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The requirement for protein *O*-mannosylation for *Ustilago maydis* virulence seems to be linked to intrinsic aspects of the infection process rather than an altered plant response

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Fungal plant pathogenesis involves complex crosstalk between fungi and their plant hosts. In the case of biotrophic fungi, the host interaction is finely controlled to maintain plant viability during infection since the fungus depends on the survival of colonized plant cells. Many proteins which participate in this process are thought to be glycosylated. Thus, defects in the glycosylation of fungal proteins might alter the normally attenuated plant response and consequently affect fungal progression. *O*-mannosyltransferases are responsible for adding mannose residues onto target proteins, with each *O*-mannosyltransferase having individual target specificities. In an earlier study, we showed that *O*-mannosylation is essential for *Ustilago maydis* virulence. We found that the loss of *O*-mannosyltransferase PMT4 was associated with a reduced formation frequency of the invasive morphogenic structure known as the appressorium, combined with a loss in their ability to penetrate plant cuticle. Here, we discuss the possible molecular causes of these phenotypes and present additional evidence, which argue against an alteration of plant response to fungal infection as the primary cause of the Δ*pmt4* phenotype.

Key words: *O*-mannosylation, corn smut, host response, appressorium development, PMT, trypan blue, reactive oxygen species (ROS) production

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Ustilago maydis is a model for plant pathogenesis which exhibits a biotrophic interaction with its host, *Zea mays*, meaning that the fungus needs to maintain the plant cells alive in order to complete its life cycle. During this process, *U. maydis* induces tumor formation on all aerial parts the maize plant. In the early stages of infection, fungal cells differentiate to form polarized infection hyphae in order to penetrate into the plant.[1]

Glycosylation is the most common eukaryotic posttranslational protein modification. The majority of proteins synthesized in the endoplasmatic reticulum, including receptors, cell wall components and secreted proteins, are glycoproteins.[2] *O*-mannosylation is a type of glycosylation which consists of the addition of mannose residues to target proteins. In *Saccharomyces cerevisiae*, this is catalyzed by members of the PMT (Protein Mannosyltransferase) family. Many of these glycoproteins are likely to participate in fungal pathogenic development since mutants with altered *O*-mannosylation patterns show reduced virulence in *Candida albicans* or *Cryptococcus neoformans*.[3,4] We have previously shown that the loss of *pmt4*, one of three *U. maydis* PMT homologues, is associated with defective appressoria production and penetration, together with the loss of *U. maydis* infectivity.[5] Two possibilities might explain the defects exhibited by *pmt4* mutants; (1) a disrupted *U. maydis*-maize pathosystem, where *O*-glycosylation defects in the *pmt4* mutant induce altered plant responses which indirectly affect *U. maydis* pathogenic development, or alternatively, (2) an intrinsic problem in the mutant fungal cells, where defects in cell wall structure, protein secretion, adhesion or sensing capacities affect fungal progression and plant infection.

An important part of a plant's reaction to fungal infection is the production of Reactive Oxygen Species (ROS), which plays an important role in regulating

fungal infection-associated plant cell death.[6] Fungi need to detoxify plant ROS in order to permit pathogenic development. A defect in ROS detoxification in *pmt4* mutants, such as has been shown for *yap1* mutant fungi,[7] or a specific ROS overproduction by plant cells in response to *pmt4* infection, could be responsible for the infection defects observed in *pmt4* mutant. Diaminobenzidine (DAB) staining can be used to detect H_2O_2 in plant tissues through its conversion to dark-brown polymers.[7] To analyze if the deletion of *pmt4* affects ROS detoxification or ROS production in the plant we performed DAB staining on plants inoculated with the wild-type, *pmt4* or *yap1* mutant strains. As expected we observed strong DAB staining for *yap1* but not for either wild-type or Δ*pmt4* inoculations (**Fig. 1A**). To confirm that the impaired infectivity of *U. maydis* Δ*pmt4* is not due to plant ROS production, we treated infected plants with DPI, an inhibitor of the plant NADPH oxidase. We found that, while the *yap1* infection defect was fully rescued by DPI treatment, but the same treatment had no effect on the infectivity of *pmt4* mutants.

As *U. maydis* is a biotrophic fungus, it is essential for it to minimize the induction of programmed cells death associated with infection.[1] The deletion of *pmt4* might alter the crosstalk between fungi and host, increasing the number of dead plant cells and thus affect infection progression. To detect necrotic plant tissues we stained inoculated plants with trypan blue.[8] We did not observe any differences in the pattern of trypan blue staining of plant cell tissues following wild-type and *pmt4* mutant infections (**Fig. 1**). In conclusion, ours observations suggest that the loss of *pmt4* does not significantly affect the plant response to *U. maydis* infection in terms of ROS production or increased cell death.

