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MPP-OA-14-274

"The Penicillium digitatum protein O–Mannosyltransferase Pmt2 is required for cell wall integrity, conidiogenesis, virulence and sensitivity to the antifungal peptide PAF26"

Harries, Eleonora; Gandía, Mónica; Carmona, Lourdes; Marcos, Jose F.

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Sincerely,

Gert HJ Kema

Senior Editor, Molecular Plant Pathology
gert.kema@wur.nl

Reviewer(s)' comments:
Referee: 1

Comments to the Author
The current work is aimed at identification and characterization the function of pmt genes in Penicillium digitatum as far as its effect on cell wall integrity, morphogenesis, virulence and sensitivity to anti-fungal protein PAF26.
Overall, this work is very well written and results are presented adequately. The findings are very interesting and shed light on the role of protein O–Mannosyltransferase in the biology of P. digitatum.
Except for minor comments , mainly in the material and methods sections, that are indicated directly in the text, this reviewers finds the manuscript in good shape and recommend its acceptance.

Referee: 2

Comments to the Author
This works shows the effect of PMT genes of Penicillium digitatum, specifically pmt2, on fungal growth and virulence on citrus fruit.
The subject is quite original and the information obtained could be useful for a future management of this disease by using antifungal peptide as PAF26. This basic research provides new and interesting information to understand the role of some of these Pmts on virulence factor of fungal postharvest pathogens.
The structure is consistent with that required by the journal.

In general, the methodology is appropriate and can achieve the objectives of the work. However, some section are explanting too much details (construction of plasmids vector, transformation, ...) and other not enough (Infection assays: variety of oranges? Harvest season? Quality parameter of fruit? Many works on fruit have demonstrated the important of quality parameter of fruit to evaluate the pathogenity of postharvest diseases. How did you made wounds? How did you evaluate conidiation index? Statistical analysis to virulence assays: Show mean ±SD is not enough (fig 7)!

In general, results are well presented and maintain a logical and appropriate sequence. However, statistical analysis is not rather adequate; For example, from relative expression results obtained in Fig 3 you chose pmt2 to continue your work; however the relative expression of both pmt2 and pmt4 at 4 pdi in (A) experiment is equal, but this behaviour was not repeated at (B) experiment. Do you believe that from these results is consistent enough to continue your study only with pmt2 and not pmt4?

The discussion can take advantage of all the results obtained by you and achieve a good discussion related to the experience of other authors.

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Molecular Plant Pathology

The *Penicillium digitatum* protein *O*–Mannosyltransferase Pmt2 is required for cell wall integrity, conidiogenesis, virulence and sensitivity to the antifungal peptide PAF26

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The *Penicillium digitatum* protein *O*–Mannosyltransferase Pmt2 is required for cell wall integrity, conidiogenesis, virulence and sensitivity to the antifungal peptide PAF26.

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Running title: *P. digitatum* protein glycosylation and virulence

Keywords: *Penicillium digitatum*, protein glycosylation, postharvest decay, citrus fruit, cell wall, antifungal peptides.

Word Count (Title, References and Supporting material not included): 6,998

SUMMARY

The activity of protein *O*-mannosyltransferases (Pmt) affects the morphogenesis and virulence of fungal pathogens. *PMT* genes have been recently shown to determine the sensitivity of *Saccharomyces cerevisiae* to the antifungal peptide PAF26. This study reports the identification and characterisation of the three *Pdpmt* genes in the citrus postharvest pathogen *Penicillium digitatum*. The *Pdpmt* genes are expressed during fungal growth and fruit infection, with the highest induction for *Pdpmt2*. *Pdpmt2* complemented the growth defect of the *S. cerevisiae* Δ*pmt2* strain. The *Pdpmt2* gene mutation in *P. digitatum* caused pleiotropic effects, including the reduction of fungal growth and virulence, while its constitutive expression had no phenotypic effect. The *Pdpmt2* null mutants also showed a distinctive colourless phenotype with a strong reduction in the number of conidia, which was associated with severe alterations in the development of conidiophores. Additional effects of the *Pdpmt2* mutation were hyphal morphological alterations, increased sensitivity to cell wall-interfering compounds, and a blockage of invasive growth. In contrast, the *Pdpmt2* mutation increased tolerance to oxidative stress and to the antifungal activity of PAF26. These data confirm the role of protein *O*-glycosylation in the PAF26-mediated antifungal mechanism present in distant fungal species. Important to future crop protection strategies, this study demonstrates that a mutation rendering fungi more resistant to an antifungal peptide results in severe deleterious effects on fungal growth and virulence.

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INTRODUCTION

Protein glycosylation is a post-translational modification conserved in organisms from yeasts to humans (Lehle *et al.*, 2006). The modification consists of the attachment of different glycan structures to specific proteins, either at an asparagine residue (N-glycosylation) or at serine or threonine residues (O-glycosylation). In fungi, protein glycosylation contributes to the function of proteins involved in important processes such as cell wall (CW) integrity, sensing environmental signals, morphogenesis and the virulence of fungal pathogens (Gentzsch and Tanner, 1996; Prill *et al.*, 2005; Olson *et al.*, 2007; Zhou *et al.*, 2007; Fernández-Álvarez *et al.*, 2012).

Protein O-mannosyltransferases (Pmts) catalyse the first step in protein O-mannosylation, transferring mannose to the hydroxyl groups of serine/threonine residues in the nascent protein chain at the lumen of the endoplasmic reticulum (Loibl and Strahl, 2013). Pmts have been identified in prokaryotes, fungi and animals but appear to be absent in plants (Girrbach and Strahl, 2003; Loibl and Strahl, 2013), which makes them a potential target for antifungal strategies in plant protection (Fernández-Álvarez *et al.*, 2009; González *et al.*, 2013). In the budding yeast *Saccharomyces cerevisiae* there are six Pmts divided in the Pmt1, Pmt2 and Pmt4 subfamilies, which differ in the number of genes in each and protein substrate specificity (Gentzsch and Tanner, 1996; Girrbach and Strahl, 2003; Loibl and Strahl, 2013). The Pmt1 and Pmt2 subfamilies are redundant, and their members form heterodimers, while Pmt4 is a single member subfamily and acts as a homodimer. The *PMT* genes in *S. cerevisiae* are not individually essential for viability, likely due to gene redundancy,

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although several combinations of double mutants are lethal (Gentzsch and Tanner, 1996; Loibl and Strahl, 2013).

