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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SUMMARY

The activity of protein O-mannosyltransferases (Pmts) affects the morphogenesis and virulence of fungal pathogens. Recently, *PMT* genes have been shown to determine the sensitivity of *Saccharomyces cerevisiae* to the antifungal peptide PAF26. This study reports the identification and characterization of the three *Pdpmt* genes in the citrus post-harvest 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* $\Delta pmt2$ strain. The *Pdpmt2* gene mutation in *P. digitatum* caused pleiotropic effects, including a reduction in fungal growth and virulence, whereas 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 distantly related 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.

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

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 of environmental signals, morphogenesis and the virulence of fungal pathogens (Fernández-Álvarez *et al.*, 2012; Gentzsch and Tanner, 1996; Olson *et al.*, 2007; Prill *et al.*, 2005; Zhou *et al.*, 2007).

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 into the Pmt1, Pmt2 and Pmt4 subfamilies, which differ in their number of genes and the 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, whereas Pmt4 is a single member subfamily and acts as a homodimer. The *PMT* genes in *S. cerevisiae* are not individually essential for viability, probably as a result of gene redundancy, although several combinations of double mutants are lethal (Gentzsch and Tanner, 1996; Loibl and Strahl, 2013).

In the fission yeast *Schizosaccharomyces pombe* and in filamentous fungi, such as *Aspergillus nidulans*, the human pathogen *Aspergillus fumigatus* or the plant pathogens *Ustilago maydis* and *Botrytis cinerea*, only one member has been identified for each Pmt subfamily (Fernández-Álvarez *et al.*, 2009; González *et al.*, 2013; Goto *et al.*, 2009; Mouyna *et al.*, 2010; Oka *et al.*, 2004; Willer *et al.*, 2005). Importantly, the *pmt2* subfamily is essential for the viability of several fungi studied so far. The requirement for *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 (Olson *et al.*, 2007; Prill *et al.*, 2005; Timpel *et al.*, 1998; Willger *et al.*,

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2009). In contrast, mutants in either *pmt1* or *pmt4* of *A. fumigatus* are as virulent as the parental strain in a murine model (Mouyna *et al.*, 2010; Zhou *et al.*, 2007). In *U. maydis*, only the disruption of *pmt4* specifically affected appressorium formation, penetration and tumour formation in maize, whereas *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 citrus fruit green mould disease and contributes up to 5%–10% of post-harvest losses in the main fruit tree crop in the world. *Penicillium digitatum* is a necrotroph and a wound pathogen that requires a pre-existing injured fruit peel to penetrate and infect. Despite this rather unspecific infection mechanism, *P. digitatum* exhibits an exquisite and high degree of host specificity. The most common method to control *P. digitatum* is the post-harvest 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 characterize the fruit–fungus interaction (Ballester *et al.*, 2011; Buron-Moles *et al.*, 2012; González-Candelas *et al.*, 2010) and fungal pathogenicity (Gandía *et al.*, 2014; Wang and Li, 2008; Zhang *et al.*, 2013a, 2013b). 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 for the control of 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 which 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, 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).

In this study, the identification and characterization of *pmt* genes from *P. digitatum* were 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 characterization of *Pdpmt2* in *P. digitatum* and its requirement for fungal growth, CW integrity, 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 *Pmts* and belong to the *Pmt1*, *Pmt2* and *Pmt4* 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 amino acids) and *PdPmt2* (744 amino acids).

The assignment of these identified genes as protein *O*-mannosyltransferases was further confirmed by phylogenetic reconstruction with homologous fungal genes (Fig. S1, see Supporting Information). The predicted amino acid sequences showed the presence of the two characteristic domains of the *Pmt* family (Fig. S2, see Supporting Information): the *PMT* domain required for *O*-mannosyltransferase activity and the mannosyltransferase IP3 ryanodine receptor domain (MIR) composed of three submotifs. In addition, the three proteins showed potential *N*-glycosylation sites, whereas 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 $\Delta pmt2$, 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* $\Delta pmt2$ (Fig. 1). *Pdpmt2* restored the growth of this yeast mutant under induction conditions (presence of galactose). In contrast, complementation was not observed in transformants obtained with *Pdpmt1* or *Pdpmt4*, which demonstrates that *Pdpmt2* is a functional orthologue of *S. cerevisiae* *PMT2*.

