The phosphatase Ptc6 is involved in virulence and MAPK signalling in *Fusarium oxysporum*

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SUMMARY

Mitogen-activated kinase (MAPK) signalling pathways are involved in several important processes related to the development and virulence of *Fusarium oxysporum*. Reversible phosphorylation of the protein members of these pathways is a major regulator of essential biological processes. Among the phosphatases involved in dephosphorylation of MAPKs, type 2C protein phosphatases (PP2Cs) play important roles regulating many developmental strategies and stress responses in yeasts. Nevertheless, the PP2C family is poorly known in filamentous fungi. The *F. oxysporum* PP2C family includes seven proteins, but only Ptc1 has been studied so far. Here we show the involvement of Ptc6 in the stress response and virulence of *F. oxysporum*. Expression analysis revealed increased expression of ptc6 in response to cell wall and oxidative stresses. Additionally, targeted inactivation of ptc6 entailed enhanced susceptibility to cell wall stresses caused by Calcofluor White (CFW). We also demonstrate that the lack of Ptc6 deregulates both the Mpk1 phosphorylation induced by CFW and, more importantly, the Fmk1 dephosphorylation induced by pH acidification of the extracellular medium, indicating that Ptc6 is involved in the regulation of these MAPKs. Finally, we showed, for the first time, the involvement of a phosphatase in the invasive growth and virulence of *F. oxysporum*.

**Keywords:** cell wall integrity, invasive growth, pathogenesis, PP2C phosphatases, stress response.

INTRODUCTION

Reversible phosphorylation of proteins, controlled by protein kinases and protein phosphatases, is a major mechanism regulating different biological processes. The three-tiered protein kinase module of mitogen-activated kinase (MAPK) is highly conserved in eukaryotes. This module is composed of a MAPK kinase kinase (MAPKKK), which in turn activates the MAPK for the downstream transmission of cellular signals (Segorbe et al., 2017; Turra et al., 2014; Widmann et al., 1999). In the budding yeast *Saccharomyces cerevisiae*, five MAPKs (Fus3, Kss1, Mpk1/Slt2, Hog1 and Smk1) regulate different signalling pathways (Chen and Thorner, 2007). By contrast, only three MAPK pathways (Fmk1, Mpk1 and Hog1, orthologues to Fus3/Kss1, Mpk1 and Hog1, respectively) have been defined in most ascomycete fungi (Turra et al., 2014). *Fusarium oxysporum* is an ascomycete that causes vascular wilt diseases in a large number of field and greenhouse crops, leading to important economic losses throughout the world (Dean et al., 2012). *Fusarium oxysporum* has also been recognized as an emerging human pathogen for immunocompromised patients (Nucci and Anaissie, 2007). An interplay among the three MAPK pathways, Fmk1, Mpk1 and Hog1, has been reported to contribute to the stress response and cross-pathogenicity in *F. oxysporum* (Segorbe et al., 2017). In a previous work, it has also been demonstrated that Fmk1 and the downstream transcription factor Ste12 are essential for plant infection (Di Pietro et al., 2001; Rispail and Di Pietro, 2009). Moreover, Segorbe and collaborators demonstrated that, similar to other fungal MAPKs, Mpk1 is important for host sensing, penetration and colonization, and thus for virulence both on plant and animal hosts (Segorbe et al., 2017; Turra et al., 2014, 2015).

