstream effectors of Cdc42 and Cdc24, the PAKs have been extensively studied (see above). Ste20 and Cla4 are two PAKs first identified and well characterized in *S. cerevisiae*, and the functions of the two kinases are required in several key aspects of cell development. Simultaneous deletion of both ste20 and cla4 is lethal, indicating that the two PAK homologues share at least one essential function (12). In addition, Ste20 and Cla4 play roles in many distinct processes. Ste20 is involved in the pheromone response pathway, the haploid invasive growth pathway, and the high-osmolarity glycerol (HOG) pathway. Cla4 regulates septin function and polarized growth, as well as cytokinesis (5). Data have linked

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Ste20 to cell polarity and cytokinesis, Cla4 to the mating response, and both to actin organization, indicating that a functional overlap between the two kinases exists (21, 25, 30). In S. pombe, Shk1 (Ste20 homologue) is essential for viability and is required for several cell functions, including mating, cytoskeletal regulation, cell cycle regulation, and response to hyperosmotic conditions, while Shk2 (Cla4 homologue), dispensable for growth, appears to be redundant to Shk1 in many functions (4, 40, 49).

In Ustilago maydis, a fungal pathogen of maize, several Rho-like GTPases have been identified, including Cdc42, Rac1, Rho1, and Rho3, of which Cdc42, Rac1, and Rho1 regulate cell separation, hyphal development, and cell polarity, respectively (7, 34, 38, 47). In addition, Cdc42, Rac1, and Rho1 have been demonstrated to interact in vitro with two known PAKs from U. maydis, Cla4 and Smu1 (31, 38). In vivo analysis has identified Cla4 as an effector of Rac1 regulating cell polarity (31). A Cdc24 homologue was identified in U. maydis; however, Cdc24 is the preferred GEF of Rac1, not Cdc42 (1, 10). A second GEF, Don1, was identified as an activator of Cdc42. It regulates the formation of the second septum between mother and daughter cells (7, 34). No downstream effector of Cdc42 has been identified, though Cla4 and Smu1 are potential candidates.

Hsl7 homologues are known to interact with the PAKs and are conserved from fungi to humans (16, 18, 32, 39). Hsl7 homologues are putative protein-arginine methyltransferases that are identified by a single methyltransferase domain and are involved in many cellular functions, including cell cycle regulation, cell morphogenesis, and response to environmental conditions (4, 33, 43, 48). Hsl7 from S. cerevisiae acts as a mitotic inducer by promoting the targeted degradation of the mitosis inhibitor protein kinase Swel. Swel degradation is required to permit the G2/M transition in the cell cycle. In S. cerevisiae, Hsl1 is localized to septins and recruits Hsl7 to those sites. Hsl7, in turn, tethers Swel to the mother bud neck, where Swel is phosphorylated by the polo kinase Cdc5 (2, 29, 43). Conversely Skb1, the Hsl7 homologue present in S. pombe, acts in concert with Shk1 as a mitotic inhibitor in a Wee1 (S. cerevisiae)-dependent fashion (19). Additionally, Hsl7 acts as a negative regulator of Ste20 in the filamentous response pathway by acting as a competitive inhibitor of the Ste20 activator Cdc42 (16).

The study presented here describes the further characterization of U. maydis Smu1 and its roles in cell length, the filamentous response to low levels of ammonium, and pathogenicity. Further, through yeast two-hybrid analyses, we identified Hsl7 as a potential interactor with Smu1. We hypothesized that Hsl7 would act as a negative regulator of Smu1 in the control of the filamentous response to low-ammonium conditions and potentially the mating response and virulence. Here we show that disruption of hsl7 leads to increases in the filamentous response to low-ammonium conditions and a mild increase in the mating response, while no defects in pathogenicity were observed. Concomitant overexpression of smu1 leads to an exacerbation of the filamentous response, as well as cell separation defects and attenuation of virulence of U. maydis.

### Materials and Methods

#### Strains and growth conditions.

The U. maydis strains and plasmids utilized in this study are listed in Table 1. S. cerevisiae strains AH109 (MATα trpl-901 leu2-3,112 ura3-52 his3-200 Gal4Δ GAL1 GAL2 GAL4:GAL1 TATA-ura3 HIS3 GAL2:GAL1 GAL7-ADE2 UR4-3 MEL1-GAL1 GAL7-TATA-ura3) and Y187 (MATα trpl-901 leu2-3,112 ura3-52 his3-200 Gal4Δ met1 gal80Δ GAL1-GAL7 TATA-ura3) were obtained from Clontech (Mountain View, CA) and S. Ellis (University of Louisville) and used for yeast two-hybrid experiments. The S. cerevisiae strains used for complementation, MYJ102 (MATα ADE2 can1-100 his3-11,15 leu2-3,112 lys2-801 trpl1-1 ura3-1 HIS3) and MYJ110 (MATα ADE2 can1-100 his3-11,15 leu2-3,112 lys2-801 trpl1-1 ura3-1-20:His3) were a gift of J. Thorner (University of California, Berkeley). Wild-type strains DLY8155 (MATα) and DLY8165 (MATα) were obtained from D. Lew (Duke University). Escherichia coli strains DH5α (Bethesda Research Laboratories, Bethesda, MD) and TOP10 (Invitrogen, Carlsbad, CA) were utilized for all cloning and subcloning needs. U. maydis strains were grown at 25°C in YEP (1% yeast extract, 2% peptone) supplemented with 2% sucrose or dextrose and SLAD (0.17% yeast nitrogen base without ammonium sulfate or amino acids [YNB] with 2% dextrose and 50 mM ammonium sulfate) (23). All liquid cultures were grown with shaking (260 rpm). Mating medium and solid medium were made with 1% activated charcoal and/or 2% agar (24). S. cerevisiae strains were grown at 30°C in YEP-dextrose or SD (0.17% YNB, 1% amino acid dropout solution) supplemented with dextrose or galactose. For complementation assays, yeast strains were grown in SD with either glucose (repressor of the GAL1 promoter) or galactose (inducer of the GAL1 promoter), E. coli strains were grown at 37°C in Luria-Bertani (6) and/or Circle Grow (PM Biomedical, LLC, Solon, OH) medium.

#### Primer design and PCR.

Primers were designed with the Primer3 program (http://frodo.wi.mit.edu/primer3/). Primers were obtained from Eurofins MWG Operon (Huntsville, AL) and are listed in Table 2. PCRs and gradient PCRs were run on a PTC100 thermal controller (MJ Research Inc., San Francisco, CA), respectively. PCRs were run with initial denaturation at 94°C for 4 min, followed by 34 cycles of a second denaturation at 94°C for 30 s, annealing at temperatures ranging from 56°C to 62°C for 30 s, and extension at 72°C for 1 min per 1,000 bp.