In the fission yeast *Schizosaccharomyces pombe* or in filamentous fungi such as *Aspergillus nidulans*, the human pathogen *Aspergillus fumigatus*, or the plant pathogens *Ustilago maydis* and *Botrytis cinerea*, only one member for each Pmt subfamily has been identified (Oka *et al.*, 2004; Willer *et al.*, 2005; Fernández-Álvarez et al., 2009; Goto *et al.*, 2009; Mouyna *et al.*, 2010; González et al., 2013). Importantly, the *pmt2* subfamily is essential for the viability of several fungi studied so far. The requirement of *pmt* genes in fungal pathogenesis is variable and depends on the specific pathosystem. Both *pmt1* and *pmt4* of the human pathogens *Candida albicans* and *Cryptococcus neoformans* are necessary for virulence to varying degrees (Timpel *et al.*, 1998; Prill et al., 2005; Olson et al., 2007; Willger *et al.*, 2009). In contrast, mutants in either *pmt1* or *pmt4* of *A. fumigatus* are as virulent as the parental strain in a murine model (Zhou et al., 2007; Mouyna et al., 2010). In *U. maydis,* only the disruption of *pmt4* specifically affected appressorium formation, penetration and tumour formation in maize, while *pmt1* disruption was dispensable for plant infection (Fernández-Álvarez et al., 2009). In *B. cinerea*, the three *pmt* genes including *pmt2* are required for the pathogenesis of different plants and plant organs (González et al., 2013).

Penicillium digitatum causes the citrus fruit green mould disease and contributes up to 5-10 % of postharvest losses in the main fruit tree crop in the world. *P. digitatum* is a necrotroph and a wound pathogen that requires a pre-existing injured fruit peel to penetrate and infect. Despite this rather unspecific

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infection mechanism, *P. digitatum* exhibits an exquisite and high degree of host specificity. The most common method to control *P. digitatum* is the postharvest application of fungicides, but their continued use has a negative impact on human health and the environment and has increased the appearance of resistance isolates. The economic interest in *P. digitatum* has promoted the use of molecular and genetic tools to characterise the fruit-fungus interaction (González-Candelas *et al.*, 2010; Ballester *et al.*, 2011; Buron-Moles *et al.*, 2012) and fungal pathogenicity (Wang and Li, 2008; Zhang *et al.*, 2013a; Zhang *et al.*, 2013b; Gandía *et al.*, 2014). These efforts are exemplified in the recent release of the genome sequence of three strains (Marcet-Houben *et al.*, 2012; Sun *et al.*, 2013).

Antimicrobial peptides (AMPs) are promising alternatives to control pathogens in plant protection (Marcos *et al.*, 2008). PAF26 belongs to the cationic and tryptophan-rich PAF series of synthetic peptides (López-García *et al.*, 2000). PAF26 is a hexapeptide that inhibits the growth of filamentous fungi including *P. digitatum* and is effective in controlling citrus fruit infection (Muñoz *et al.*, 2007a). Previous studies with the model fungi *S. cerevisiae* and *Neurospora crassa* have revealed insights into the mode of PAF26 antifungal action (Muñoz *et al.*, 2012; Muñoz *et al.*, 2013b). PAF26 is a cell penetrating antifungal peptide that first interacts with the fungal cell envelope and then translocates to the cell interior where it kills the fungal cell (reviewed in Muñoz *et al.*, 2013a). In *S. cerevisiae*, PAF26 treatment induces genes encoding highly glycosylated CW proteins (López-García *et al.*, 2010), and the disruption of protein N- and O-glycosylation genes results in enhanced tolerance to PAF26 (Harries *et al.*, 2013).

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In this study, the identification and characterisation of *pmt* genes from *P. digitatum* was conducted. One member from each *pmt1*, *pmt2* and *pmt4* subfamily was identified and shown to be expressed during fungal growth and citrus infection. The study reports the functional characterisation of *Pdpmt2* in *P. digitatum* and its requirement for fungal growth, virulence and sensitivity to PAF26.

RESULTS

Identification and sequence analysis of Pmts in *P. digitatum*

The *Pdpmt1* (GenBank accession number KC757712), *Pdpmt2* (KC757713) and *Pdpmt4* (KC757714) genes each encode a putative protein O-mannosyltransferase and were identified in a *P. digitatum* genomic library, similarly to chitin synthase genes (*chs*) (Gandía *et al.*, 2012). The total mRNA was reverse transcribed, and the corresponding cDNAs were cloned. The coding sequences of the three genomic sequences and cDNAs show homology to other fungal Pmt and belong to the Pmt1, 2 and 4 subfamilies, respectively. BLASTP searches identified the three Pmts in the recently reported *P. digitatum* genomes (Marcet-Houben et al., 2012). Our deduced PdPmt1 and PdPmt4 proteins contain 937 and 776 amino acid residues and are identical to PDIG_19890 and PDIG_11480, respectively, which are annotated as putative protein mannosyltransferases in the *P. digitatum* PHI26 genome. In contrast, the amino acid sequence of PdPmt2 differed from the annotated PDIG_43220. Our cDNA analysis found changes in the intron position, explaining the difference between PDIG_43220 (737 aa) and PdPmt2 (744 aa).

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The assignment of these identified genes as protein O-mannosyltransferases was further confirmed by phylogenetic re-construction with homologous fungal genes (Supporting Fig. S1). The predicted amino acid sequences showed the presence of the two characteristic domains of the Pmt family (Supporting Fig. S2): the PMT domain required for O-mannosyltransferase activity and the mannosyltransferase IP3 ryanodine receptor domain (MIR) composed of three sub-motifs. In addition, the three proteins showed potential N-glycosylation sites, while only the larger PdPmt1 sequence has potential O-glycosylation sites located at the C-terminus.

Functional complementation was conducted to confirm the identity of the *Pdpmt* genes. The *S. cerevisiae pmt* mutants, particularly Δ*pmt2*, have defects in CW and exhibit increased sensitivity to the chitin binding fluorophore calcofluor white (CFW) (Harries et al., 2013; Loibl and Strahl, 2013). We tested whether the *P. digitatum* genes complemented the growth defect in the presence of CFW in the *S. cerevisiae pmt* mutants. Reliable data were only obtained with *S. cerevisiae* Δ*pmt2* (Fig. 1). *Pdpmt2* restored the growth of this yeast mutant under induction conditions (the presence of galactose). In contrast, complementation was not observed in transformants obtained with *Pdpmt1* or *Pdpmt4,* which demonstrates that *Pdpmt2* is a functional ortholog of *S. cerevisiae PMT2*.

Characterisation of *Pdpmt* gene expression

The changes in *Pdpmt* gene expression were analysed by qRT-PCR during axenic growth and fungal infection of citrus fruit. The three *pmt* genes were expressed during growth in liquid PDB and PDA plates. First, the expression

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among the three *Pdpmt* genes was compared. The absolute quantification of the molar concentrations of *pmt*-specific cDNAs was conducted. *Pdpmt4* showed the lowest expression and was used as the reference to quantify the expression of the other *pmt* genes (Fig. 2A). *Pdpmt1* was the most highly expressed and was twenty-fold higher than *Pdpmt4* and ten-fold higher than *Pdpmt2*.