MAPK pathways are regulated by phosphorylation and protein phosphatase-mediated dephosphorylation. Protein phosphorylation and dephosphorylation generally occur in serine, threonine or tyrosine residues. Based on their substrate specificities, protein phosphatases are classified into two major groups: serine/threonine phosphatases and tyrosine phosphatases (PTPs). There are two super-families of serine/threonine phosphatases, phosphoprotein phosphatases (PPPs) and metal-dependent protein phosphatases (PPMs) (Cohen, 1989). The PPP family contains three subtypes of phosphatases: PP1, PP2A and PP2B. The PPM family involves type 2C protein phosphatases (PP2Cs) and pyruvate dehydrogenase phosphatases (*Arino et al., 2011; Lammers and Lavi, 2007*). PP2Cs are monomeric enzymes that require metal cations Mg^{2+} or Mn^{2+} for their activities. The role of PP2Cs has been extensively studied in...
S. cerevisiae, where seven PP2Cs, PTC1–PTC7, have been shown to be involved in different developmental and stress response pathways (Cheng et al., 1999; Guo et al., 2017a,b; Jiang et al., 2001; Ruan et al., 2007). Saccharomyces cerevisiae PP2C orthologues also play significant roles in medically important fungi such as Candida albicans (Fan et al., 2009; Feng et al., 2010; Hanaoka et al., 2008; Wang et al., 2007; Yu et al., 2010; Zhao et al., 2010) and the opportunistic human pathogen Aspergillus fumigatus (Winkelstroter et al., 2015a,b). Members of the PP2C family have also been described in filamentous fungi, such as the model organism Neurospora crassa (Ghosh et al., 2014) and several plant pathogens such as Fusarium graminearum (Jiang et al., 2010, 2011), F. oxysporum (Lemos et al., 2018) and Botrytis cinerea (Yang et al., 2013). In recent years, several groups have undertaken the study of PP2Cs in different fungal species. Recently, a study including 144 sequences from 22 fungal species has been reported, defining five major groups of PP2Cs related to S. cerevisiae PTC1–PTC7 phosphatases (Arino et al., 2011). Most studies concerning PP2C proteins describe the role of fungal PP2Cs in MAPK signalling, stating the role of PTC1 in the high-osmolarity glycerol (HOG) response and cell wall integrity pathways (Arino et al., 2011; Jiang et al., 2011; Lemos et al., 2018; Liu et al., 2011; Yang et al., 2013). PTC1 has additional functions in tRNA splicing, sporulation, heat-shock and lithium tolerance, the target of rapamycin (TOR) pathway and vacuole distribution during cell division (Gonzalez et al., 2006, 2009; Jin et al., 2009; Lemos et al., 2018; Robinson et al., 1994; Ruiz et al., 2006; Shiozaki et al., 1994). PTC2 and PTC3 are also involved in the S. cerevisiae HOG pathway (Young et al., 2002). The putative orthologue of both PTC2 and PTC3 in A. fumigatus, PtcB, is involved in MpKα phosphorylation (Sit2/Mpk1 orthologue), suggesting a role of this phosphatase in the cell wall integrity pathway (Winkelstroter et al., 2015a). In the case of B. cinerea, BcPtc3 negatively regulates phosphorylation of BcSak1 (the orthologue of HOG1) (Yang et al., 2013). Moreover, S. cerevisiae, PTC2 and PTC3 are the major phosphatases dephosphorylating CDC28, contributing with 90% of the total CDK dephosphorylation activity (Cheng et al., 1999). Little is known about PTC4, a phosphatase absent in filamentous fungi (Arino et al., 2011). Phosphatases PTC5, PTC6 and PTC7 are located in S. cerevisiae mitochondria. PTC5 and PTC6, but not PTC7, in concert with protein kinases PKP1 and PKP2, regulate the reversible phosphorylation of PDA1, the E1 alpha subunit of the PDH complex in S. cerevisiae (Gey et al., 2008; Krause-Buchholz et al., 2006; Tal et al., 2007). Additionally, PTC6 regulates positively SLT2 signalling in S. cerevisiae (Sharmin et al., 2015) and is required for mitochondrial autophagy as well as for rapamycin and caffeine sensitivity (Gey et al., 2008; Ruan et al., 2007; Sakumoto et al., 2002). CaPTC6 and CaPTC7 are phosphatases involved in C. albicans tolerance to azoles (Zhao et al., 2012). To date, little is known about the role of Ptc6 in filamentous fungi. Fusarium graminearum mutants lacking Ptc6 did not show significant phenotypical changes (Jiang et al., 2011). Here we demonstrate for the first time the role of Ptc6 protein phosphatase in the pH-mediated regulation of Fmk1 phosphorylation in F. oxysporum. We also show that Ptc6 contributes to invasive growth and complete disease symptom development of F. oxysporum on tomato plants. In addition, we show that mutants lacking Ptc6 became less tolerant to cell wall stress compounds and were delayed in the activation of the cell wall integrity pathway.

RESULTS

Fusarium oxysporum genome contains a phosphatase induced by stress that is orthologous to the PTC6 gene of S. cerevisiae

A previous analysis of F. oxysporum f. sp. lycopersici genome revealed the presence of seven genes belonging to the PP2C family of phosphatases, orthologues to the S. cerevisiae PP2C genes (Lemos et al., 2018). The PTC6 orthologous gene, FOXG_07912, contains no introns and codes for a putative 662 amino acid protein.

As a first approach to unravel the biological role of Ptc6, we conducted gene expression analyses under different stress conditions produced by the exposure to different compounds, including osmotic (sorbitol and KCl), membrane and cell wall [sodium decyl sulphate (SDS)], and Calcofluor White (CFW) and oxidative (menadione) stressors. We also tested ptc6 expression levels after pH shift from 7.0 to 5.0. Significantly increased ptc6 mRNA levels were detected after induction by all tested compounds, especially after pH shift or in response to membrane (SDS), oxidative (menadione) and cell wall (CFW) stress compounds (Fig. 1). These results drove us to further investigate the implication of Ptc6 in the response of F. oxysporum to different cell stress related to MAPK pathways.