### Table 1. U. maydis strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungal strains&lt;sup&gt;a&lt;/sup&gt;</td>
<td>WT&lt;sup&gt;a&lt;/sup&gt; FB1</td>
<td>a b1</td>
</tr>
<tr>
<td></td>
<td>Δhsl7 a1 mutant</td>
<td>a b1 hsl7::cbx&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Δ&lt;sub&gt;187-2832&lt;/sub&gt;hsl7 a1 mutant</td>
<td>a b1 hsl7:: Jug&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Δ&lt;sub&gt;hsl7&lt;/sub&gt; a1 mutant</td>
<td>a b1 p&lt;sub&gt;α&lt;/sub&gt;&lt;sub&gt;α&lt;/sub&gt;hsl7 hsl7&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Δ&lt;sub&gt;shl1&lt;/sub&gt; a1 mutant</td>
<td>a b1 smu1:: cbx&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Δ&lt;sub&gt;187-2832&lt;/sub&gt;hsl7 Δ&lt;sub&gt;shl1&lt;/sub&gt; a1 mutant</td>
<td>a b1 hsl7:: Jug&lt;sup&gt;R&lt;/sup&gt; smu1:: cbx&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Δ&lt;sub&gt;187-2832&lt;/sub&gt;hsl7 Δ&lt;sub&gt;shl1&lt;/sub&gt; a1 mutant</td>
<td>a b1 hsl7:: Jug&lt;sup&gt;R&lt;/sup&gt; P&lt;sub&gt;α&lt;/sub&gt;α smu1&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>WT FB2</td>
<td>a b2</td>
</tr>
<tr>
<td></td>
<td>Δhsl7 a2 mutant</td>
<td>a b2 hsl7::cbx&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Δ&lt;sub&gt;187-2832&lt;/sub&gt;hsl7 a2 mutant</td>
<td>a b2 hsl7:: Jug&lt;sup&gt;R&lt;/sup&gt;</td>
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<td></td>
<td>Δ&lt;sub&gt;shl1&lt;/sub&gt; a2 mutant</td>
<td>a b2 smu1:: cbx&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Δ&lt;sub&gt;187-2832&lt;/sub&gt;hsl7 Δ&lt;sub&gt;shl1&lt;/sub&gt; a2 mutant</td>
<td>a b2 hsl7:: Jug&lt;sup&gt;R&lt;/sup&gt; smu1:: cbx&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Δ&lt;sub&gt;187-2832&lt;/sub&gt;hsl7 Δ&lt;sub&gt;shl1&lt;/sub&gt; a2 mutant</td>
<td>a b2 hsl7:: Jug&lt;sup&gt;R&lt;/sup&gt; P&lt;sub&gt;α&lt;/sub&gt;α smu1&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> All mutant strains were made with the wild-type background strains listed.

<sup>b</sup> WT, wild type.
A final extension step of 72°C for 2 min longer than the cycle extension was used to complete the reaction.

Alignments of Hsl7/Skb1 homologues. Amino acid sequences from five Hsl7 homologues found in the Entrez Gene Database (http://www.ncbi.nlm.nih.gov/gene) were analyzed using the BLASTP tool from the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences used were from Homo sapiens (PM57; isoform A, accession no. NP_001034708.1, and isoform B, accession no. NP_001084480), S. pombe (SKB1; accession no. CAH197909), and Homo sapiens (Hsl7; accession no. NP_000691). Alignments of the predicted Methyltransferase 12 domain were performed using the ClustalW multiple-sequence alignment tool (http://web.ebi.ac.uk/Tools/msa/clustalw2/).

Genetic manipulation and vector construction. PCR products were separated by gel electrophoresis through 0.6% agarose (Agarose LE; USB Corp., Cleveland, OH) and purified using the Wizard miniprep kit (Promega, Madison, WI, USA). Plasmid DNA was purified using the GeneClean III kit (MP Biomedicals, Solon, OH, USA) and purified using the EcoR1 site of the pGADT7 prey vector of the BD Matchmaker Library Construction and Screening Kits user manual (BD Biosciences). Two vectors were used, pGBKT7 ("bait" vector with the GAL4 DNA-binding domain) and pGADT7 ("prey" vector with the GAL4 DNA activation domain). Initial experiments examined the interaction of Smu1 as bait and a U. maydis cDNA library as prey (38). In directed assays, the hsl7 ORF was cloned after the Gal4 promoter into the EcoRI site of the pGADT7 prey vector and thereby fused to the activation domain of the Gal4 transcription factor. The smu1 ORF was cloned after the Gal4 promoter into the BamHI and NotI sites of the pGBK7 bait vector and thus fused to the DNA-binding domain of the Gal4 transcription factor. Both the pGA-hsl7 and pGSM-smu1 vectors were cotransformed into the AH109 yeast background strain, plated onto double-dropout medium (DDO), and subsequently restreaked onto a more stringent selective medium, quadruple-dropout medium (QDO). A positive interaction was assessed after 3 days of growth on Trp-, Leu-, His-, and Ade-lacking synthetic dropout medium (QDO). A similar experiment was conducted with the Cl4a and Hsl7 ORFs. As a control, each gene was cloned separately into the bait and prey vectors so that the interactions could be examined without bias toward the vector in which the genes resided. The outcomes were consistent when the genes were switched between the bait and prey vectors. In addition, as negative controls, we examined the growth on DDO and QDO of AH109 strains transformed with each construct alone or in tandem with an “empty” bait or prey vector. In all cases, strains containing a single plasmid failed to grow on DDO or QDO, while all those transformed with any bait and prey vector-construct combination grew well on DDO.

RNA isolation and expression analysis. RNA isolation and expression analysis. RNA isolation and expression analysis.
RESULTS

Smu1 plays a role in cell length and filamentation as a response to low-ammonium conditions. Previous work (44) determined that Smu1 was involved in the mating response pathway, differentially influencing pheromone expression and pathogenicity, with disruption mutants showing a more severe effect in the a2 b2 mating type background. It was also observed that Smu1 played a subtle role in the filamentous response to low-ammonium conditions (44). To further explore this, as well as possible effects on cell morphology, smu1 disruption and overexpression strains were generated in the FB genetic background.

\[\Delta smu1\] mutant strains generated in the FB background exhibited a reduction in the mating response primarily in the a2 b2 mating type background, as was demonstrated previously by Smith et al. for another wild-type genetic background (44). Overexpression of the smu1 ORF produced a subtle increase in the mating response in the a1 b1 mating type background (see Fig. S1 in the supplemental material). Building upon these results, we found that the \(\Delta smu1\) mutant strains showed a decrease in cell length, while the \(smu1^{Tef}\) (constitutively overexpressed smu1) mutant strains displayed elongated cell morphology in comparison to the wild-type progenitor cells (Table 3). The decrease in cell length was statistically significant in only the a2 b2 background. The increase in cell length in the \(smu1^{Tef}\) mutant was observed and statistically significant only in the a1 b1 background. Comparisons of the a1 b1 and a2 b2 smu1 mutant strains indicated that the differences in cell length were significant for both the disruption and overexpression strains (Table 4).

In \(S. pombe\), the Smu1 homolog Shk1 plays a role in cell cycle regulation, producing cell length fluctuations when \(shk1\) expression is altered (19). To explore additional roles Smu1 might play in the cell cycle in \(U. maydis\), the growth rates of \(\Delta smu1\) and \(smu1^{Tef}\) mutant strains were examined. \(\Delta smu1\), \(smu1^{Tef}\), and wild-type cells were grown in YEPS medium, and direct cell counts were taken at three different time points. The cell counts were plotted, and rates of growth were determined and analyzed (described in Materials and Methods). Examination of rates of growth of the smu1 mutant strains indicated that most mutants were not significantly different from their wild-type progenitors. Since such mutants sustained differences in cell length without differences in growth rate (as measured by cell doubling time).

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of isolates</th>
<th>Length (μm)</th>
<th>Comparison</th>
<th>(P) valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT a1 b1</td>
<td>91</td>
<td>19.27 ± 0.43</td>
<td>WT a1 b1 vs WT a2 b2</td>
<td>NS</td>
</tr>
<tr>
<td>WT a2 b2</td>
<td>213</td>
<td>19.14 ± 0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\Delta smu1/\Delta hsl7) a1 mutant</td>
<td>167</td>
<td>25.22 ± 0.45</td>
<td>WT a1 b1 vs (\Delta smu1/\Delta hsl7) a1</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>(hsl7^{Tef}/\Delta smu1) a1 mutant</td>
<td>227</td>
<td>18.66 ± 0.27</td>
<td>WT a1 b1 vs (hsl7^{Tef}/\Delta smu1) a1</td>
<td>NS</td>
</tr>
<tr>
<td>(\Delta smu1/\Delta hsl7) a1 mutant</td>
<td>380</td>
<td>18.64 ± 0.16</td>
<td>WT a1 b1 vs (\Delta smu1/\Delta hsl7) a1</td>
<td>NS</td>
</tr>
<tr>
<td>(hsl7^{Tef}/\Delta smu1) a1 mutant</td>
<td>308</td>
<td>21.75 ± 0.28</td>
<td>WT a1 b1 vs (hsl7^{Tef}/\Delta smu1) a1</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>(\Delta smu1/\Delta hsl7) smu1a2/(\Delta hsl7) a2 mutant</td>
<td>221</td>
<td>25.25 ± 0.33</td>
<td>WT a1 b1 vs (\Delta smu1/\Delta hsl7) smu1a2/(\Delta hsl7) a2</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>(hsl7^{Tef}/\Delta smu1) a2 mutant</td>
<td>143</td>
<td>27.86 ± 0.45</td>
<td>WT a1 b1 vs (hsl7^{Tef}/\Delta smu1) a2</td>
<td>(&lt;0.001)</td>
</tr>
</tbody>
</table>

\(a\) Cell length values are averages ± standard errors.

\(b\) Statistical analysis was performed using a Student’s t test. NS, not significant.

\(c\) WT, wild type.
by direct cell count), this is consistent with cell cycle differences in the respective mutants (45). The only significant differences in growth rate were between the a1 b1 smu1Otef mutant and its FB1 a1 b1 progenitor and between the a2 b2 Δsmu1 mutant and its FB2 a2 b2 progenitor strain (see Table S1 in the supplemental material). Another possible cause of the cell elongation in FB2 a2 b2/H11003 ammonium medium. On YEPS at both (as described in Materials and Methods). Strains were grown of their wild-type progenitors via examination of microcolonies on morphology of mentous response to low-ammonium conditions (44). The col-
tion and deposition.

Data, Smu1 does not appear to play a role in cell wall localization and deposition.

Previous work indicated that Smu1 plays a role in the filamentous response to low-ammonium conditions (44). The col-

ony morphology of smu1 mutant strains was compared to that of their wild-type progenitors via examination of microcolonies (as described in Materials and Methods). Strains were grown on either YEPS or low-ammonium (SLAD; 50 μM ammon-

ium) medium. On YEPS at both ×20 and ×60 magnifications, neither the Δsmu1 nor the smu1Otef mutant strain displayed any obvious phenotypic difference from the wild-type progenitor strain (see Fig. S2 in the supplemental material). When grown on SLAD, there appeared to be a difference in the filamentous response between the wild-type FB1 a1 b1 and FB2 a2 b2 backgrounds. Microcolonies of FB1 and FB2 indicate that FB1 has increased filament production on low-ammonium medium in comparison to FB2. Keeping this in mind, the following comparisons were made only within the same genetic background. The Δsmu1 mutant strains displayed a subtle decrease in filament formation compared to the appropi-

ate wild-type a2 b2 control strain, while in the a1 b1 background, on average, half as many filaments formed in the mutant as in the wild type (see Fig. S3). Also, filaments of the Δsmu1 mutant strains were generally shorter than those of their wild-type progenitors (52 μm versus 80 μm, respectively). The smu1Otef mutant strain filaments were longer (120 μm) than those observed in the wild-type progenitor cells (80 μm), and the effect, in this case, was more prominent in the a1 b1 mutant background strain, where the mutant had roughly 2.5 times the number of filaments (see Fig. S3). Overall, these results indicate that Smu1 plays a role in cell length and the filamentous response pathway to low-ammonium conditions, in addition to the mating response in U. maydis.

Identification of a U. maydis Hsl7 homolog and comparisons with those of other fungi. In order to identify proteins poten-
tially interacting with Smu1, we screened a cDNA library using yeast two-hybrid analyses, and this suggested that Smu1 inter-

acts physically with an Hsl7 homologue (data not shown). To further confirm this, directed yeast two-hybrid analysis was conducted after inspection of the newly available U. maydis genome databases (Broad Institute [www.broadinstitute.org /annotation/genome/ustilago_maydis/] and MumDB [http://mips.helmholtz-muenchen.de/genres/proj/Ustilago] websites). This investigation provided the likely coding region of this puta-
tive protein-arginine N-methyltransferase homolog (um15057). The predicted complete um15057 ORF was then cloned into the bait vector and used for yeast two-hybrid assays with Smu1 in the prey vector. As shown in Fig. 1, a positive interaction was exhibited by colony growth on QDO, whereas proteins known not to interact failed to provide such growth. This is a strong indication that Hsl7 and Smu1 potentially interact in vivo, as was observed for the corresponding homologues in both S. cerevisiae and S. pombe (16, 18). A similar directed yeast two-

hybrid analysis investigated the possibility that Hsl7 interacts with another U. maydis PAK, Cla4. In this case, no interaction was detected (Fig. 1, compare bottom two images).

