The O-Mannosyltransferase PMT4 Is Essential for Normal Appressorium Formation and Penetration in *Ustilago maydis*

Alfonso Fernández-Álvarez, Alberto Elías-Villalobos, and José I. Ibeas

Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide-Consejo Superior de Investigaciones Científicas, 41013 Sevilla, Spain

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-mannosyltransferase 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 adherence 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
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 Um11220, Um10749, and Um05433 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 dendogram 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 Δpmt1 and FB2 Δpmt1, or FB1 Δpmt4 and FB2 Δpmt4. 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 Δpmt1 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 (Man$_2$-Man$_3$) (Lussier et al., 1997a, 1997b), and MNN1 family proteins attach a fourth (Man$_4$) 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, Ax1p2p, 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; Willig 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).

Evidence for the PMT4 pathway in U. maydis was found in 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 *Δpmt2/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* (Gentzsch 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 *Δpmt1* and FB2 *Δpmt1* or compatible *pmt4* deletion mutants (FB1 *Δpmt4* and FB2 *Δpmt4*) (see Methods). Disease progression was evaluated 10 d after infection. The mixture of compatible *Δpmt1* 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 *Δpmt4* 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 *Δpmt4* mutants were also unable to form tumors in plants infected through the stigma, which presents a much weaker barrier to infection than leaf tissues (Snetsealaar 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 *Δpmt4* with the wild-type strain FB2 or FB2 *Δpmt4* 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 *Δpmt4* 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 (Bölkert 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* mutants *ogm1Δ* and *ogm4Δ*. 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 ogm1Δ*, 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 *ogm1Δ* 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* *ogm4Δ* strain forms aggregates when grown in liquid medium. The ability of *U. maydis* *pmt* genes to complement *ogm4Δ* was tested by transforming the mutant *ogm4Δ* 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

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No. of Plants</th>
<th>Tumor Formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1 × FB2</td>
<td>73</td>
<td>86.3</td>
</tr>
<tr>
<td>FB1 <em>Δpmt1</em> × FB2 <em>Δpmt1</em></td>
<td>38</td>
<td>89.5</td>
</tr>
<tr>
<td>FB1 <em>Δpmt4</em> × FB2 <em>Δpmt4</em></td>
<td>94</td>
<td>0.0</td>
</tr>
<tr>
<td>FB1 × FB2 <em>Δpmt4</em></td>
<td>32</td>
<td>87.5</td>
</tr>
<tr>
<td>FB1 <em>Δkre2</em> × FB2 <em>Δkre2</em></td>
<td>30</td>
<td>90.0</td>
</tr>
<tr>
<td>FB1 <em>Δktr3</em> × FB2 <em>Δktr3</em></td>
<td>34</td>
<td>88.2</td>
</tr>
<tr>
<td>FB1 <em>Δkre2</em> <em>Δktr3</em> × FB2 <em>Δkre2</em> <em>Δktr3</em></td>
<td>33</td>
<td>87.9</td>
</tr>
<tr>
<td>FB1 <em>Δmnn1</em> <em>Δmmt4</em> × FB1 <em>Δmnn1</em> <em>Δmmt4</em></td>
<td>29</td>
<td>93.1</td>
</tr>
<tr>
<td>SG200</td>
<td>84</td>
<td>91.7</td>
</tr>
<tr>
<td>SG200 <em>Δpmt4</em></td>
<td>75</td>
<td>0.0</td>
</tr>
</tbody>
</table>

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 *Δpmt4*, 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...
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

Figure 2. U. maydis PMTs Complement S. pombe ogm1Δ and ogm4Δ Mutants.

(A) Functional complementation of S. pombe ogm1Δ. Serial dilutions of wild-type S. pombe leu1-32 strain transformed with pREP41X and the ogm1 Δ 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 ogm1Δ mutant resulting in single cell growth.

(B) S. pombe ogm4Δ complementation. The ogm4Δ mutant cells form aggregates. The ogm4Δ 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 ogm4Δ mutant resulting in single cell growth.