A statistically significant increase in the *Pdpmt2* and *Pdpmt4* mRNA levels was observed during submerged growth of mycelium in PDB (Fig. 2B). The three *pmt* genes were induced during growth on PDA plates (Fig. 2C) in correlation with the production of conidia, which starts at day 2 and reaches maximum at day 7. The effect on *Pdpmt* expression under osmotic and oxidative stress conditions was also analysed. Our data indicate a differential regulation in the three genes since *Pdpmt2* was clearly induced in PDA plates containing 1 mM H_2O_2 or 1.2 M sorbitol while *Pdpmt1* was repressed (Fig. 2D).

The expression of *pmt* genes during the infection of citrus fruit was determined in experimental inoculations. Fig. 3 shows the results obtained from two independent experiments in which fruits from different orchards and harvesting season were used. None of the three *pmt* genes were detected at early stages of infection (1 day post-inoculation, dpi). From 2 to 7 dpi, an overall increase in *Pdpmt2* and *Pdpmt4* mRNA during the infection process was observed. The induction was more pronounced and statistically significant in *Pdpmt2*, which reached 5-10-fold induction at 7 dpi (Fig. 3A and B). This induction correlated with conidia production on infected fruit, which started at 4-5 dpi (Fig. 3C).

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Disruption and constitutive expression of *Pdpmt2* in *P. digitatum*

Our gene expression results show that *Pdpmt2* is the most responsive of the three *pmt* genes during axenic growth and infection progress. To characterise the functional roles of *Pdpmt2*, we generated *P. digitatum* gene disruption and constitutive expression mutants using *Agrobacterium tumefaciens*-mediated transformation (ATMT).

Gene disruption by homologous recombination was conducted using the pGKO2 vector (Khang *et al*., 2006) that contains the HSV*tk* gene as a negative marker to favour the selection of homologous recombinants (Fig. S3). Four independent transformants (PDEH508, PDEH510, PDEH515 and PDE525) with a distinctive phenotype were obtained (see Fig. 4A below). The detailed characterization of transformant PDEH515 is shown (Fig. S3). PDEH515 underwent a homologous recombination event at the *Pdpmt2* locus as demonstrated by Southern blot and PCR analyses. As the internal negative control in further experiments, we selected one transformant (PDEH540) with an ectopic integration of the T-DNA.

We obtained transformants for *Pdpmt2* constitutive expression under the strong *gpdA* promoter from *A. nidulans*. Three of them (PDEH58, PDEH59 and PDEH67) were selected for further analysis (Fig. S4). Southern hybridisation showed that PDEH59 is a transformant that harbours multiple copies of the constitutive expression construct. Gene expression studies confirmed that the null PDEH515 mutant did not express *Pdpmt2*; however, PDEH59 displayed six-fold increased expression compared with the parental strain (Fig. S5).

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The *Pdpmt2* mutation results in growth defects and conidiation deficiency in *P. digitatum*

The *Pdpmt2* mutation affected *P. digitatum* colony morphology (Fig. 4). The mutants showed a white fungal colony contrasting with the typical olive green colour exhibited by the parental strain (Fig. 4A) and grew significantly slower, reaching only ~40 % of the parental diameter (Fig. 4B). This growth defect was almost completely reversed by osmotic stabilisation with 1 M sorbitol (Fig. 4B). In addition, mutants displayed a “fluffy” phenotype, with white cotton-like masses of vegetative cells that suggest conidiation defects. The production of conidia from PDEH515 was severely reduced by three orders of magnitude (Fig. 4C), and obtaining sufficient conidia to perform our experiments was difficult; therefore, 10-12 plates had to be pooled each time. Moreover, the concentrated PDEH515 conidia solutions appeared colourless, and conidia viability dropped much quicker than the parental strain (data not shown). Interestingly, the conidiation defect was not recovered when PDEH515 was grown under osmotic stabilization (Fig. 4C).

Due to these phenotypes, we characterised the morphology of PDEH515 by fluorescence microscopy after CFW staining. At early stages of germ tube elongation, the PDEH515 mutant presented more septa, which were intensively stained and had shorter interseptal distances than PHI26 (Fig. 5A). The PDEH515 mutant also showed defects in polarity as demonstrated by tip dichotomous branching. Older mycelium (24 or 48 h) showed short interseptal distances that presented intense CFW staining (Fig. 5B), and dichotomous

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apical ends, in which the presence of CW thickening and globular balloon-like structures was common (see higher magnification images in Fig. 5B).

The conidiophore structure formed by PDEH515 was also abnormal (Fig. 5C). Typical phialides (p, Fig. 5C) above metulaes (m) observed in the PHI26 strain were difficult to distinguish in the mutant. PDEH515 only showed cells resembling elongated phialides at a number lower than the parental strain. Putative phialides had at most 1-2 small round cells at the tip with intense CFW staining, which likely correspond to the few conidia produced by this mutant. In PDEH515, the long conidia strings common in the parental strain were not observed. The microscopy characterisation of the constitutive expressors and the PDEH540 transformant did not reveal any of these defects (data not shown). Overall, these results demonstrate that *Pdpmt2* is required for normal mycelial growth and conidiation in *P. digitatum.*

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Effects of the *Pdpmt2* deletion on the sensitivity of PDEH515 to diverse compounds and stresses

We examined the sensitivity of the transformants to different compounds. PDEH515 had increased sensitivity to CW-damaging agents such as CFW or Congo red (CR) and to the cell membrane destabilising compound sodium dodecyl sulphate (SDS), indicating a defective CW (Fig. S6). None of the constitutive expression transformants showed changes in susceptibility to these compounds (data not shown).

The Δ*pmt2* mutant of *S. cerevisiae* is tolerant to the antifungal peptide PAF26 (Harries et al., 2013). The sensitivity of *Pdpmt2* transformants to PAF26

activity was tested in two different assays (Fig. 6). PDEH515 was able to grow on agarose solid medium containing 32 µM PAF26, while the parental strain and the constitutive expressors were not able to grow on this medium (Fig. 6A). Dose-response curves in liquid culture confirmed the higher tolerance of PDEH515 (Fig. 6B). Specifically, PAF26 had an IC_{50} of 1.8 µM and a minimal inhibitory concentration (MIC) of 4 µM against the parental strain, while PAF26 concentrations as high as 32 µM did not reduce PDEH515 growth after 48 h of culture.

Pdpmt2 is required for _P. digitatum_ full virulence against citrus fruit

Fruit inoculation assays were performed to analyse the role of *Pdpmt2* in pathogenicity and virulence. At a high inoculum dose (10^5 conidia mL^{-1}, Fig. S7), the incidence of infected wounds inoculated with PHI26 increased along time and reached 100 % at 5 dpi. The PDEH540, PDEH59 and PDEH67 strains did not differ significantly from PHI26 in the infection incidence, rate of progression of maceration area and sporulation. However, the *Pdpmt2* deletion mutant PDEH515 showed a clear delay in disease progression compared with the other strains. The reduction of disease incidence on fruits infected with PDEH515 was more evident at a lower inoculum dose (10^4 conidia mL^{-1}) (Fig. 7). In this case, the incidence of disease caused by PHI26 reached only ~70 % at 7 dpi, while PDEH515 inoculation resulted in just ~10 % of infected wounds. Lesion diameter measurements evidenced that at early infection (i.e., 2-5 dpi), PDEH515 lesions enlarged slower than the rest of the strains (Fig. 7B and S7B). Significantly, none of the PDEH515-infected wounds showed the typical colouration of the green mould caused by *P. digitatum* (Fig. 7C and S7D). The

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appearance of white mycelium was rare in the case of PDEH515, and infection was only noticeable by tissue maceration, even in lesions of size comparable with the lesions caused by PHI26 (Fig. 7C).