While further work will needed to exclude other types of plant response such as an altered interaction with defense related proteins (PR)[9] or jasmonate (JA) signaling,[10] in the absence of evidence to the contrary, our results seem to point to the alternative hypothesis where altered *O*-glycosylation in *pmt4* mutants affects the intrinsic capacity of *U. maydis* to complete the pathogenic program. Such intrinsic defects could manifest themselves in several ways e.g., cell wall structure or protein secretion.

The fungal cell wall is the primary contact surface between the fungus and plant.[11] Alterations in cell wall integrity is a characteristic phenotype of PMT mutants,[12] and could cause the reduced production of appressoria and the inability of those appressoria that do form to penetrate shown in *U. maydis pmt4* mutants. We previously analyzed the state of cell wall integrity in *pmt4* mutant by studying cell response to thermal, oxidative, osmotic and salt-based stresses. *U. maydis pmt4* mutant showed slight sensitivity to SDS, which has previously been linked to cell wall integrity defects.[4] Chitin is one of the main components of fungi cell wall[13] and lectins from *Triticum vulgaris* (WGA) and calcofluor-white staining might be used to reveal cell wall chitin content defects. However, using these techniques, we have not observed any differences in the staining patterns between wild-type and *pmt4* mutant strains (unpublished observations). We are currently analyzing the cell wall structure of *pmt4* mutants by electron microscopy. Preliminary results using scanning electron microscopy have so far not revealed any differences in cell wall appressorium (unpublished observations) but further studies using transmission electron microscopy will be necessary to confirm these findings.

Adhesion to hydrophobic surfaces is required for appressorium formation in *U. maydis*[14] and is also essential for appressorium-mediated penetration in other phytopathogenic fungi.[15] Glycosylation mutants in *C. albicans* have been shown to affect host adhesion.[16] It is possible that *U. maydis pmt4* mutants do not properly form appressoria, and those that are formed are not able to penetrate the plant because of an adhesion defect. To test this possibility, we are currently performing cell adhesion assays studying the capacity of cell-solid surface and cell-cell adhesion. Our preliminary observations suggest that *pmt4* colonies do indeed have a reduced capacity to adhere to agar in solid medium and may also have an altered invasive growth capacity, although their cell to cell adhesion capacity does not seem to be affected (unpublished observations). Hydrophobicity as well as chemical signals from plant must be sensed by fungi to induce normal appressoria development,[14] and the membrane receptors involved in this signaling are usually glycosylated, thus a defect in protein glycosylation might produce sensing defects. Preliminary studies on appresorium formation in vitro, where appresorium formation is induced by only a hydrophobic surface and a chemical signal, such as 12-hydroxystearic or 16-hydroxy-hexadecanoic acid, have shown that *pmt4* mutants exhibit similar appressoria defects as occur in plant infections (unpublished observations). This type of assay will enable us to analyze how the *pmt4* mutation affects the sensing of physical and chemicals conditions, and to identify which signal/s (if any) are not properly detected in *pmt4* mutants. Although further investigation is required to define the molecular basis for the failure normal appressorium development in *pmt4* mutants, our preliminary results strongly point towards the abnormal signal sensing and adhesion as important causes contributing to the failure pathogenic development in *pmt4* deletion mutants.

The *U. maydis* genome contains a large number of putative secreted proteins which could be related to the infection process.[17] In *U. maydis* the secretion of cell wall degrading enzymes are likely to be required for appressorium-mediated penetration and their glycosylation is probably required for their function. We have analyzed the hydrolytic activity of *pmt4* mutant fungi on several substrates, but so far, we have not observed any differences in hydrolytic activity compared to wild type cells. However, we cannot exclude the possibility that some other Ustilago specific cell wall degrading activity could be responsible for the *pmt4* penetration phenotype. We are now working on an in silico search for putative PMT4 targets proteins[18] and analyzing their role in *U. maydis* pathogenic program.