Characterization of *Pdpmt* gene expression

The changes in *Pdpmt* gene expression were analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) during axenic growth and fungal infection of citrus fruit. The three

Fig. 1 Complementation assay of the *Saccharomyces cerevisiae* $\Delta pmt2$ mutant with the *Penicillium digitatum* *Pdpmt* genes. Yeast cells transformed with each pGREG505 plasmid (indicated on the left-hand side) were grown at 30 °C for 9 h in synthetic complete medium lacking leucine (SC-Leu) containing either 0.2% glucose (Glu) or 0.2% galactose (Gal). Serial 10-fold dilutions of these cells at 0.1 OD₆₀₀ were dotted onto SC-Leu plates with Glu or Gal supplemented with 12.5 µg/mL calcofluor white (CFW) as indicated, and incubated at 30 °C for 3 days. Note that the *S. cerevisiae* $\Delta pmt2$ strain presents high sensitivity to CFW under repression conditions (+Glu) and that this phenotype is rescued only with *Pdpmt2* under inductive conditions (+Gal).

pmt genes were expressed during growth in liquid potato dextrose broth (PDB) and potato dextrose agar (PDA) plates. First, the expression 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: 20-fold higher than *Pdpmt4* and 10-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 started at day 2 and reached a maximum at day 7. The effect on *Pdpmt* expression under osmotic and oxidative stress conditions was also analysed. Our data indicated a differential regulation in the three genes, as *Pdpmt2* was clearly induced in PDA plates containing 1 mM H₂O₂ or 1.2 M sorbitol, whereas *Pdpmt1* was repressed (Fig. 2D).

The expression of *pmt* genes during the infection of citrus fruit was determined in experimental inoculations. Figure 3 shows the results obtained from two independent experiments in which fruits from different orchards and harvesting seasons were used. None of the three *pmt* genes were detected at the 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, B). This induction correlated with conidia production on infected fruit, which started at 4–5 dpi (Fig. 3C).

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

Our gene expression results showed that *Pdpmt2* was the most responsive of the three *pmt* genes during axenic growth and infection progress. To characterize the functional roles of *Pdpmt2*,

we generated *P. digitatum* gene disruption and constitutive expression mutants using *Agrobacterium tumefaciens*-mediated transformation.

Gene disruption by homologous recombination was conducted using the pGKO2 vector (Khang *et al.*, 2006) which contains the HSVtk gene as a negative marker to favour the selection of homologous recombinants (Fig. S3, see Supporting Information). Four independent transformants (PDEH508, PDEH510, PDEH515 and PDE525) with a distinctive phenotype were obtained (Fig. 4A). The detailed characterization of transformant PDEH515 is shown in 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 (PDEH58, PDEH59 and PDEH67) were selected for further analysis (Fig. S4, see Supporting Information). Southern hybridization 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, see Supporting Information).

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 more slowly, reaching only ~40% of the parental diameter (Fig. 4B). This growth defect was almost completely reversed by osmotic stabilization with 1 M sorbitol (Fig. 4B). In addition, mutants displayed a 'fluffy' phenotype, with

Fig. 2 Quantification of *Pdpmt* gene expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (A) Comparison of *Pdpmt* gene expression after 2 days of growth in potato dextrose broth (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 days of growth as indicated. (C) Relative expression of *Pdpmt* genes in solid medium (potato dextrose agar, PDA) after 2, 4 or 7 days 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 H₂O₂ or sorbitol as indicated. The graphs in (B–D) show the relative expression normalized independently for each gene to the samples indicated by an inverted triangle. In all graphs, the bars represent the means $\pm$ standard deviations (SDs) of three replicates. Statistically significant differences are labelled with an asterisk ($P < 0.05$).

white cotton-like masses of vegetative cells, suggesting conidiation defects. The production of conidia from PDEH515 was severely reduced by three orders of magnitude (Fig. 4C), and to obtain 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 more rapidly than that of the parental strain (data not shown). Interestingly, the conidiation defect was not recovered when PDEH515 was grown under osmotic stabilization (Fig. 4C).

As a result of these phenotypes, we characterized 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 those of 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 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 metulae (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 that in the parental strain. Putative phialides had, at most, one or two small round cells at the tip with intense CFW staining, which probably correspond to the few conidia produced by this mutant. In PDEH515, the long conidia strings common in the parental strain were not observed. The microscopic characterization 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*.