We explored additional properties of PDEH515 that may be related to the plant-pathogen interaction. Reactive oxygen species (ROS) are produced by plant tissues in response to pathogen infection. PDEH515 grew better than the parental strain and the other transformants in the presence of hydrogen peroxide (H_2O_2) (Fig. 8). Consistent with a role in mediating oxidative stress, the multicopy constitutive expressor PDEH59 had increased susceptibility to H_2O_2.

An assay for penetration through cellophane membranes (Prados Rosales and Di Pietro, 2008) was used to test the invasive growth capacity of PDEH515 (Fig. 9). Growth of the parental PHI26 strain on the membranes for 3 days was sufficient to allow penetration through the membranes and the invasion of the PDA underneath. In contrast, the PDEH515 mutant was unable to penetrate cellophane membranes even after 7 days of growth, demonstrating a blockage of invasive growth capacity.

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DISCUSSION

This study reports the identification of one member of each *pmt* subfamily in the postharvest pathogen *P. digitatum*, consistent with reports that showed the existence of only one ortholog for each *pmt* subfamily in other filamentous fungi and in the fission yeast. Functional analysis by complementation of yeast Δ*pmt* mutants demonstrated that *PdPmt2* is orthologous to *PMT2* from *S. cerevisiae*. Both Pmt2 proteins from *P. digitatum* and *S. cerevisiae* have 52 % amino acid

identity, the highest identity value between any Pmt from these two fungi. This is the first study that complements a yeast Δ*pmt2* mutant and uses CFW sensitivity as a complementation assay. Previous heterologous complementation had been demonstrated for *CaPMT1* in the Δ*pmt1* mutant of *S. cerevisiae* (Timpel et al., 1998) and for *pmt1* and *pmt4 U. maydis* genes in *S. pombe* (Fernández-Álvarez et al., 2009). Overall, these findings confirm the functional conservation within each subfamily of *pmt* genes among distantly related fungi.

Detailed characterisation of fungal *pmt* gene expression has been previously described in *A. nidulans* (Oka et al., 2004; Goto et al., 2009), *C. neoformans* (Willger et al., 2009), *B. cinerea* (González et al., 2013) and the yeast *C. albicans* (Timpel *et al.*, 2000; Prill et al., 2005). Overall, the three *pmt* subfamilies are all expressed under different growth conditions as is also the case in *P. digitatum*. Our study adds the comparison of gene expression among the three *Pdpmt*, which demonstrated that *Pdpmt1* has the highest expression, one order of magnitude higher than *Pdpmt2* or *Pdpmt4*. Our data also indicate that differential regulation of the three *Pdpmt* genes exists. For example, *Pdpmt2* is the most responsive of the three and is induced during submerged and aerial growth, after osmotic and oxidative stresses, and during fungal infection. In contrast, the *pmt4* gene of *B. cinerea* is the most induced during tomato leave infection, while *pmt2* remains constant or even declines (González et al., 2013). In spite of its high induction, the *pmt2* deletion in PDEH515 was not compensated by the overexpression of any of the other two genes (Fig. S5), similarly to the findings in the *C. neoformans pmt1* and *pmt4* mutants (Willger et al., 2009).

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The *Pdpmt2* gene is neccesary for growth, conidiogenesis and cell wall integrity

This study demonstrates that the disruption of *pmt2* has a viable phenotype in *P. digitatum*. Viable mutants of the Pmt2 subfamily have also been described in *A. nidulans*, *Aspergillus awamori*, *B. cinerea* and *C. neoformans* (Oka et al., 2004; Oka *et al.*, 2005; Kriangkripipat and Momany, 2009; González et al., 2013; Shimizu *et al.*, 2014). In the pathogens *C. albicans*, *A. fumigatus,* and *U. maydis*, *pmt2* appears to be an essential gene; therefore, the functional significance of null mutations could not be assayed (Prill et al., 2005; Willger et al., 2009; Mouyna et al., 2010). The selection of transformants in our study required osmotic stabilisation likely due to the compromised CW integrity of the mutants. Functional complementation of PDEH515 with the native *Pdpmt2* could not be demonstrated because of its low conidia recovery and defective CW, which made the ATMT of this strain not possible after several attempts (data not shown).

The PDEH515 mutant displayed a reduction in radial growth and severe morphological defects. Significantly, young germlings showed dichotomous tip branching, suggesting defects in polarity. Early characterisation of polarity mutants in fungi identified *pmt* genes, as it was the case of the *swoA* mutant in *A. nidulans* (Shaw and Momany, 2002). The reduction of *pmt2* expression in *A. fumigatus* also demonstrated the involvement of this gene in hyphal polarity (Fang *et al.*, 2010).

The Δ*pmtA* mutants of *A. nidulans* and *A. awamori* also presented growth reduction and defects in mycelium morphology with balloon-like structures (Oka

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et al., 2004; Oka et al., 2005), similar to our observations with PDEH515. These globular structures have also been described in null chitin synthase gene mutants involved in fungal CW synthesis (Martín-Urdíroz *et al.*, 2008; Gandía et al., 2014). These morphological defects reveal abnormal CW synthesis, producing cell enlargements with altered chitin content. The PDEH515 mutant showed high sensitivity to compounds that target the CW, and the growth defect was reversed with the addition of sorbitol. Studies have revealed that defective protein glycosylation has a negative impact on the sensitivity to CW-interfering compounds and CW integrity, which is restored with the addition of osmotic stabilisers (Gentzsch and Tanner, 1996; Goto et al., 2009; Kriangkripipat and Momany, 2009; Mouyna et al., 2010; González et al., 2013). Our gene expression analysis demonstrated the induction of *Pdpmt2* and *Pdpmt4* expression under osmotic stress (Fig. 3C) and the repression of *chs* genes (Gandía et al., 2012), indicating that the CW is remodelled under osmotic adaptation, which is likely related to the restoration of PDEH515 growth in the presence of sorbitol.