Ptc6 contributes to conidiation, germination and stress response in F. oxysporum

To gain insight into the function of Ptc6, targeted null mutants were generated by replacement of the entire coding region with the hygromycin resistance cassette, using the split-marker technique (Catlett et al., 2003) (Fig. S1A). Hygromycin-resistant transformants were analysed by PCR using an appropriate combination of primers (Table S1). Putative mutants lacking the
wild-type ptc6 locus were identified (Fig. S1B). Southern blot hybridization analysis of transformants confirmed the replacement of a 5.1-kb HindIII fragment, corresponding to the wild-type ptc6 allele, by a fragment of 6.0 kb, corresponding to homologous recombinants containing a single copy of the deletion construct (Δptc6-1 and Δptc6-2 mutants) (Fig. S1C). Complemented strains were obtained by co-transformation of Δptc6-1 protoplasts with the phleomycin resistance cassette and the wild-type ptc6 allele. Transformants containing the wild-type ptc6 allele (Δptc6+ptc6) were identified both by PCR and Southern blot analyses (Fig. S1D,E). Transformant Δptc6+ptc6-6 was selected for further experiments since it showed a single insertion event, although located at a non-homologous region.

To test the involvement of Ptc6 in vegetative hyphal growth, the colony diameter of the different strains was measured on yeast-peptone-glucose agar (YPGA) medium. A slight, but not significant, reduction was observed in the growth of two independent Δptc6 mutants (Δptc6-1 and Δptc6-2) in comparison to the wild-type colonies or the Δptc6+ptc6 complemented strain (data not shown). Moreover, similar to the previously reported Δptc1 mutant (Lemos et al., 2018), we observed a 20% reduction in germination in both Δptc6 independent mutants in comparison to the wild-type strain (Fig. 2A). By contrast, increased conidiation was shown by both Δptc6 independent mutants (Fig. 2B). Complementation of the Δptc6 mutant with the wild-type ptc6 allele partially restored these phenotypes. These results suggest a role of Ptc6 in both developmental processes.

In order to further study the involvement of Ptc6 in the F. oxysporum stress response, we analysed the growth of the Δptc6 mutant on YPGA medium supplemented with different stress compounds. Sensitivity of the Δptc6 mutant to membrane (SDS), cell wall (CFW) and oxidative (menadione) stress compounds was increased compared to the wild-type strain or the complemented transformant. No differences in tolerance to osmotic stress (sorbitol and KCl) was shown (Fig. 3A). These results together with the expression analysis suggest that Ptc6 participates in the cell wall and oxidative stress pathways, both regulated by Mpk1.

Previous studies have shown that filamentous fungi respond to fludioxonil by activation of the HOG MAPK pathway, and that Hog1 hyperactivation becomes deleterious to the fungus (Kilani and Fillinger, 2016). In a previous work, the Δptc1 mutant showed increased sensitivity to fludioxonil compared to the wild-type strain, in agreement with the constitutive phosphorylation of Hog1 in this background (Lemos et al., 2018). In the case of F. oxysporum Δptc6 we found that mutants were more tolerant to fludioxonil than the wild-type strain (Fig. 3B). By contrast, tolerance to iprodione remained unaltered.

**Ptc6 contributes to basal phosphorylation levels of F. oxysporum MAPKs**

Recent studies reported that three MAPK pathways (Hog1, Mpk1 and Fmk1) contribute, combined or individually, to the regulation of fungal growth, stress response and virulence of F. oxysporum (Segorbe et al., 2017). Moreover, PP2Cs were described as strong regulators of MAPK pathways in fungi (Arino et al., 2011; Yang et al., 2013). Additionally, we previously demonstrated that F. oxysporum Ptc1 negatively regulates the Hog1 pathway (Lemos et al., 2018).
In order to understand the role of Ptc6 in the regulation of MAPK pathways, we analysed Hog1, Mpk1 and Fmk1 phosphorylation levels in the Δptc6 mutant compared to the wild-type strain. Western blot analysis of proteins isolated from 15-h grown germlings revealed a significant increased basal phosphorylation level of Fmk1 in the Δptc6 mutant, suggesting that Ptc6 might be a negative regulator of the Fmk1 pathway (Fig. 4). In addition, a significant decreased phosphorylation level of Hog1 was observed in the Δptc6 mutant. No significant changes in Mpk1 phosphorylation status were detected in the mutant strains. These results suggest that Ptc6 participates in the regulation of basal phosphorylation levels of Fmk1 and Hog1 MAPK pathways in *F. oxysporum*.

**The *F. oxysporum* cell wall integrity pathway is modulated by Ptc6**

The results shown above suggest that Ptc6 plays an important role in *F. oxysporum* tolerance to cell wall stresses induced by CFW. To deepen the analysis of the function of Ptc6 in the Mpk1 response cascade, we monitored the phosphorylation status of Mpk1 in response to CFW exposure. While a significant increase in Mpk1 phosphorylation was observed in the wild-type strain in response to the stressor, no change in Mpk1 phosphorylation levels was detected in the Δptc6 mutant (Fig. 5A).