A BLASTX comparison of the predicted protein showed similarity to various hsl7/skb1 homologs. Primers were de-
signed from um15057 to amplify a 2.9-kb ORF from cDNA. BLASTP comparison to other hsl7/skb1 homologues showed the greatest similarity to the human protein PRMT5 (isoform A [accession no. NP_001034708.1] and isof orm B [accession no. NP_006100.2]; identities, 207/473 [43%]; positives, 277/473 [58%]; gaps, 41/473 [8%]). S. pombe protein Skb1 (accession no. CAAT7909; identities, 215/507 [42%]; positives, 290/507 [57%]; gaps, 33/507 [6%]), and X. laevis protein Hsl7 (accession no. NP_001084480; identities, 201/471 [42%]; positives,
273/471 [57%]; gaps, 41/471 [8%]). Similarity to S. cerevisiae protein Hsl7 was also seen (accession no. NP_009691; identities, 205/660 [31%]; positives, 303/660 [45%]; gaps, 130/660 [19%]). A search of potential domains in the sequence from U. maydis yielded only one hit, the Methyltransf_12 domain (see Fig. S4 in the supplemental material). Multiple-alignment analysis of this region showed the highest similarity among the homologues with PRMT5Is isoforms A and B from H. sapiens and Hsl7 from X. laevis (identities, 64/105 [60%]; positives, 77/105 [73%]; gaps, 4/105 [3%]; identities, 63/105 [60%]; positives, 79/105 [75%]; gaps, 4/105 [3%], respectively). S. pombe Skb1 was next in similarity (identities, 53/99 [53%]; positives, 64/99 [64%]; gaps, 4/99 [4%]), followed by Hsl7 from S. cerevisiae (identities, 47/106 [44%]; positives, 62/106 [58%]; gaps, 4/106 [3%]).

To explore if Hsl7 from U. maydis is functionally conserved compared to other Hsl7 homologues, the U. maydis homolog was expressed in S. cerevisiae strains lacking hsl7. The U. maydis hsl7 homolog was cloned after the GAL1 promoter into yeast expression plasmid pYES and introduced into S. cerevisiae strains MJY102 (MATa Hsl7) and MJY110 (MATa Δhsl7). The Δhsl7 mutant S. cerevisiae strains display an elongated bud morphology due to a G2/M delay (43). When expressed, hsl7 from U. maydis alleviated the elongated bud morphology of MJY102 and MJY110 (see Fig. S5 in the supplemental material), indicating that Hsl7 from U. maydis is functionally related to Hsl7 from S. cerevisiae and may play a role in cell cycle regulation.

Disruption of hsl7 increases cell length and the filamentous response under low-ammonium conditions. Two disruption constructs were created using an $\delta f$-based approach by inserting either a carboxin or hygromycin resistance cassette between an upstream and a downstream flank of genomic DNA. First, a partial-deletion strain (retaining the first 810 bp of the ORF) was generated. To confirm that the phenotypes observed in such mutants were not strictly associated with the partial-deletion construct, a complete-deletion construct was also generated and no differences in phenotypes were seen in the corresponding mutants compared to those found previously. In the remainder of this work, we thus report the results for the partial-disruption strains (Δ2832hsl7). The fact that deleting hsl7 in haploid strains still allowed cell growth and reproduction indicates that Hsl7 is not essential for cell viability.

Disruption strains (Δ2832hsl7) displayed elongated cell morphology, while overexpression of the hsl7 ORF showed a decrease in cell length compared to that of the wild-type strains (Table 3). Statistical evidence indicated that the elongated cell length of the disruption strains is significantly different from that of the wild-type progenitor strains, independently of the mating type background. The cell length decrease was significant in the a2 b2 hsl7mutant strain only, but there was a similar trend in the a1 b1 background as well. The comparison of the hsl7mutant strains indicated that the difference in cell length between the mutant strains of different mating types was significant (Table 4). This potentially adds Hsl7 to a growing list of proteins (e.g., Smu1 [44], Cla4 [31], and Rhol [38]) whose expression differentially affects the different mating type backgrounds.

The Hsl7 homologues in both S. cerevisiae and S. pombe play roles in cell cycle regulation which ultimately affect cell length and morphology (18, 19, 43). To explore this, the growth rates of Δ2832hsl7 and hsl7mutant strains were determined. Examination of rates of growth of the hsl7 mutant strains indicated that only the a1 b1 hsl7mutant strain was significantly different from that of its wild-type progenitor strain (see Table S1 in the supplemental material). In addition, staining of both the Δ2832hsl7 and hsl7mutant strains with CFW and WGA did not show any aberrations in cell wall material localization and deposition, nor were the strains sensitive to the cell wall antagonists CFW and CR (CFW and CR data are shown in Fig. S6 in the supplemental material).

The Hsl7 homologues of S. cerevisiae and S. pombe are involved in the response to environmental conditions regulating colony morphologies (4, 16). Examination of YEPS microcolonies at ×20 magnification indicated that the Δ2832hsl7 mutant strains displayed a phenotype that could be described as “directional growth,” where groups of cells parallel to one another run in a similar direction, different from that of another group of parallel cells (Fig. 2, inset). At ×60 magnification, Δ2832hsl7mutant strains were observed to have elongated cellular morphology similar to the increased length of individual Δ2832hsl7 mutant cells, in addition to the directional-growth phenotype (Fig. 2). Wild-type progenitor cells did not show this directional-growth phenotype, displaying an unorganized distribution and arrangement of cells. Overexpression of hsl7 did not result in any observable phenotypic difference from the wild-type strains when the strains were grown on YEPS as microcolonies (see Fig. S2 in the supplemental material). Δ2832hsl7 mutant strains grown on SLAD as microcolonies produced the same directional-growth phenotype observed for these strains on YEPS medium when viewed at ×20 magnification (Fig. 3, inset). At ×60 magnification, elongated, highly branched filaments were evident. The filaments were longer (up to 160 μm versus 80 μm for the wild type) and more highly branched than those observed in the wild-type progenitor cells. A dramatic reduction in filament formation was observed for hsl7mutant strains on SLAD when viewed at both ×20 and ×60 magnifications (Fig. 3).

To determine if Hsl7 is involved in the production of filaments as a response to pheromone, plate mating assays were utilized. Assessment of mating ability relies on the production of white aerial hyphae on the black background of YPD-activated charcoal medium. Combinations of wild-type, Δ2832hsl7mutant, and hsl7mutant strains with opposite mating backgrounds were mixed and spotted onto YPD-activated charcoal medium. The a1 b1 Δ2832hsl7 mutant strain displayed a subtle increase in the production of aerial hyphae, while no increase was observed in the a2 b2 Δ2832hsl7 mutant strain. No differences in mating abilities were observed in the hsl7mutant mutant strains (see Fig. S1 in the supplemental material). The mating assay indicates that absence of Hsl7 appears to increase the mating response, with the effect most prominent in the a1 b1 mating type background.

Disruption of smu1 in a Δ2832hsl7 background does not rescue any of the phenotypes associated with the Δ2832hsl7 background. Double disruption mutants were created in order to determine if there was a genetic interaction between Hsl7 and Smu1 in vivo. In both the a1 b1 and a2 b2 Δ2832hsl7 backgrounds, an smu1 disruption construct was introduced by the same method previously described and was confirmed by their growth characteristics (see Table S1 in the supplemental material).
PCR. The disruption of smu1 in the \( \Delta_{810-2832, hsl7} \) background did not attenuate the elongated cellular phenotype of the \( \Delta_{810-2832, hsl7} \) mutant strains (Table 3), nor did it have any effect on the rate of growth (see Table S1 in the supplemental material). Staining of the cell walls of the double disruption strains with CFW and WGA did not reveal any defect in the localization and deposition of cell wall material, and they were not sensitive to the cell wall antagonists CFW and CR (data not shown, but see Fig. S6 in the supplemental material).

Examination of microcolony morphologies indicated that disruption of smu1 in the \( \Delta_{810-2832, hsl7} \) background did not alleviate the directional-growth phenotype previously observed in the \( \Delta_{810-2832, hsl7} \) mutant strains. In SLAD microcolonies, \( \Delta_{810-2832, hsl7} \) smu1 mutant strains exhibited the same increase in the filamentous response to SLAD medium (Fig. 5A) as seen in the \( \Delta_{810-2832, hsl7} \) mutant strains. Examination of rates of growth of the \( \Delta_{810-2832, hsl7} \) smu1Otef mutant strains indicated that the a1 b1/\( \Delta_{810-2832, hsl7} \) smu1Otef mutant did not differ significantly from that of its wild-type progenitor in growth rate. This suggests that Smu1 may be involved in cell cycle regulation in the a1 b1 mating type background, since its overexpression tends to increase cell length, at least in the context of the hsl7 deletion. On the other hand, the growth rate of the a2 b2 \( \Delta_{810-2832, hsl7} \) smu1Otef mutant strain was significantly different from that of the FB2 a2 b2 strain (see Table S1 in the supplemental material). In the a2 b2 background, the \( \Delta_{810-2832, hsl7} \) smu1Otef mutant strain displayed mother-daughter cell separation defects (Fig. 6). To explore the cell separation defects observed in the a2 b2 \( \Delta_{810-2832, hsl7} \) smu1Otef mutant strain, two cell morphology aspects were examined: cell wall localization...
and number of nuclei. To this end, the cells were stained with
WGA, CFW, and DAPI (nucleic acid stain; DNA specific). Staining
with WGA and CFW indicated no aberrations in
the localization and deposition of cell wall material in the
\( \Delta _{a10-2832} \text{hsl7 smu1}^{\text{Otef}} \) mutant strains, yet the a2 b2 strain
failed to separate correctly, with random cross wall septa
interspersed in the cells (Fig. 6). Visualization of a1 b1
\( \Delta _{a10-2832} \text{hsl7 smu1}^{\text{Otef}} \) mutant cells also stained with DAPI
exhibited a single, centrally located bright spot representing
the nucleus (Fig. 6). The a2 b2 \( \Delta _{a10-2832} \text{hsl7 smu1}^{\text{Otef}} \) mutant strain
displayed several nuclei separated into individual compartments
by cross wall septa (Fig. 6, arrows). In addition, the \( \Delta _{a10-2832} \text{hsl7 smu1}^{\text{Otef}} \) mutant strains were not
sensitive to the cell wall antagonists CFW and CR, indicating
normal cell wall integrity (data not shown, but see Fig. S6 in
the supplemental material).

To further characterize the defect in mother-daughter cell
separation, microcolony morphologies on YEPS medium
were examined. The \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}} \) mutant strains exhibited
filamentous microcolony morphologies, most notably for the
a2 b2 strain at \( \times 20 \) magnification, which differed from all
other FB2 a2 b2 strains examined (Fig. 4A, inset). At \( \times 60 \)
magnification, we observed directional growth of the a1 b1
\( \Delta _{a10-2832} \text{hsl7 smu1}^{\text{Otef}} \) mutant strain, similar to that of the
\( \Delta _{a10-2832} \) \text{hsl7} and \( \Delta _{a10-2832} \text{smu1} \) a1 b1 mutant strains.
But unlike either the \( \Delta _{a10-2832} \) \text{hsl7} or the \( \Delta _{a10-2832} \) \text{smu1}
a1 b1 mutant, cells of the a1 b1 \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}} \) mutant strain
were beginning to form hyphae (Fig. 4A, arrows, right panels). The a2 b2 \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}} \) mutant strain exhibited filamentous growth and what appeared to be multiple
areas of random cytoplasmic evacuation within each filament
(Fig. 4A, arrows, middle right and bottom right expanded
panel). CFW staining of an a2 b2 \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}} \) mutant
strain microcolony identified septated hyphae (Fig. 4B, arrows).
In the mating reaction, the a1 b1 \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}} \) mutant strain displayed an increase in the mating response
compared to that of wild-type strains, while the a2 b2 mating
type-specific mutant strain exhibited a positive mating reaction
in the absence of the opposite mating partner (circled in Fig.
S1 in the supplemental material).

\( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}} \) mutant strains display a hyperfilamentous
response to SLAD medium. Observations at \( \times 20 \)
magnification of \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}} \) mutant strain microcolonies on SLAD medium
detected hyperfilamentous growth that was more pronounced in the a2 b2 mating type
background (Fig. 5A, inset). At \( \times 60 \) magnification, long branched
filaments were observed in the a1 b1 \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}}
mutant strain. The a2 b2 \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}} \) mutant strain
filaments were much longer but not as highly branched as those of the a1 b1 \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}}
strain. The filaments also exhibited dramatic regions of cytoplasmic evacuation
more extensive than what was observed on YEPS medium (Fig. 5A, arrows). Staining of the
microcolonies of the \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}} \) mutant strains with WGA indicated chitin
delocalization throughout the length of the filaments of both the a1 b1
and a2 b2 mutant strains (Fig. 5B).

To examine cell wall deposition in the \( \Delta _{a10-2832} \) \text{hsl7 smu1}^{\text{Otef}}
mutant strains, the strains were grown in liquid SLAD. The
strains were grown overnight due to their slower growth in
SLAD medium than in YEPS medium and stained with CFW,
WGA, and DAPI. Wild-type cells and, with one exception,
cells of the previously described mutant strains did not form

FIG. 3. Filamentous response of \text{hsl7} mutants to low-ammonium conditions. \( \Delta _{a10-2832} \) \text{hsl7} mutant strains were increased in filament length and
branching and displayed directional growth when SLAD microcolonies were examined. \text{hsl7}^{\text{Otef}} mutant strains displayed a decrease in both the
number and the length of filaments, with filamentation rarely observed. Scale bars: 50 \( \mu \)m in the \( \times 20 \) inset image, 40 \( \mu \)m in the \( \times 60 \) image. WT, wild type.
filaments in liquid SLAD as opposed to on solid SLAD medium. Surprisingly, the \( H9004 \) \( cl4 \) mutant produced hyphae in liquid SLAD (discussed below). Additionally, no aberrations in cell wall localization and deposition or number of nuclei were observed when cells were stained with CFW, WGA, and DAPI (see Fig. S7 in the supplemental material). Under the same conditions, the \( H9004 \) \( 810-283\text{hsl7 smu1Otef} \) mutant strains displayed true hyphae or pseudohyphae. Chitin delocalization, indicated by WGA staining, was observed in both \( H9004 \) \( 810-283\text{hsl7 smu1Otef} \) mutant strains. The \( a1 b1 \) \( H9004 \) \( 810-283\text{hsl7 smu1Otef} \) mutant strain exhibited filamentous growth with random areas of cytoplasmic evacuation within the filaments (A, arrows, middle right and bottom right expanded panel). Scale bars: 50 \( \mu \)m in the \( \times 20 \) inset images, 40 \( \mu \)m in the \( \times 60 \) images. Staining of microcolonies of the \( a2 b2 \) \( H9004 \) \( 810-283\text{hsl7 smu1Otef} \) mutant strain with CFW indicated that the filaments are septated hyphae (B, arrows). CFW stains chitin and \( \beta \)-glucan. Scale bars: 20 \( \mu \)m.