A distinguishable phenotypic characteristic of the PDEH515 mutant was the white colony associated with reduced conidiation and abnormal development of conidiophores. A high induction of *Pdpmt2* was observed during conditions that produce high conidiation such as growth on PDA plates and fruit infection (Fig. 3B and 4). Studies in *A. nidulans* and *A. fumigatus* have also demonstrated a reduction of conidia in *pmt* mutants (Goto et al., 2009; Kriangkripipat and Momany, 2009; Fang et al., 2010; Mouyna et al., 2010). A more extreme phenotype was found in the *B. cinerea* Δ*pmt2*, which was reported to be completely unable to sporulate (González et al., 2013). The *wetA* mutants of *A.*

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nidulans and *P. chrysogenum* also produce colourless and unstable conidia (Prade and Timberlake, 1994). WetA is a key protein in the regulatory pathway of conidiation and also a serine and threonine-rich (STR) protein with numerous potential O-glycosylation sites. Potentially, the conidiation-related phenotype of PDEH515 could be at least partially a consequence of abnormal WetA glycosylation and function, which affect conidia development. The alteration of PDEH515 conidiophore structure at the very early stages of development (Fig. 6C) supports a regulatory effect.

Involvement of Pmt in the virulence of *P. digitatum* on citrus fruit

The O-glycosylation of proteins has been linked to the virulence of fungal pathogens of humans and plants (Lengeler *et al.*, 2008; Fernández-Álvarez et al., 2009). The involvement of *pmt* genes in fungal pathogenesis depends on the specific fungus-host interaction. However, most of the null mutants in *pmt* genes whose pathogenesis has been studied belong to the Pmt1/Pmt4 subfamilies, due to the difficulties in obtaining viable *pmt2* mutants. We have shown that *Pdpmt2* disruption produces a reduction in virulence and no conidiation on fully infected oranges. The *B. cinerea* Δ*Bcpmt2* mutant displayed reduced growth and no conidiation in axenic culture as well as defects in the adhesion and penetration to host tissue that are associated with reduced virulence (González et al., 2013). Defective cellular adhesion was also found in the Δ*pmt4* mutant of *U. maydis* (Fernández-Álvarez et al., 2012).

The slow growth and defective CW of PDEH515 surely limits fruit tissue colonisation and explains the decrease in virulence. However, the severe reduction of virulence and absence of visible hyphal development on fruit also

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indicate that additional factors may be involved. The production of ROS in infected citrus fruit is a well characterised host defence response that is suppressed by *P. digitatum* during infection (Macarisin *et al.*, 2007). Indeed, our data revealed that *Pdpmt* genes are induced by H_2O_2 and indicate that the absence of any of these genes would reduce fitness under oxidative stress. Unexpectedly, the contrary result was found, and *Pdpmt2* disruption produced a slight increase in tolerance to ROS that goes against an involvement of ROS in the reduction of virulence of the PDEH515 mutant. Similarly, the inhibition of plant ROS production did not restore pathogenicity of the *U. maydis* Δ*pmt4* mutant in maize (Fernández-Álvarez et al., 2009).

Most sensor proteins from MAPK (mitogen activated protein kinase) signalling cascades are cell surface proteins that are glycosylated (Rispail *et al.*, 2009; Lien *et al.*, 2013). In fungal pathogens, MAPK pathways control morphogenesis, disease progression and virulence (Román *et al.*, 2007). Not surprisingly, the defective glycosylation of sensor proteins affects the pathogenesis of fungal plant pathogens. Msb2 is a STR sensor protein in the high osmolarity glycerol (HOG) pathway, and its defective glycosylation activates the pathway constitutively (Tatebayashi *et al.*, 2007; Yang *et al.*, 2009). In *P. digitatum*, null mutants of the MAPK Os2/Hog1 have reduced virulence, indicating that a functional HOG pathway is required for full infection capacity (Wang *et al.*, 2014). The *U. maydis* Δ*pmt4* mutant produced defects in the glycosylation of Msb2, which in this fungus also activates a MAPK involved in appressorium formation (Fernández-Álvarez et al., 2012). Msb2 also signals the filamentous and invasive growth pathway in fungi (Román et al., 2007) and is necessary for the invasive growth and pathogenesis of *F. oxysporum* on

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tomato plants (Pérez-Nadales and Di Pietro, 2011). Invasive growth was completely blocked in the *P. digitatum pmt2* mutant, thus providing additional reasons to explain the reduction of virulence.

Future work will aim to identify the specific CW and surface sensor proteins that are O-glycosylated by PdPmt2 in *P. digitatum* and analyse its involvement in morphogenesis, sensitivity to PAF peptides and pathogenesis as well as the cross-talk among these processes.

Protein glycosylation genes determine the activity of PAF26 against *P. digitatum* and *S. cerevisiae*

In this study, we demonstrated that PDEH515 conidia are tolerant to PAF26. Our previous results with *S. cerevisiae* showed enhanced tolerance to PAF26 in several deletion mutants of protein glycosylation genes, including *PMT1-6* (Harries et al., 2013). The resistance to PAF26 in *S. cerevisiae* and *P. digitatum pmt2* mutants demonstrates that protein O-glycosylation has a role in the antifungal action of this peptide that is conserved between distantly related fungi. Previous studies, mostly conducted in yeast, have found tolerance to other antifungal peptides and proteins in protein glycosylation mutants (Ibeas *et al.*, 2000; Koo *et al.*, 2004; Harris *et al.*, 2009), demonstrating that proper protein glycosylation is required for full activity of a number of antifungal peptides and proteins, including PAF26.

Glycan patterns from CW proteins are proposed to act as docking moieties for the binding (and therefore activity) of antifungal peptides (Harris et al., 2009; Marcos and Gandía, 2009). The deletion of the glycosylated highly abundant

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CW protein Flo11 abolished the binding of PAF26 to wine “flor” strains of yeast, which supports this view (Bou Zeidan *et al.*, 2013). The glycosylated domain of Msb2 from *C. albicans* is capable of binding to the antifungal peptides histatin 5 and LL-37, and its release into the medium protects cells by sequestering the peptide (Szafranski-Schneider *et al.*, 2012). PAF104 is a heptapeptide derived from extension of the PAF26 sequence (Muñoz *et al.*, 2007b) that specifically blocks appressorium formation and reduces virulence in the rice blast pathogen *Magnaporthe oryzae* (Rebollar and López-García, 2013). PAF104 represses the expression of *Momsb2*, which encodes the glycosylated Msb2 and is involved in the Pmk1/Kss1 MAPK pathway that regulates appressorium formation in *M. oryzae* (Liu *et al.*, 2011), demonstrating that PAF peptides affect a MAPK pathway involved in a plant-pathogen interaction, fungal penetration and infection. Small antifungal proteins and peptides activate MAPK stress signalling pathways as a response to their antifungal action (Hayes *et al.*, 2014); however, whether PAF26 binding to the cell surface influences the activity of the glycosylated Msb2 and/or MAPK pathways in *P. digitatum* remains unknown.

In summary, this study shows the critical role of *Pdpmt2* for growth, conidiation and virulence of *P. digitatum* and demonstrates that *Pdpmt2* determines the susceptibility to antifungal peptides exemplified by PAF26. Therefore, a gene mutation that increases resistance to an antifungal compound results in detrimental effects to fungal fitness and virulence. This observation is relevant for the future use of antifungal peptides in crop protection.