To further study the role of Ptc6 in the regulation of the cell wall integrity pathway, we performed gene expression analyses of downstream components of the Mpk1 cascade reported to be activated in response to CFW (Boorsma et al., 2004; Sanz et al., 2017), including the own Mpk1 MAPK, the Gfa1 (the major controller of chitin synthesis) (Lagorce et al., 2002) and the Chs3 chitin synthase (Shaw et al., 1991). A significant reduction in the transcript levels of the three genes analysed was observed in the Δptc6 mutant compared to the wild-type strain (Fig. 5B). Together these results support the involvement of Ptc6 in the regulation of Mpk1 pathway in response to the cell wall stress produced by CFW.
Previous studies revealed that a functional MAPK signalling cascade is required for fungal pathogenicity on plants (Turra et al., 2014; Xu and Hamer, 1996). Furthermore, the Fmk1 MAPK cascade controls invasive growth and is essential for the infection of tomato plants in F. oxysporum (Di Pietro et al., 2001). Although the mechanisms regulating MAPK signalling during the plant–fungus interaction remain unknown, a recent work reported that during the initial steps of infection F. oxysporum produces a rapid alkalization of the surrounding medium (Masachis et al., 2016). These authors also demonstrated that the increase of extracellular pH from 5 to 7 generates a rapid phosphorylation of Fmk1, while a further decrease to pH 5 results in Fmk1 dephosphorylation.

To further evaluate the involvement of Ptc6 in the regulation of the Fmk1 pathway, we tested the invasive growth ability of the Δptc6 mutant using a cellophane penetration assay at
different pH conditions, which defines experimentally the ability of the fungus to invade a solid surface, mimicking the initial steps on the infection process (Prados Rosales and Di Pietro, 2008; Perez-Nadales and Di Pietro, 2011). As reported previously (Masachis et al., 2016), the wild-type strain was able to invade the solid substrate at pH 7 but not under acidic conditions (pH 5) (Fig. 6). By contrast, the absence of functional Ptc6 allowed the fungus to invade the underlying solid substrate and develop a colony even at pH 5, suggesting that the invasive growth pathway was active at pH 5 in the Δptc6 mutant. Complementation of the mutant with the wild-type allele restored the phenotype.

To investigate the role of Ptc6 in the regulation of the Fmk1 cascade, we monitored the Fmk1 phosphorylation status in the Δptc6 mutant in response to pH changes by western blot analyses of proteins from germlings grown at pH 7 for 15 h or after 5 min shift to pH 5. As previously shown, the change of extracellular pH resulted in a rapid Fmk1 dephosphorylation in the wild-type strain (Masachis et al., 2016) (Fig. 7). However, no significant differences in the Fmk1 phosphorylation level were detected after extracellular acidification in the Δptc6 mutant. These results support the role of Ptc6 in the regulation of F. oxysporum Fmk1 pathway in response to extracellular pH changes.

**Ptc6 is necessary for the full virulence of F. oxysporum on tomato plants**

Since Fmk1 and Mpk1 are essential for the infection of tomato plants in *F. oxysporum* (Di Pietro et al., 2001; Segorbe et al., 2017), virulence of the Δptc6 mutant was evaluated by inoculating the roots of tomato seedlings with microconidial suspensions of two independent Δptc6 mutants, the wild-type strain and the complemented Δptc6+ptc6 transformant. Plants inoculated with the wild-type strain showed characteristic wilt symptoms, disease severity increased steadily throughout the experiment and all the plants were dead 30 days after inoculation (Fig. 8). Surprisingly, the two independent Δptc6 mutants showed significant reduced virulence compared to the wild-type strain, showing a 40% plant survival at the end of the experiment (30 days post-inoculation). Complementation of the mutant strain with a functional copy of the gene partially restored the wild-type virulence. As stated above, complementation of the mutant strain was accomplished by the insertion of a wild-type allele in an unknown genomic region that might be differentially activated in response to different stimuli. This could explain the different complementation levels of the phenotypes tested for the ptc6 mutant. Although these results were unexpected, the differences observed in mortality indicate unequivocally that Ptc6 contributes to virulence of *F. oxysporum*.

**DISCUSSION**

PP2Cs are involved in a wide variety of key cellular processes in both pathogenic and nonpathogenic fungi, such as cell wall integrity, filamentous growth, virulence and environmental stress responses. PP2Cs are also involved in cell cycle progression, metabolism and cell death (Arino et al., 2011; Lammers and Lavi, 2007). In a previous study, we identified seven putative phosphatases in the genome of *F. oxysporum*: single orthologues to *S. cerevisiae* PTC1, PTC3 and PTC6 and two predicted orthologues to PTC5 and PTC7, respectively, as previously described in *F. graminearum* (Jiang et al., 2011; Lemos et al., 2018). Nevertheless, there is not much information regarding PP2Cs in filamentous fungi. Previous studies demonstrated the involvement of PtcB in the osmotic stress response of *A. fumigatus* (Winkelstroter et al., 2015a) and the contribution of BcPtc1 and BcPtc3 in virulence of *B. cinerea* (Yang et al., 2013). In a recent work, we demonstrated that *F. oxysporum* Ptc1 participates in the dephosphorylation of Hog1 and Mpk1 MAPKs, thus regulating the HOG pathway.
and CWI pathways, respectively (Lemos et al., 2018). Loss of Ptc1 produced no alteration in the symptom disease progress in tomato plants, suggesting that Ptc1 is not important for pathogenesis in *F. oxysporum*. However, *F. graminearum* Ptc1 and Ptc3 have been reported to contribute to pathogenesis on wheat plants (Jiang et al., 2010, 2011). Although functions of Ptc1 and Ptc3 have been studied in several fungi, no information is available to date concerning Ptc6 functions in filamentous fungi. This is the first study showing the involvement of Ptc6 in growth, virulence and stress response in *F. oxysporum*.