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

Fig. 4 Colony morphology and growth of *Penicillium digitatum* parental and mutant strains. (A) *Penicillium digitatum* strain colonies grown on potato dextrose agar (PDA) plates at 24 °C 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 (filled symbols and full lines) and PDA plates supplemented with 1 M sorbitol (open symbols and broken lines). Data show the mean $\pm$ standard deviation (SD) of three replicates 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 $\pm$ SD of three replicates of the conidia/mL produced per plate.

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 showed increased sensitivity to CW-damaging agents, such as CFW and Congo red (CR), and to the cell membrane destabilizing compound sodium dodecyl sulfate (SDS),

Fig. 5 Comparative fluorescence microscopy analysis of *Penicillium digitatum* PHI26 and PDEH515 strains. (A) Germlings of PHI26 and PDEH515 after 16 h of incubation at 24 °C of conidial suspension in potato dextrose broth (PDB). (B) Mycelium of PHI26 and PDEH515 grown on slides. (C) Conidiophore structure formed on slides after 72 h of growth. Images show the differential interference contrast (DIC) bright fields and the corresponding fluorescence fields of samples stained with calcofluor white (CFW) to visualize the cell wall (CW). Letters on the images indicate the septum (s), the balloon-like structures (b) formed in the mycelium and the different cell types of conidiophore: rame (r), metulae (m), phialide (f) and conidium (c).

indicating a defective CW (Fig. S6, see Supporting Information). None of the constitutive expression transformants showed changes in susceptibility to these compounds (data not shown).

The $\Delta pmt2$ 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 μ M PAF26, whereas 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 μ M and a minimal inhibitory concentration (MIC) of

Fig. 6 Antifungal activity of the PAF26 peptide against *Penicillium digitatum* strains. (A) In total, 5 μ L of three serial five-fold dilutions (2.5×10^4 , 5×10^3 and 10^3 conidia/mL, indicated by the upper triangle) from the parental PHI26, deletion mutant PDEH515, ectopic transformant PDEH540 and two constitutive transformants PDEH59 and PDEH67 were grown at 24 °C for 7 days on potato dextrose (PD) agarose 24-well plates with either no peptide (control) or containing 16 or 32 μ M 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 (full line) with 50% inhibitory concentration (IC_{50}) of 1.8 μ M ($r = 0.9553$).

4 μ M against the parental strain, whereas PAF26 concentrations as high as 32 μ M 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; Fig. S7, see Supporting Information), the incidence of infected wounds inoculated with PHI26 increased over

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 in disease incidence on fruits infected with PDEH515 was more evident at a lower inoculum dose (10^4 conidia/mL) (Fig. 7). In this case, the incidence of disease caused by PHI26 reached only ~70% at 7 dpi, whereas PDEH515 inoculation resulted in just ~10% of infected wounds. Lesion diameter measurements showed that, at early infection (i.e. 2–5 dpi), PDEH515 lesions enlarged more slowly than those of the rest of the strains (Figs 7B and S7B). Significantly, none of the PDEH515-infected wounds showed the typical coloration of the green mould caused by *P. digitatum* (Figs 7C and S7D). The appearance of white mycelium was rare in the case of PDEH515, and infection was only noticeable by tissue maceration, even in lesions of a size comparable with those 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 more strongly 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 showed 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 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.

DISCUSSION

This study reports the identification of one member of each *pmt* subfamily in the post-harvest pathogen *P. digitatum*, consistent with reports that showed the existence of only one orthologue for each *pmt* subfamily in other filamentous fungi and in the fission yeast. Functional analysis by complementation of yeast Δpmt 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 *Pmts* from these two fungi. This is the first study that complements a yeast $\Delta pmt2$ mutant and uses CFW sensitivity as a complementation assay. Previous heterologous complementation has been demonstrated for *CaPMT1* in the $\Delta pmt1$ mutant of *S. cerevisiae* (Timpel *et al.*, 1998) and for *pmt1* and *pmt4* *U. maydis* genes in *Sc. pombe* (Fernández-Álvarez *et al.*,

Fig. 7 Virulence assays of *Penicillium digitatum* parental PHI26 or mutant PDEH515 on orange fruit at low inoculum dose (10^4 conidia/mL). (A) Incidence of infection determined as the percentage of infected wounds. (B) Lesion diameter of the infected wounds. In (A) and (B), data show the mean $\pm$ standard deviation (SD) of three replicates at each day post-inoculation (dpi). In (A), the incidence of infection of PDEH515 was different from that of PHI26 at 4, 5, 6 and 7 dpi ($P < 0.05$, Student's *t*-test). (C) Representative images of three oranges infected with each fungal strain at 7 dpi.