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EXPERIMENTAL PROCEDURES

Fungal strains and culture conditions

The parental strain used was the wild type *Penicillium digitatum* PHI26 (CECT20796), whose genome sequence is available (Marcet-Houben et al., 2012) (WGS project AKCT01). *P. digitatum* strains were grown at 24 ºC either on potato dextrose agar (PDA) (Difco, #213400) plates or in potato dextrose broth (PDB) (Difco, #254920) with shaking. Conidia were collected from plates. To determine colony growth, 5 µL of 1 x 10^5 conidia mL^{-1} were deposited on PDA plates, and the colony diameter was measured. Conidiation was quantified on plates grown for 12 days. Cellophane invasion assays were performed as previously described (Prados Rosales and Di Pietro, 2008) by placing autoclaved cellophane membranes on PDA plates and depositing conidial suspensions on membranes.

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Sequence analysis of *Pdpmt* genes

Pdpmt coding sequences were obtained from a *P. digitatum* fosmid genomic library (Gandía et al., 2012), and sequenced by chromosome walking. To determine the position and size of the introns in each *Pdpmt,* RNA was extracted from the PHI26 strain as reported (Gandía et al., 2012) and reverse transcribed using SuperScript III reverse transcriptase (Invitrogen, #18080-044) into first-strand cDNA, which was then PCR amplified using specific primer pairs, cloned into pGEM-T (Promega #A1360) and sequenced. All the oligonucleotides used in this work are detailed in Supporting Table S1.

S. cerevisiae **complementation assays**

Functional complementation assays in the *S. cerevisiae* Δ*pmt2* strain were conducted using the pGREG505 plasmid following the Drag and Drop cloning system (Jansen *et al*., 2005). The coding sequences for each *Pdpmt* gene were amplified from plasmids containing cDNAs using primers designed to place each *Pdpmt* under the *GAL1* inducible promoter. The pGREG505_PdPmt1, pGREG505_PdPmt2 and pGREG505_PdPmt4 plasmids derived from pGREG505 were recovered from the BY4741 yeast strain (*MAT*a *his3*Δ*1 leu2*Δ0 *met15*Δ0 *ura*Δ0) selected on synthetic complete (SC) medium lacking Leu (SC-Leu) and confirmed by sequencing. The plasmids were transformed in the genetic background of the Δ*pmt2* mutant (BY4741 *pmt2*Δ::*KanMX4*) and selected in SC-Leu.

Sensitivity assays to CFW (Sigma-Aldrich #F3543) were performed to determine functional complementation. The transformants were grown over night in liquid SC-Leu with 2 % glucose at 30 ºC. The cultures were refreshed to an OD_{600} of 0.2, pelleted by centrifugation, washed twice in sterile water and inoculated in 5 mL of either liquid SC-Leu with 2 % glucose (for *GAL1* repression) or 2 % galactose (for *GAL1* induction). After 9 h of incubation at 30 ºC, the cells were diluted to an OD_{600} of 0.1 and ten-fold serial dilutions were dotted onto SC-Leu plates containing: (i) 2 % glucose, (ii) 2 % glucose, 12.5 µg mL^{-1} CFW, or (iii) 2 % galactose, 12.5 µg mL^{-1} CFW. The plates were incubated at 30 ºC for 3 days.

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qRT-PCR analysis

To determine the expression of the *Pdpmt* genes, total RNA extraction, reverse transcription and quantitative PCR reactions were performed as reported (Gandía et al., 2012). For expression normalisation, the β-tubulin gene, ribosomal protein L18a, and 18S rRNA were simultaneously used as references as previously described.

Construction of plasmid vectors for fungal transformation

Genomic DNA was extracted from *P. digitatum* as reported (Marcet-Houben et al., 2012). PCR reactions were performed with specific primers and the AccuPrime High Fidelity polymerase (Invitrogen #12346-086). The *Pdpmt2* disruption construct (pGKO2_Pdpmt2) was generated by fusion PCR (Szewczyk *et al.*, 2006) and cloned in the binary vector pGKO2 (Khang et al., 2006) (see construct details in Supporting Information Fig. S3). The *Pdpmt2* constitutive expression construct (pBHt2_Pdpmt2) was designed with the full length *Pdpmt2* coding sequence obtained by PCR amplification from genomic DNA and cloned under the control of the *A. nidulans* glyceraldehyde triphosphate dehydrogenase (*gpdA*) promoter into vector pBHt2 (Mullins *et al.*, 2001) (Supporting Information Fig. S4).

Agrobacterium tumefaciens*-mediated transformation of *P. digitatum

Agrobacterium tumefaciens-mediated transformation of *P. digitatum* PHI26 was performed as previously described (Mullins et al., 2001; Khang et al., 2006; Michielse *et al.*, 2008; Gandía et al., 2014) with minor modifications. *A. tumefaciens* carrying each transformation vector (pGKO2_Pdpmt2 or

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pBHt2_Pdpmt2) was induced with 200 µM acetosyringone. Freshly prepared *A. tumefaciens* and conidia of *P. digitatum* were co-cultured at 24 ºC for 2 days on nitrocellulose filters. The filters were transferred to PDA plates containing 50 µg mL^{-1} hygromicin (Invivogen #ant-hm-5). The plates were incubated for 2 days at 24 ºC or until Hyg^R colonies appeared. In the case of the disruption construct (pGKO2_Pdpmt2), the plates containing Hyg^R transformant colonies were washed with sterile water, and the wash was plated on PDA supplemented with 10 µM 5-Fluoro-2-deoxyuridine (F2dU) (Sigma-Aldrich #F0503) and incubated for 2-7 days at 24 ºC. F2dU is transformed by the herpes simple virus thiamine kinase gene (HSV*tk*) encoded in pGKO2 in a toxic compound for negative selection against ectopic transformants (Khang et al., 2006). Resistant transformants were checked for Hyg^R and subjected to monosporic culturing for molecular analysis.

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Molecular characterisation of *P. digitatum* transformants

Genomic DNA was digested with *Eco*RI and analysed by Southern blot using the non-isotopic digoxigenin-labelling kit (DIG-High Prime DNA Labelling and Detection Starter Kit II, Roche). Transformants were further confirmed by PCR with genomic DNA with gene specific primer pairs (see Supporting Figs. S3 and S4).

Fluorescence microscopy analyses

The morphology of each fungal strain was visualised with fluorescence microscopy (microscope E90i, Nikon) after staining with 50 µg mL^{-1} CFW. Representative images observed in the 40x objective were captured by the NIS-

Elements BR v2.3 program (Nikon). For sample preparation and fixation, we followed previously described methods (Harris *et al.*, 1994).

Sensitivity to antifungal compounds

In total, 5 μL of serial 10-fold dilutions of 1 x 10^5 conidia mL^{-1} of *P. digitatum* strains were inoculated on PDA plates supplemented with CFW, SDS (Sigma-Aldrich #L4509), CR (Sigma-Aldrich #C6767) or H_2O_2.