**Ptc6 contributes to cell integrity through the regulation of Mpk1 MAPK phosphorylation level**

In a previous study we reported the involvement of Ptc1 in tolerance to cell wall damage-inducing compounds, associated with unbalanced phosphorylation levels of Mpk1 (Lemos et al., 2018). It is well known that cell wall damage causes activation of Mpk1/Slt2 mitogen-activated pathway, demonstrating a relationship between Ptc1 and the regulation of the CWI pathway (Arino et al., 2011; Levin, 2005). The inactivation of both PTC1 and the MAPK SLT2 in yeast provoked cells extremely sensitive to cell wall damage agents (Gonzalez et al., 2006). Additionally, PTC6 was reported to participate in the activation of the SLT2-mediated cell wall integrity (Sharmin et al., 2015).

Here we show that the expression of *F. oxysporum* ptc6 increased in response to SDS- and CFW-induced cell wall stress. Furthermore, loss of Ptc6 produced a significantly increased sensitivity to CFW. Moreover, we found changes in the phosphorylation level of Mpk1 in response to CFW exposure, similar to the behaviour of strains lacking Ptc1 in response to SDS (Lemos et al., 2018). Taken together, our results suggest that the diminished tolerance to CFW shown by the Δptc6 mutant is related to the unbalanced phosphorylation level of Mpk1 triggered by CFW.

**Ptc6 contributes to the pH-mediated regulation of Fmk1 phosphorylation**

The Fmk1 MAPK pathway is highly conserved in fungi and is essential for infection in an important number of plant pathogens (Turra et al., 2014). Recent work in *F. oxysporum* reported that extracellular alkalization activates the Fmk1 invasive growth MAPK, leading to tomato plant infection. The surrounding root alkalization is induced by the export of the rapid alkalization factor (RALF) peptide, which stimulates the activation of the conserved Fmk1 MAPK cascade (Masachis et al., 2016). The rapid phosphorylation of Fmk1 induces the expression of Ste12 transcription factor which, in turn, activates the transcription of genes involved in degradation of the tomato cell wall (Rispail and Di Pietro, 2009).

Our results demonstrate that, in contrast to the wild-type strain, the Δptc6 mutant was able to invade the solid substrate even at acidic pH and showed higher Fmk1 basal
phosphorylation levels together with an unbalanced dephosphorylation status of Fmk1 in response to extracellular pH changes. Furthermore, we demonstrated an increased ptc6 expression level after pH shift to acidic conditions, supporting a crucial role of this phosphatase in the regulation of Fmk1-mediated invasive growth in *F. oxysporum*. To our knowledge this is the first report of a PP2C involved in invasive growth in filamentous fungi.

**Participation of Ptc6 in the interplay between Mpk1 and Fmk1 modulates *F. oxysporum* virulence**

The *F. oxysporum* Fmk1 MAPK pathway is essential for virulence-related functions such as invasive growth on cellophane membranes, root adhesion, colonization of tomato plants, growth on tomato fruits and secretion of cell wall-degrading enzymes (CWDE) (Di Pietro et al., 2001; Perez-Nadales and Di Pietro, 2011; Prados Rosales and Di Pietro, 2008; Rispail and Di Pietro, 2009; Segorbe et al., 2017). Moreover, Segorbe et al. (2017) reported that some of the altered virulence-related functions observed in the Δfmk1 strain were also deficient in strains lacking Hog1 or Mpk1 MAPKs, suggesting that Hog1 and Mpk1 contribute to plant infection through additional Fmk1-independent functions. Our results indicate that the phosphatase activity of Ptc6 contributes negatively to Fmk1 and positively to Mpk1 pathways. The ability of the Δptc6 mutant to invasively grow through cellophane membranes in acidic conditions, together with the unbalanced dephosphorylation level of Fmk1, suggests that the Δptc6 mutant should be more virulent than the wild-type strain on tomato plants. Surprisingly, we found a significant increase in the survival rate of plants infected with the Δptc6 mutant compared to those infected with the wild-type strain. We suggest that the balanced phosphorylation levels of both Mpk1 and Fmk1 kinases is necessary for the successful infection of tomato plants, as was reported previously (Segorbe et al., 2017). Recently, it was demonstrated that nutrient sensing is mediated by Fmk1 while pheomere sensing is regulated by Mpk1, showing the contribution of both MAPKs to the chemotropic growth of *F. oxysporum* germlings towards the plant roots, the previous step to the invasion of root tissues (Turra et al., 2015).

In summary, our results demonstrate that Ptc6 phosphatase plays a crucial role in the regulation of MAPK signalling affecting virulence in *F. oxysporum*.