2009). Overall, these findings confirm the functional conservation within each subfamily of *pmt* genes among distantly related fungi.

Detailed characterization of fungal *pmt* gene expression has been described previously in *A. nidulans* (Goto *et al.*, 2009; Oka *et al.*, 2004), *Cr. neoformans* (Willger *et al.*, 2009), *B. cinerea*

(González *et al.*, 2013) and the yeast *C. albicans* (Prill *et al.*, 2005; Timpel *et al.*, 2000). 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 *Pdpmts*, which demonstrated that *Pdpmt1* has the highest expression, one order of magnitude higher than that of *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 leaf infection, whereas *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), similar to the findings in the *Cr. neoformans* *pmt1* and *pmt4* mutants (Willger *et al.*, 2009).

The *Pdpmt2* gene is necessary for growth, conidiogenesis and CW 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 *Cr. neoformans* (González *et al.*, 2013; Kriangkripipat and Momany, 2009; Oka *et al.*, 2004, 2005; 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 (Mouyna *et al.*, 2010; Prill *et al.*, 2005; Willger *et al.*, 2009). The selection of transformants in our study required osmotic stabilization, probably because of the compromised CW integrity of the mutants. Functional complementation of PDEH515 with the native *Pdpmt2* could not be demonstrated because of its low conidial recovery and defective CW, which made the *A. tumefaciens*-mediated transformation 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 characterization of polarity mutants in fungi identified *pmt* genes, as was the case for the *swaA* mutant in *A. nidulans* (Shaw and Momany, 2002). The reduction in *pmt2* expression in *A. fumigatus* also demonstrated the involvement of this gene in hyphal polarity (Fang *et al.*, 2010).

The $\Delta pmtA$ mutants of *A. nidulans* and *A. awamori* also presented growth reduction and defects in mycelium morphology with balloon-like structures (Oka *et al.*, 2004, 2005), similar to our observations with PDEH515. These globular structures have also been described in null *chs* mutants involved in fungal CW synthesis (Gandía *et al.*, 2014; Martín-Urdiroz *et al.*, 2008). These

Fig. 8 Sensitivity of *Penicillium digitatum* strains to hydrogen peroxide (H_2O_2). In total, 5 μL of three serial 10-fold dilutions (10^5 , 10^4 and 10^3 conidia/mL) of the same strains as in Fig 6 were applied to potato dextrose agar (PDA) plates supplemented with three different concentrations of H_2O_2 . The plates were incubated at 24 °C for 4 days.

Fig. 9 Invasive growth of *Penicillium digitatum* PHI26 parental and PDEH515 mutant strains. The top diagram shows a scheme to follow the experimental procedure. In total, 5 μL of 2.5×10^4 conidia/mL were grown for 3, 5 or 7 days on potato dextrose agar (PDA) plates covered by a cellophane membrane (A), the cellophane membrane with the colony was removed (B) and, finally, the plate was incubated for four additional days (C).

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 stabilizers (Gentzsch and Tanner, 1996; González *et al.*, 2013; Goto *et al.*, 2009; Kriangkripiat and Momany, 2009; Mouyna *et al.*, 2010). Our gene expression analysis demonstrated the induction of *Pdpmt2* and *Pdpmt4* expression under osmotic stress (Fig. 3C) and the repression of *chs* genes (Gandia *et al.*, 2012), indicating that the CW is remodelled under osmotic adaptation,

which is probably 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 the abnormal development of conidiophores. A high induction of *Pdpmt2* was observed during conditions that produced high conidiation, such as growth on PDA plates and fruit infection (Figs 3B and 4). Studies in *A. nidulans* and *A. fumigatus* have also demonstrated a reduction in conidia in *pmt* mutants (Fang *et al.*, 2010; Goto *et al.*, 2009; Kriangkripiat and Momany, 2009; Mouyna *et al.*, 2010). A more extreme phenotype was found in the *B. cinerea* $\Delta pmt2$, which was reported to be completely unable to sporulate (González *et al.*, 2013). The *wetA* mutants of *A. nidulans*

and *Penicillium 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 (Fernández-Álvarez *et al.*, 2009; Lengeler *et al.*, 2008). 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, because of 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* $\Delta Bcpmt2$ mutant displayed reduced growth and no conidiation in axenic culture, as well as defects in the adhesion and penetration to host tissue that were associated with reduced virulence (González *et al.*, 2013). Defective cellular adhesion was also found in the $\Delta pmt4$ mutant of *U. maydis* (Fernández-Álvarez *et al.*, 2012).