PAF26 (amino acid sequence RKKWFW) was synthesised and provided at >95 % purity by GenScript Corporation (Piscataway, NJ, USA). Two types of assays were used to evaluate the antifungal activity of PAF26 against *P. digitatum* strains. First, the strains were grown on 25 % (one fourth diluted) potato dextrose (PD) plates solidified with 1.25 % agarose and supplemented with either 16 or 32 μM PAF26. Second, dose response experiments were conducted in 5 % PDB using a 96-well microtiter plate assay as previously described (Muñoz *et al.*, 2006).

Infection assays

P. digitatum inoculation on freshly harvested orange fruits (*Citrus sinensis*) and the collection of fruit peel tissue samples were conducted as previously described (González-Candelas et al., 2010). At different times after inoculation, the incidence of infected wounds, diameter of lesion and conidiation index were recorded. Three replicas of five fruits each (four wounds per fruit) were used.

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FIGURE LEGENDS

Fig. 1. Complementation assay of the *S. cerevisiae* Δ*pmt2* mutant with the *P. digitatum Pdpmt* genes. Yeast cells transformed with each pGREG505 plasmid (indicated at the left side) were grown at 30 ºC for 9 h in SC-Leu medium containing either 0.2 % glucose (Glu) or 0.2 % galactose (Gal). Serial ten-fold dilutions of these cells at 0.1 OD_{600} were dotted onto SC-Leu plates with Glu or Gal supplemented with 12.5 µg mL^{-1} CFW as indicated and incubated at 30 ºC for 3 days. Note that the *S. cerevisiae* Δ*pmt2* strain presents high sensitivity to CFW under repression conditions (+ Glu) and that this phenotype is rescued only with *Pdpmt2* under the inductive condition (+ Gal).

Fig. 2. Quantification of *Pdpmt* gene expression by qRT-PCR. (A) Comparison of *Pdpmt* gene expression after 2 days (d) of growth in PDB. The Ct values obtained by qRT-PCR were transformed to molar amounts of cDNA through equations obtained from standard curves (obtained by amplification of serial two-fold dilutions of equimolar amounts of each *Pdpmt* amplicon) and normalized to the lowest expression of *Pdpmt4* (marked with an inverted triangle). (B) Relative expression of *Pdpmt* genes in submerged culture (PDB) after 1, 2, 3 or 6 d of growth as indicated. (C) Relative expression of *Pdpmt* genes in solid medium (PDA) after 2, 4 or 7 d of growth as indicated. (D) Relative expression of *Pdpmt* genes under oxidative or osmotic stress. Graphs show the relative expression at day 4 of growth in PDA (control) and PDA supplemented with H_2O_2 or sorbitol as indicated. The (B), (C) and (D) graphs show the relative expression normalised independently for each gene to the samples indicated by an inverted triangle. In all graphs, the bars represent the

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means ± standard deviations (SD) of three replicates. Statistically significant differences are labelled with an asterisk ($p < 0.05$).

Fig. 3. Relative expression of *Pdpmt* genes during orange fruit infection. (A) and (B) graphs show the relative expression at each day post-inoculation (dpi) in two independent experiments. The values were normalised independently for each gene to the value at 2 dpi. Statistically significant differences are labelled with an asterisk ($p < 0.05$). (C) Representative images for the progress of fungal colonisation on infected fruit.

Fig. 4. Colony morphology and growth of *P. digitatum* parental and mutant strains. (A) *P. digitatum* strain colonies grown on PDA plates at 24 ºC for 7 days as indicated: parental strain PHI26, mutants PDEH508, PDEH510, PDEH515 and PDEH525, and the ectopic transformant PDEH540. (B) Growth of PHI26 (triangles), PDEH515 (circles) and PDEH540 (squares) on PDA plates (black symbols and straight lines) and PDA plates supplemented with 1 M sorbitol (white symbols and stripped lines). Data show the mean ± SD of three replicas of the diameter registered daily. (C) Conidia production of PHI26, PDEH515 and PDEH540 on PDA plates and PDA plates supplemented with 1 M sorbitol. Data show the mean ± SD of three replicas of the conidia mL^{-1} produced per plate.

Fig. 5. Comparative fluorescence microscopy analysis of *P. digitatum* PHI26 and PDEH515 strains. (A) Germlings of PHI26 and PDEH515 after 16 h of incubation at 24 ºC of conidial suspension in PDB. (B) Mycelium of PHI26 and PDEH515 grown on slides. (C) Conidiophore structure formed on slides after 72 h of growth. Images show the DIC bright fields and the corresponding fluorescence fields of samples stained with CFW to visualize the CW. Letters on

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the images indicate the septum (s), the balloon-like structures (b) formed in the mycelium, and the different cell types of conidiophores; rame (r), metulae (m), phialide (f) and conidium (c).

Fig. 6. Antifungal activity of the PAF26 peptide against *P. digitatum* strains. (A) In total, 5 µL of three serial five-fold dilutions (2.5 x 10^4, 5 x 10^3, and 10^3 conidia mL^{-1}, indicated by the upper triangle) from the parental PHI26, the deletion mutant PDEH515, the ectopic transformant PDEH540 and the two constitutive transformants PDEH59 and PDEH67 were grown at 24 ºC for 7 days on PD agarose 24-well plates with either no peptide (control) or containing 16 µM or 32 µM PAF32 as indicated. (B) Dose response experiment of the growth of *P. digitatum* PHI26 (grey circles) or PDEH515 (open triangles) exposed to increasing PAF26 concentrations. The values of triplicate samples are shown. The values corresponding to PHI26 were adjusted to a four parameter sigmoidal curve (black line) with 50 % inhibitory concentration (IC_{50}) of 1.8 µM (r = 0.9553).

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Fig. 8. Sensitivity of *P. digitatum* strains to hydrogen peroxide. In total, 5 µL of three serial ten-fold dilutions (10^5, 10^4 and 10^3 conidia mL^{-1}) of the same strains

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SUPPORTING INFORMATION

Supporting Fig. S1. Phylogenetic analysis of *P. digitatum* PdPmt1, PdPmt2, PdPmt4 and homologous sequences in fungi.

Supporting Fig. S2. Domain organisation of Pmt proteins from *P. digitatum*.

Supporting Fig. S3. Disruption of the *Pdpmt2* gene in *P. digitatum*.

Supporting Fig. S4. Constitutive expression of the *Pdpmt2* gene in *P. digitatum*.

Supporting Fig. S5. Relative quantification of *Pdpmt* gene expression in the *P. digitatum* strains.

Supporting Fig. S6. Sensitivity of the *P. digitatum* strains to different antifungal compounds.

Supporting Fig. S7. Virulence assays on orange fruits of *P. digitatum* strains at high inoculum dose (10^5 conidia mL^{-1}).

Supporting Table S1. Oligonucleotide primers used in this work.