(C) Quantification of pmt4 functional complementation of ogm4Δ. 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.
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 (Wille 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 Δkre2 Δktr3 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 Δkre2, Δktr3, and Δkre2 Δktr3 strains to the compounds and conditions previously assayed for the PMT mutants. As shown in Figure 3E, Δkre2 mutants demonstrated enhanced sensitivity to SDS, while their reaction to other drugs was normal. As Δkre2 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 the addition of further mannose residues by members of the PMT mutants. As shown in Figure 3E, Δkre2 mutants demonstrated enhanced sensitivity to SDS, while their reaction to other drugs was normal. As Δkre2 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 the 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 (Wille 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 Δkre2 Δktr3 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 Δkre2, Δktr3, and Δkre2 Δktr3 strains to the compounds and conditions previously assayed for the PMT mutants. As shown in Figure 3E, Δkre2 mutants demonstrated enhanced sensitivity to SDS, while their reaction to other drugs was normal. As Δkre2 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 the addition of further mannose residues by members of the PMT mutants. As shown in Figure 3E, Δkre2 mutants demonstrated enhanced sensitivity to SDS, while their reaction to other drugs was normal. As Δkre2 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 the addition of further mannose residues by members of the PMT mutants. As shown in Figure 3E, Δkre2 mutants demonstrated enhanced sensitivity to SDS, while their reaction to other drugs was normal. As Δkre2 mutants exhibit normal infectivity, it would suggest that pmt4 mutant sensitivity to SDS is unlikely to be responsible for its pathogenic defect.
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 ∆pmt4 strain because a compensatory response has been described in C. albicans pmt mutants (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 ∆pmt4 and FB2 ∆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 ∆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 ∆pmt4 YFP cells or equal numbers of SG200YFP and SG200 ∆pmt4 CFP strains. We then quantified the number of appressorium formation as described by Flor-Parr et al. (2006). In the quantitative comparison shown in the Figure 5B, we noticed that appressorium formation was reduced fivefold in SG200 ∆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 appressorium quantification process, we observed that in those cases where appressorium did form in ∆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 appressorium 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 appressorium morphology of wild-type U. maydis during the first stages of infection (Figure 5C). When we performed the same analysis on plants inoculated with ∆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 ∆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 appressorium 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 Δgas1 strain, which is able to penetrate plant cuticle, but its growth arrests after surface penetration (Schirawski et al., 2005). While wild-type and Δgas1 hyphae can be seen to invade beyond the epidermal cell layer, ∆pmt4 mutants were never observed to reach this stage of the infection process (Figure 6), resulting in all the ∆pmt4 hyphae being localized close to the plant surface. We measured the

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 μg of total RNA were loaded per lane. 18s RNA was used as a loading control.
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 ± 1.13 μm, in the gas1 mutant cells the last septum was localized 10.97 ± 3.06 μm from the tip, while in the case of the Δpmt4 strains, it was 17.62 ± 4.56 μm (Table 2). These results confirm that appressorium-mediated penetration in pmt4 mutant is defective.

Figure 5. The Deletion of pmt4 Affects Appressoria Production.

(A) Filament formation of the solopathogenic SG200 and SG200 Δpmt4 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 Δpmt4 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 Δpmt4, and SG200 YFP and SG200 CFP Δpmt4 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.
Taken together, our observations suggest that \textit{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 \textit{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). \textit{U. maydis yap1}, a gene involved in H$_2$O$_2$ 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 \textit{Dpmt4} cells is somehow related to problems in H$_2$O$_2$ detoxification, 0.5 mM DPI was added to the \textit{Dpmt4} cell culture during inoculation. The suppression of ROS generation by treatment with DPI did not restore the phenotype of the \textit{Dpmt4} strain as it did on the \textit{Dyap1} strain (Figure 7). Thus, consistent with the normal sensitivity to H$_2$O$_2$ (see Supplemental Figure 7 online), the virulence phenotype of \textit{U. maydis Dpmt4} 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>
<thead>
<tr>
<th>Inoculum</th>
<th>No. of Appressorium-Mediated Penetrations/No. of Appressorium</th>
<th>Percentage</th>
<th>Distance from Hyphal Tip to the Closest Visible Septum (μm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1 × FB2</td>
<td>96/97</td>
<td>98.97</td>
<td>6.15 ± 1.13</td>
<td>49</td>
</tr>
<tr>
<td>FB1 \textit{Dgas1} × FB2 \textit{Dgas1}</td>
<td>46/47</td>
<td>97.87</td>
<td>10.97 ± 3.06</td>
<td>29</td>
</tr>
<tr>
<td>FB1 \textit{Dpmt4} × FB2 \textit{Dpmt4}</td>
<td>0/73</td>
<td>0.00</td>
<td>17.62 ± 4.56</td>
<td>47</td>
</tr>
</tbody>
</table>

Maize seedlings were infected with the strains indicated above. Two days after infection, leaf samples were stained with Chlorozole 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).
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 planta, with a drastic reduction in appressorium formation and, more importantly, those appressoria that do form are nonfunctional in planta.