The slow growth and defective CW of PDEH515 surely limits fruit tissue colonization and explains the decrease in virulence. However, the severe reduction in virulence and absence of visible hyphal development on fruit also indicate that additional factors may be involved. The production of ROS in infected citrus fruit is a well-characterized host defence response that is suppressed by *P. digitatum* during infection (Macarasin *et al.*, 2007). Indeed, our data revealed that *Pdpmt* genes are induced by H₂O₂ and indicated 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* $\Delta pmt4$ mutant in maize (Fernández-Álvarez *et al.*, 2009).

Most sensor proteins from mitogen-activated protein kinase (MAPK) signalling cascades are cell surface proteins that are glycosylated (Lien *et al.*, 2013; Rispaill *et al.*, 2009). 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* $\Delta pmt4$ 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 *Fusarium oxysporum* on 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 in 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 their 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 have 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 plays 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 (Harris *et al.*, 2009; Ibeas *et al.*, 2000; Koo *et al.*, 2004), demonstrating that appropriate protein glycosylation is required for the full activity of a number of antifungal peptides and proteins, including PAF26.

Glycan patterns from CW proteins have been 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 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 sequestration of 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.

EXPERIMENTAL PROCEDURES

Fungal strains and culture conditions

The parental strain used was the wild-type *P. digitatum* PHI26 (CECT20796), whose genome sequence is available (Marcet-Houben *et al.*, 2012) (WGS project AKCT01). *Penicillium digitatum* strains were grown at 24 °C either on PDA (Difco, Detroit, MI, USA, #213400) plates or in PDB (Difco, #254920) with shaking. Conidia were collected from plates, dispersed in water, filtered and counted in a haemocytometer. To determine colony growth, 5 µL of 1×10^5 conidia/mL were deposited on PDA plates, and the colony diameter was measured daily. Conidiation was quantified on plates grown for 12 days. Cellophane invasion assays were performed as described previously (Prados Rosales and Di Pietro, 2008) by placing autoclaved cellophane membranes on PDA plates and depositing 5 µL of 2.5×10^4 conidia/mL on the membranes. Plates were grown for 3, 5 or 7 days, the cellophane membranes with the colonies were removed and, finally, the plates were incubated for four additional days.

Sequence analysis of *Pdpmt* genes

Pdpmt coding sequences were obtained from a *P. digitatum* fosmid genomic library (Gandia *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 by Gandia *et al.* (2012) and reverse transcribed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA, #18080-044) into first-strand cDNA, which was then PCR amplified using specific primer pairs, cloned into pGEM-T (Promega, Madison, WI, USA, #A1360) and sequenced. All the oligonucleotides used in this work are detailed in Table S1 (see Supporting Information).

Saccharomyces cerevisiae complementation assays

Functional complementation assays in the *S. cerevisiae* $\Delta pmt2$ 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 (*MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura Δ 0*) selected on synthetic complete medium lacking leucine (SC-Leu) and confirmed by sequencing. SC-Leu is 0.6% yeast nitrogen base (Invitrogen #Q300-09), 2% glucose and 0.16% yeast synthetic drop-out medium without leucine (Sigma-Aldrich, St. Louis, MO, USA, #Y1376). The plasmids were transformed in the genetic background of the $\Delta pmt2$ 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 overnight in liquid SC-Leu with 2% glucose at 30 °C. The cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.2 in 5 mL, pelleted by centrifugation, washed twice in sterile water and resuspended 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 °C, the cells were diluted to an OD₆₀₀ of 0.1 and 5 µL of ten-fold serial dilutions were dotted onto SC-Leu plates containing: (i) 2% glucose; (ii) 2% glucose, 12.5 µg/mL CFW; or (iii) 2% galactose, 12.5 µg/mL CFW. The plates were incubated at 30 °C for 3 days.

qRT-PCR analysis

To determine the expression of the *Pdpmt* genes, total RNA extraction, reverse transcription and quantitative PCRs were performed as reported by Gandia *et al.* (2012). For expression normalization, the β -tubulin gene, ribosomal protein L18a and 18S rRNA were simultaneously used as references as described previously. The Relative Expression Software Tool (REST) (Pfaffl *et al.*, 2002) was used to determine the statistical significance of the relative expression changes of target genes normalized to the reference genes.