**EXPERIMENTAL PROCEDURES**

**Fungal isolates and cultures conditions**

*Fusarium oxysporum* f. sp. *lycopersici* wild-type strain 4287 race 2 (FGSC 9935) was obtained from J. Tello, Universidad de Almeria, Spain. Fungal strains were stored at −80 °C as microconidial suspensions in 30% glycerol. For microconidia and mycelium production, cultures were grown at 28 °C with shaking at 170 rpm on liquid potato dextrose broth (PDB) (Di Pietro and Roncero, 1998) containing the appropriate antibiotic when necessary. For germling production, fresh microconidia were incubated in YPG (0.3% yeast extract, 1% peptone, 2% glucose) for 15 h. When necessary, the pH of the medium was buffered using 100 mM of 2-(N-morpholino)ethanesulfonic acid (MES) and adjusted with HCl or NaOH.

**Targeted gene deletion and gene complementation**

Targeted gene deletion was carried out by replacement of the *ptc6* gene (FOXG_07912) with the hygromycin B resistance (HygR) marker cassette using the split-marker methodology (Catlett et al., 2003). Two overlapping gene deletion constructs were generated (Fig. S1A). One contained the promoter sequence of *ptc6*, obtained by amplification with the specific primer pair Ptc6-1/Ptc6-2-TrpC (Table S1), fused to the 3’ end (approximately 75%) of the HygR cassette (Carroll et al., 1994) and amplified using primer pair trpC-8B and hyg-G (Table S1). The second fragment contained the terminator region of the target gene, obtained by amplification with the specific primer pair Ptc6-3-gpdA/Ptc6-4 (Table S1), fused to the 5’ end (approximately 75%) of the HygR cassette and amplified using primer pair gpdA-15B and hyg-Y (Table S1). These two overlapping DNA fragments were used to transform protoplasts of *F. oxysporum* f. sp. *lycopersici* 4287 wild-type strain, as reported previously (Punt and van den Hondel, 1992; Di Pietro and Roncero, 1998). HygR transformants were subjected to two rounds of monoclonal isolation. Deletion mutants were initially identified by PCR (Fig. S1B) and the homologous recombination events were confirmed by Southern analysis of selected transformants (Fig. S1C).

For complementation of the Δptc6 mutant, a 4.7 kb fragment containing the *ptc6* gene (including promoter and terminator regions) was amplified from genomic DNA using primer pair Ptc6-1/Ptc6-4 (Table S1). A 2.5-kb fragment containing the pheomycin resistance (PhlR) cassette was amplified from plasmid pAN8.1 (Punt and van den Hondel, 1992) using primers gpdA-15B and trpC-8B (Table S1). Both fragments were used in the proportion 3:1 to co-transform protoplasts of strain Δptc6-1. The resulting PhlR transformants were subjected to two rounds of monoclonal isolation and the complementation events were identified by both PCR (Fig. S1D) and Southern blot analysis (Fig. S1E).

**Nucleic acid manipulation and quantitative real-time reverse transcription-PCR analyses**

Total RNA and genomic DNA were extracted from *F. oxysporum* mycelium following previously reported protocols (Chomczynski...
and Sacchi, 1987; Raeder and Broda, 1985). For the analysis of ptc6 gene expression, total RNA was isolated from germlings of the wild-type strain grown on PDB for 15 h and subsequently treated during 1 h with SDS (0.0125% w/v), CFW (50 µg/mL), menadione (20 µg/mL), KCl (1.2 M) and sorbitol (1.25 M), or from germlings grown for 15 h on PDB at pH 7 and then shifted to PDB at pH 5 for 30 min. For the expression analysis of genes downstream of Mpk1, total RNA was isolated from germlings of the different strains grown on YPGA for 15 h and subsequently treated for 30 min with CFW (50 µg/mL). The quality and quantity of extracted nucleic acids were determined by running aliquots in ethidium bromide-stained agarose gels and by spectrophotometric analysis in an ND-1000 spectrophotometer (NanoDrop Technologies Wilmington, DE, USA). PCR experiments were routinely performed in a Bio-Rad T100 thermal cycler, using BioTaq polymerase (Bioline, London, UK) and Expand High Fidelity polymerase (Roche Diagnostics, Basel, Switzerland) when necessary. The labelling of probes was performed using the non-isotopic labelling digoxigenin kit (Roche Diagnostics) using primer pair Ptc6-7/Ptc6-8 (Table S1). Southern blot analyses were carried out as described by Di Pietro and Roncero (1998).