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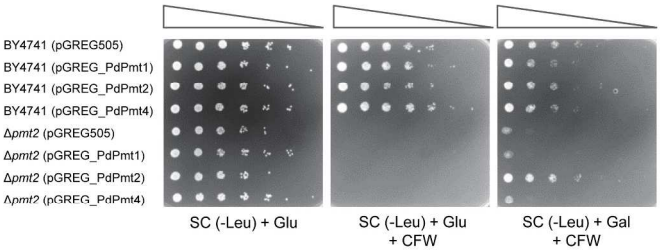
Figure 1 (Harries *et al*., 2014)



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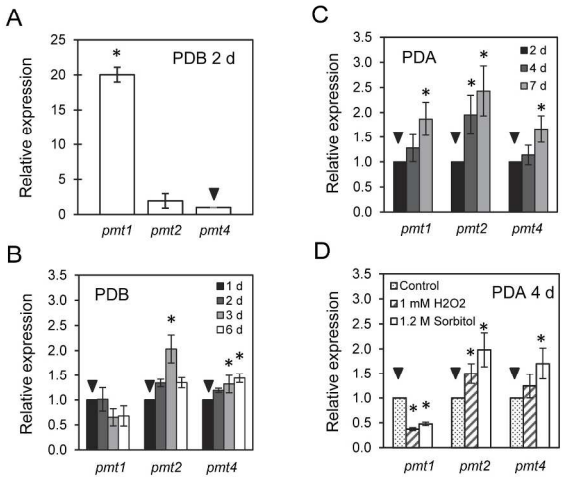

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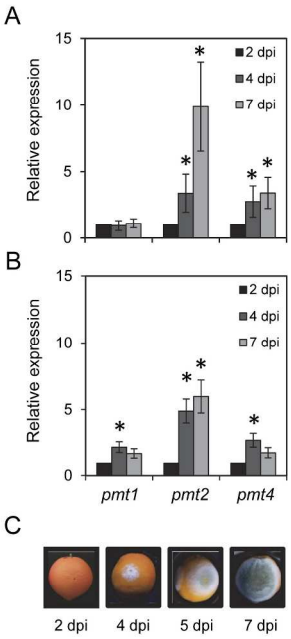

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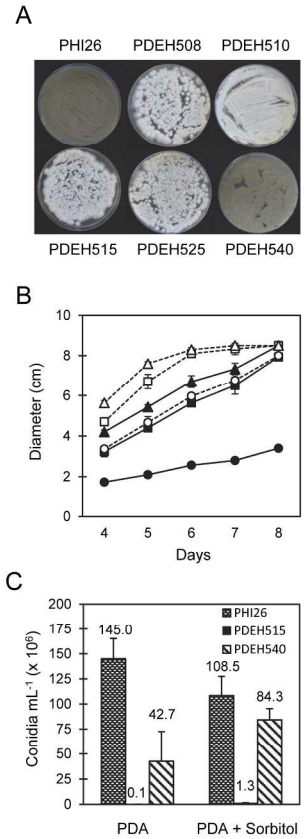

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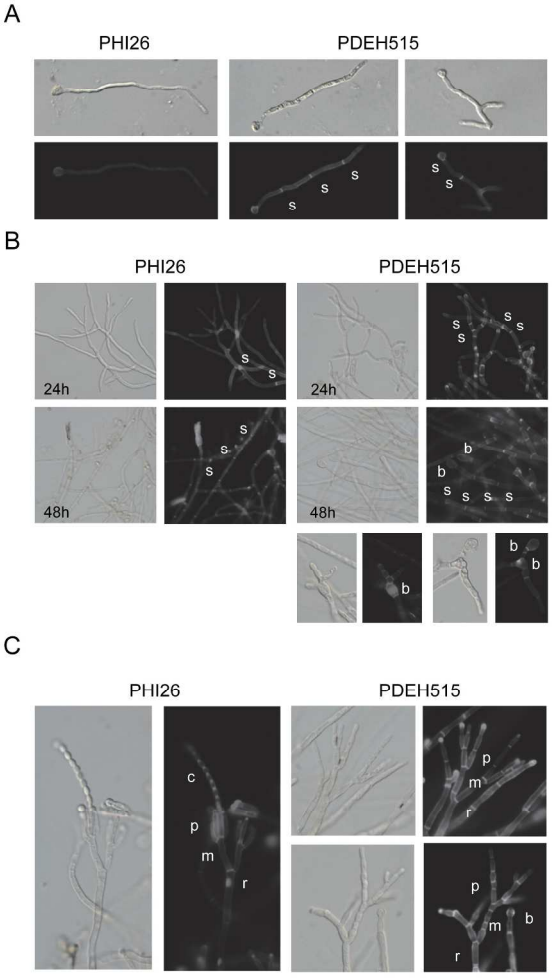

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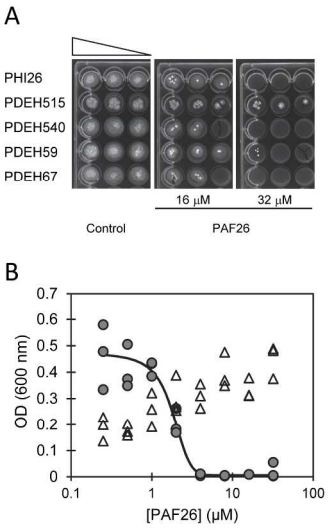

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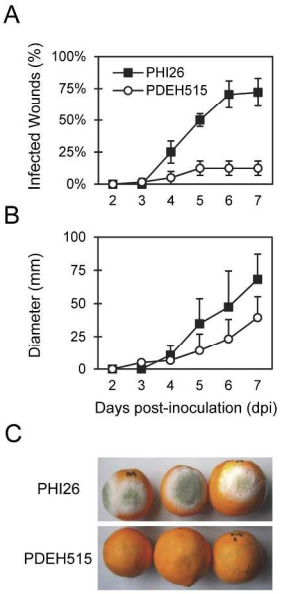

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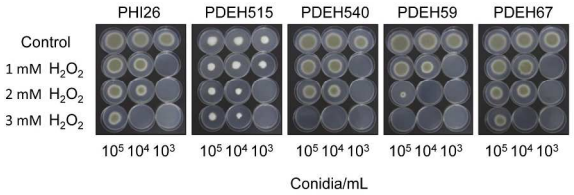

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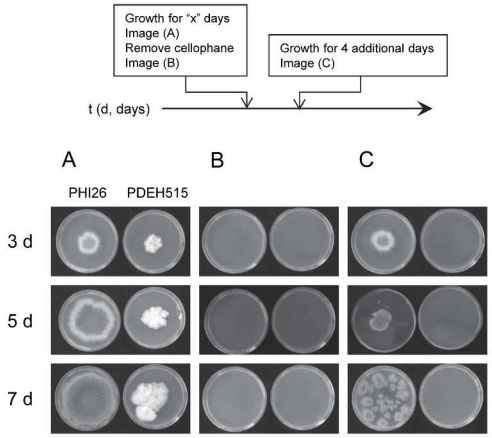

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