

The O-Mannosyltransferase PMT4 Is Essential for Normal Appressorium Formation and Penetration in *Ustilago maydis* ^{W|O|A}

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In *Saccharomyces cerevisiae*, the PMT, KRE2/MNT1, and MNN1 mannosyltransferase protein families catalyze the steps of the O-mannosylation pathway, sequentially adding mannoses to target proteins. We have identified members of all three families and analyzed their roles in pathogenesis of the maize smut fungus *Ustilago maydis*. Furthermore, we have shown that PMT4, one of the three PMT family members in *U. maydis*, is essential for tumor formation in *Zea mays*. Significantly, PMT4 seems to be required only for pathogenesis and is dispensable for other aspects of the *U. maydis* life cycle. We subsequently show that the deletion of *pmt4* results in a strong reduction in the frequency of appressorium formation, with the few appressoria that do form lacking the capacity to penetrate the plant cuticle. Our findings suggest that the O-mannosylation pathway plays a key role in the posttranslational modification of proteins involved in the pathogenic development of *U. maydis*. The fact that PMT homologs are not found in plants may open new avenues for the development of fungal control strategies. Moreover, the discovery of a highly specific requirement for a single O-mannosyltransferase should aid in the identification of the proteins directly involved in fungal plant penetration, thus leading to a better understanding of plant–fungi interactions.

INTRODUCTION

In many fungal pathogens, a key step in plant infection is penetration of the host plant cuticle, an important physical barrier against plant pathogens. To overcome this barrier, fungi have developed different mechanisms ranging from penetration through natural openings, such as stomata, to the formation of appressoria (Howard and Valent, 1996; Dean, 1997; Talbot, 2003), which are specialized structures that use mechanisms including osmotic pressure, hydrolytic enzymes, and the generation of reactive oxygen intermediates to break into host plant tissue (Howard et al., 1991; Egan et al., 2007; Skamnioti and Gurr, 2007).

Ustilago maydis is a basidiomycete fungus with nonpathogenic haploid yeast-like and infective dikaryon filamentous stages of its life cycle. This fungus belongs to the Ustilaginales group of plant pathogens that can infect around 4000 species of angiosperms and has an important impact on agriculture. Particularly, *U. maydis* causes the smut disease of maize (*Zea mays*), one of the world's major cereal crops. This disease results in stunted plant growth leading to high economic losses (Martinez-Espinoza et al., 2002). This fact, combined with its ability to be

easily cultured and genetically manipulated in the laboratory, makes *U. maydis* an excellent model for the molecular study of vegetal pathogenesis (Bolker, 2001; Kamper et al., 2006). In the *U. maydis* life cycle (Figure 1A), spore germination results in the formation of sexually differentiated, saprophytic yeast cells. Sexual compatibility is determined by the presence of two independent loci: locus *a*, which encodes a pheromone-receptor system, and locus *b*, which encodes a transcription factor composed of the *bE/bW* heterodimer that triggers growth of infectious hyphae (Feldbrugge et al., 2004). Mating between two sexually compatible cells leads to *bE/bW* complex formation, which initiates filamentation and appressorium-mediated plant penetration (Snetselaar and Mims, 1992). Once inside the plant, *U. maydis* colonizes plant tissues by forming hyphae, which by the end of the infection process, causes the formation of tumors in all green parts of the plant. Fungal hyphae continue to proliferate inside these tumors until finally differentiating to form diploid spores (Banuett and Herskowitz, 1996).

Understanding the molecular basis of appressorium-based plant penetration is of great interest in the development of antifungal strategies. Fungal adhesion to hard, hydrophobic surfaces and the presence of various chemical signals are required for effective appressorium formation and plant penetration (Howard et al., 1991; Lee and Dean, 1993; Gilbert et al., 1996). Cell wall glycoproteins have been identified as fungal adhesives and have been implicated in host cell adhesion in many organisms (Gaur and Klotz, 1997; Buck and Andrews, 1999; Frieman et al., 2002; Sheppard et al., 2004). These studies show that blocking access to glycoproteins with sugar group binding compounds, such as concanavalin A, interferes with the

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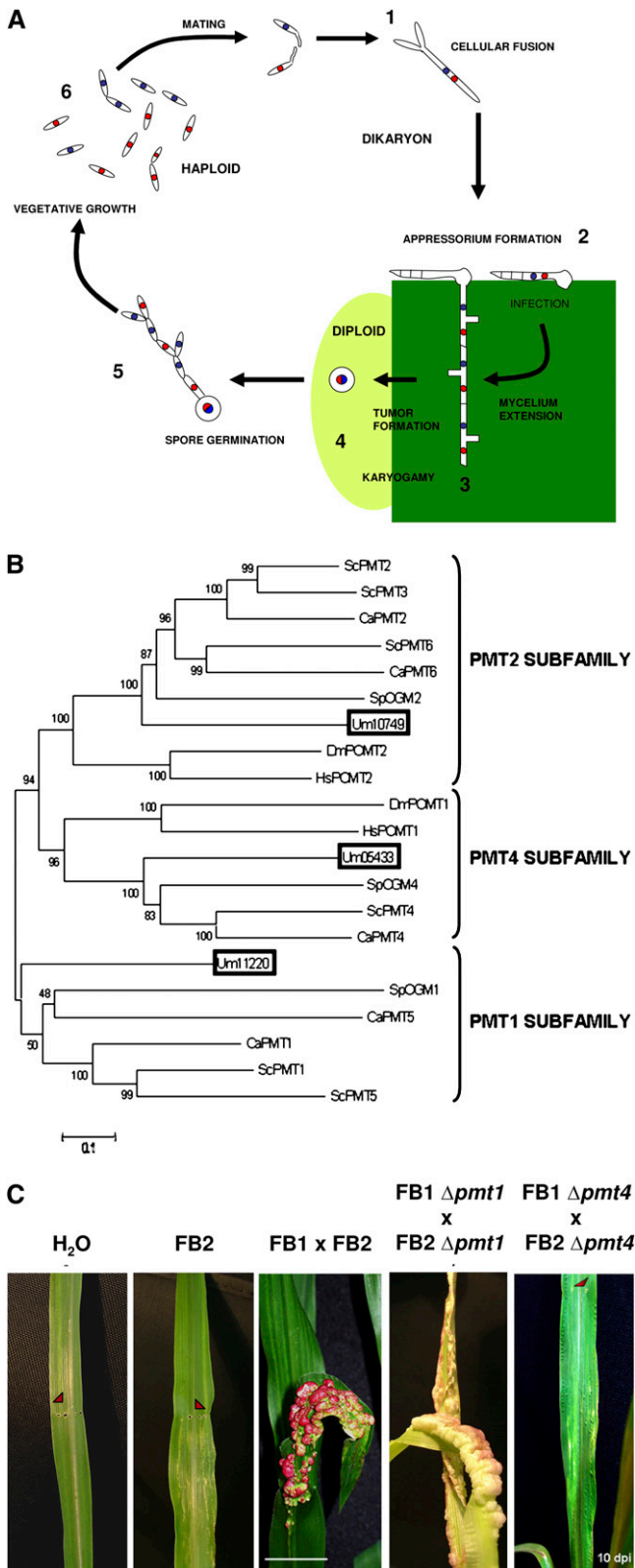


Figure 1. The *U. maydis* PMT Family.

(A) Life cycle of *U. maydis*. Developmental stages of the *U. maydis* life

adhesive capacity of fungal cells (Buck and Andrews, 1999). Thus, current evidence suggests that protein glycosylation plays a key role in the adhesive properties of fungal cells (Buurman et al., 1998; Huang et al., 2003).

Glycosylation is an extremely common and highly conserved type of protein modification involving the addition of carbohydrate residues to target proteins (Willer et al., 2003). It plays a critical role in determining the structure and function of numerous secreted and membrane-bound proteins. This type of protein modification is highly regulated and is mediated by the activity of protein- and site-specific enzymes. There are different types of glycosylation, the most frequent being *N*- and *O*-glycosylation, which differ from each other in the amino acid residue where the carbohydrate moieties are attached, as well as in the final structure of the carbohydrate complex. In eukaryotic cells, *N*- and *O*-glycosylation occurs within the endoplasmic reticulum and Golgi apparatus and can take place as either a cotranslational or posttranslational process (Strahl-Bolsinger et al., 1999). Both pathways are highly conserved between species from prokaryotes to humans, including plants, although they can differ in the class, number, and location of carbohydrate residues added (Burda and Aebi, 1999; Willer et al., 2003).

O-mannosylation is a type of *O*-glycosylation absent from plants that is characterized by the addition of mannose residues to target proteins. Over the last decade, many roles have been described for fungal *O*-mannosylation, including in cell wall integrity, cell morphology, and the stability, sorting, and localization of proteins (Strahl-Bolsinger et al., 1999; Hirayama et al., 2008; Lommel and Strahl, 2009). The initial *O*-mannosyltransferase reaction is performed by protein mannosyltransferases (PMTs), which are integral endoplasmic reticulum membrane proteins that add the first mannose to Ser or Thr residues of target proteins. Subsequently, as *O*-mannosylated proteins move into the Golgi apparatus, glycosylation continues with the addition of more mannoses

cycle: 1, fusion of two haploid cells; 2, dikaryon filament invades plant cells to form the appressorium; 3, *U. maydis* proliferates and differentiates within tumor tissue; 4, the fungus produces diploid spores; 5, spores undergo meiosis; 6, *U. maydis* enters its haploid phase.

(B) Dendrogram of PMT proteins. PMT proteins of *S. cerevisiae* (ScPMT), *S. pombe* (SpOGM), *C. albicans* (CaPMT), *D. melanogaster* (DmPOMT), and *H. sapiens* (HsPOMT) were compared with the Urm12220, Urm10749, and Urm05433 proteins (boxed). The black bar represents an evolutionary distance of 0.1 amino acid substitutions per site. Bootstrap support values are given at branch nodes. The phylogenetic analysis showed that a single putative *U. maydis* PMT protein was assigned to each of the three PMT subfamilies. A text file of the alignment used to generate this dendrogram is available as Supplemental Data Set 1 online.

(C) Effects of *pmt4* deletion on pathogenicity. Seven-day-old maize seedlings were inoculated with water, FB2, a cross of FB1 and FB2 wild-type strains, FB1 $\Delta pmt1$ and FB2 $\Delta pmt1$, or FB1 $\Delta pmt4$ and FB2 $\Delta pmt4$. Arrows point to the injection punctures. Ten days after infection (dpi), anthocyanin production and tumor formation were observed on plants infected with the wild-type and $\Delta pmt1$ crosses, while no infection symptoms were detected on plants inoculated with the *pmt4* mutant strains. Bar = 2 cm.

in yeast or a linear heterosaccharide consisting of *N*-acetylglucosamine, galactose, and neuraminic acid in higher eukaryotes. The latter stage of *O*-mannosylation in the Golgi has been poorly characterized in most species except for *Saccharomyces cerevisiae*. In this yeast, the KRE2/MNT1 protein family is responsible for the addition of the second and third mannose residues (Man₂-Man₃) (Lussier et al., 1997a, 1997b), and MNN1 family proteins attach a fourth (Man₄) and possibly additional mannose residues (Romero et al., 1999).

The PMT family was initially described in *S. cerevisiae* (Gentzsch and Tanner, 1996) and has been shown to be widely conserved in other fungi such as *Schizosaccharomyces pombe* (Willer et al., 2005) or *Candida albicans* (Prill et al., 2005). The PMT family can be grouped into three subfamilies: PMT1, PMT2, and PMT4, with the number of members in each subfamily varying between species. In *S. cerevisiae*, PMT proteins can interact to form dimers: heterodimers between PMT1 (Pmt1p and Pmt5p) and PMT2 (Pmt2p and Pmt3p) subfamily members and homodimers between the sole representative of the PMT4 subfamily (Girrbach and Strahl, 2003). PMT dimers show substrate-specific enzymatic activity. In accordance with this, it has been described that some *S. cerevisiae* proteins, such as Kre9p, Cts1p, Bar1p, Pir2p, and Aga2p, are exclusively *O*-mannosylated by the heterodimer PMT1/PMT2 (Gentzsch and Tanner, 1997), while proteins such as Kex2p, Gas1p, Axl2p, and Fus1p are modified only by PMT4 (Gentzsch and Tanner, 1997; Proszynski et al., 2004). By contrast, other proteins, such as members of the WSC family, Mid2p and Ccw5p/Pir4p, are *O*-mannosylated by both complexes (Ecker et al., 2003; Lommel et al., 2004). In order to determine other PMT targets, *in silico* studies based on the general characteristics of *O*-mannosylated proteins have been initiated (Hutzler et al., 2007). Mutations in PMT family proteins have been linked to defects in *S. pombe* morphogenesis (Willer et al., 2005), reduced pathogenicity of *C. albicans* (Timpel et al., 2000; Prill et al., 2005) and *Cryptococcus neoformans* (Olson et al., 2007; Willger et al., 2009), or, in the case of humans, to the Walter-Warburg syndrome (Beltran-Valero de Bernabe et al., 2002; Lengeler et al., 2008).

Evidence suggesting a role for *N*-glycosylation in *U. maydis* infectivity has come from work that showed that an enzyme implicated in the *N*-glycosylation pathway, the glucosidase II GAS1, is required for fungal pathogenicity (Schirawski et al., 2005). These observations suggest that glycosylation may be important for the pathogenic development of *U. maydis*. However, up to now, *O*-mannosylation pathway enzymes have not been characterized in phytopathogenic fungi nor has the role of protein *O*-mannosylation in plant pathogenesis been analyzed. Here, we describe a detailed characterization of the role of the *O*-mannosylation pathway in fungal phytopathogenesis, using *U. maydis* as a model organism. We identify several putative members of the *O*-mannosylation pathway in *U. maydis* and determine which are required for plant infectivity. Significantly, we show that the loss of one of these enzymes, PMT4, affects the ability of *U. maydis* to form functional appressoria and blocks plant cuticle penetration. We discuss our findings and explore the potential of this pathway as a target for antifungal strategies.

RESULTS

The *U. maydis* PMT Family Consists of Three Members

To identify PMT family proteins encoded by the *U. maydis* genome, we performed a protein BLAST search at the National Center for Biotechnology (NCBI) of the 6786 proteins defined in the MIPS *U. maydis* protein database using *S. cerevisiae* PMT proteins (Pmt1p-Pmt7p) as query sequences. We found three putative PMT protein homologs: Um11220, a protein of 956 amino acids with an amino acid identity of 43% to Sc Pmt1p (172/400; in an alignment of 400 amino acids, 172 are the same at equivalent positions); Um10749, of 787 amino acids with 46% identity (328/712) to Pmt2p; and Um05433 of 777 amino acids, with 43% identity (314/721) with respect to Pmt4p (see Supplemental Table 1 online).

Hydropathy analysis (Kyte and Doolittle, 1982) of Um11220, Um10749, and Um05433 suggest that they conserve the characteristic structure of *O*-mannosyltransferase proteins of two large transmembrane domains, located in their N- and C-terminal regions and separated by a central hydrophilic region (Strahl-Bolsinger and Scheinost, 1999). In addition to the topological distribution, the putative *U. maydis* PMT proteins conserve the number of predicted transmembrane domains with respect to *S. cerevisiae* PMTs: 11 for Um11220 and nine for Um10749 and Um05433 (see Supplemental Figure 1 online).

Next, we analyzed our candidate proteins for the presence of two characteristic PMT motifs: the PMT domain, a region implicated in PMT complex formation and *O*-mannosyltransferase activity, found within the N-terminal transmembrane region, and the MIR domain (Mannosyltransferase, IP3, RyReceptor) linked solely to transferase activity and composed of submotifs A, B, and C, located in the hydrophilic central region (Girrbach et al., 2000). We found that the PMT domain is conserved in all three putative *U. maydis* PMT homologs with amino acid similarities of between 53 and 74% with respect to PMT domains in other organisms. Similarly, we found regions with homology to the MIR domain submotifs in all three *U. maydis* PMTs (with amino acid similarity of between 48 and 64%). Um11220, Um10749, and Um05433 all possess the conserved initial Leu-His-Ser-His sequence of submotif A, while Um05433 also has a specific seven-amino acid insertion (Tyr-Asn-Asn-Gly-Arg-Ile-Ser) within submotif A, which is present in Sc Pmt4p, Sp OGM4, and Cn PMT4 (Olson et al., 2007; see Supplemental Figure 2 online).

To analyze the phylogenetic relationships between the three putative *U. maydis* PMTs and their homologs in other organisms, we performed phylogenetic tree analysis using CLUSTAL and MEGA (see Methods and Supplemental Data Set 1 online). Figure 1B shows a phylogenetic tree comparing *S. cerevisiae*, *C. albicans*, *S. pombe*, *Drosophila melanogaster*, and *Homo sapiens* PMT families together with the three putative *U. maydis* proteins. Our analysis grouped each of the three putative *U. maydis* PMTs into one of the PMT subfamilies. Thus, consistent with their hydropathy profiles, conserved sequence motifs, and our phylogenetic analysis, we can provisionally designate Um11220, Um10749, and Um05433 as PMT1, PMT2, and PMT4, respectively.

PMT4 Is Required for Virulence on Maize

To ascertain if *O*-mannosylation has a role in *U. maydis* plant pathogenesis, we generated single deletion mutants of the putative PMT family members in FB1 (*a1b1*) and FB2 (*a2b2*) wild-type strains (see Methods). Strains carrying single deletions in either *pmt1* or *pmt4*, as well as the combined double *pmt1 pmt4* mutant, were viable and showed normal growth rates and mating (Figure 3; see Supplemental Figure 3 online). By contrast, the loss of *pmt2* was lethal in both FB1 and FB2 strains. The requirement of *pmt2* was confirmed by spore analysis of the diploid strain FBD11 $\Delta pmt2/pmt2$ (see Supplemental Figure 3 online). Our results concur with those previously obtained for members of the PMT2 subfamily in *S. cerevisiae*, *C. albicans*, and *S. pombe* (Gentsch and Tanner, 1996; Prill et al., 2005; Willer et al., 2005) and thus reaffirm the requirement of the PMT2 subfamily for fungal viability.

To test if Um PMT1 and PMT4 are required for virulence, we compared leaves from 7-d-old maize seedlings inoculated with a mixture of FB1 and FB2 wild-type strains with those inoculated with either mixtures of compatible *pmt1* deletion mutants (FB1 $\Delta pmt1$ and FB2 $\Delta pmt1$) or compatible *pmt4* deletion mutants (FB1 $\Delta pmt4$ and FB2 $\Delta pmt4$) (see Methods). Disease progression was evaluated 10 d after infection. The mixture of compatible $\Delta pmt1$ strains caused disease symptoms comparable to the wild-type strains (chlorosis, anthocyanin induction, and tumor formation) (Figure 1C, Table 1). By contrast, the mixture of compatible $\Delta pmt4$ strains did not show any disease symptoms, with the exception of chlorosis, which is a consequence of the inoculation process (Figure 1C). Infection assay results are shown in Table 1. Similar results were obtained 15 and 25 d after infection, ruling out the possibility that our results reflect a delay in the development of fungal pathogenesis. Significantly, we found that $\Delta pmt4$ mutants were also unable to form tumors in plants infected through the stigma, which presents a much weaker barrier to infection than leaf tissues (Snetselaar and Mims, 1993) (see Supplemental Figure 4 online).

To test if the *pmt4* alleles present in FB1 and FB2 are both functional and required for virulence, we infected plants with combinations of either FB1 $\Delta pmt4$ with the wild-type strain FB2

or FB2 $\Delta pmt4$ with the wild-type strain FB1. Infections with both combinations caused normal disease symptoms (Table 1). To exclude the possibility that our results reflect a cell fusion defect of the $\Delta pmt4$ strains, we generated a deletion of *pmt4* in the solopathogenic SG200 strain (*a1 mfa2 bE1bW2*), which is able to infect plants without mating as it possesses both *a* and *b* loci active (Bölker et al., 1995). Deletion of *pmt4* in SG200 also resulted in a total loss of infectivity (Table 1). Taken together, these results confirm that PMT4 is required for the pathogenic development of *U. maydis*.

Um PMT1 and PMT4 Encode Protein *O*-Mannosyltransferases

To verify the *O*-mannosyltransferase activity of the putative *U. maydis* PMT homologs, we tested them for their capacity to complement *S. pombe pmt* mutants *ogm1* Δ and *ogm4* Δ . For this purpose, the open reading frames of *pmt1*, *pmt2*, and *pmt4* were cloned into pREP41X (*LEU2 ars1+*) (Maundrell, 1993) to generate the plasmids pREP41X-Umpmt1, pREP41X-Umpmt2, and pREP41X-Umpmt4 (see Methods). All three plasmids, as well as the empty vector, were transformed into *S. pombe ogm1* Δ , which is defective in vitro and in vivo for *O*-mannosyltransferase activity (Willer et al., 2005). The loss of *ogm1* affects viability at 28°C and even more so at 36°C. At 28°C, *U. maydis pmt1*, *pmt2*, and *pmt4* were able to partially complement the *ogm1* Δ mutant. However, at 36°C, only *pmt1* was able to complement this phenotype, suggesting that Um PMT1 represents a functional homolog of Sp OGM1 (Figure 2A). The *S. pombe ogm4* Δ strain forms aggregates when grown in liquid medium. The ability of *U. maydis pmt* genes to complement *ogm4* Δ was tested by transforming the mutant *ogm4* Δ strain and growing transformants in EMM media at 28°C to exponential phase and then studying their cellular aggregation state by optical microscopy. Under these conditions, only pREP41X-Umpmt4 was able to completely restore the single cell growth shown by the wild-type strain (Figure 2B; see Figure 2C for quantification). These findings indicate that *U. maydis* PMT1 and PMT4 are functional homologs of their *S. pombe* equivalents, OGM1 and OGM4, respectively. Moreover, it confirms our phylogenetic-based assignment of PMT family members.

Table 1. Pathogenicity Assays

Inoculum	No. of Plants	Tumor Formation (%)
FB1 \times FB2	73	86.3
FB1 $\Delta pmt1$ \times FB2 $\Delta pmt1$	38	89.5
FB1 $\Delta pmt4$ \times FB2 $\Delta pmt4$	94	0.0
FB1 \times FB2 $\Delta pmt4$	33	87.9
FB1 $\Delta pmt4$ \times FB2	32	87.5
FB1 $\Delta kre2$ \times FB2 $\Delta kre2$	30	90.0
FB1 $\Delta ktr3$ \times FB2 $\Delta ktr3$	34	88.2
FB1 $\Delta kre2 \Delta ktr3$ \times FB2 $\Delta kre2 \Delta ktr3$	33	87.9
FB1 $\Delta mnn1 \Delta mnt4$ \times FB1 $\Delta mnn1 \Delta mnt4$	29	93.1
SG200	84	91.7
SG200 $\Delta pmt4$	75	0.0

Seven-day-old maize seedlings were inoculated with the strains indicated, and the tumor formation was scored 10 d after infection.

PMT4 Is Required Only for the Pathogenic Phase of the *U. maydis* Life Cycle

The loss of PMTs from *S. cerevisiae*, *S. pombe*, and *C. albicans* causes several defects, such as abnormal morphology, cellular aggregation, alterations in polar growth, and hypersensitivity to antifungal drugs, among others (Proszynski et al., 2004; Prill et al., 2005; Willer et al., 2005). To determine the effects of the deletion of *pmt4* on *U. maydis* biology, we performed an in-depth analysis of $\Delta pmt4$, looking at the different phases of the *U. maydis* life cycle.

The deletion of *pmt4* from haploid FB1 and FB2 strains did not show any significant differences in morphology, cellular aggregation, or cell cycle progression compared with wild-type strains when grown in liquid (Figures 3A and 3B; see Supplemental Figure 5 online) or solid culture. To detect mating defects previously described in *S. cerevisiae* (Proszynski et al., 2004), we cocultivated

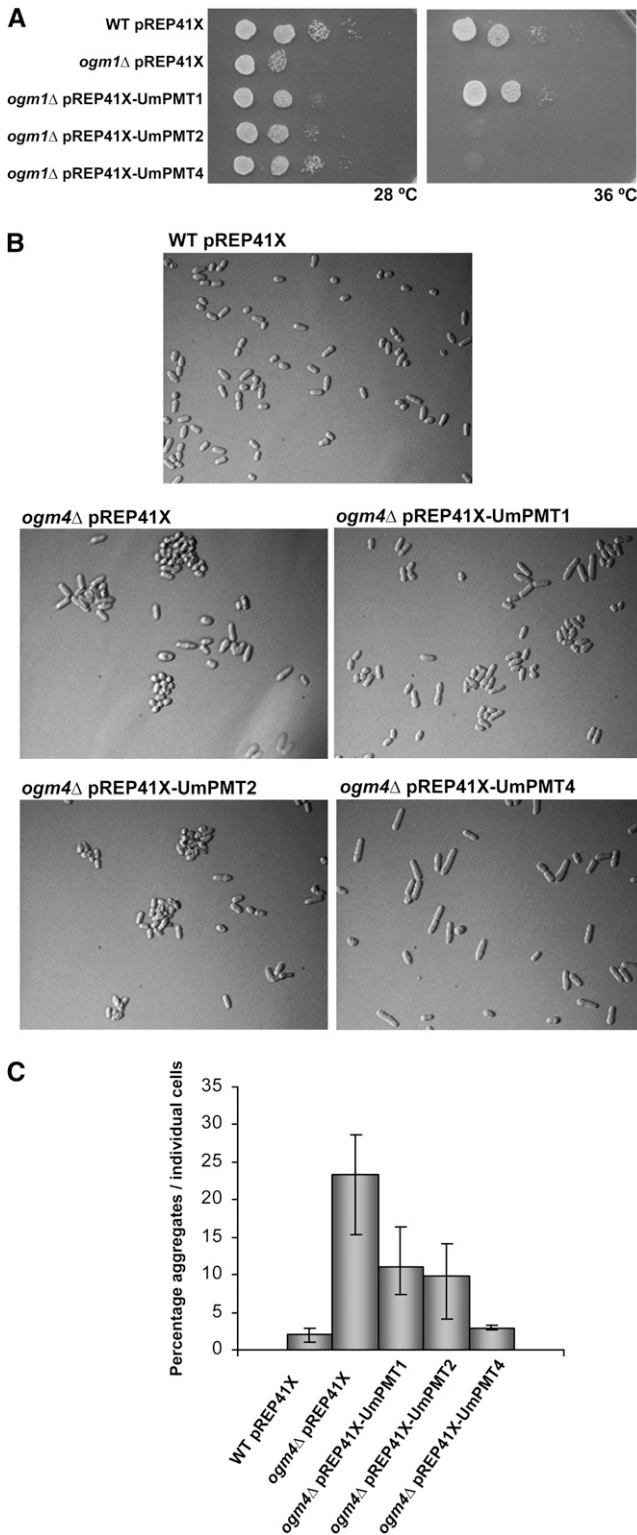


Figure 2. *U. maydis* PMTs Complement *S. pombe* *ogm1*Δ and *ogm4*Δ Mutants.

(A) Functional complementation of *S. pombe* *ogm1*Δ. Serial dilutions of wild-type *S. pombe* *leu1-32* strain transformed with pREP41X and the

a mixture of the sexually compatible strains FB1 *Δpmt4* and FB2 *Δpmt4* on charcoal-containing agar plates. Normal formation of dikaryon filaments, recognized as white fuzzy mycelium, was observed (Figure 3C). These results suggest that PMT4 does not have an important role in vegetative growth or mating.

To determine the role of PMT4 in polar growth, as has been described for *C. albicans* PMT mutants (Prill et al., 2005), we generated a *pmt4* deletion in the haploid strain AB33 (Brachmann et al., 2001), which harbors the compatible *bE2/bW1* genes under the control of the nitrate-inducible *nar1* promoter. In nitrate-containing medium, AB33 enters the filamentation program. Under inducing conditions, AB33 *Δpmt4* shows a normal polar growth in infectious hyphae when compared with the wild-type strain (Figure 3D). Thus, we can conclude that *pmt4* is not required for *b*-dependent filament formation.

The consequences of the deletion of *pmt4* on the production of secreted hydrolytic enzymes were also investigated as O-glycosylation has been shown to be required for fungal host interactions (Lehle et al., 2006). On-plate cellulase, pectinase, and amylase activity assays (see Methods) revealed no difference in the activity of these secreted enzymes between *pmt4* mutant fungi and wild-type cells (see Supplemental Figure 6 online).

Finally, to establish the stress response of the *Δpmt4* strain, we tested its resistance to thermal (34 and 36°C), antifungal (congo red, chlorpromazine, calcofluor white, and caffeine), oxidative (H₂O₂), osmotic (SDS and sorbitol), and salt-based (NaCl and CaCl₂) stresses. We performed a serial dilution patch test of *Δpmt4*, *Δpmt1*, and control strains grown for 2 d at 28°C on YPD agar plates containing the compounds and conditions mentioned above. *Pmt1* and *pmt4* mutants did not exhibit enhanced sensitivity to any of these agents or conditions (see Supplemental Figure 7 online; data not shown) except for increased sensitivity to SDS by the *Δpmt4* strain (Figure 3E). SDS sensitivity has also been observed in *C. neoformans* *pmt4* mutants and was linked to a failure of cell wall integrity (Olson et al., 2007).

PMT4 Is the Only Putative O-Mannosyltransferase in the *U. maydis* Genome Essential for Pathogenesis

Our findings suggest that PMT4-mediated O-mannosylation may play a specific role in pathogenesis. In *S. cerevisiae*, the

*ogm1*Δ strain transformed with either pREP41X (control), pREP41X-Um*pmt1*, pREP41X-Um*pmt2*, or pREP41X-Um*pmt4* are shown after 3 d at 28 or 36°C. *ogm1*Δ cells were viable at 36°C if transformed with *U. maydis* *pmt1*. Um *pmt1*, *pmt2*, and *pmt4* were able to partially complement the *ogm1*Δ mutant at 28°C.

(B) *S. pombe* *ogm4*Δ complementation. The *ogm4*Δ mutant cells form aggregates. The *ogm4*Δ mutants transformed with pREP41X *LEU2* (empty vector) or with the vector containing either *pmt1*, *pmt2*, or *pmt4* were grown to exponential phase at 28°C and photographed. *pmt4* complemented the phenotype of the *ogm4*Δ mutant resulting in single cell growth.

(C) Quantification of *pmt4* functional complementation of *ogm4*Δ. The quantification shows the percentage of aggregates relative to individual cells. Three independent samples were analyzed for each strain (number of cells for each sample ≥100). Error bars represent SE of the mean.

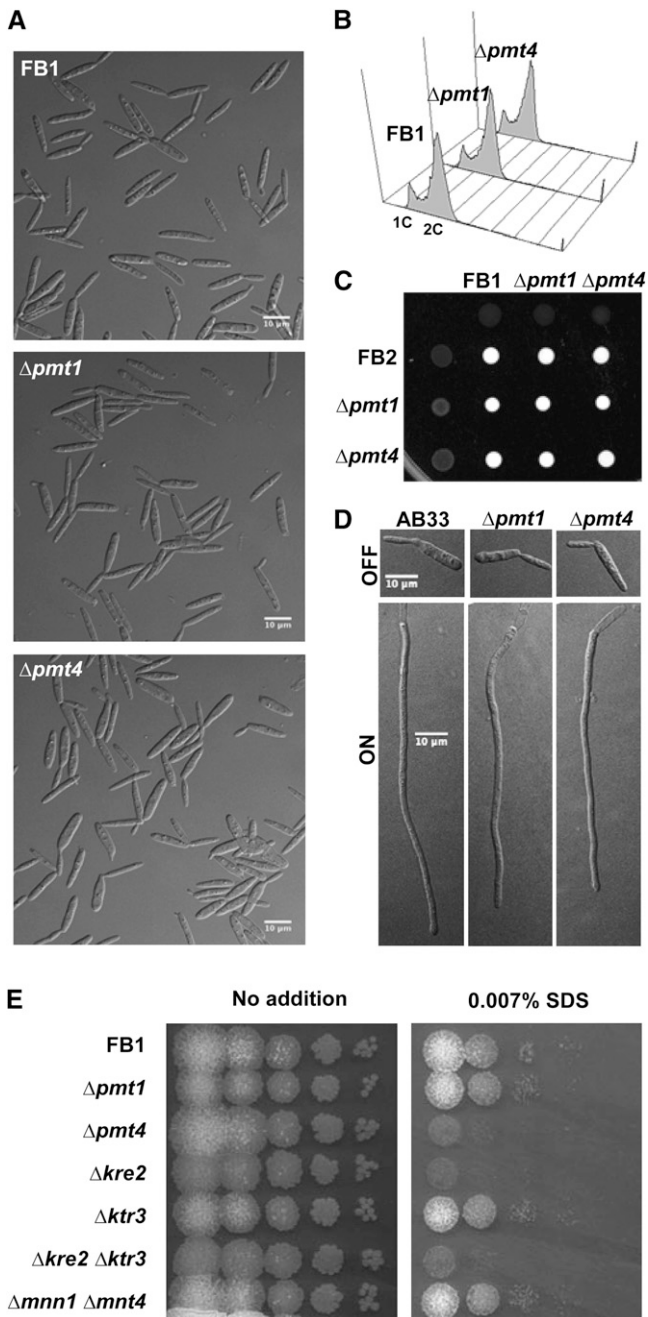


Figure 3. *U. maydis* $\Delta pmt4$ Phenotypes.

(A) Cell morphology and aggregation states of wild-type (FB1), *pmt1* mutant (FB1 $\Delta pmt1$), and *pmt4* mutant (FB1 $\Delta pmt4$) strains. Cells were grown in 1% glucose complete medium CMD to exponential phase at 28°C.

(B) Flow cytometry analysis. The DNA content of cell populations of FB1 and FB1 $\Delta pmt1$ and FB1 $\Delta pmt4$ strains growing in CMD medium to exponential phase was measured. The relative 1C to 2C (haploid to diploid) DNA contents in *pmt1* and *pmt4* mutants are similar to that of the wild-type strain.

(C) Mating and dikaryont filament formation. Wild-type (FB1 and FB2), $\Delta pmt1$ (FB1 $\Delta pmt1$ and FB2 $\Delta pmt1$), and $\Delta pmt4$ (FB1 $\Delta pmt4$ and FB2

initial PMT-catalyzed *O*-mannosylation step is a prerequisite for the addition of further mannose residues by members of the KRE2/MNT1 family, which add second and third mannose residues to target proteins (Willer et al., 2003). Thus, we wanted to determine if the *U. maydis* equivalents of the KRE2/MNT1 family might also have a role in pathogenesis. With this aim, we searched for the putative KRE2/MNT1 family protein homologs in *U. maydis* using a method similar to that performed for the PMT family. The *S. cerevisiae* KRE2/MNT1 family comprises nine members: Kre2p, Ktr1p, Ktr2p, Ktr3p, Ktr4p, Ktr5p, Ktr6p, Ktr7p, and Yur1p. However, we found only two putative members in the *U. maydis* genome: Um01154 with 52% identity (in an alignment of 315 amino acids, 165 are the same at equivalent positions) with respect to *Sc* Kre2p; and Um01821 with 47% identity (143/290) to *Sc* Ktr3p (more data in Supplemental Table 1 online). We assigned Um01154 and Um01821 as putative homologs of Kre2p and Ktr3p, respectively. We generated single deletions of *kre2* and *ktr3* and the double $\Delta kre2 \Delta ktr3$ mutant in FB1 and FB2 wild-type strains. To assay the virulence of these mutants, we inoculated a mixture of compatible mutants or wild-type strains on 7-d-old maize seedlings (Table 1). All mutants tested caused normal disease symptoms on plants, suggesting that *U. maydis* KRE2 and KTR3 are not required for virulence.

Our observation of SDS sensitivity in the *U. maydis* *pmt4* mutant suggests that *pmt4* may have a role in maintaining cell wall integrity. We were interested in knowing whether the KRE2/MNT1 family could also be involved in this process. We performed a sensitivity study of $\Delta kre2$, $\Delta ktr3$, and $\Delta kre2 \Delta ktr3$ strains to the compounds and conditions previously assayed for the PMT mutants. As shown in Figure 3E, $\Delta kre2$ mutants demonstrated enhanced sensitivity to SDS, while their reaction to other drugs was normal. As $\Delta kre2$ mutants exhibit normal infectivity, it would suggest that *pmt4* mutant sensitivity to SDS is unlikely to be responsible for its pathogenic defect.

To complete the study of the *U. maydis* *O*-mannosylation pathway, we investigated the role of the MNN1 family, which adds the fourth mannose in *S. cerevisiae*. The *Sc* MNN1 family consists of six members: Mnn1p, Mnn2p, Mnn5p, Mnt2p, Mnt3p, and Mnt4p. As for the KRE2/MNT1 family, we identified only two putative members in the *U. maydis* genome: Um05152 with 30%

$\Delta pmt4$) strains, either as pure cultures or as a cross of compatible strains, were spotted on charcoal-containing agar plates and incubated at 25°C for 24 h. Fuzzy spots of white filaments indicate dikaryotic hyphae formation. The crosses of *pmt1* and *pmt4* mutants resulted in normal dikaryont formation.

(D) Polar growth. AB33 strain harboring *bE/bW* compatible alleles under the *nar* promoter and $\Delta pmt1$ and $\Delta pmt4$ derivative strains were induced to express *bE/bW* by switching from ammonium-containing (OFF) to nitrate-containing (ON) medium. Six hours after induction, wild-type and mutants were able to develop *b*-dependent filaments.

(E) SDS sensitivity of *O*-mannosylation pathway mutants. A series of 10-fold dilutions of FB1, $\Delta pmt1$, $\Delta pmt4$, $\Delta kre2$, $\Delta ktr3$, $\Delta kre2 \Delta ktr3$, and $\Delta mnn1 \Delta mnt4$ strains were spotted on YPD agar plates containing 0.007% SDS detergent and incubated at 28°C during 2 d. *pmt4* and *kre2* single mutants and the *kre2 ktr3* double mutant showed increased sensitivity to SDS.

(48/157) identity with respect to Mnn1p and Um05153 with 24% identity to Mnt4p (68/274), and these were therefore renamed to Um *mnn1* and *mnt4*, respectively (see Supplemental Table 1 online). We generated single deletions of *mnn1* and *mnt4* and the double mutant $\Delta mnn1 \Delta mnt4$ in FB1 and FB2 strains. All mutants showed normal responses to the conditions previously tested for *kre2* and *ktr3* mutants, including SDS (double mutant in Figure 3E). To analyze the role of MNN1 family proteins in *U. maydis* virulence, we inoculated maize plants with mixtures of compatible mutant strains. As expected, we obtained normal tumor formation 10 d after infection (Table 1). Thus, *U. maydis* members of the MNN1 family are not required for pathogenesis or SDS resistance.

In summary, PMT4 is the only identified O-mannosyltransferase enzyme that is required for *U. maydis* pathogenesis.

Um *pmt* Expression Is Not Dependent on the *bE/bW* Heterodimer

Our data suggest that Um *pmt4* is primarily required for fungal virulence. Therefore, we looked to see if its expression, and that of other *pmt* family members, is regulated by the *b* locus-controlled pathogenicity program. We analyzed *pmt* gene expression by RNA gel blot. For this purpose, we used the AB33 strain, whose *b*-pathway becomes activated in nitrate-containing medium (Brachmann et al., 2001). We included an AB34 strain harboring the noncompatible *bE2/bW2* combination (Brachmann et al., 2001) and FB2 as control strains. Our expression analysis, shown in Figure 4, suggests that *pmt4* is not a *b*-regulated gene. Thus, we conclude that *pmt4* expression is not dependent on the presence of an active *bE/bW* heterodimer. Analysis of *pmt1* and *pmt2* expression under the same conditions showed that their expression levels also did not change following *b*-locus activation. In addition, we analyzed *pmt* expression in the AB33 $\Delta pmt4$ strain because a compensatory response has been described in *C. albicans* *pmt* mutants

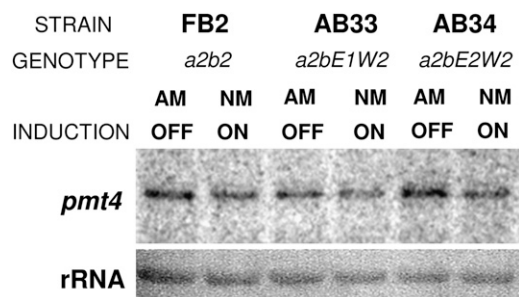


Figure 4. RNA Gel Blot Analysis of the Strains FB2, AB33, and AB34 after Induction of *b*-Gene Expression.

Cells of haploid FB2, AB33, and AB34 strains were grown under repressing conditions on EMM-containing ammonium (ammonium medium [AM]) as the sole nitrogen source to OD₆₀₀ 0.5 and then shifted to inducing conditions (nitrate medium [NM]) and incubated for 6 h. Expression was also analyzed on AM as a control. In all cases, 10 μ g of total RNA were loaded per lane. 18s RNA was used as a loading control.

(Cantero et al., 2007). By contrast, we found that during both yeast-like and filamentous growth of *U. maydis*, the expression levels of the other *pmt* genes are independent of the presence of *pmt4* (data not shown).

PMT4 Is Essential for Functional Appressorium Formation

To investigate which step of pathogenic development might be affected in *pmt4* mutants, we inoculated a mixture of FB1 $\Delta pmt4$ and FB2 $\Delta pmt4$ strains and observed fungal cells on the plant surface. Cells were stained with calcofluor white 15 h after plant inoculation to study filamentation and appressorium production capability. In *pmt4* mutant strains, we observed normal filament formation but with a significantly lower frequency of appressorium formation. We obtained similar results comparing SG200 and SG200 $\Delta pmt4$ strains. Normal *pmt4* mutant filament formation on charcoal-containing medium (see Methods) can be seen in the Figure 5A.

To confirm these observations we coinoculated plants with a mixture containing equal numbers of SG200CFP and SG200 $\Delta pmt4$ YFP cells or equal numbers of SG200YFP and SG200 $\Delta pmt4$ CFP strains. We then quantified the number of appressoria as described by Flor-Parra et al. (2006). In the quantitative comparison shown in the Figure 5B, we noticed that appressorium formation was reduced fivefold in SG200 $\Delta pmt4$ compared with SG200, irrespective of the combination analyzed. Our findings strongly suggest that Um PMT4 plays an important role in appressorium formation.

Interestingly, during the appressoria quantification process, we observed that in those cases where appressoria did form in $\Delta pmt4$ mutants, their morphology appeared abnormal (data not shown; see below). Moreover, we did not see the formation of clamp-like cells, which are typical of mycelium extensions in planta. To better visualize appressoria morphology in these mutants, we used DeltaVision deconvolution microscopy, combining a large number of optical sections from unfixed samples (see Methods). Using this method, we could visualize surface filament and appressoria morphology of wild-type *U. maydis* during the first stages of infection (Figure 5C). When we performed the same analysis on plants inoculated with $\Delta pmt4$ *U. maydis*, we found that mutant appressoria were unable to form the structures associated with plant penetration in the wild type, including clamp-like cells (Figure 5D), indicating that $\Delta pmt4$ might be defective in its ability to penetrate plant tissues.

To confirm that the deletion of *pmt4* affects *U. maydis* plant penetration, we used the Chlorazol Black E staining procedure to visualize invading hyphae (Brachmann et al., 2003). This technique has the advantage that it allows us to simultaneously visualize both appressoria and plant cells. In this experiment, we analyzed appressoria morphology 2 d after inoculation to confirm that the results seen in Figure 5 reflect a genuine failure of plant penetration. In parallel, we analyzed the $\Delta gas1$ strain, which is able to penetrate plant cuticle, but its growth arrests after surface penetration (Schirawski et al., 2005). While wild-type and $\Delta gas1$ hyphae can be seen to invade beyond the epidermal cell layer, $\Delta pmt4$ mutants were never observed to reach this stage of the infection process (Figure 6), resulting in all the $\Delta pmt4$ hyphae being localized close to the plant surface. We measured the

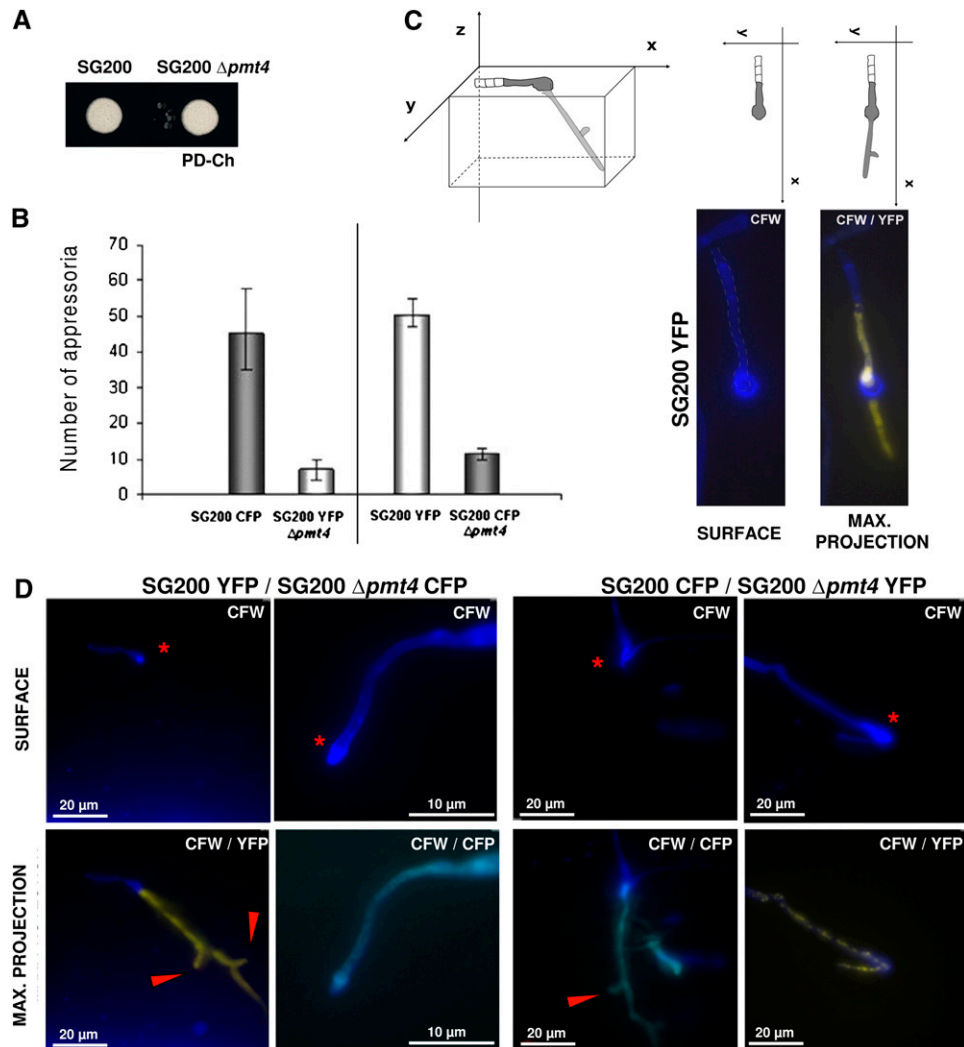


Figure 5. The Deletion of *pmt4* Affects Appressoria Production.

(A) Filament formation of the solopathogenic SG200 and SG200 $\Delta pmt4$ strains that were spotted on PD-charcoal (PD-Ch) plates and incubated at 25°C for 24 h. The fuzzy spots indicate filament formation.

(B) Appressoria production was reduced in *pmt4* mutants. A mixture of equal numbers of cells of SG200 and SG200 $\Delta pmt4$ strains tagged with CFP and YFP under the *OMA* promoter were used to inoculate 7-d-old maize seedlings. After 15 h, we scored appressoria formation by scoring for cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) fluorescence. Three independent samples were analyzed. The error bars represent SE. The deletion of *pmt4* produced a decrease in appressoria formation.

(C) Visualization of appressorium morphology by deconvolution microscopy. Schematic XYZ drawing representing appressorium plant penetration. In the XY plane, we can see the appressorium structure on the leaf surface. Below, we showed an image without deconvolution. On the right, a series of images taken at different Z planes were reconstructed using maxim projection during deconvolution (see Methods for more details) to visualize fungal progression through plant tissue.

(D) Clamp-like cells are absent 24 h after infection in the *pmt4* mutant. A mixture of equal numbers of cells for SG200 CFP and SG200 YFP $\Delta pmt4$, and SG200 YFP and SG200 CFP $\Delta pmt4$ strains were inoculated on 7-d-old maize seedlings. Twenty-four hours after infection, cells were observed by deconvolution microscopy in three channels: CFW, YFP, and CFP to identify each one of the strains. Deconvolved images of inoculated plants show that the deletion of *pmt4* affects its capacity to form clamp-like cells, a structure associated with plant tissue penetration. Red asterisks indicate the site of penetration, and clamp cells in the wild type are marked with arrowheads.

distance from the hypha tips to the closest visible septa 2 d after infection in leaves stained with CBE. This distance correlates with the level of penetration because the new septa formed at the posterior end of the cytoplasm are probably necessary for penetration (Schirawski et al., 2005) (Figure 6). In the case of

the wild-type strain, the mean distance was $6.15 \pm 1.13 \mu\text{m}$, in the *gas1* mutant cells the last septum was localized $10.97 \pm 3.06 \mu\text{m}$ from the tip, while in the case of the $\Delta pmt4$ strains, it was $17.62 \pm 4.56 \mu\text{m}$ (Table 2). These results confirm that appressorium-mediated penetration in *pmt4* mutant is defective.

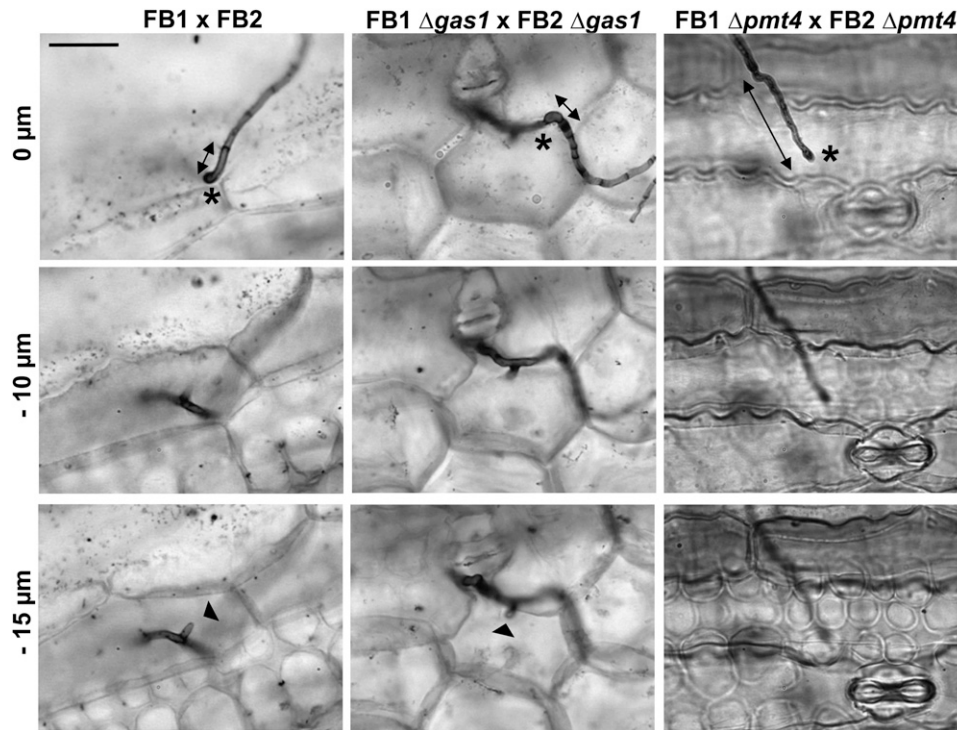


Figure 6. *Δpmt4* Mutants Are Unable to Penetrate into the Plant.

Maize seedlings were infected with a mixture of compatible FB1 and FB2, FB1 *Δgas1* and FB2 *Δgas1*, and FB1 *Δpmt4* and FB2 *Δpmt4* strains. Two days after infection, leaf samples were stained with Chlorazole Black E and analyzed by light microscopy. The z axis image projections show the site of penetration (marked with an asterisk) and the hyphae invading into plant cells (narrow point of the clamp-like cells). *Δgas1* mutant (identified by stronger staining of the septum closest the hyphae tip) and wild-type strains, but not *Δpmt4* mutants, penetrate into the plant. Distance from hyphal tip to the closest visible septum is indicated by double-headed arrows. Bar = 15 μm.

Taken together, our observations suggest that *pmt4* plays a key role in fungal pathogenesis through roles in appressorium formation and plant penetration.

The Inhibition of Plant ROS Production Does Not Restore the Virulence of the *pmt4* Mutant

It is already known that plants respond to pathogen attack by producing, among other compounds, reactive oxygen species (ROS) at the site of attempted invasion. Due to their toxicity and importance in plant defense responses, ROS are essential for the progression of fungal infection (Wojtaszek, 1997; Apel and Hirt, 2004). *U. maydis yap1*, a gene involved in H₂O₂ detoxification, is required for virulence, and the addition of diphenyleneiodonium (DPI), an inhibitor of the plant NADPH oxidase, restores the normal infectious growth in plant tissues (Molina and Kahmann, 2007). To determine whether the infection defect shown by *Δpmt4* cells is somehow related to problems in H₂O₂ detoxification, 0.5 μM DPI was added to the *Δpmt4* cell culture during inoculation. The suppression of ROS generation by treatment with DPI did not restore the phenotype of the *Δpmt4* strain as it did on the *Δyap1* strain (Figure 7). Thus, consistent with the normal sensitivity to H₂O₂ (see Supplemental Figure 7 online), the virulence phenotype of *U. maydis Δpmt4* is not caused by an enhanced sensitivity to plant ROS production.

DISCUSSION

The fungal cell wall has historically been considered as a key target for antifungal drugs since it is in intimate contact with host cells, mediating cell interactions and eliciting host immune responses. β-Glucans and chitin, as major components of

Table 2. Quantification of Appressoria and Plant Penetration

Inoculum	No. of Appressorium-Mediated Penetrations/ No. of Appressoria	Percentage	Distance from Hyphal Tip to the Closest Visible Septum (μm)	n
FB1 × FB2	96/97	98.97	6.15 ± 1.13	49
FB1 <i>Δgas1</i> × FB2 <i>Δgas1</i>	46/47	97.87	10.97 ± 3.06	29
FB1 <i>Δpmt4</i> × FB2 <i>Δpmt4</i>	0/73	0.00	17.62 ± 4.56	47

Maize seedlings were infected with the strains indicated above. Two days after infection, leaf samples were stained with Chlorazole Black E and analyzed by light microscopy. Analysis was based on assays of three independent pools of plants with the total number of appressoria scored shown (n).

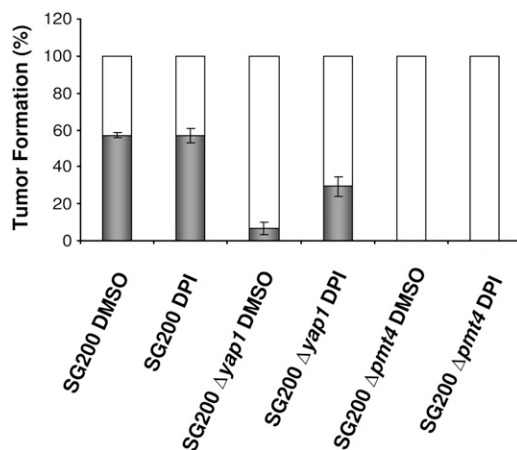


Figure 7. Virulence of *U. maydis* $\Delta pmt4$ Is Not Restored Using DPI.

Maize seedlings were inoculated with SG200 and *yap1* and *pmt4* mutants to which DMSO or DPI dissolved in DMSO were added. Symptoms were scored 14 d after infection. Three independent assays were performed and the average percentage of tumor formation is indicated (number of plants for each assay ≥ 30). Error bars represent SE.

fungal cell walls, have been selected as the main targets, and many studies on their synthesis and function in fungal infection have been undertaken. Glycoproteins are also major components of the fungal cell wall, involved in its structural and cellular properties, such as cell adhesion, cell fusion, and molecular uptake (Bowman and Free, 2006). Glycoproteins have also been considered as potential targets of antifungal compounds (Ernst and Prill, 2001), and many studies of cell wall glycoproteins and the glycosylation process are now in progress to determine their role in infective mechanisms and their potential as antifungal targets.

In this study, we have analyzed the consequences of deleting all of the putative mannosyltransferases involved in the protein *O*-mannosylation pathway present in the *U. maydis* genome. Of the mannosyltransferases tested, only PMT4 was specifically required for *U. maydis* infectivity. Given the high level of identity between members of the three *O*-mannosyltransferase families and the absence of other genes with significant similarity, we conclude that PMT4 is the only *O*-mannosyltransferase essential for the *U. maydis* virulence. The deletion of *pmt4* is associated with a drastic reduction in appressorium formation and, more importantly, those appressoria that do form are nonfunctional in terms of plant penetration. Thus, protein *O*-mannosylation can now be considered a protein modification process essential for *U. maydis* virulence and, as PMT family proteins are not conserved in plants (Rouabhia et al., 2005; Bowman and Free, 2006), they may represent an interesting target for future antifungal drugs.

***U. maydis* as a Phytopathogenic Model for Studying Protein *O*-Glycosylation**

Fungal members of the PMT family, which are involved in the addition of the first mannose monomer to Ser/Thr residues of

nascent proteins, have been best characterized in *S. cerevisiae*. There are seven known PMT members in *S. cerevisiae*, grouped into three subfamilies, PMT1, PMT2, and PMT4. By contrast, only three members have been found in the *U. maydis* genome, one for each of these subfamilies. Three PMT family members have also been described in *S. pombe* (Willer et al., 2005), *C. neoformans* (Olson et al., 2007; Willger et al., 2009), and *Aspergillus fumigatus* (Zhou et al., 2007). The increased number of PMT proteins in *S. cerevisiae* probably originates from a duplication event during evolution (reviewed in Dujon, 2006). In *C. albicans*, the PMT family contains five nonfunctionally redundant members, of which two new isoforms seem to have acquired new biological roles, such as host cell invasion (Prill et al., 2005). Our results show that *U. maydis* PMT proteins not only show sequence homology with three conserved PMT subfamilies, but also share their functionality and target specificity as shown by our cross-complementation assay in *S. pombe*. We also found that *U. maydis* possesses fewer genes of families implicated in subsequent rounds of *O*-mannosylation. In the case of the KRE2/MNT1 family, which is responsible for the addition of the second and third mannose residues to target proteins in *S. cerevisiae*, *U. maydis* contains only two members compared with the nine members described for the budding yeast. Similarly, only two members have been found belonging to the *U. maydis* MNN1 family, which is responsible for the addition of the fourth mannose residue, while there are six members described for *S. cerevisiae*. The reduced complexity of this process in *U. maydis* promises to make it an excellent model to better understand fungal *O*-mannosylation and its role in phytopathogenicity.

Biological Significance of the PMT Family

In most fungal and animal cells, protein *O*-mannosylation is involved in a wide variety of essential cellular processes (Lommel and Strahl, 2009). Our findings suggest that a significant part of the *U. maydis* protein *O*-mannosylation machinery is dispensable for its viability. Although the deletion of *pmt2* in *U. maydis* is lethal, as it is in *S. pombe* (Willer et al., 2005), the viability of simultaneous *U. maydis* *pmt1* and *pmt4* deletions contrasts greatly to fission yeast. In *S. cerevisiae*, *O*-mannosylation determines the solubilization of misfolded proteins in the endoplasmic reticulum (Nakatsukasa et al., 2004), the maintenance of cell polarity (Lommel et al., 2004; Proszynski et al., 2004), and the cell wall response (Gentzsch and Tanner, 1996; Lommel and Strahl, 2009). By contrast, no appreciable defects in cell morphology, cell polarity, or drug response were observed in *U. maydis* $\Delta pmt1$ and $\Delta pmt4$ strains. Our findings strongly suggest that protein *O*-mannosylation mediated by *pmt1* and *pmt4* is not essential for saprophytic growth in *U. maydis*.

Effects of Protein *O*-Mannosylation on Cell Wall Integrity and Pathogenesis in *U. maydis*

We focused our analysis toward the role of *O*-mannosyltransferase proteins on cell wall integrity and pathogenesis. In Figure 8, we have summarized the roles of the *O*-mannosylation protein

families in these contexts. Individual deletions of six of the seven putative *U. maydis* O-mannosyltransferases, as well as double deletions of enzymes belonging to the KRE2/MNT1 and MNN1 family, did not affect cell growth. Only the deletion of *pmt4* and *Kre2* resulted in slight cell wall integrity defects, as revealed by sensitivity to SDS in these two mutants. Of these two genes, only *pmt4* seems to be involved in pathogenesis. Thus, defects in cell wall integrity do not correlate with a defect in pathogenesis nor is the addition of the first mannose always required for virulence. By deleting any of the PMT proteins, we prevent the addition of the whole mannose moiety structure to their particular target proteins, but only those that depend on PMT4 O-mannosyltransferase seem to specifically affect pathogenesis. This finding is not surprising since specific targets of PMT4 and phenotypes exclusively depending on this O-mannosyltransferase have been described previously (Gentsch and Tanner, 1997; Proszynski et al., 2004). Thus, we can postulate that one or more targets of Um PMT4 are important for virulence. A similar case was observed for the Um KRE2/MNT1 family proteins where deletion of *kre2* but not *ltr3* affected cell wall integrity. This could be because the targets of both proteins are different, and only the ones affected by KRE2 are involved in cell wall structure. Alternatively, it could be because in *U. maydis*, KRE2 is the enzyme primarily responsible for the addition of the second mannose, while KTR3 is involved in the addition of the third residue as has been suggested for *C. albicans* (Buurman et al., 1998). The fact that the deletion of genes from KRE2/MNT1 or MNN1 families does not affect pathogenesis suggests that PMT4 target proteins might only have one mannose residue added or that a single mannose is sufficient to confer to these proteins their proper tertiary structure required for functionality in the infection program.

So far, PMT4 targets have been best characterized in *S. cerevisiae*. Of these targets, we have identified *U. maydis* homologs for Sc Kex2p (Um02843), Sc Gas1p (Um01640), and less clearly for Sc Axl2p (Um01289). In budding yeast, these genes have been implicated in pheromone maturation, cell wall assembly, and axial budding of haploid cells, respectively. Given that

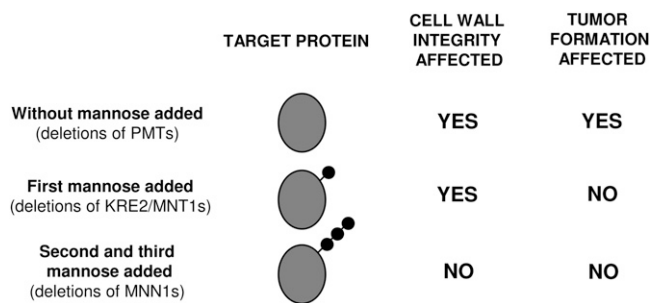


Figure 8. Schematic Model of the Effects on Cell Wall Integrity and Pathogenesis of Protein O-Mannosylation in *U. maydis*.

Deletions of *pmt* family members in *U. maydis* alter both cell wall integrity and maize tumor formation. Elimination of KRE2/MNT1 family members suggests they are essential for maintaining cell wall integrity but not for virulence. The addition of the first three mannose residues is sufficient for the fungus to exhibit normal growth and pathogenic development.

Table 3. *U. maydis* Strains Used in This Study

Strain	Relevant Genotype	Reference
FB1	<i>a1 b1</i>	Banuett and Herskowitz (1989)
FB2	<i>a2 b2</i>	Banuett and Herskowitz (1989)
FBD11	<i>a1 a2 b1 b2</i>	Banuett and Herskowitz (1989)
SG200	<i>a1 mfa2 bW2 bE1</i>	Bölker et al. (1995)
AB33	<i>a2 Pnar:bW2 Pnar:bE1</i>	Brachmann et al. (2001)
AB34	<i>a2 Pnar:bW2 Pnar:bE2</i>	Brachmann et al. (2001)
HBU13	<i>a1 b1 Δgas1</i>	Schirawski et al. (2005)
HBU14	<i>a2 b2 Δgas1</i>	Schirawski et al. (2005)
SG200CFP	<i>a1 mfa2 bW2 bE1 P_{OMA}:CFP</i>	Flor-Parra et al. (2006)
SG200YFP	<i>a1 mfa2 bW2 bE1 P_{OMA}:YFP</i>	Flor-Parra et al. (2006)
SG200 Δyap1	<i>a1 mfa2 bW2 bE1 Δyap1</i>	Molina and Kahmann (2007)
UMG1	<i>a1 b1 Δpmt1</i>	This work
UMG2	<i>a2 b2 Δpmt1</i>	This work
UMG3	<i>a1 a2 b1 b2 Δpmt2</i>	This work
UMG4	<i>a1 b1 Δpmt4</i>	This work
UMG5	<i>a2 b2 Δpmt4</i>	This work
UMG6	<i>a2 Pnar:bW2 Pnar:bE1 Δpmt4</i>	This work
UMG7	<i>a1 b1 Δkre2</i>	This work
UMG8	<i>a2 b2 Δkre2</i>	This work
UMG9	<i>a1 b1 Δktr3</i>	This work
UMG10	<i>a2 b2 Δktr3</i>	This work
UMG11	<i>a1 b1 Δkre2 Δktr3</i>	This work
UMG12	<i>a2 b2 Δkre2 Δktr3</i>	This work
UMG13	<i>a1 b1 Δmnn1</i>	This work
UMG14	<i>a2 b2 Δmnn1</i>	This work
UMG15	<i>a1 b1 Δmnt4</i>	This work
UMG16	<i>a2 b2 Δmnt4</i>	This work
UMG17	<i>a1 b1 Δmnn1 Δmnt4</i>	This work
UMG18	<i>a2 b2 Δmnn1 Δmnt4</i>	This work
UMG19	<i>a1 mfa2 bW2 bE1 Δpmt4</i>	This work
UMG20	<i>a1 mfa2 bW2 bE1 Δpmt4 P_{OMA}:CFP</i>	This work
UMG21	<i>a1 mfa2 bW2 bE1 Δpmt4 P_{OMA}:YFP</i>	This work
UMG22	<i>a2 Pnar:bW2 Pnar:bE1 Δpmt1</i>	This work

the deletion of *pmt4* does not affect cell cycle progression, mating, or polar growth, it seems unlikely that these proteins are responsible for the phenotypes we observe in *U. maydis*, although we are currently working to test this experimentally. Sc Fus1p, Sc Opy2p, and Sc Msb2p, components of the yeast filamentous growth mitogen-activated protein kinase pathway, have recently been described as additional PMT4 targets (Yang et al., 2009). We found that *U. maydis* contains only some elements of the yeast filamentous growth mitogen-activated protein kinase pathway. We have identified a *U. maydis* homolog for Sc Msb2p (Um01513), but none were found for Sc Fus1p or Sc Opy2p. Although we cannot currently exclude a role for any of these genes without further investigation, we think that the *pmt4*

deletion phenotype is more likely to be caused by other, as yet unidentified, PMT4 protein targets, which are more directly implicated in pathogenic development. Thus, the identification of additional PMT4 target proteins is now a priority.

Why Are *pmt4* Mutants Unable to Form a Normal Number of Appressoria and Penetrate the Plant Cuticle?

We have shown that the loss of PMT4 results in a strong decrease in the number of appressoria on the plant surface and that no fungal plant penetration occurs. We can postulate a number of different explanations. (1) Adhesion has been shown to be required for appressorium formation and has an important role in appressorium-mediated penetration. Thus, the mannan fraction of cell wall proteins that are glycosylated by PMT4 might mediate fungal adhesion to the plant surface, similar to the situation in *C. albicans*, in which *pmt6* is required for adhesion to human epithelium (Timpel et al., 2000). (2) In other organisms, PMT mutations affect cell wall composition, increasing or decreasing the amount of β 1-2 and β 1-3 glucans, chitin, and glycoproteins. For example, *C. albicans pmt1* mutants show increased levels of chitin (Kapteyn et al., 2000), while Ca *pmt4* mutants have increased levels of β 1,3-glucan compared with wild-type cells (Prill et al., 2005). Similar changes in *U. maydis* could affect appressorium formation by yielding either hypha without appressoria or malformed appressoria unable to penetrate the plant tissues. (3) Another possibility is that alterations in the amount of the glycosylation in cell wall proteins could affect the resistance of the fungus to pathogenesis-related proteins produced by the plant and, therefore, its virulence (Narasimhan et al., 2003). (4) A fourth possibility is that the loss of infectivity reflects a problem in the protein secretion process during appressorium formation and plant penetration. Although the production and function of secreted hydrolytic enzymes, such as cellulose, pectinase, or amylase, seem not to be affected in Um *pmt4* mutants, the *U. maydis* genome contains ~400 putative secreted proteins, any of which could be related to pathogenic processes or plant-fungal crosstalk (Kamper et al., 2006). The function of many of these proteins remains unknown, but it is likely that some of them could play a role in appressorium formation and host penetration. Sequence analysis of these proteins has shown that many of them are rich in Ser and Thr, which are typical residues targeted for O-mannosylation. Moreover, glycosylation is required for sorting, stability, and localization of secreted proteins. A defect in the secretion of any of these proteins caused by the loss of PMT4 could result in defects during appressorium formation and penetration into the plant.

Our results have shown that PMT4 is required for *U. maydis* virulence by affecting appressorium formation but is not essential for asexual growth. Significantly, while the PMT protein family is conserved in most fungi, it is not conserved in plants, making PMT4 a very attractive target for antifungal agents. Moreover, the PMT4 target protein(s) that are required for *U. maydis* virulence would also be potential targets for antifungal treatments. We are now initiating a search for PMT4 target proteins that could be implicated in plant pathogenesis through an in silico search as described by Hutzler et al. (2007).

METHODS

Strains, Growth Conditions, and Plasmids

Escherichia coli DH5 α and pGEM-T easy (Promega) were used for cloning purposes. *Schizosaccharomyces pombe* strains FY527 (h⁻, his3-D1, leu1-32, ura4-D18, and ade6-M216; Forsburg Lab), SBY88 (isogenic to FY527 except *ogm4::hisG*), and SBY90 (isogenic to FY527 except *ogm1::hisG*) (Willer et al., 2005) were grown in EMM medium supplemented with the necessary amino acids and glucose (2%, w/v) (Mitchison, 1970; modified by Nurse, 1975). All *Ustilago maydis* strains are described in Table 3. *U. maydis* cells were grown at 28°C in liquid YPD (2% bacto-peptone, 1% yeast extract, and 2% glucose) medium or solid YPD agar. Induction of the *nar* promoter in AB33 and its derivatives was done according to the protocol described by Brachmann et al. (2001). Mating assays were performed by cospotting compatible strains onto PD plates containing 1% charcoal (PD charcoal) and incubating them at 25°C for 1 to 2 d (Gillissen et al., 1992).

Generation of Mutant *U. maydis* Strains

Deletion constructs were generated according to Kamper (2004). To generate single deletion mutants of *pmt1*, *pmt2*, *pmt4*, *kre2*, *ktr3*, *mnn1*, and *mnt4* genes, fragments of the 5' and 3' flank of their open reading frames were generated by PCR on *U. maydis* FB1 genomic DNA with the following primer combinations: UmPMT1K05-1/UmPMT1K05-2 and UmPMT1K03-1/UmPMT1K03-2; UmPMT2K05-1/UmPMT2K05-2 and UmPMT2K03-1/UmPMT2K03-2; UmPMT4K05-1/UmPMT4K05-2 and UmPMT4K03-1/UmPMT4K03-2; UmKRE2K05-1/UmKRE2K05-2 and UmKRE2K03-1/UmKRE2K03-2; UmKTR3K05-1/UmKTR3K05-2 and UmKTR3K03-1/UmKTR3K03-2; UmMNN1K05-1/UmMNN1K05-2 and UmMNN1K03-1/UmMNN1K03-2; UmMNT4K05-1/UmMNT4K05-2 and UmMNT4K03-1/UmMNT4K03-2 (sequences in Supplemental Table 2 online). These fragments were digested with *Sfi*I and ligated into the 1.9-Kb *Sfi*I carboxin resistance cassette. To select double mutants, we used a 2.7-kb *Sfi*I hygromycin resistance cassette as described previously (Brachmann et al., 2004).

S. pombe PMTs Complementation

Fragments containing the open reading frames of *pmt1*, *pmt2*, and *pmt4* *U. maydis* genes were amplified by PCR using FB1 genomic DNA as template and the following primer combinations: 5'-AGGATCCATGAGTAGCCAGCTGCTAACAGC-3'/5'-ACCCGGTTCAGTAGATGTTCCAGCTCTTACG-3', 5'-ACTCGAGATGGCGTCGGTAGGCCAGACAGC-3'/5'-ACCCGGTTACAGCTCCTTGAGAATGACTTGTCG-3' and 5'-AGGATCCATGGTTCGACGCTACAAAAGC-3'/5'-ACCCGGTTCACITTGCAAAGTGGAGCG-3', respectively. These fragments were cloned into the expression vector pREP41X, obtaining the plasmids pREP41X-Umpmt1, pREP41X-Umpmt2, and pREP41X-Umpmt4. Transformed *S. pombe* cells were grown on EMM lacking Leu. As a control, the strains were transformed with the empty pREP41X vector. Cells were grown at 28°C in EMM liquid media supplemented with adenine, histidine, and uracil to exponential phase. Five microliters of 10-fold dilutions of each culture were then spotted onto EMM plates with the appropriate supplements and incubated for 3 d at 28 or 36°C.

Phylogenetic Analysis

PMT sequences were downloaded from the MIPS *U. maydis* database (<http://mips.gsf.de/genre/proj/ustilago/>) and NCBI (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). Multiple sequence alignments were made with CLUSTAL 2.0.2 using the default settings. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4

(Tamura et al., 2007) with the minimum evolution algorithms using 1000 bootstrap replications. Motif analysis was performed using Pfam from Sanger Institute (<http://pfam.sanger.ac.uk/>).

Pathogenicity Assays

Pathogenicity assays were performed as described by Kamper et al. (2006). *U. maydis* cultures were grown to exponential phase and concentrated to an OD₆₀₀ of 3, washed two times in water, and injected into young maize (*Zea mays*) seedlings (Early Golden Bantam) or directly through stigma into 2-week-old maize seedlings (var. Gaspar Flint). Tumor formation was quantified 7 to 25 d after infection. In the DPI treatment, DPI solved in DMSO was added at 0.5 μM directly to the *U. maydis* strains prior to inoculation. As a control, DMSO alone was added to the strains (Molina and Kahmann, 2007).

Flow Cytometry

To analyze the *pmt4* mutant DNA content, we used the protocol described by Garcia-Muse et al. (2003). Cells were grown in CMD overnight at 28°C and then placed into fresh CMD medium and incubated to an OD₆₀₀ of 0.8. Prior to analysis, cells were harvested, washed twice with cold water, fixed in 70% ethanol overnight, and resuspended in 50 mM sodium citrate, pH 7.5. Cellular RNA was destroyed by incubation with RNase A (0.25 mg/mL) at 50°C for 1 h and then proteinase K (1 mg/mL) was added and the cells were incubated for one more hour at 50°C.

Cells were stained at 4°C with propidium iodide (16 μg/mL). The fluorescence of 10,000 cells was measured using a FACSCalibur flow cytometer (Becton Dickinson) with a 530/30 bandpass filter.

Thermal Resistance and Drug Treatment Assays

To ascertain the effects of chemical agents on *pmt* mutants, we grew the strains to exponential phase in YPD at 28°C. Then, we performed a serial dilution patch test of Um *pmt* mutants and control strains on YPD agar plates with NaCl (to 0.25 and 0.5 M), congo red (to 15 and 30 μg/mL), chlorpromazine (50 and 75 μM), caffeine (1.5 and 3 mM), sorbitol (to 0.5, 1, and 1.5 M), calcofluor white (50 μM), SDS (0.005 to 0.01%), H₂O₂ (0.5 to 10 mM), and CaCl₂ (100 μM) growing at 28°C for 2 d. For these sensitivity studies, analytical grade reagents from Sigma-Aldrich were used. To analyze the response to thermal stress, we incubated the YPD agar plates at 34°C for 2 d.

Enzyme Secretion Assays

Cells were grown to exponential phase in YPD at 28°C. Then, we performed a patch test of Um *pmt* mutants and control strains on YPD agar plates with pectin, carboxymethyl-cellulose (CMC), and starch. Pectin was precipitated with 0.1% Ruthenium Red. CMC was stained with 0.1% congo red and starch precipitates with 100% ethanol. Pectin medium contains 0.1% ammonium sulfate, 0.1% sucrose, 0.25% pectin from citrus fruit, and 25 mM phosphate buffer, pH 7. CMC medium contains 0.25% carboxymethyl-cellulose, 0.1% ammonium sulfate, 0.1% sucrose, and 25 mM phosphate buffer, pH 7. Starch medium contains 0.25% starch from potato, 0.1% ammonium sulfate, 0.1% sucrose, and 25 mM phosphate buffer, pH 7.

Calcofluor White and Chlorazol Black E Staining

To analyze the prepenetration stages of *U. maydis* using fluorescence microscopy, cells were stained by adding calcofluor white on infected leaves using Fluorescent Brightener 28 (Sigma-Aldrich). Postpenetration

stages were studied by optical microscopy of CBE-stained leaf samples as previously described (Brachmann et al., 2003).

Microscopy

U. maydis cells were incubated in WGA staining solution (10 μg/mL WGA) for 30 min and washed in 1× PBS, pH 7.4. Cells were examined using a Leica fluorescence microscope, equipped with a PlanApo ×100 lens. Analysis of the prepenetration stages was done using a Deltavision wide-field microscope (Applied Precision). Image deconvolution was performed using z-series of between 7 and 23 focal planes, acquired at 0.5-μm intervals. Image processing was performed using Adobe Photoshop CS2 and Canvas 8.0 (Deneba).

DNA and RNA Procedures

Molecular biology techniques were used as described by Sambrook et al. (1989). *U. maydis* DNA was isolated following the protocol of Hoffman and Winston (1987). Standard *U. maydis* transformation procedure was used (Schulz et al., 1990). *S. pombe* molecular genetic techniques were used as described by Moreno et al. (1991). In the expression analysis, cells were washed with cold water, and total RNA was isolated with the Qiagen RNeasy mini kit, separated by formaldehyde denaturing agarose gel electrophoresis, and transferred overnight by capillary action to nylon membranes. To generate the probe for *Umpmt1* a 0.429-kb fragment was generated with the primer combination UmPMT1KO3-1/5'-ACGTCCTTGAGAATGACTTGTCGG; for *Umpmt2*, a 0.297-kb fragment was generated with the primer combination 5'-GTACGTCGCCAACTCGCTGCG-3'/5'-CCAGCTTACGCCACTTGAGG-3'; in the case of *Umpmt4*, a 0.303-kb fragment was generated using the primer combination 5'-UmPMT4KO3-1/5'-AAAGTGGAGCGTCCAGGATGG-3' (see Supplemental Table 2 online). Radioactive bands were visualized and quantified using a Molecular Dynamics PhosphorImager.

Accession Numbers

U. maydis PMT sequence data can be found in the GenBank/EMBL data libraries under accession numbers XP_762320 for *pmt1* (Um11220), XP_761621 for *pmt2* (Um10749), XP_761580 for *pmt4* (Um05433), XP_757301 for *kre2* (Um01154), XP_757968 for *ktr3* (Um01821), XP_761299 for *mnn1* (Um05152), and XP_761300 for *mnt4* (Um05153). *S. cerevisiae* PMT sequence data can be found under CAA64917 for *Pmt1p*, AAC04934 for *Pmt2p*, CAA58728 for *Pmt3p*, CAA58729 for *Pmt4p*, CAA98661 for *Pmt5p*, CAA97226 for *Pmt6p*, and Q06644 for *Pmt7p*. *C. albicans* PMT sequence under EEQ47067 for *pmt1*, EEQ44716.1 for *pmt2*, EEQ46011.1 for *pmt4*, EEQ43985 for *pmt5*, and EEQ45029 for *pmt6*. *Drosophila melanogaster* POMT sequence under NP_524025 for *pomt1* and NP_569858 for *pomt2*. Finally, *H. sapiens* POMT sequence data can be found under NP_009102.3 for *pomt1* and NP_037514.2 for *pomt2*.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Hydropathy Profiles of *U. maydis* PMT1, PMT2, and PMT4 with Regard to Sc *Pmt1p* Using a Window of 17 Amino Acids.

Supplemental Figure 2. Conservation of PMT Sequence Motifs in *U. maydis* PMTs.

Supplemental Figure 3. *U. maydis pmt1 pmt4* Double Mutants Are Viable, While the Loss of *pmt2* Is Lethal.

Supplemental Figure 4. Effects of *pmt4* Deletion on Pathogenicity in Plants Infected through Stigma.

Supplemental Figure 5. Budding Morphology and Cell Growth of $\Delta pmt1$ and $\Delta pmt4$ Strains Following WGA Staining.

Supplemental Figure 6. Assay of Hydrolytic Enzyme Secretion.

Supplemental Figure 7. *Pmt4* Disruption Results in Normal Sensitivity to Reactive Oxygen Species.

Supplemental Table 1. Annotation of O-Mannosyltransferases in *U. maydis*.

Supplemental Table 2. Primers Used in This Study.

Supplemental Data Set 1. Alignments of PMT Proteins Produced by CLUSTAL 2.0.2.

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