**FIG. 4.** \( \Delta 810-283\text{hsl7 smu1} \) mutant strains produce the directional-growth phenotype on rich medium, while \( \Delta 810-283\text{hsl7 smu1Otef} \) mutant strains are filamentous. Microcolonies of \( \Delta 810-283\text{hsl7 smu1} \) mutant cells displayed the directional-growth phenotype of parallel groups of cells growing in a specific direction (A). Microcolonies of the \( a1 b1 \) \( \Delta 810-283\text{hsl7 smu1Otef} \) mutant strain displayed the directional-growth phenotype with the cells dramatically elongated (see arrows, top right panel). The \( a1 b1 \) \( \Delta 810-283\text{hsl7 smu1Otef} \) mutant strain exhibited filamentous growth with random areas of cytoplasmic evacuation within the filaments (A, arrows, middle right and bottom right expanded panel). Scale bars: 50 \( \mu \)m in the \( \times 20 \) inset images, 40 \( \mu \)m in the \( \times 60 \) images. Staining of microcolonies of the \( a2 b2 \) \( \Delta 810-283\text{hsl7 smu1Otef} \) mutant strain with CFW indicated that the filaments are septated hyphae (B, arrows). CFW stains chitin and \( \beta \)-glucan. Scale bars: 20 \( \mu \)m.

filaments in liquid SLAD as opposed to on solid SLAD medium. Surprisingly, the \( \Delta cl4 \) mutant produced hyphae in liquid SLAD (discussed below). Additionally, no aberrations in cell wall localization and deposition or number of nuclei were observed when cells were stained with CFW, WGA, and DAPI (see Fig. S7 in the supplemental material). Under the same conditions, the \( \Delta 810-283\text{hsl7 smu1Otef} \) mutant strains displayed true hyphae or pseudohyphae. Chitin delocalization, indicated by WGA staining, was observed in both \( \Delta 810-283\text{hsl7 smu1Otef} \) mutant strains. The \( a1 b1 \) \( \Delta 810-283\text{hsl7 smu1Otef} \) mutant strain displayed extremely long filamentous cells, containing no more than one cross wall and a single brightly stained nucleus per filamentous section of the cell under CFW and DAPI staining (Fig. 7, arrows). However, the \( a2 b2 \) \( \Delta 810-283\text{hsl7 smu1Otef} \) mutant strain exhibited bubbled hyphae growing in a bipolar fashion, separated by randomly interspersed cross wall septa. These bubbled hyphae were multinucleated and appeared similar to pseudohyphae with the cross walls separating the multiple nuclei to a single nucleus per compartment. However, the cross walls were irregularly separated, with several forming in the middle of cell-like structures, while other septa were detected between two bubbled regions of the structure (Fig. 7, see large arrows). Overall, these data lead to the conclusion that, together, Hsl7 and Smu1 play a role in the regulation of the filamentous response and cell separation under low-ammonium conditions. The only other strain to exhibit filamentous growth in liquid SLAD was the \( cl4 \) mutant (see Fig. S8 in the supplemental material).

**\( \Delta 810-283\text{hsl7 smu1Otef} \) mutant strains are attenuated in virulence.** To assess the roles of Hsl7 and Smu1 in *U. maydis* virulence, compatible mutant strains of opposite mating types were mixed and injected into maize seedlings (8 days postplanting). Three independent trials were performed and measurements of virulence were taken at 7, 10, 14, 17, and 21 dpi.
The cross of an a1 b1 and an a2 b2 strain of a single mutant background was compared in each case to a wild-type progenitor cross. The mutant backgrounds /H9004 810-283 hsl7, hsl7Otef, /H9004 smu1, smu1Otef, /H9004 810-283 hsl7, and /H9004 810-283 hsl7 smu1Otef were examined. The virulence of each infection was measured by a disease index by ranking the severity of the disease symptoms on a scale of 0 to 5 (see Materials and Methods for an explanation). The results at 21 dpi are presented both in a percentage of symptom formation graph (13) and as an average of the disease index. Statistical analysis

**FIG. 5.** hsl7 smu1 double mutants are increased in filament formation on SLAD. Δ_{610-283,hsl7 Δsmu1} a1 mutant cells showed increased filament length compared to that of the wild-type (WT) cells (approximately 160 μm versus 80 μm for the wild type) and branching, as well as displaying directional growth in SLAD microcolonies (A). Microcolonies of the Δ_{610-283,hsl7 smu1Otef} mutant strains displayed extremely elongated filaments above and beyond those of all previously examined strains. The a2 b2 Δ_{610-283,hsl7 smu1Otef} mutant strain also exhibited random areas of cytoplasmic evacuation within the filaments (A, arrows). Scale bars: 50 μm in the ×20 inset image, 40 μm in the ×60 image. Staining of microcolonies of the Δ_{610-283,hsl7 smu1Otef} mutant strains with rhodamine-labeled WGA indicated chitin localization defects (B). WGA stains chitin. Scale bars: 20 μm.

The cross of an a1 b1 and an a2 b2 strain of a single mutant background was compared in each case to a wild-type progenitor cross. The mutant backgrounds Δ_{610-283,hsl7, hsl7Otef, Δsmu1, smu1Otef, Δ610-283,hsl7 Δsmu1, and Δ610-283,hsl7 smu1Otef} were examined. The virulence of each infection was measured by a disease index by ranking the severity of the disease symptoms on a scale of 0 to 5 (see Materials and Methods for an explanation). The results at 21 dpi are presented both in a percentage of symptom formation graph (13) and as an average of the disease index. Statistical analysis

### TABLE 5. Cell length differences in Δ_{610-283,hsl7 smu1Otef} mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of isolates</th>
<th>Length (μm)</th>
<th>Comparison</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ_{610-283,hsl7 a1}</td>
<td>167</td>
<td>25.22 ± 0.45</td>
<td>Δ_{610-283,hsl7 a1} vs Δ_{610-283,hsl7 a2}</td>
<td>NS'</td>
</tr>
<tr>
<td>smu1Otef a1 mutant</td>
<td>308</td>
<td>21.75 ± 0.28</td>
<td>Δ_{610-283,hsl7 a1} vs Δ_{610-283,hsl7 smu1Otef} a1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ_{610-283,hsl7 smu1Otef} a1 mutant</td>
<td>143</td>
<td>27.86 ± 0.45</td>
<td>smu1Otef a1 vs Δ_{610-283,hsl7 smu1Otef} a1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ_{610-283,hsl7 a2}</td>
<td>406</td>
<td>24.78 ± 0.32</td>
<td>Δ_{610-283,hsl7 a2} vs Δ_{610-283,hsl7 smu1Otef} a2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>smu1Otef a2 mutant</td>
<td>224</td>
<td>19.66 ± 0.30</td>
<td>smu1Otef a2 vs Δ_{610-283,hsl7 smu1Otef} a2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ_{610-283,hsl7 smu1Otef} a2 mutant</td>
<td>89</td>
<td>27.33 ± 0.86</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Cell length values are averages ± standard errors.