For quantitative reverse transcription-PCR (RT-qPCR), RNA samples were treated with DNase I (Roche Diagnostics). Two micrograms of total RNA were used to synthesize the first-strand cDNA by Transcriptor cDNA Master (Roche Diagnostics). Genomic DNA contamination and quality of cDNA samples were analysed by PCR using primer pair Act-7q and Act-9q (flanking an intron on the actin gene) (Table S1) and Expand High Fidelity Taq DNA polymerase (Roche Diagnostics). cDNA samples were diluted 1:1 in ultrapure deionized water for RT-qPCR assays. RT-qPCR was performed using 7.5 µL FastStart Essential DNA Green master (Roche Diagnostics), 5 µL of cDNA (1:1) and 0.3 µL of each gene-specific primer in a final volume of 15 µL. Gene-specific primers were designed to flank introns (Table S1). The following PCR programme was used for all reactions: an initial step of denaturation (10 min, 95 °C), followed by 40 cycles of 10 s at 95 °C, 10 s at 62 °C and 20 s at 72 °C, 5 s at 80 °C (Plate read), in a CFX-Connect Real Time System (Bio-Rad, Hercules, CA, USA). Transcript levels were calculated by comparative ∆ΔCt (Livak and Schmittgen, 2001) and normalized to the actin gene. Expression values were represented as time-fold increase relative to the control samples in each experiment. All experiments included three independent biological replicates and three technical replicates for each biological sample. Data were subjected to statistical significance test (t-test). Differences were considered significant with P < 0.05 or P < 0.01.

Protein manipulation and western blot analyses
Total proteins were isolated from germlings of the different strains grown for 15 h on YPGA and subsequently treated for 15 min with 50 µg/mL CFW or from germlings grown for 15 h on PDB at pH 7 and after 5 min shift to PDB at pH 5.

Protein extraction and western blot analyses were carried out as described previously (Lemos et al., 2018; Mechin et al., 2007) using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XPV R rabbit monoclonal antibody (mAb) (no. 4370), horseradish peroxidase (HRP)-conjugated anti-rabbit (no. 7074) and LumiGLO monoclonal antibody (no. 7003), all from Cell Signaling Technology (Danvers, MA, USA), to detect both phosphorylated Fmk1 and Mpk1 MAPKs. Unphosphorylated Fmk1 and Mpk1 were detected using antiFus3 (gN-19) (sc6772) and anti-Mpk1 (gN-19) (sc-6802), respectively, and donkey anti-goat IgG-HRP (sc2020) as secondary antibody (Santa Cruz Biotechnology, Heidelberg, Germany). Phosphorylated Hog1 was detected using the Phospho-p38 MAPK (Erk1/2) (Thr180/Tyr182) rabbit monoclonal antibody (no. 92115) (Cell Signaling Technology). Unphosphorylated Hog1 was detected using anti-Hog1 rabbit monoclonal antibody (γ-215) (sc-9079) (Santa Cruz Biotechnology). Anti-mouse-α-tubulin antibody (sc69971) and goat anti-rat IgG HRP secondary antibody (sc2006) (Santa Cruz Biotechnology) were used to detect the loading control α-tubulin. Hybridizing bands were visualized using ECL Select Western Blotting Detection Reagent (GE Healthcare, Madrid, Spain) on a LAS-3000 Intelligent Dark-box (Fuji Photo Film Co, Tokyo, Japan). Band intensity was quantified using Image J v. 1.08 software (NIH, Maryland, USA). All experiments included three independent biological replicates. Data were subjected to statistical significance test (t-test). Differences were considered significant with P < 0.05 or P < 0.01.

Colony growth, conidiation and germination assays
For growth assays, serial dilutions of freshly obtained microconidia were transferred to YPGA (0.3% yeast extract, 1% tryptone, 2% glucose, 2% agar). For stress tolerance analyses, stress compounds were added at the indicated concentrations: SDS (0.0125% w/v), CFW (50 µg/mL), Congo Red (CR) (50 µg/mL), menadione (20 µg/mL), NaCl (1.2 M), KCl (1.2 M) and sorbitol (1.25 M). For fungicide assays, 5 × 104 fresh microconidia were transferred to YPGA plates containing fludioxonil (20 µg/mL) or iprodione (20 µg/mL).

For conidiation assays 5 × 105/mL fresh microconidia were inoculated in PDB and incubated for 48 h at 28 °C with shaking (170 rpm). Aliquots of 1 mL were taken from each replicate to perform quantification of the conidia on a Leica DMR microscope using a haemocytometer (Thoma 250) (Marienfield, Lauda-Königshofen, Germany). Germination was measured 13 h after inoculation of 3.19 × 105 conidia in 1 mL of germination medium (0.05% KCl, 0.05% MgSO4, 0.05% KH2PO4, 0.1% NaN3, 2% sucrose) (Vitale et al., 2019). One hundred events (germinated or ungerminated microconidia) were counted by sample.
using a Leica DMR microscope. Germination rate was calculated using the following formula: germination (%) = (germinated microconidia/total microconidia) × 100. Statistical analysis (t-test) was performed to compare data from the Δptc6 mutant or the Δptc6+ptc6 complemented transformant to the wild-type strain. Differences were considered significant with P < 0.05 or P < 0.01.

Invasive growth analysis

Invasive growth assays on cellophane membranes were performed as described previously (Lopez-Berges et al., 2010; Prados Rosales and Di Pietro, 2008) with some modifications, using solid minimal medium (MM) (0.05% MgSO₄·7H₂O, 0.1% KH₂PO₄, 0.05% KCl, 0.2% NaNO₃, 3% sucrose, 0.2% agar) (Puhalla, 1968) and buffered with 100 mM MES. MM plates buffered to pH 5 or 7 were covered with sterile 8 mm diameter cellophane sheets. Five-microlitre water droplets containing 5 × 10⁴ microconidia were spotted onto the centre of the plates. After 3 days incubation at 28 °C, cellophane sheets containing the fungal colonies were carefully removed and the plates were further incubated for 24 additional hours at 28 °C. Plates were imaged before and after cellophane removal. All experiments included three replicates and were performed twice.

Plant infection assays

Tomato root infection assays were performed as described by Di Pietro et al. (2001) using the susceptible cultivar Monika (Syngenta Seeds, Almeria, Spain). Two-week-old tomato seedlings were inoculated by submerging roots in a suspension of 5 × 10⁶ conidia/mL, planted on vermiculite and maintained in a growth chamber at 28 °C with a photoperiod of 14 h light/10 h darkness. Ten plants were used for each treatment. Plant survival was recorded for 30 days. A log-rank (Mantel–Cox) test was used to assess the statistical significance of the differences in survival among groups. Data were plotted using the software GraphPad Prism v. 5 (GraphPad Software, La Jolla, CA, USA). P < 0.05 was considered to be significant. Experiments were performed three times with similar results. The data presented are from one representative experiment.

Protein domain prediction

Prediction of protein domains was performed using the algorithm ScanProsite (ExPASY, Swiss Institute Bionformatics, Switzerland).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Schematic representation of the ptc6 wild type and mutant loci and molecular analysis of Δptc6 mutants and Δptc6+ptc6 complemented strains. (A) Strategy for targeted replacement of the ptc6 coding region using the split marker technique and the hygromycin resistance (HygR) cassette as selection marker. Black arrow heads indicate the primer pairs used for amplification of both overlapping fragments (dashed lines). DNA fragment used as probe for Southern hybridization analyses is indicated. HindIII restriction sites are shown. (B) Identification of putative Δptc6 mutants by PCR. Primers Ptc6-11q/Ptc6-12q were used to amplify a fragment of 200 bp corresponding to the ptc6 ORF. PCR products were analysed by electrophoresis on a 0.7% agarose gel. The absence of the wild-type (WT) 200 bp fragment was observed in strains #1 and #2, while it was still present in the ectopic transformant Ect #6. (C) Southern analysis of Fusarium oxysporum WT strain, Δptc6 mutants (#) and the ectopic transformant Ect #6. gDNAs were digested with HindIII and hybridized to the probe indicated in panel A. The 5.1 kb WT fragment was replaced by a 6 kb fragment in Δptc6 mutants, but not in the ectopic transformant E#6. (D) PCR analysis of Δptc6+ptc6 complemented strains using primer pair Ptc6-11q/Ptc6-12q. Recovery of the WT 200 bp band was observed in strains #1, #3, #5 and #6. (E) Southern analysis of Δptc6+ptc6 complemented strains. gDNA from the indicated strains was digested with HindIII and hybridized to the probe indicated in panel A. A 6 kb fragment corresponding to the Δptc6 allele and additional bands, corresponding to heterologous insertion of the ptc6 gene in the genome, were observed in all the strains tested. Transformant Δptc6+ptc6 #6 was selected for further experiments since it showed a single insertion event.

Fig. S2 Original western blot images of proteins extracted from mycelia grown on yeast-peptone-glucose agar (YPGA) and after Calcofluor White (CFW) treatment. Protein extracts from the wild-type strain (WT) and the Δptc6 mutant cultured for 15 h in YPGA (0) and subsequently treated during 15 minutes with CFW (15) were subjected to immunoblot analysis using (A) α-tubulin, (B) α-P-Mpk1, (C) α-Mpk1, (D) α-P-Fmk1, (E) α-Fmk1, (F) α-P-Hog1 and (G) α-Hog1 monoclonal antibodies. I, II and III indicate three biological replicates of each sample. In panels A, B, C and D replicates I and II were loaded on the same gel, whereas replica III was loaded on a separate gel. In panels E, F and G the three biological replicates were loaded on the same gel. Asterisks indicate bands used in figure 4 (*) and 5 (**).

Fig. S3 Original western blot images of proteins extracted from mycelia grown in potato dextrose broth (PDB) pH 7 and after shift to pH 5. Protein extracts from the wild-type strain (WT) and the Δptc6 mutant cultured for 15 h in PDB (0) and subsequently treated during 15 minutes with CFW (15) were subjected to immunoblot analysis using (A) α-tubulin, (B) α-P-Fmk1, (C) α-Mpk1, (D) α-P-Fmk1, (E) α-Fmk1, (F) α-P-Hog1 and (G) α-Hog1 monoclonal antibodies. I, II and III indicate three biological replicates of each sample. Replicates I and II were loaded on the same gel, whereas replicates III was loaded on a separate gel. Asterisks indicate bands used in Fig. 7.

Table S1 Oligonucleotides used in this study.