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

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; Williger 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 endoplasmatic reticulum (Nakatsukasa et al., 2004), the maintenance of cell polarity (Lommel et al., 2004; Proszyński 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 Δpmt1 and Δpmt4 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 family...
families in these contexts. Individual deletions of six of the seven putative \textit{U. maydis} \textit{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 \textit{pmt4} and \textit{kre2} resulted in slight cell wall integrity defects, as revealed by sensitivity to SDS in these two mutants. Of these two genes, only \textit{pmt4} seems to be involved in pathogenesis. Thus, defects in cell wall integrity do not correlate with a defect in pathogenesis nor is it 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 \textit{O}-mannosyltransferase seem to specifically affect pathogenesis. This finding is not surprising since specific targets of PMT4 and phenotypes exclusively depending on this \textit{O}-mannosyltransferase have been described previously (Gentzsch and Tanner, 1997; Proszynski et al., 2004). Thus, we can postulate that one or more targets of \textit{U. maydis} \textit{pmt4} are important for virulence. The addition of the first three mannose residues is sufficient for these proteins 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 \textit{S. cerevisiae}. Of these targets, we have identified \textit{U. maydis} homologs for \textit{Sc} Kex2p (Um02843), \textit{Sc} Gas1p (Um01640), and less clearly for \textit{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 the deletion of \textit{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 \textit{U. maydis}, although we are currently working to test this experimentally. \textit{Sc} Fus1p, \textit{Sc} Opy2p, and \textit{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 \textit{U. maydis} contains only some elements of the yeast filamentous growth mitogen-activated protein kinase pathway. We have identified a \textit{U. maydis} homolog for \textit{Sc} Msb2p (Um01513), but none were found for \textit{Sc} Fus1p or \textit{Sc} Opy2p. Although we cannot currently exclude a role for any of these genes without further investigation, we think that the \textit{pmt4}