*b Statistical analysis was performed using a Student t test.

*c NS, not significant.
plants exhibiting only chlorosis (Fig. 8). Also, the creases in plant death and increases in disease-free plants or ground dramatically reduces virulence. In the onset of disease attenuation in the decreased virulence at 7 dpi. This indicates that there is a delay that the examined to determine if the decreases in virulence at 21 dpi, while the become significantly different from wild-type strains until 10 dpi, but overexpression of Hsl7 does not rescue the decrease in virulence of the Δhsl7 strains, but overexpression of smu1 in a Δhsl7 background dramatically reduces virulence.

DISCUSSION

In the process of further examining the roles of Smu1, the Ste20 homologue in U. maydis, we cloned and characterized Hsl7, the sole protein-arginine methyltransferase homologue in U. maydis. Moreover, we investigated the genetic interaction of Hsl7 with Smu1. Hsl7 shares many roles identified in both Hs1 and Skb1 from S. cerevisiae and S. pombe, respectively. Both Hsl7 and Skb1 have been shown to play a role in cell cycle regulation in S. cerevisiae and S. pombe, respectively, although they have opposite effects on the G2/M transition (2, 18, 19, 32, 43). Hsl7 and Skb1 also play roles in the response to environmental stresses (4, 16). In S. cerevisiae, Hsl7 plays a role in the filamentous response to low-ammonium conditions, purportedly by acting as a negative regulator of Ste20 (16). However, an additional study points to Hsl7 regulating the filamentous response to low-ammonium conditions via regulation of Swe1 and/or cyclic AMP/protein kinase A) (29). Subsequent loss of swe1 eliminated this enhanced filamentous response. The ability of ste20 mutants to suppress hsl7 mutant phenotypes could be explained by Ste20 playing an indirect role in cell cycle regulation. Such regulation could occur through actin polarization and septin formation (12, 23, 25, 29). In contrast, in fission yeast, Shk1 acts in parallel to the MAPK pathway in morphological responses but is predicted to act directly through the MAPK module in regard to the mating response.
Here, Skb1 acts upon Shk1 in a positive manner, regulating the cell cycle and the response to hyperosmotic conditions (4, 18, 19). We have demonstrated in U. maydis that Hsl7 negatively regulates cell length and the filamentous response to low-ammonium conditions, partially with Smu1. However, the effects of these two proteins on such phenotypes are opposite to one another. An increase in cell length and the filamentous response was observed in the \( \Delta a1b1 H9004 810 2832hsl7 \) and \( \Delta a2b2 H9004 810 2832hsl7 smu1Otef \) mutant strains, with only the \( \Delta a1b1 H9004 810 2832hsl7 \) mutant strains displaying the directional-growth phenotype. A decrease in cell length and a reduction in the filamentous response on low-ammonium medium were seen in the \( \Delta a1b1 H9004 810 2832hsl7 \) and \( \Delta a2b2 H9004 810 2832hsl7 smu1Otef \) mutant strains, primarily in the \( \Delta a2b2 H9004 810 2832hsl7 smu1Otef \) mutant strains. An increase in cell-to-cell adhesion may explain the directional-growth phenotype. Flo11, a cell surface flocculin involved in cell-cell adhesion, is required for haploid invasive growth, as well as diploid pseudohyphal development in S. cerevisiae (37). Mutating hsl7 in S. cerevisiae enhances agar invasion, as well as filamentous growth, independently of flo11 expression. In U. maydis, Hsl7 could play a similar role, repressing cell-to-cell adhesion that may be required for filament formation. Another possible explanation of the directional-growth phenotype is a defect in cell separation. In U. maydis cells, budding alternates between the poles of cells, where one daughter cell buds and completely separates from the mother cell before the next daughter cell begins budding from the opposite pole (26). If mother and daughter cells do not correctly separate, then the cells will remain connected and grow in a linear fashion, mimicking pseudohyphal cells in appearance. The seemingly random cross wall septa identified by staining of \( \Delta hsl7 \) mutants (Fig. 4B) are consistent with such separation defects.

Unlike the situation in both S. cerevisiae and S. pombe (4, 16, 18, 19), disruption of Smu1 in the \( a1b1 \Delta 810-2832hsl7 \) mutant strain alleviates only the subtle increase in the mating response observed but no other Hsl7-associated phenotypes in this background. Disruption of hsl7 while concomitantly overexpressing smu1 has a negative effect on mother-daughter cell separation. It is possible that the \( a2b2 \Delta 810-2832hsl7 smu1Otef \) mutant strain does not correctly produce a primary septum, preventing normal mother-daughter cell separation. In addition, \( a2b2 \Delta 810-2832hsl7 smu1Otef \) cells do not undergo a unipolar growth pattern but rather undergo a bipolar growth pattern. This bipolar growth is different from budding that alternates between the poles of wild-type cells in that two daughter cells appear to bud simultaneously at both poles of the mother cell. It is unclear when each daughter cell goes through bud formation in relation to the other, but it is clear that the daughter cells are not separating from the mother cell. This strain produces predominately mother cells with a central septum, and subsequent daughter cells then bud from both poles. Interestingly, Cdc42, which also localizes to the primary septum, is present in mother cells but not in daughter cells, suggesting a role in cell separation.

**FIG. 7.** \( \Delta 810-2832hsl7 smu1Otef \) mutant strains form filaments in liquid SLAD. Wild-type (WT) cells do not form filaments in liquid SLAD. \( a1b1 \Delta 810-2832hsl7 smu1Otef \) mutant cells exhibit long filaments with a single, centrally located nucleus and septa (large arrows mark septa, small arrows mark nuclei). \( a2b2 \Delta 810-2832hsl7 smu1Otef \) cells exhibit a “bubbled” hyphal phenotype with randomly spaced septa (CFW/DAPI, large arrows mark septa, small arrows mark nuclei) that separate the individual nuclei into compartments. Both \( \Delta 810-2832hsl7 smu1Otef \) mutant strains display chitin delocalization (WGA). CFW stains β-glucan, WGA stains chitin, and DAPI stains nuclear DNA. Scale bars: 10 μm.
dispensable for polarized growth and filament formation (34).