Construction of plasmid vectors for fungal transformation

The *Pdpmt2* disruption construct (pGKO2_Pdpmt2) was generated by fusion PCR (Szewczyk *et al.*, 2006) and cloned into the binary vector pGKO2 (Khang *et al.*, 2006) (see construct details in Fig. S3). The *Pdpmt2* constitutive expression construct (pBht2_Pdpmt2) was designed with the full-length *Pdpmt2* coding sequence cloned under the control of the *A. nidulans* glyceraldehyde triphosphate dehydrogenase (*gpdA*) promoter into the vector pBht2 (Mullins *et al.*, 2001) (Fig. S4).

Agrobacterium tumefaciens-mediated transformation of *P. digitatum*

Agrobacterium tumefaciens-mediated transformation of *P. digitatum* PHI26 was performed as described previously (Gandia *et al.*, 2014; Khang *et al.*, 2006; Michielse *et al.*, 2008; Mullins *et al.*, 2001) with minor modifications. Induced *A. tumefaciens* carrying each transformation vector (pGKO2_Pdpmt2 or pBht2_Pdpmt2) and conidia of *P. digitatum* were

co-cultured at 24 °C for 2 days on nitrocellulose filters. The filters were transferred to PDA plates containing 50 µg/mL hygromycin (Invivogen, San Diego, CA, USA, #ant-hm-5), and incubated for 2 days at 24 °C. The plates were then washed with sterile water, and the wash was plated on PDA plates supplemented with 50 µg/mL hygromycin, which were incubated at 24 °C until Hyg^R colonies appeared. In the case of the disruption construct (pGKO2_Pdpmt2), the plates containing Hyg^R transformant colonies were washed again 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 °C. F2dU is transformed by the herpes simplex virus thiamine kinase gene (HSVtk) 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 monospore culture for molecular analysis.

Molecular characterization of *P. digitatum* transformants

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

Fluorescence microscopy analyses

The morphology of each fungal strain was visualized with fluorescence microscopy (microscope E90i, Nikon Chiyoda, TO, Japan) after staining with 50 µg/mL CFW. Representative images observed in the 40× 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 1×10^5 conidia/mL of *P. digitatum* strains were inoculated on PDA plates supplemented with CFW, SDS (Sigma-Aldrich #L4509), CR (Sigma-Aldrich #C6767) or H₂O₂.

PAF26 (amino acid sequence RKKWFW) was synthesized and provided at >95% purity by GenScript Corporation (Piscataway, NJ, USA). Two types of assay 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 microtitre plate assay as described previously (Muñoz *et al.*, 2006).

Infection assays

Penicillium digitatum inoculation on fully mature and freshly harvested orange fruits (*Citrus sinensis* L. Osbeck cv. Navelina) and the collection of fruit peel tissue samples were conducted as described previously (González-Candelas *et al.*, 2010). Fruits were wounded by making punctures (depth, 3 mm) with a nail at four sites around the equator, and

immediately inoculated by depositing 5 µL of conidial suspension on each wound. Each treatment (fungal strain) consisted of three replicates of five fruits each (four wounds per fruit). Three parameters were recorded daily: the percentage of infected wounds per replicate, the diameter of the lesion of infected wounds and the percentage of wounds per replicate that showed the green colour typical of conidiation (conidiation index). The mean ± standard deviation of each parameter was calculated.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

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

Fig. S2 Domain organization of Pmt proteins from *Penicillium digitatum*.

Fig. S3 Disruption of the *Pdpmt2* gene in *Penicillium digitatum*.

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

Fig. S5 Relative quantification of *Pdpmt* gene expression in *Penicillium digitatum* strains.

Fig. S6 Sensitivity of *Penicillium digitatum* strains to different antifungal compounds.

Fig. S7 Virulence assays on orange fruits of *Penicillium digitatum* strains at high inoculum dose (10^5 conidia/mL).

Table S1 Oligonucleotide primers used in this work.