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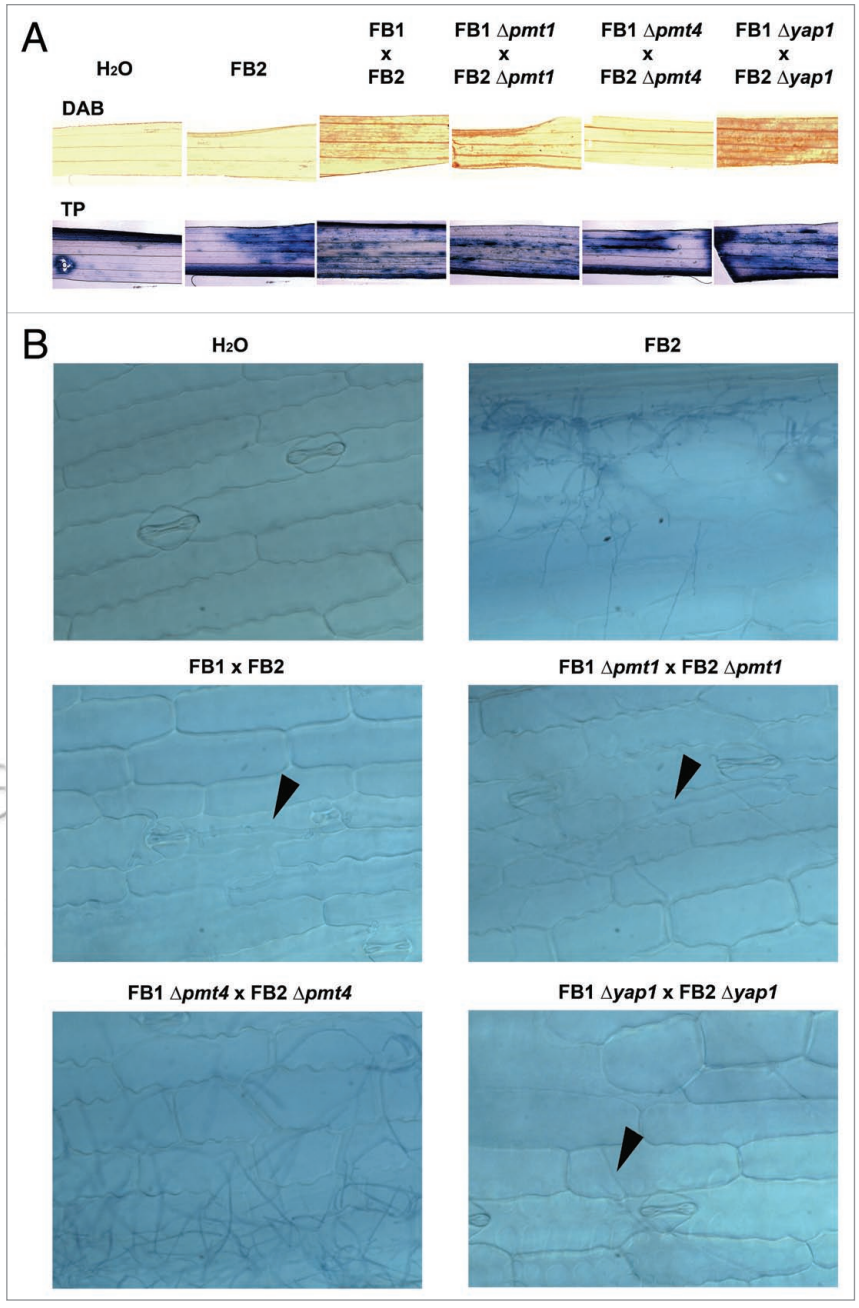

Figure 1. The deletion of *pmt4* does not affect host normal response. Plant ROS production and epidermal cell death are features of a pathogenic fungal infection. These responses can be monitored by staining with diaminobenzidine (DAB) and trypan blue, respectively. (A) Infected leaves with compatible wild-type strains, *pmt1*, *pmt4* and *yap1* mutants, and FB2 and H_2O as controls, were stained with solution of DAB or trypan blue. For DAB treatment, leaves were stained with DAB solution (1 mg/mL) for 16 h under darkness. Leaves were decolorized with ethanol. Brown polymerization products indicate a reaction of DAB with H_2O_2. Only *yap1* mutant infected leaves showed strong DAB staining. For the trypan blue assay, infected tissues were covered with trypan blue solution (0.01 g/mL), the staining solution was boiled for one minute on the plant and left overnight in the staining solution, chloral hydrate was used for washing. No differences were observed between plants infected with compatible wild-type or *pmt1*, *pmt4* and *yap1* mutants strains. (B) Optical microscopy confirms the absence of necrotic tissues with trypan blue staining. Arrows point to hyphae invading into the plant, for the cases where there is no plant penetration (FB2 and *pmt4* mutants) hyphae are localized on the plant surface.