In yeast, Ste20 is a downstream effector of Cdc42 in the filamentous responsive MAPK pathway (30). An interaction through yeast two-hybrid analysis indicated that in U. maydis, Cdc42 interacts with the CRIB domain of Smu1 in vitro (31). It is possible that Smu1 is a downstream effector of Cdc42 in U. maydis, potentially regulating mating pheromone expression and the filamentous response on SLAD.

The situation in S. cerevisiae may serve to explain the inability of Δs10-2832hsl7 smu10mut mutant strains to undergo mother-daughter cell separation. In U. maydis, Hsl7 would be acting as a negative regulator of Smu1. Lack of negative regulation by Hsl7 while overexpressing Smu1 promotes filament formation. The hyperactivity of Smu1 on downstream targets may enhance filamentous growth. Also, Smu1 may play a role in reorganizing the actin cytoskeleton, promoting cell elongation. This could also explain the defect in cell separation. Smu1 may be important in the creation or stability of the actomyosin ring, and loss of smu1 leads to defects in cell separation. However, these ideas fail to explain the inability of smu1 disruption mutants to rescue phenotypes associated with loss of hsl7 or vice versa, with the exception of the mild increase in the mating response of the Δa1 b1 Δs10-2832hsl7 mutant strain. Alternatively, the loss of hsl7 could potentially delay cell cycle progression, while overexpression of smu1 promotes the filamentous response. Acting on the cell cycle, Hsl7 could promote entry into mitosis by targeting Wee1 for degradation. Absence of Hsl7 would delay the cell cycle, leading to an increase in cell length, as is observed in several S. cerevisiae strains, where Hsl7 plays a role in targeting Swe1 for degradation, promoting G2/M transition (43). This hypothesis is supported by the complementation by Hsl7 from U. maydis of the elongated bud morphology of Δhsl7 mutant S. cerevisiae strains, potentially alleviating the G2/M delay. It is also supported by the increase in cell length of the Δhsl7 mutants without a concomitant change in growth rate (see Table S1 in the supplemental material). The stabilization of Swe1 by the absence of Hsl7 in S. cerevisiae is offset by the activity of the phosphatase Mih1, making the changes in the cell cycle caused by Swe1 stability minute (45). The cell cycle delay in the absence of Hsl7 from U. maydis may act in concert with the overexpression of smu1, leading to exacerbation of the filamentous response to low-ammonium conditions and filament formation in liquid low-ammonium medium with respect to the wild type. On the other hand, overexpression of both hsl7 and smu1 in the a1 b1 mating type background does lead to a significant increase in growth rate. Also, loss of smu1 and the Δs10-2832hsl7 smu10mut mutant strain in the a2 b2 mating type background showed a significant reduction in the rate of growth. These results may mean that the direct counting method is not robust enough to pick up...
subtle changes in the rate of growth. Additional analyses, such as flow cytometry or quantitative RT-PCR of cyclin levels may be needed to dissect the roles Smu1 and Hsl7 play in cell cycle regulation with greater precision.

We have shown that the Δcla4 mutant also produced filaments in both liquid and solid SLAD. In *U. maydis*, Cla4 has been identified as the downstream effector of Rac1, regulating bud formation and morphology, cell separation, and polar growth (31, 34). Moreover, deletion of either *cla4* or *rac1* eliminates *b* mating type locus-dependent filamentation. It has been hypothesized that Rac1, through Cla4, acts as the master regulator of *b*-dependent filament formation (34, 38). However, in liquid, as in solid, SLAD, Δcla4 mutant strains were able to produce filaments, indicating that cla4 is dispensable for filament formation in SLAD (see Fig. S8 in the supplemental material). The same cannot be said for Rac1 activity. Cells lacking *rac1* are unable to form filaments, while overexpression of *rac1* leads to hyphal growth (34). Yet, neither mutant strain formed filaments in liquid SLAD (see Fig. S8). Cla4 could limit the filamentous response to low-ammonium conditions by one of two means, (i) sequestering one or more components in the filamentous response pathway (potentially Cdc42) or (ii) promoting bud emergence and normal cell cycle progression where deletion of *cla4* indirectly promotes filamentous growth by failing to promote cell separation (via binding to Rac1). Additional pathways would then promote increased polar growth, leading to the filamentous response. These results imply that Cla4 may not be the only downstream effector of Rac1 or, equally, that Cla4 may have other upstream activators regulating septin assembly and proper cell separation. Potential candidates include Cdc42 and Rh01 (38).

It is interesting that the two PAKs Cla4 and Smu1 have opposite roles in the filamentous response pathway, where deletion of *cla4* alone or overexpression of *smu1* in a Δ*sl7* background leads to a hyperfilamentous response to low-ammonium conditions. A functional overlap between Cla4 and Smu1 may exist in *U. maydis*, as is observed in *S. cerevisiae* (12, 14, 23, 27). Deletion of both ste20 and *cla4* is lethal, indicating that Ste20 and Cla4 share at least one essential function (12). Here we have demonstrated that although the absence of Cla4 or the overabundance of Smu1, with concomitant Hsl7 absence, is not lethal to *U. maydis*, either genetic configuration results in a hyperfilamentous response to low-ammonium conditions. Perhaps the interaction between Hsl7 and Smu1 is a convergence of independent roles creating the exacerbated response. The lack of a clear genetic interaction between Hsl7 and Smu1 points to two possible ways these two gene products regulate the filamentous response to SLAD. Hsl7 and Smu1 may be involved in separate pathways that culminate in regulation of the filamentous response to SLAD. Here, Hsl7 could play a role in cell cycle regulation, promoting *G2/M* transition, and Smu1 could play a role in the filamentous response to SLAD, potentially though the MAPK pathway. The delay in *G2/M* transition created by the removal of Hsl7 primes the cells for the filamentous response to low-ammonium conditions, while simultaneous overexpression of Smu1 leads to a hyperfilamentous response. It is also conceivable that Hsl7 and Smu1 do interact together in the filamentous response to SLAD. The interaction may play a role in actin dynamics, leading to changes in cell size and cell separation defects, potentially through the MAPK pathway. In both cases, the differences seen in the two mating backgrounds are probably due to differential gene expression in these contexts. In fact, RT-PCR indicates (see Fig. S9 in the supplemental material) that *hsl7* is more highly expressed in the FB1 *a1 b1* background than in the FB2 *a2 b2* background. In contrast, *smu1* is normally more highly expressed in FB2. The coding regions of *hsl7*, *smu1*, and *cla4* are identical in the *a1 b1* and the *a2 b2* mating type background strains (data not shown). Thus, differences in phenotype in these distinct backgrounds or another could be due to noncoding regions of the respective genes or to signaling related to the respective mating type loci. The interaction between Hsl7 and Smu1, as well as the loss of *cla4*, indicates that a complex network of protein interactions regulates the process of filamentation in *U. maydis*, both in response to environmental stimuli and mating and for pathogenicity.

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