![Table 3. \textit{U. maydis} Strains Used in This Study](image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1</td>
<td>\textit{a1 b1}</td>
<td>Banuett and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herskovitz (1989)</td>
</tr>
<tr>
<td>FB2</td>
<td>\textit{a2 b2}</td>
<td>Banuett and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herskovitz (1989)</td>
</tr>
<tr>
<td>FBD11</td>
<td>\textit{a1 a2 b1 b2}</td>
<td>Banuett and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herskovitz (1989)</td>
</tr>
<tr>
<td>SG200</td>
<td>\textit{a1 mfa2 bW2 bE1}</td>
<td>Böker et al. (1995)</td>
</tr>
<tr>
<td>AB33</td>
<td>\textit{a2 Pnar:bw2 Pnar:bE1}</td>
<td>Brachmann et al. (2001)</td>
</tr>
<tr>
<td>AB34</td>
<td>\textit{a2 Pnar:bw2 Pnar:bE2}</td>
<td>Brachmann et al. (2001)</td>
</tr>
<tr>
<td>HBU13</td>
<td>\textit{a1 b1 gma1}</td>
<td>Schirawski et al. (2005)</td>
</tr>
<tr>
<td>HBU14</td>
<td>\textit{a2 b2 gma1}</td>
<td>Schirawski et al. (2005)</td>
</tr>
<tr>
<td>SG200CFP</td>
<td>\textit{a1 mfa2 bw2 bE1}</td>
<td>Flor-Parra et al. (2006)</td>
</tr>
<tr>
<td>P_{OMA1}CFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SG200YFP</td>
<td>\textit{a1 mfa2 bw2 bE1}</td>
<td>Flor-Parra et al. (2006)</td>
</tr>
<tr>
<td>P_{OMA1}YFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SG200 \textit{Aynp1}</td>
<td>\textit{a1 mfa2 bw2 bE1 Aynp1}</td>
<td>Molina and Kahmann (2007)</td>
</tr>
<tr>
<td>UMG1</td>
<td>\textit{a1 b1 Dpm1}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG2</td>
<td>\textit{a2 b1 Dpm1}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG3</td>
<td>\textit{a1 a2 b1 b2 Dpmt2}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG4</td>
<td>\textit{a1 \textit{b} \textit{a} \textit{pmt4}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG5</td>
<td>\textit{a2 b2 \textit{pmt4}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG6</td>
<td>\textit{a2 Pnar:bw2 Pnar:bE1}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG7</td>
<td>\textit{a1 b1 \textit{k} \textit{kre2}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG8</td>
<td>\textit{a2 b2 \textit{k} \textit{kre2}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG9</td>
<td>\textit{a1 b1 \textit{k} \textit{kre3}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG10</td>
<td>\textit{a2 b2 \textit{k} \textit{kre3}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG11</td>
<td>\textit{a1 b1 \textit{k} \textit{kre2} \textit{k} \textit{kre3}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG12</td>
<td>\textit{a2 b2 \textit{k} \textit{kre2} \textit{k} \textit{kre3}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG13</td>
<td>\textit{a1 b1 \textit{mnn1}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG14</td>
<td>\textit{a2 b1 \textit{mnn1}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG15</td>
<td>\textit{a1 b1 \textit{mnn4}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG16</td>
<td>\textit{a2 b2 \textit{mnn4}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG17</td>
<td>\textit{a1 b1 \textit{mnn1} \textit{mnn4}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG18</td>
<td>\textit{a2 b2 \textit{mnn1} \textit{mnn4}}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG19</td>
<td>\textit{a1 mfa2 bw2 bE1}</td>
<td>This work</td>
</tr>
<tr>
<td>UMG20</td>
<td>\textit{a1 mfa2 bw2 bE1 \textit{pmt4}}</td>
<td>This work</td>
</tr>
<tr>
<td>P_{OMA1}CFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UMG21</td>
<td>\textit{a1 mfa2 bw2 bE1}</td>
<td>This work</td>
</tr>
<tr>
<td>P_{OMA1}YFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UMG22</td>
<td>\textit{a2 Pnar:bw2 Pnar:bE1}</td>
<td>This work</td>
</tr>
<tr>
<td>P_{OMA1}YFP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Figure 8. Schematic Model of the Effects on Cell Wall Integrity and Pathogenesis of Protein \textit{O}-Mannosylation in \textit{U. maydis}.](image)
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 hypa without appressorium 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 penetration. Although the production and function of secreted hydrolytic enzymes, such as cellulose, pectinase, or amylase, seem not to be affected in U. maydis mutants, the cellulose, pectinase, or amylase, seem not to be affected in U. maydis and function of secreted hydrolytic enzymes, such as reflects a problem in the protein secretion process during ap- et al., 2003). (4) A fourth possibility is that the loss of infectivity produced by the plant and, therefore, its virulence (Narasimhan the resistance of the fungus to pathogenesis-related proteins

METHODS

Strains, Growth Conditions, and Plasmids

Escherichia coli DH5α and pGEM-T easy (Promega) were used for cloning purposes. Schizosaccharomyces pombe strains FY527 (his, his3-d1, leu1-32, ura4-D18, and ade6-M216; Forbsurg 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% bactopeptone, 1% yeast extract, and 2% glucose) medium or solid YPD agar. Induction of the npr promoter in AB33 and its derivatives was done according to the protocol described by Brachmann et al. (2001). Mating assays were performed by cross-pollinating 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’ flanks of their open reading frames were generated by PCR on U. maydis FB1 genomic DNA with the following primer combinations: UmPMT1KO5-1/UmPMT1KO5-2 and UmPMT1KO3-1/UmPMT1KO3-2; UmPMT2KO5-1/UmPMT2KO5-2 and UmPMT2KO3-1/UmPMT2KO3-2; UmPMT4KO5-1/UmPMT4KO5-2 and UmPMT4KO3-1/UmPMT4KO3-2; UmKRE2KO5-1/UmKRE2KO5-2 and UmKRE2KO3-1/UmKRE2KO3-2; UmKTR3KO5-1/UmKTR3KO5-2 and UmKTR3KO3-1/UmKTR3KO3-2; UmMNN1KO5-1/UmMNN1KO5-2 and UmMNN1KO3-1/UmMNN1KO3-2; UmMT4KO5-1/UmMT4KO5-2 and UmMT4KO3-1/UmMT4KO3-2 (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’-AGATCATGAG-TAGCCCGTGCTAAACG-3’/5’-ACCCGGGTCACTTTGCAAG-3’ and 3’-ACTGAGATGGCGCTGCGTGAAGCGCAGACAGC-3’/5’-CCGGGTTACTACGGTCTTGGAGAATGACTTGCG-3’ and 5’-AGG-CTCAGTGGTCGCTACAAAGC-3’/5’-ACCCGGGTTACCTGCTTGGCAAGACCG-3’ respectively. These fragments were cloned into the expression vector pREP41X, obtaining the plasmids pREP41X-Ummt1, 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 2.02 using the default settings. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4
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 OD$_{600}$ 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), chlorpromazine (50 and 75 μM), caffeine (1.5 and 1.5 M), calcofluor white (50 μM), SDS (0.005 to 0.01%), H$_2$O$_2$ (0.5 to 10 mM), and CaCl$_2$ (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.

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 Leica DMR 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-AGTCCTTGAGAATGACTTGTCGG; for _Umpmt2_, a 0.297-kb fragment was generated with the primer combination 5’-GTAGCTGGCAACAATCGTGG-3’/5’-CAAGCTTACG-CACCTGGA-3’; in the case of _Umpmt4_, a 0.303-kb fragment was generated using the primer combination 5’-UmPMT4KO3-1/-5-AAA-GTGGAGGTCCAGGATGG-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_75730 for _pmt2_ (Um11220), XP_761621 for _pmt2_ (Um10749), XP_761580 for _pmt4_ (Um05433), XP_757301 for _ktr3_ (Um01154), XP_757968 for _ktr3_ (Um01821), XP_761299 for _pmt1_ (Um05153), and XP_761300 for _pmt6_ (Um05153).

_S. pombe_ PMT sequence data can be found under accession numbers XP_757968 for _pomt1_ (Um10749), XP_757968 for _pomt2_ (Um01154), XP_757968 for _pomt3_ (Um01821), XP_761299 for _pomt1_ (Um05153), and XP_761300 for _pomt6_ (Um05153).

_S. cerevisiae_ PMT sequence data can be found under accession numbers AAC493985 for _pmt1_ and AAC493985 for _pmt6_. _Drosophila melanogaster_ PMT sequence under accession number NP_524025 for _pomt1_ and NP_569858 for _pomt2_. Finally, _H. sapiens_ PMT sequence data can be found under accession numbers 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.** Leaky Phenotypes 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_.

**Supplemental Figure 3.** _U. maydis_ _pmt1_ _pmt2_ Double Mutants Are Viable, While the Loss of _pmt2_ Is Lethal.

(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/).
Supplemental Figure 4. Effects of pmt4 Deletion on Pathogenicity in Plants Infected through Stigma.

Supplemental Figure 5. Budding Morphology and Cell Growth of Δpmt1 and Δpmt4 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.

ACKNOWLEDGMENTS

We thank all members of the genetics area for useful discussion, José Pérez Martín and his group for technical assistance, and Angel M. Carrión and John R. Pearson for his critical reading of the manuscript. We thank all of the Ustilago community for providing strains and plasmids and S. Strahl-Bolsinger for the S. pombe strains. This work was supported by Ministerio de Ciencia e Innovación Grant BIO2007-60531. A.F.-A. and A.E.-V. were supported by fellowships from Ministerio de Ciencia e Innovación. Centro Andaluz de Biología del Desarrollo is institutionally supported by Consejo Superior de Investigaciones Científicas, Universidad Pablo de Olavide, and Junta de Andalucía.

Received January 21, 2009; revised September 7, 2009; accepted October 5, 2009; published October 30, 2009.

REFERENCES


The $O$-Mannosyltransferase PMT4 Is Essential for Normal Appressorium Formation and Penetration in *Ustilago maydis*

Alfonso Fernández-Álvarez, Alberto Elías-Villalobos and José I. Ibeas

*Plant Cell*; originally published online October 30, 2009; DOI 10.1105/tpc.109.065839

This information is current as of June 29, 2017

<table>
<thead>
<tr>
<th>Supplemental Data</th>
<th>/content/suppl/2009/10/09/tpc.109.065839.DC1.html</th>
</tr>
</thead>
<tbody>
<tr>
<td>eTOCs</td>
<td>Sign up for eTOCs at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a></td>
</tr>
<tr>
<td>CiteTrack Alerts</td>
<td>Sign up for CiteTrack Alerts at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a></td>
</tr>
<tr>
<td>Subscription Information</td>
<td>Subscription Information for <em>The Plant Cell</em> and <em>Plant Physiology</em> is available at: <a href="http://www.aspb.org/publications/subscriptions.cfm">http://www.aspb.org/publications/subscriptions.cfm</a></td>
</tr>
</tbody>
</table>

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY