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Three *Fusarium oxysporum* mitogen-activated protein kinases (MAPKs) have distinct and complementary roles in stress adaptation and cross-kingdom pathogenicity

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SUMMARY

Mitogen-activated protein kinase (MAPK) cascades mediate cellular responses to environmental signals. Previous studies in the fungal pathogen *Fusarium oxysporum* have revealed a crucial role of Fmk1, the MAPK orthologous to *Saccharomyces cerevisiae* Fus3/Kss1, in vegetative hyphal fusion and plant infection. Here, we genetically dissected the individual and combined contributions of the three MAPKs Fmk1, Mpk1 and Hog1 in the regulation of development, stress response and virulence of *F. oxysporum* on plant and animal hosts. Mutants lacking Fmk1 or Mpk1 were affected in reactive oxygen species (ROS) homeostasis and impaired in hyphal fusion and aggregation. Loss of Mpk1 also led to increased sensitivity to cell wall and heat stress, which was exacerbated by simultaneous inactivation of Fmk1, suggesting that both MAPKs contribute to cellular adaptation to high temperature, a prerequisite for mammalian pathogens. Deletion of Hog1 caused increased sensitivity to hyperosmotic stress and resulted in partial rescue of the restricted colony growth phenotype of the *mpk1*Δ mutant. Infection assays on tomato plants and the invertebrate animal host *Galleria mellonella* revealed distinct and additive contributions of the different MAPKs to virulence. Our results indicate that positive and negative cross-talk between the three MAPK pathways regulates stress adaptation, development and virulence in the cross-kingdom pathogen *F. oxysporum*.

Keywords: cross-talk, development, *Fusarium oxysporum*, MAPKs, stress response, virulence.

INTRODUCTION

Fungal pathogens can sense and respond to a wide variety of stimuli from the environment and their host organisms. Among the key pathways functioning in the molecular perception of these cues are mitogen-activated protein kinase (MAPK) cascades, a family of evolutionarily conserved three-tiered protein kinase modules composed of a MAPK kinase kinase (MAPKKK) that phosphorylates the downstream MAPK kinase (MAPKK), which, in turn, activates the MAPK for the downstream transmission of cellular signals (Turrà *et al.*, 2014; Widmann *et al.*, 1999). In the budding yeast *Saccharomyces cerevisiae*, five MAPKs have been reported, Fus3, Kss1, Mpk1/Slt2, Hog1 and Smk1, which regulate pheromone response, filamentation and invasive growth, cell wall integrity, high-osmolarity stress response and spore wall assembly, respectively (Chen and Thorner, 2007). By contrast, most ascomycete fungi only possess three MAPKs which are orthologous to Fus3/Kss1, Mpk1 and Hog1 (Turrà *et al.*, 2014).

Components of the Fus3/Kss1 MAPK cascade are broadly conserved regulators of infection-related morphogenesis and invasive growth in plant-pathogenic fungi. For example, in the rice pathogen *Magnaporthe oryzae*, deletion of the orthologous MAPK Pmk1 leads to impaired formation of appressoria and loss of virulence (Xu and Hamer, 1996). Similarly, mutants in the orthologous MAPKs of phylogenetically distant phytopathogens, including both appressoria-forming and appressoria-non-forming species, fail to infect the host even when applied directly on wounded plant tissues (Di Pietro *et al.*, 2001; Jenczmionka *et al.*, 2003; Lev *et al.*, 1999; Mey *et al.*, 2002b; Ruiz-Roldán *et al.*, 2001; Takano *et al.*, 2000). Thus, loss of virulence in these mutants is caused by a general defect in invasive growth and host penetration.

The MAPK Mpk1 is required for fungal cell wall remodelling and integrity, which are critical during cell cycle progression and in response to cell wall stress. Both processes are highly relevant for fungal pathogens during interaction with their host (Brown *et al.*, 2014; Levin, 2005). Deletion of *mpk1* orthologues in different pathogen species results in hypersensitivity to cell wall-damaging agents, such as Congo red (CR) or calcofluor white (CFW), as well as to plant defence compounds, such as chitinases, glucanases, antimicrobial peptides or phytoalexins (Hou *et al.*, 2002; Joubert *et al.*, 2011; Mehrabi *et al.*, 2006; Mey *et al.*, 2002a; Ramamoorthy *et al.*, 2007; Valiante *et al.*, 2015; Xu *et al.*, 1998). Importantly, *mpk1* mutants also show defects in host sensing, penetration and colonization, leading to decreased virulence on plant and animal hosts (Diez-Orejas *et al.*, 1997; Igbaria *et al.*, 2008; Kojima *et al.*, 2002; Kraus *et al.*, 2003; Mey *et al.*, 2002a; Rui and Hahn, 2007; Turrà *et al.*, 2015; Xu *et al.*,

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1998). In addition to pathogenicity, the Mpk1 cascade regulates developmental processes, such as hyphal growth on solid surfaces and vegetative hyphal fusion (Hou *et al.*, 2002; Maerz *et al.*, 2008; Turrà *et al.*, 2015; Xu *et al.*, 1998).

The third fungal MAPK, Hog1 (high-osmolarity glycerol), plays a pivotal role in the adaptive response to hyperosmotic stress and mediates sensitivity to certain fungicides, such as phenylpyrroles and dicarboximides (Dixon *et al.*, 1999; Igbaria *et al.*, 2008; Lin and Chung, 2010; Park *et al.*, 2004; Segmuller *et al.*, 2007; Van Thuat *et al.*, 2012). Hog1 orthologues contribute to virulence in a number of phytopathogens (Igbaria *et al.*, 2008; Turrà *et al.*, 2014; Van Thuat *et al.*, 2012), but are dispensable in others (Dixon *et al.*, 1999). Importantly, they appear to be universally required for infection in fungal pathogens of mammals and insects, as reported in the dimorphic fungi *Cryptococcus neoformans* and *Candida albicans*, in which Hog1 controls morphogenetic differentiation and resistance to phagocytic killing (Alonso-Monge *et al.*, 1999; Arana *et al.*, 2007; Bahn *et al.*, 2005; Jin *et al.*, 2012). However, the exact role of Hog1 in fungal virulence is largely unknown.

The soil-inhabiting ascomycete *Fusarium oxysporum* causes vascular wilt disease in a large number of field and glasshouse crops, leading to economically important losses worldwide (Dean *et al.*, 2012). *Fusarium oxysporum* has also been reported as an emerging human pathogen that can cause lethal systemic infections in immunocompromised individuals (Nucci and Anaissie, 2007). Previous work has established that Fmk1, the *F. oxysporum* MAPK orthologous to *S. cerevisiae* Fus3/Kss1, as well as its downstream transcription factor Ste12, are essential for invasive hyphal growth and plant infection (Di Pietro *et al.*, 2001; Rispail and Di Pietro, 2009). Moreover, Fmk1 regulates a number of virulence-related processes, such as hyphal adhesion, vegetative hyphal fusion, the production of plant cell wall-degrading enzymes (CWDEs) and the chemotropic sensing of nutrients (Di Pietro *et al.*, 2001; Prados-Rosales and Di Pietro, 2008; Turrà and Di Pietro, 2015; Turrà *et al.*, 2015). Interestingly, despite the critical role in plant infection, Fmk1, as well as its orthologues in other fungal pathogens, are largely dispensable for virulence on mammalian and invertebrate hosts (Csank *et al.*, 1998; Davidson *et al.*, 2003; Navarro-Velasco *et al.*, 2011; Ortoneda *et al.*, 2004).

Previous studies performed in a number of species suggested that fungal MAPK cascades have broadly conserved, but also species-specific, functions in pathogenicity. The aim of the present study was to systematically dissect the role of the three MAPKs Fmk1, Mpk1 and Hog1 in development, stress response and virulence of *F. oxysporum* on plant and animal hosts. In order to investigate putative cross-talk interactions, a set of mutant strains was generated in which the different MAPK genes were deleted either individually or in combination. Our results reveal both unique and shared functions of *F. oxysporum* Fmk1, Mpk1 and Hog1, and suggest that the three MAPKs contribute in a coordinate manner to the remarkable physiological and pathogenic versatility of this cross-kingdom fungal pathogen.

RESULTS

The three *F. oxysporum* MAPKs differentially contribute to cell wall, hyperosmotic, oxidative and heat stress responses

To explore the biological roles of the three MAPKs Fmk1, Mpk1 and Hog1, we obtained single- and double-deletion mutants in all possible combinations. As *F. oxysporum* Fmk1 and Mpk1 had been identified in previous studies, the corresponding single and double mutants were already available (Di Pietro *et al.*, 2001; Turrà *et al.*, 2015). BLASTp analysis of the *F. oxysporum* genome database identified a single *hog1* orthologue, *FOXG_06318*, encoding a predicted 358-amino-acid protein showing 77% identity with *S. cerevisiae* Hog1. The *hog1*Δ single and double mutants were obtained by replacing the entire coding region with the hygromycin resistance cassette in the wild-type and *fmk1*Δ background or with the phleomycin resistance cassette in the *mpk1*Δ background (Fig. S1A, see Supporting Information). Polymerase chain reaction (PCR) analysis with gene-specific primers identified several putative transformants producing amplification patterns indicative of homologous gene replacement. Southern blot analysis of these transformants confirmed the replacement of a 10-kb *Eco*RI fragment, corresponding to the wild-type *hog1* allele, with a fragment of 7 or 5.5 kb in the wild-type and *fmk1*Δ background (Fig. S1B,C) or *mpk1*Δ background (Fig. S1D), respectively. Complemented strains (*hog1*Δ+*hog1*) were obtained by re-insertion of a 5-kb DNA fragment encompassing the complete *hog1* gene into protoplasts of the *hog1*Δ strain by co-transformation with the phleomycin resistance marker. PCR with gene-specific primers yielded an amplification product identical to that obtained from the wild-type strain in five independent transformants, but not in the *hog1*Δ mutant, suggesting that these strains re-integrated an intact copy of the gene (Fig. S1E).

To test the role of the different MAPK pathways in vegetative hyphal growth, the colony diameter was measured in two different media: nutrient-poor minimal medium (MM) and nutrient-rich YPD (3% yeast extract, 1% peptone, 2% glucose and 1.5% agar) medium. The *fmk1*Δ and *mpk1*Δ mutants exhibited a significantly slower growth rate at the optimum temperature (28 °C), particularly on MM, and this phenotype was exacerbated in the *fmk1*Δ *mpk1*Δ double mutant (Fig. S2A–C, see Supporting Information). At sublethal high temperature (34 °C), hyphal growth was severely restricted in all fungal strains, but loss of Hog1 or Mpk1, either alone or in combination with any of the other MAPKs, exacerbated the growth defect at high temperature (Fig. 1A,B).

In an earlier study, increased sensitivity of the *F. oxysporum* *mpk1*Δ mutant towards the cell wall-targeting compounds CFW

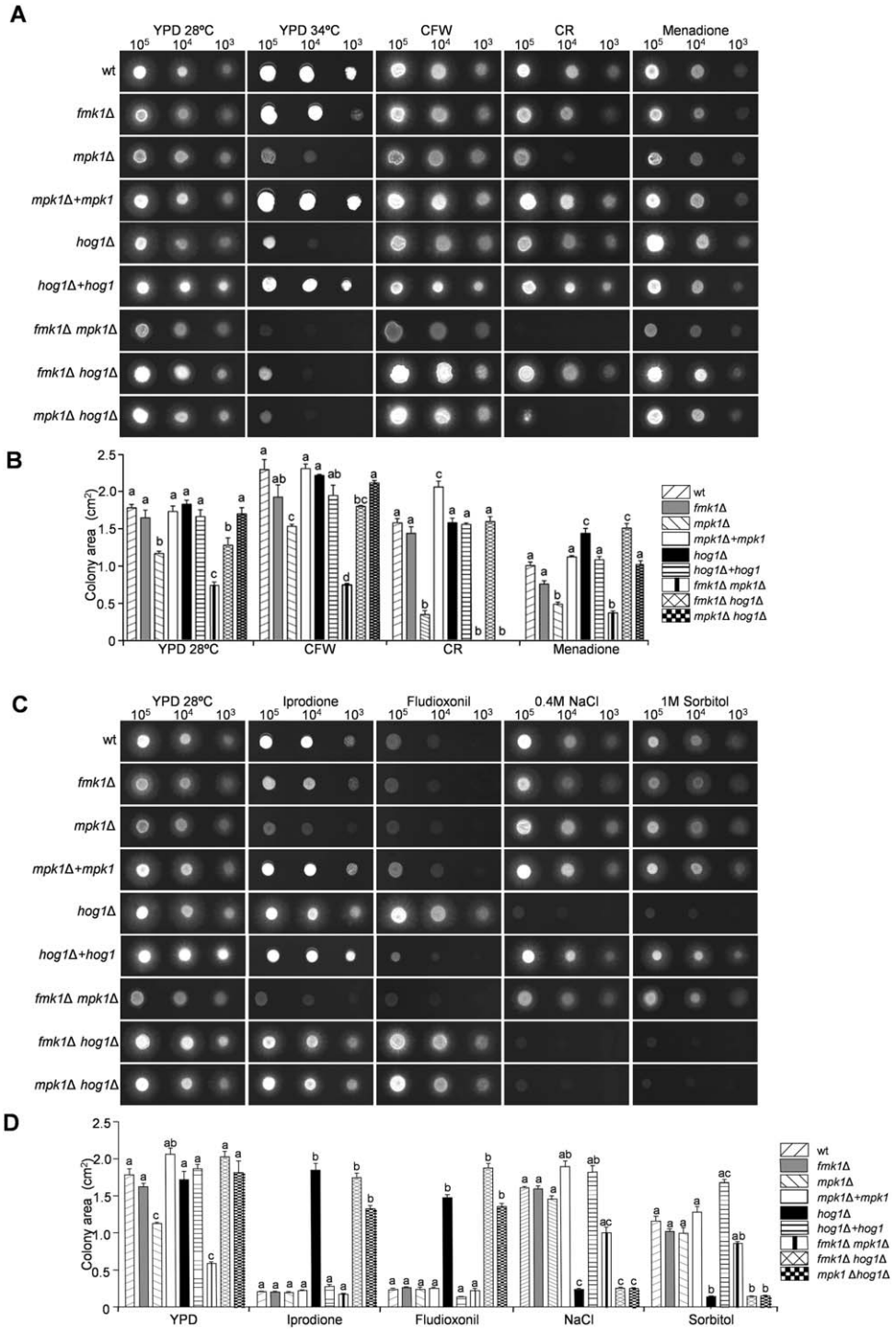

Fig. 1 Stress response phenotypes of *Fusarium oxysporum* mitogen-activated protein kinase (MAPK) mutants. (A, C) Colony phenotypes of the indicated strains grown on YPD (3% yeast extract, 1% peptone, 2% glucose and 1.5% agar) medium at different temperatures (28 or 34 °C) or at 28 °C in the presence of the indicated compounds. Plates were spot inoculated with the indicated number of microconidia, incubated for 2 days at 28 °C or 4 days at 34 °C, and scanned. Bar, 1 cm. (B, D) Quantitative analysis of the colony area (cm^2) performed using MultiGauge software. Data were analysed and plotted with the program GraphPad Prism version 5. Error bars represent standard deviations of colony areas calculated from four independent replicates. Values with the same letter are not significantly different according to Dunnett's multiple comparison test ($P \leq 0.05$). CFW, calcofluor white; CR, Congo red; wt, wild-type.

and CR was reported (Turrà *et al.*, 2015). Here, we confirmed this phenotype which clearly distinguished *mpk1*Δ from *fmk1*Δ and *hog1*Δ (Fig. 1A,B). Interestingly, double deletion of *mpk1* and *fmk1* genes resulted in an additional growth reduction compared with either of the single mutants, suggesting that both MAPKs contribute independently to the cell wall stress response. Furthermore, the *fmk1*Δ *hog1*Δ and *mpk1*Δ *hog1*Δ double mutants were more sensitive to cell wall stress compounds and showed increased phosphorylation of Mpk1 and Fmk1 MAPKs, respectively, compared with the single mutants (Figs 1A,B and 2A–C). However, this phenotype was only visible on CFW for the *fmk1*Δ *hog1*Δ and on CR for the *mpk1*Δ *hog1*Δ strain.

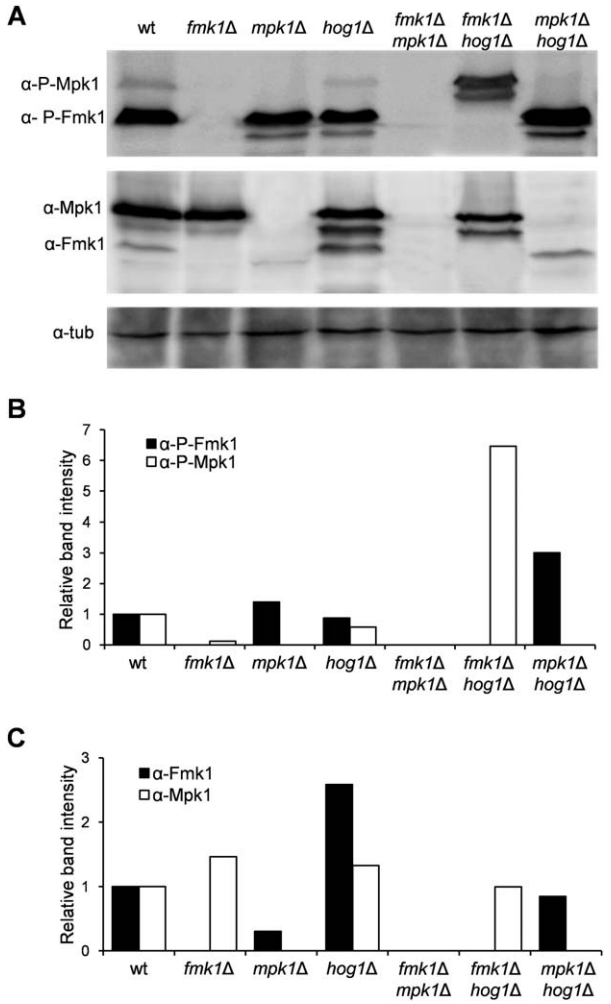

Fig. 2 Phosphorylation of Fmk1 and Mpk1 is altered in mitogen-activated protein kinase (MAPK) deletion mutants of *Fusarium oxysporum*. (A) Protein extracts from fungal mycelium of the indicated strains were subjected to immunoblot analysis with anti-phospho-p44/42 MAPK antibody (α-P-Fmk1 and α-P-Mpk1) or anti-Fus3 and anti-Mpk1 antibodies (α-Fmk1 and α-Mpk1). Anti-α-tubulin antibody (α-tub) was used as loading control. (B, C) The graph shows the ratio of P-Fmk1 and P-Mpk1 (B) or Fmk1 and Mpk1 (C) band intensities normalized to the tubulin control and expressed relative to wild-type levels.

Interestingly, loss of *hog1* in the *mpk1*Δ background largely rescued the colony growth defect on YPD, both in the absence and presence of CFW (Fig. 1A,B).

In response to oxidative stress caused by menadione, contrasting effects on colony growth were observed in the different mutants (Fig. 1A,B). Whereas loss of Mpk1 resulted in significant growth reduction, the mutants lacking Hog1 showed enhanced growth under these conditions. In line with this, growth of the *mpk1*Δ *hog1*Δ double mutant was comparable with that of the wild-type strain, suggesting a compensatory effect of the two mutations.

As reported previously in a number of fungal pathogens (Dixon *et al.*, 1999; Igbaria *et al.*, 2008; Lin and Chung, 2010; Park *et al.*, 2004; Segmuller *et al.*, 2007; Van Thuat *et al.*, 2012), single and double mutants lacking Hog1 displayed increased resistance to the dicarboximide and phenylpyrrole fungicides iprodione and fludioxonil, and were highly sensitive to hyperosmotic stress (Fig. 1C,D). Unexpectedly, the *fmk1*Δ *mpk1*Δ double mutant showed an opposite phenotype, namely decreased resistance to the fungicides and increased resistance to hyperosmotic stress (Fig. 1C,D). These results suggest that Fmk1 and Mpk1 may act antagonistically to Hog1. This idea was further supported by the finding that hyperosmotic stress, which activates the Hog1 cascade, reversed the restricted colony growth phenotype of the *mpk1*Δ and *fmk1*Δ *mpk1*Δ mutants (Fig. 1C,D).

Reactive oxygen species (ROS) production in the different fungal strains was examined by staining the fungal colony with 4-nitroblue tetrazolium chloride (NBT). In the wild-type strain, formazan precipitates, which are indicative of the production of superoxide, were typically more intense at the colony periphery and more abundant on nutrient-rich medium (Fig. S2B). By contrast, the *fmk1*Δ and *mpk1*Δ single and double mutants showed a different pattern, with higher superoxide production at the colony centre. The same phenotype was found in *fmk1*Δ *hog1*Δ and *mpk1*Δ *hog1*Δ double mutants. Detailed observation of the colony margin confirmed the presence of high ROS levels, particularly on rich medium, in *fmk1*Δ and *mpk1*Δ, but not in the *hog1*Δ mutant (Fig. S2B). These results suggest that the different MAPK pathways play distinct roles in the regulation of ROS generation during hyphal development and colony establishment.

Fmk1 and Mpk1 are required for vegetative hyphal fusion and formation of mycelial aggregates

A previous study has revealed that Fmk1 is required for vegetative hyphal fusion of *F. oxysporum* (Prados-Rosales and Di Pietro, 2008). In line with this, we found that *fmk1*Δ strains were impaired in hyphal fusion (Table 1). In addition, *mpk1*Δ mutants also failed to undergo hyphal fusion, as did all the double mutants lacking either one or both of these MAPKs. By contrast, *hog1*Δ mutants performed hyphal fusion as efficiently as the wild-type strain. Moreover, the addition of specific Hog1 activators or inhibitors to the wild-type strain had no significant effect on the hyphal fusion rate (Fig. S3A–C, see Supporting Information). Taken together, these results indicate that Hog1 is not involved in the regulation of vegetative hyphal fusion.

During the growth of *F. oxysporum* in liquid medium, hyphal adhesion and fusion lead to the formation of macroscopically visible hyphal networks (Di Pietro *et al.*, 2001; Lopez-Berges *et al.*, 2010; Prados-Rosales and Di Pietro, 2008). Although the wild-type strain produced dense hyphal aggregates on sodium nitrate, the *fmk1*Δ, *mpk1*Δ, *fmk1*Δ *mpk1*Δ and *fmk1*Δ *hog1*Δ mutants

Table 1 Fmk1 and Mpk1, but not Hog1, are required for vegetative hyphal fusion (VHF) in *Fusarium oxysporum*.

Strain	VHF
wt	+
*fmk1*Δ	−
*fmk1*Δ + *fmk1*	+
*mpk1*Δ	−
*mpk1*Δ + *mpk1*	+
*hog1*Δ#14	+
*hog1*Δ#15	+
*fmk1*Δ *mpk1*Δ	−
*fmk1*Δ *hog1*Δ	−
*mpk1*Δ *hog1*Δ	−

Presence (+) or absence (−) of VHF was determined by spreading microconidia on top of a plate containing 4 mL of solid minimal medium. After 14 h at 28°C, VHF events were observed microscopically. Three hundred germlings were examined for each isolate and each experiment was repeated at least three times.

failed to produce such networks, most likely as a consequence of impaired hyphal fusion (Fig. 3A). Interestingly, aggregate formation was partly restored in the hyphal fusion-defective *mpk1*Δ *hog1*Δ strain. Moreover, Fmk1 phosphorylation was increased in the *mpk1*Δ *hog1*Δ mutant in comparison with the wild-type (Fig. 2A,B), indicating a possible negative role of Hog1 in Fmk1-mediated hyphal adhesion. As reported previously (Lopez-Berges *et al.*, 2010), the wild-type strain failed to produce aggregates in the presence of ammonium. Moreover, none of the tested mutants formed hyphal aggregates under these conditions.

The three *F. oxysporum* MAPKs contribute to different extents to infectious growth and virulence on plant and animal hosts

Fmk1 has been shown previously to control infectious growth functions in *F. oxysporum*, including the ability to invade and colonize living fruit tissue (Di Pietro *et al.*, 2001; Prados-Rosales and Di Pietro, 2008). Here, we found that both *mpk1*Δ and *hog1*Δ, as well as the double mutants, also exhibited significantly reduced invasive growth on apple slices, although some degree of fruit tissue colonization around the inoculation site was still visible (Figs 3B and S4A, see Supporting Information).

Concomitant with the defects in infectious growth, the *fmk1*Δ mutants also exhibited decreased extracellular pectinolytic activity, an important trait associated with the virulence of *F. oxysporum* on tomato plants (Bravo Ruiz *et al.*, 2016; Delgado-Jarana *et al.*, 2005). In contrast with the *fmk1*Δ mutant, which was

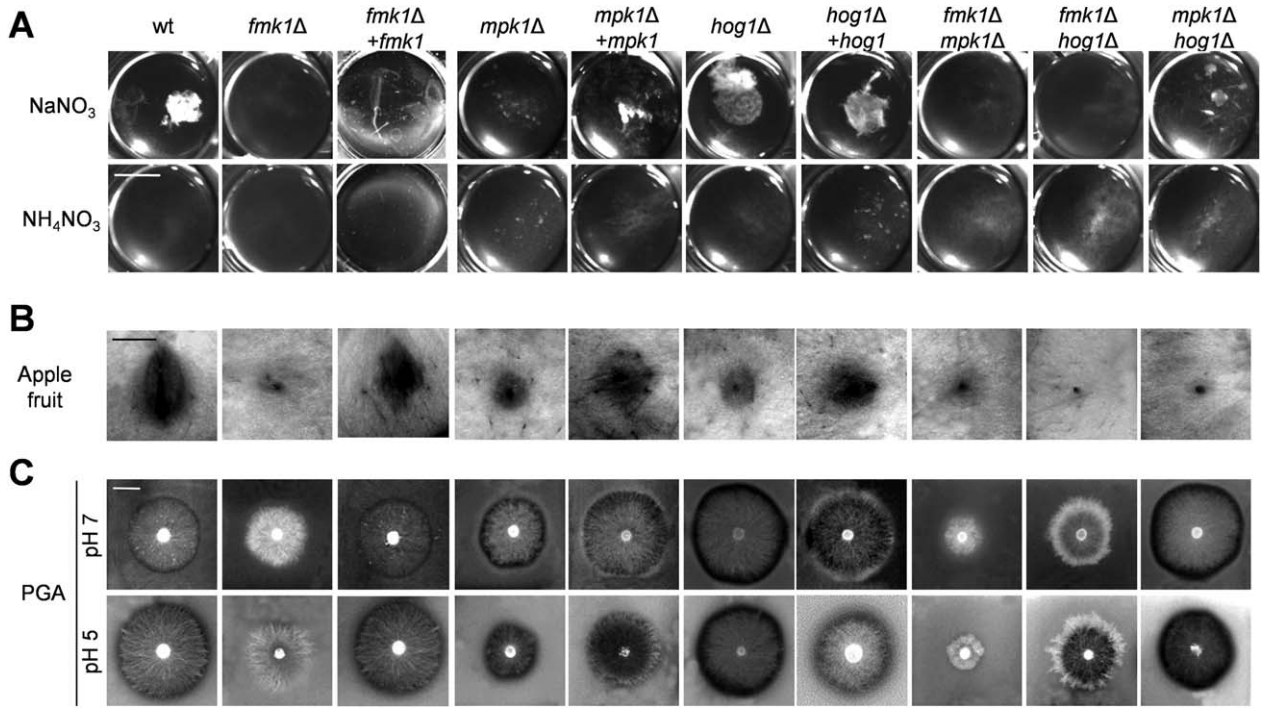

Fig. 3 The mitogen-activated protein kinases (MAPKs) Fmk1, Mpk1 and Hog1 regulate different virulence-associated traits. (A) Hyphal aggregates forming after 36 h of growth in potato dextrose broth (PDB) diluted 1 : 50 with water and supplemented with 25 mM of the indicated nitrogen source. Fungal cultures were vortexed to dissociate weakly adhered hyphae, transferred to a multiwell plate and observed using a binocular microscope. (B) Invasive growth on apple fruit inoculated with microconidia of the indicated strains and incubated at 28 °C for 4 days. (C) The production of extracellular polygalacturonase activity was determined by the inoculating plates containing polygalacturonic acid (PGA) with microconidia of the indicated strains. Enzymatic activity was visualized as a contrasting halo underneath the fungal colony after 3 days of growth at 28 °C.

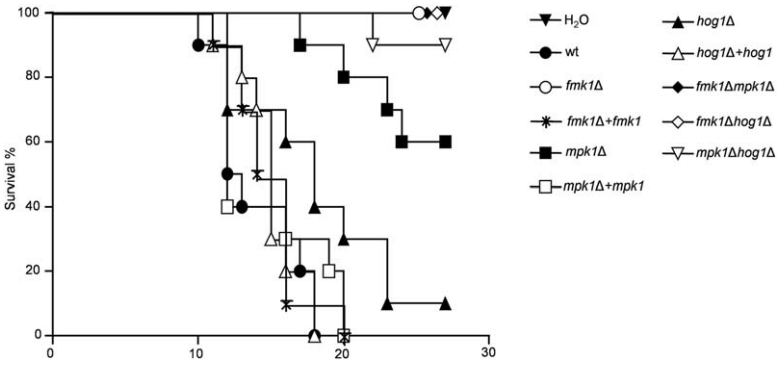

Fig. 4 Fmk1, Mpk1 and Hog1 mitogen-activated protein kinases (MAPKs) differentially contribute to the virulence of *Fusarium oxysporum* on tomato plants. Kaplan–Meier plots showing survival of tomato plants (cv. Monica) inoculated by dipping roots into a suspension of 5×10^6 microconidia/mL of the indicated fungal strains. Experiments were performed at least three times with similar results. Percentage survival of tomato plants was plotted for 30 days. Data shown are from one representative experiment.

impaired in clear halo production on polygalacturonic acid (PGA) medium, the *mpk1*Δ and *hog1*Δ mutants produced a larger clear halo surrounding the colony (Figs 3C and S4B). This phenotype was most pronounced in the *hog1*Δ and *mpk1*Δ *hog1*Δ strains grown at pH 5. Loss of Fmk1 in all genetic backgrounds was invariably associated with a decrease in pectinolytic activity. Thus, while Fmk1 acts as a positive regulator of pectinolytic activity, Mpk1 and Hog1 appear to negatively regulate this process.

We next tested the role of the different MAPKs in the ability of *F. oxysporum* to cause disease on tomato plants. Plants inoculated with the wild-type strain showed a progressive increase in wilt symptoms, and most were dead by 18 days after inoculation (Fig. 4; Table S3, see Supporting Information). By contrast, plants inoculated with single or double mutants lacking the *fmk1* gene failed to develop disease symptoms and remained alive even 30 days after inoculation ($P < 0.0001$ versus wild-type). These results confirm the previous finding that Fmk1 is absolutely essential for the virulence of *F. oxysporum* on tomato (Di Pietro *et al.*, 2001). The *mpk1*Δ and *hog1*Δ mutants showed a partial, but significant, decrease in virulence, which was more pronounced in *mpk1*Δ (*mpk1*Δ, $P < 0.0001$; *hog1*Δ, $P = 0.0214$), and was fully restored on complementation with the corresponding wild-type allele. The progression of disease symptoms in the plants inoculated with these mutants was largely arrested after 15 days, leading to survival of a higher number of plants. Importantly, simultaneous loss of Mpk1 and Hog1 caused an even stronger attenuation of mortality compared with each single mutant ($P < 0.0002$ versus *hog1*Δ; not significant versus *mpk1*Δ). Thus, these two MAPKs appear to have distinct and complementary functions during plant infection.

Previous work has established that the tomato-pathogenic *F. oxysporum* isolate used in this study can kill immunodepressed mice (Ortoneda *et al.*, 2004) as well as larvae of the wax moth *Galleria mellonella*, a non-vertebrate infection model which has been widely used to study fungal pathogens of humans (Mylonakis *et al.*, 2007; Navarro-Velasco *et al.*, 2011). The injection of microconidia of the wild-type or the *fmk1*Δ mutant into the haemocoel of *G. mellonella* resulted in rapid killing of the larvae (Fig. 5A–C; Table S4, see Supporting Information). By contrast, the *mpk1*Δ and *hog1*Δ mutants showed a significant decrease in virulence (*mpk1*Δ, $P < 0.0001$; *hog1*Δ, $P = 0.0031$ versus wild-type), which was fully reversed in the complemented strains. The severity of killing was further reduced in the different double MAPK mutants, with more than 50% of the larvae surviving 7 days after inoculation, although the additive effect was only significant in the *mpk1*Δ *hog1*Δ strain ($P = 0.0078$ versus *mpk1*Δ; $P = 0.0258$ versus *hog1*Δ). These results suggest that Mpk1 and Hog1 have distinct and additive virulence functions in this invertebrate animal infection model.

DISCUSSION

Three conserved MAPK modules regulate key physiological and developmental processes in fungi (Hamel *et al.*, 2012; Rispail *et al.*, 2009; Turrà *et al.*, 2014; Zhao *et al.*, 2007). Although each MAPK pathway has distinct cellular functions, increasing evidence suggests that cross-talk between different MAPKs is essential for the generation of an optimally orchestrated response (Fey *et al.*, 2012; Frei dit Frey *et al.*, 2014; Igbaria *et al.*, 2008; Niba *et al.*, 2013; Saito, 2010). Here, we have genetically dissected the contribution of each of the three MAPKs, Fmk1, Mpk1, and Hog1, in the economically important fungal pathogen *F. oxysporum*. Our study reveals both unique and combined functions in the regulation of developmental processes, such as hyphal growth, fusion and aggregation, as well as in the response to various stresses and during infection of plant and animal hosts (Fig. 6).

Function of Fmk1, Mpk1 and Hog1 MAPKs in stress responses

The ability of fungi to occupy a wide range of ecological niches exposes these organisms to a variety of challenges, including high temperature, cell wall, oxidative and hyperosmotic stresses. The severity of these external insults is likely to be further exacerbated during host infection (Brown *et al.*, 2014; Egan *et al.*, 2007; Yang *et al.*, 2015). For instance, the release of ROS has been reported as a first line of defence against fungal invasion in plants and animals (Brown *et al.*, 2014; Mellersh *et al.*, 2002; Nicola *et al.*,

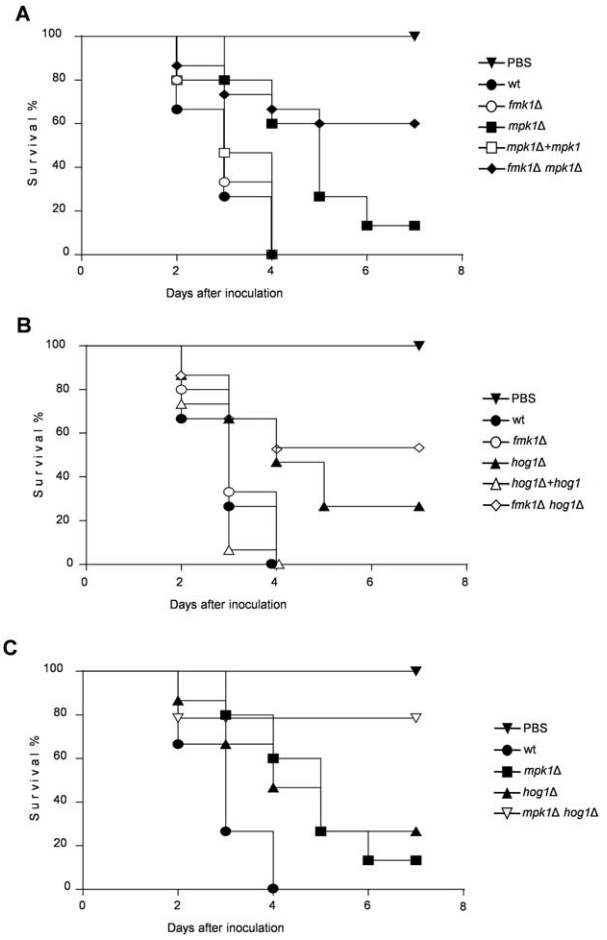

Fig. 5 Fmk1, Mpk1 and Hog1 mitogen-activated protein kinases (MAPKs) differentially contribute to the virulence of *Fusarium oxysporum* on the animal host *Galleria mellonella*. A–C. Kaplan–Meier plots showing survival of *G. mellonella* larvae after injection of 1.6×10^3 microconidia of the indicated *F. oxysporum* strains into the haemocoel and incubation at 30 °C. Percentage survival was plotted for 7 days. All experiments were performed at least four times with similar results. Data shown are from one representative experiment.

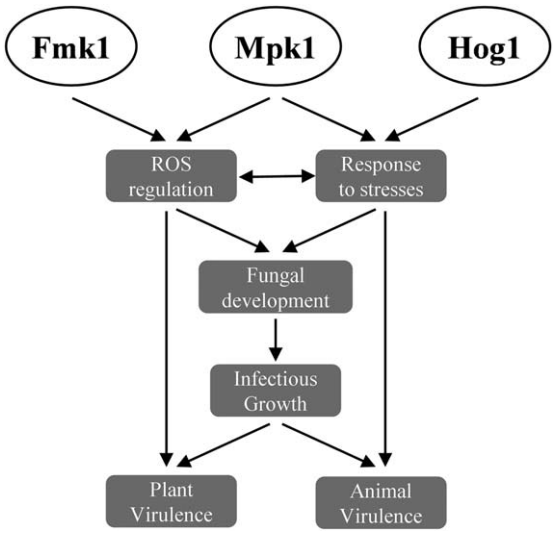

Fig. 6 Schematic diagram showing the roles of Fmk1, Mpk1 and Hog1 mitogen-activated protein kinases (MAPKs) in the stress response, development and virulence of *Fusarium oxysporum*. Mpk1 and Hog1 regulate cellular adaptation to different types of stress, whereas Fmk1 and Mpk1 jointly contribute to reactive oxygen species (ROS) homeostasis. Both processes impact on fungal development and infectious growth, and either directly or indirectly affect virulence on plant and animal hosts.

2008). Accordingly, transcriptomic and proteomic surveys in a number of fungal pathogen species have revealed host-induced activation of stress-related genes inside the plant or animal tissue (Fradin *et al.*, 2005; Irmer *et al.*, 2015; Lorenz *et al.*, 2004; Rubin-Bejerano *et al.*, 2003; Yang *et al.*, 2015). Hog1 has been implicated in cellular adaptation to hyperosmotic, heat, oxidative and citric acid stresses (Alonso-Monge *et al.*, 2003; Bilsland *et al.*, 2004; Lawrence *et al.*, 2004; Saito and Posas, 2012; Winkler *et al.*, 2002) Here, we found that deletion of the *hog1* gene in *F. oxysporum* caused increased sensitivity to hyperosmotic stress and enhanced resistance to the fungicides iprodione and fludioxonil. Both phenotypes are Hog1 specific, as they were neither alleviated nor exacerbated by the additional deletion of other MAPKs. The *hog1*Δ mutant also showed increased resistance towards menadione, whereas the *mpk1*Δ mutant exhibited higher sensitivity. These phenotypes were reversed in the double mutant, suggesting that Hog1 and Mpk1 might have opposite functions in the menadione-triggered oxidative stress response. Importantly, *hog1*Δ mutants in the closely related species *Fusarium graminearum* also exhibited increased resistance to oxidants (Van Thuat *et al.*, 2012). However, in other fungal pathogens, such as *Cochliobolus heterostrophus* and *C. albicans*, loss of Hog1 led to increased sensitivity to oxidative stress (Alonso-Monge *et al.*, 2003; Igbaria *et al.*, 2008), suggesting that oxidant and hyperosmotic stress response signalling may have evolutionarily diverged in different fungal species.

Heat shock responses are important for the virulence of bacterial and fungal pathogens of humans (Klancnik *et al.*, 2014; Shapiro and Cowen, 2012). We hypothesized that resistance to high temperature should be a prerequisite for the ability of a cross-kingdom pathogen, such as *F. oxysporum*, to infect both plant and mammalian hosts. In *S. cerevisiae*, heat stress triggers the coordinated activation of Mpk1 and Hog1, which includes the regulation of specific tyrosine protein phosphatases that block inappropriate cross-talk between both pathways (Kamada *et al.*, 1995; Winkler *et al.*, 2002). Similarly, our results revealed a cooperative role of Mpk1 and Hog1 in the heat stress response of *F. oxysporum*, as the *mpk1*Δ *hog1*Δ double mutant was significantly more affected by high temperature than either single mutant. In addition, we found evidence for a minor contribution of Fmk1 to the heat stress response, given that the *fmk1*Δ *mpk1*Δ double mutant was more sensitive than the *mpk1*Δ strain.

Thus, all three MAPKs appear to contribute to the efficient cellular adaptation of *F. oxysporum* to high temperatures. Recently, changes in the phosphorylation pattern of the three MAPKs on heat shock were observed in the human pathogen *C. albicans* as part of a long-term thermal adaptation programme associated with cell wall remodelling (Leach *et al.*, 2012).

In *S. cerevisiae* and in a number of plant-pathogenic fungi, Mpk1 is required for the adaptive response to cell wall stress (Levin, 2005; Turrà *et al.*, 2014). *Fusarium oxysporum* mutants lacking Mpk1 or its upstream components Rho1, Bck1 or Mkk2 exhibit increased sensitivity to CR and CFW, and display dramatically restricted colony growth on solid surfaces (Martínez-Rocha *et al.*, 2008; Turrà *et al.*, 2015). Here, we noted that the restricted growth phenotype of the *mpk1*Δ mutant was reversed by additional deletion of *fmk1* and, in particular, *hog1*. Moreover, partial reversal of the CFW sensitivity phenotype of *mpk1*Δ was observed in the *mpk1*Δ *hog1*Δ double mutant. Taken together, our results demonstrate that each of the three *F. oxysporum* MAPKs makes important contributions to cellular stress adaptation, and suggest that cross-talk between different MAPK pathways is required for the orchestration of cellular responses to hostile environments.

Evidence for ROS-mediated developmental roles of Fmk1 and Mpk1 MAPKs

Recent findings have suggested that the precise spatio-temporal control of ROS production in fungal cells drives a number of developmental processes, such as germination, vegetative hyphal fusion and differentiation of sexual fruiting bodies or penetration structures (Dirschnabel *et al.*, 2014; Egan *et al.*, 2007; Lara-Ortiz *et al.*, 2003; Roca *et al.*, 2012; Takemoto *et al.*, 2007). Here, we observed a correlation between the defect of different MAPK mutants to fine tune ROS production and their failure to perform vegetative hyphal fusion or to produce hyphal aggregates. Thus, *fmk1*Δ and *mpk1*Δ, but not *hog1*Δ, mutants showed abnormally increased ROS levels and were impaired in vegetative hyphal fusion and aggregation. Both processes are of relevance, as they occur during the early infection stages of *F. oxysporum* (Prados-Rosales and Di Pietro, 2008). Our findings are in line with previous studies proposing that the Fmk1 and Mpk1 pathways regulate ROS production by controlling the expression of NADPH oxidase (Nox) genes (Dirschnabel *et al.*, 2014; Lalucque *et al.*, 2012; Segmuller *et al.*, 2008), and that deletion of Nox1 or its regulator NoxR leads to increased ROS production and impaired hyphal fusion (Dirschnabel *et al.*, 2014; Kayano *et al.*, 2013; Roca *et al.*, 2012). It is interesting to note that Fmk1 and Mpk1 are essential for hyphal fusion (Hou *et al.*, 2002; Maerz *et al.*, 2008; Pandey *et al.*, 2004; Prados-Rosales and Di Pietro, 2008), which is repressed on rich media (Ishikawa *et al.*, 2010; Lopez-Berges *et al.*, 2010; Roca *et al.*, 2012), and that ROS generation in fungal mycelium is also regulated by nutrients, being more abundant in nutrient-rich conditions. Thus, Fmk1 and Mpk1 may play a role in both nutrient sensing and in the regulation of developmental processes promoted by nutrient deprivation.

In contrast with *Neurospora crassa*, in which mutants in components of the Hog1 pathway are impaired in hyphal fusion (Maerz *et al.*, 2008), *hog1* deletion in *F. oxysporum* did not affect ROS levels, hyphal fusion and aggregation. This further supports the idea that the Hog1 MAPK might have undergone evolutionary specialization in different fungal species, as further evidenced by the finding that this MAPK is dispensable for the oxidative stress response of *F. oxysporum*.

The formation of hyphal aggregates was abolished in the *fmk1*Δ and *mpk1*Δ mutants, and partly restored in the *mpk1*Δ *hog1*Δ, but not *fmk1*Δ *hog1*Δ, double mutants. These results suggest that aggregation of *F. oxysporum* hyphae may involve both fusion-dependent and fusion-independent mechanisms. The latter could be related to cell surface adhesiveness, as shown in *C. albicans*, where aggregation of yeast cells during the formation of biofilms is mediated by agglutinins and glycosylphosphatidylinositol-anchored glycoproteins (Chandra *et al.*, 2001; Granger *et al.*, 2005). Analysis of agglutinin gene orthologues and their role in the cell adhesiveness of *F. oxysporum* should provide further insights into this process.

Fmk1, Mpk1 and Hog1 MAPKs regulate distinct virulence functions

In *F. oxysporum*, the MAPK Fmk1 is essential for multiple virulence-related traits on plants, such as root adhesion, penetration of cellophane membranes, infectious growth on fruit tissue and secretion of CWDEs, as well as for successful colonization of the host plant tomato (Di Pietro *et al.*, 2001; Perez-Nadales and Di Pietro, 2011; Prados-Rosales and Di Pietro, 2008; Rispail and Di Pietro, 2009). Here, we found that some of the Fmk1-controlled functions linked to invasive growth are also altered in the *mpk1*Δ and *hog1*Δ mutants. For example, although the *mpk1*Δ and *hog1*Δ mutants showed increased secretion of polygalacturonase activity *in vitro*, their ability to colonize apple fruit tissue and to infect tomato plants was reduced significantly. Thus, although Fmk1 regulates a wide subset of virulence-related functions, Mpk1 and Hog1 contribute to plant infection through additional Fmk1-independent functions. Strikingly, despite its fundamental contribution to virulence on plants, Fmk1 is dispensable for the pathogenicity of *F. oxysporum* on invertebrate and mammalian animal hosts (Navarro-Velasco *et al.*, 2011; Ortoneda *et al.*, 2004). By contrast, we showed here that both Mpk1 and Hog1 MAPKs are required for full virulence of *F. oxysporum* on *G. mellonella*. These findings are in line with studies in *Cr. neoformans*, *C. albicans* and *C. glabrata*, showing that mutants lacking Mpk1 display attenuated virulence in mouse models, whereas *mpk1*-overexpressing strains exhibit an increased survival rate in mice

(Diez-Orejas *et al.*, 1997; Kraus *et al.*, 2003; Miyazaki *et al.*, 2010). However, deletion of Hog1 or its upstream MAPKK Pbs2 in *Cr. neoformans*, *C. albicans* or *Metarhizium acridum* caused attenuated virulence in mouse and insect models, respectively (Bahn *et al.*, 2005; Cheetham *et al.*, 2011; Jin *et al.*, 2012).

Interestingly, simultaneous deletion of *fmk1* in the *mpk1*Δ and *hog1*Δ mutant backgrounds led to a further loss in virulence on *Galleria* compared with the single mutants. The most likely explanation for this outcome is that the virulence of *F. oxysporum* on animal hosts is controlled by multiple MAPK pathways with distinct and additive inputs. Previous studies have revealed that simultaneous deletion of components of the filamentation/invasive growth MAPK cascade and the cyclic adenosine monophosphate (cAMP)–protein kinase A pathway in *C. albicans* or *F. oxysporum* impaired virulence on immunodepressed mice to a much greater extent than individual mutation of each component, suggesting complementary functions of the two cell signalling cascades in fungal virulence (Lo *et al.*, 1997; Prados-Rosales *et al.*, 2006).

In summary, our study provides new evidence for distinct and cooperative roles of Fmk1, Mpk1 and Hog1 MAPKs in the virulence of *F. oxysporum* on tomato plants and on *G. mellonella* larvae (Fig. 6). This finding may have practical applications, as it suggests that combinations of compounds that simultaneously target different MAPK signalling pathways should represent a novel and promising strategy for the treatment of fungal infections in different pathosystems. Further studies are required to understand how environmental cues trigger changes in the phosphorylation or subcellular localization of the three MAPKs, and how cross-talk between these and other cell signalling pathways generates the appropriate outputs, to allow successful adaptation of this cross-kingdom fungal pathogen to phylogenetically distant and biologically diverse host organisms.

EXPERIMENTAL PROCEDURES

Fungal isolates and culture conditions

Fusarium oxysporum f. sp. *lycopersici* wild-type isolate 4287 (FGSC 9935) was used in all experiments (Table S1, see Supporting Information). The generation and molecular characterization of *F. oxysporum fmk1*Δ, *mpk1*Δ and *fmk1*Δ *mpk1*Δ mutants have been described previously (Di Pietro *et al.*, 2001; Turrà *et al.*, 2015). All fungal strains were stored as microconidial suspensions at −80 °C with 30% glycerol. For the extraction of genomic DNA and microconidia production, cultures were grown in potato dextrose broth (PDB; Difco, Madrid, Spain) at 28 °C with shaking at 170 rpm (Di Pietro *et al.*, 1998).

Quantification of vegetative hyphal fusion and hyphal aggregation events

To observe germling fusion events, microconidia from 5-day-old cultures were suspended in water, and their concentration was adjusted to 7×10^7 mL^{-1}. Fifty microliters of conidial suspension were spread with the help of 10 glass beads (0.5 cm) on top of a plate containing 4 mL of solid MM (Di Pietro *et al.*, 1998) supplemented with 25 mM $NaNO_3$. To test the effect of Hog1 activators/inhibitors on vegetative hyphal fusion, a 5-μL drop of NaCl (0, 0.4, 0.8, 1.2 M), the inhibitor SB202190 [30 μM in dimethylsulfoxide (DMSO); Sigma-Aldrich, Madrid, Spain] or DMSO as a control was added on top of the medium. Plates were incubated for 14 h at 28 °C and vegetative hyphal fusion events were counted in an Olympus BH-2 microscope (Olympus Iberia, Barcelona, Spain) using differential interference contrast imaging (400× magnification). Three hundred germlings were examined for each isolate and each experiment was repeated at least three times. The number of germ tubes involved in vegetative hyphal fusion was scored and expressed as a percentage of fusing hyphae over the total number of counted hyphae. For macroscopic analysis of hyphal aggregates, fungal strains were grown for 36 h in PDB diluted 1 : 50 with water and supplemented with 25 mM of the indicated nitrogen source, and observed using a Leica binocular microscope (Leica Microsistemas S.L.U., Barcelona, Spain). Photographs were recorded with a Leica DC 300F digital camera.

Nucleic acid manipulations, targeted gene deletion and gene complementation

Targeted gene replacement of the *hog1* gene in the wild-type and different mutant backgrounds was performed as reported previously (Lopez-Berges *et al.*, 2010) using the split-marker method with the hygromycin or phleomycin resistance cassettes and complementation of the mutants by co-transformation with the phleomycin resistance cassette. The strategy for gene deletion and the oligonucleotides used to generate PCR fragments for gene replacement, complementation or identification of the mutants are shown in Fig. S1 and Table S2 (see Supporting Information). PCRs were routinely performed with the High Fidelity Template PCR system (Roche Diagnostics, Barcelona, Spain) using an MJ Mini personal thermal cycler (Bio-Rad, Alcobendas, Spain). The amplified flanking sequences were PCR fused with partially overlapping truncated versions of the hygromycin B (Hyg^r) or phleomycin (Phleo^r) resistance cassettes. Transformants were purified by monoconidial isolation and analysed by PCR and Southern blot to verify homologous insertion of the construct. For complementation of the osmosensitive *hog1* deletion mutant, transformed protoplasts were incubated overnight in osmotic medium (1.2 M $MgSO_4$, 100 mM Na_2HPO_4, pH 5.8) before applying the selective agent phleomycin. Transformants showing wild-type colony phenotypes in the presence of 0.4 M NaCl or iprodione were analysed by PCR to confirm the presence of the wild-type *hog1* allele.

Western blot analysis

Microconidia were germinated for 15 h in PDB. Protein extraction and western blot analysis from mycelial samples were performed as described previously (Masachis *et al.*, 2016) using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit monoclonal antibody (mAb) (no. 4370), horseradish peroxidase (HRP)-conjugated anti-rabbit (no. 7074) and LumiGLO Reagent (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 anti-Fus3 (γN-19) (sc6772) and anti-Mpk1 (γN-19) (sc-6802), respectively, and donkey anti-goat IgG-HRP (sc2020) as secondary antibody (Santa Cruz

Biotechnology, Heidelberg, Germany). Anti-mouse-α-tubulin antibody (sc69971) and goat anti-rat IgGHRP secondary antibody (sc2006) (Santa Cruz Biotechnology) were used to detect the loading control α-tubulin. Hybridizing bands were visualized using the ECL Select western blotting detection reagent (GE Healthcare, Madrid, Spain). Band intensity was quantified using MultiGauge V3.0 software (Fuji, Tokyo, Japan).

Colony growth assays

For phenotypic analysis of colony growth, drops containing serial dilutions (2×10^5, 2×10^4 and 2×10^3 mL^{-1}) of freshly obtained microconidia were spotted onto agar plates with complete rich medium (YPD). For cell wall and oxidative stress assays, 5 μg/mL CR prepared in water, 5 μg/mL CFW prepared in 0.5% KOH, 83% glycerol (both from Sigma-Aldrich) and 10 μg/mL menadione in DMSO were added to 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-buffered YPD plates, pH 6.5 (Ram and Klis, 2006). For fungicide resistance assays, 1 mg/mL stock solutions of fludioxonil and iprodione in DMSO or ethanol, respectively, were added to YPD medium to a final concentration of 10 μg/mL (Rispail and Di Pietro, 2010). For hyperosmotic stress assays, YPD plates were supplemented with 0.4 M NaCl or 1 M sorbitol. Plates were incubated for 2 days at 28 °C and imaged, except for heat stress assays in which they were incubated for 4 days at 34 °C. The colony area was measured using MultiGauge software (Fuji, Tokyo, Japan). Data were analysed and plotted with the program GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA).

For superoxide detection, freshly obtained microconidia were spotted onto YPD or MM. Plates were incubated at 28 °C for 5 days and then stained with 3 mL of an aqueous solution of 2.5 mM NBT (Roche Life Sciences, Barcelona, Spain) for 30 min. The reaction was stopped by the addition of ethanol, and the pattern of formazan staining was observed on the whole colony or on individual hyphae by cutting out a small square (about 1×1 cm^2) of an agar block at the edge of the colony and imaging in a Zeiss Axio Imager M2 microscope (Zeiss, Barcelona, Spain). Images were captured with an Evolve Photometrics digital camera (Photometrics, Tucson, AZ, USA) using Axiovision 4.8 software (Zeiss).

All experiments were performed at least three times with similar results. The data presented are from one representative experiment.

Assays for virulence-related phenotypes

Assays for polygalacturonase production were carried out as described previously (Rispail and Di Pietro, 2009) on PGA medium (0.5% PGA, 2% glucose, 0.2% ammonium sulfate, 1.5% agar) buffered with 100 mM MES to pH 5 or 7. The quantification of extracellular pectinolytic activity was performed by subtracting the area of the clear halo surrounding the colony margin from the area of the colony. Invasive growth on apple fruit slices (variety Golden Delicious) was performed as described by López-Berges *et al.* (2009). Halo, colony and necrotic areas were quantified using MultiGauge V3.0 software.

Galleria mellonella infection assays

Galleria mellonella infection assays were carried out as described by Navarro-Velasco *et al.* (2011). Briefly, larvae were inoculated with 0.8 μL of a microconidial suspension (1.6×10^5 conidia/larva). Twenty larvae per strain were inoculated, and survival was checked daily. A Kaplan–Meier test was used to assess the statistical significance of differences in survival among groups. Data were plotted using the software GraphPad Prism version 5. $P < 0.05$ was considered to be significant. Experiments were performed four times with similar results. The data presented are from one representative experiment.

Tomato plant infection assays

Tomato root infection assays were performed as described by Di Pietro *et al.* (2001) using the susceptible cultivar Monika (kindly provided by Syngenta Seeds, Almeria, Spain). Briefly, 2-week-old tomato seedlings were inoculated by submerging roots for 30 min in a suspension of 5×10^6 microconidia/mL of the different *F. oxysporum* strains, planted in vermiculite and maintained in a growth chamber (28 °C; photoperiod 14 h light/10 h dark). Fifteen plants were used for each treatment. Plant survival was recorded for 30 days. A Kaplan–Meier test was used to assess the statistical significance of the differences in survival among groups. Data were plotted using the software GraphPad Prism version 5. $P < 0.05$ was considered to be significant. Experiments were performed three times with similar results. The data presented are from one representative experiment.

Bioinformatic prediction of Hog1 and accession numbers

The predicted *F. oxysporum* Hog1 protein was identified in the *Fusarium* Comparative Database at the Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html) using a BLASTp search with the *S. cerevisiae* Hog1 protein sequence. Sequence data are found in the *Fusarium* Comparative Database under accession number FOXG_06318.

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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 Targeted deletion of the *Fusarium oxysporum hog1* gene. (A) Physical maps of the *F. oxysporum hog1* locus and the split-marker gene replacement constructs obtained by fusion polymerase chain reaction (PCR). The relative positions of the primers used for PCR are indicated. *Hygr*, hygromycin resistance gene; *Phleor*, phleomycin resistance gene; P*gpdA, gpdA* promoter; T*trpC, TrpC* terminator (both from *Aspergillus nidulans*). Discontinuous lines represent the fragments used for co-transformation. (B–D) Southern blot analysis of putative *hog1*Δ (B), *fmk1*Δ *hog1*Δ (C) and *mpk1*Δ *hog1*Δ (D) transformants. Genomic DNA of the wild-type (wt) strain and five individual transformants was treated with the indicated restriction enzyme, separated on a 0.7% agarose gel, transferred to a nylon membrane and hybridized with the DNA probe indicated. (E) PCR amplification of genomic DNA of the wt strain, the *hog1*Δ mutant and four independent *hog1*Δ + *hog1* complemented strains, using primers Hog1F2 and Hog1stop. M, molecular size markers.

Fig. S2 Superoxide production phenotypes caused by single and double deletion of mitogen-activated protein kinase (MAPK) genes. (A, B) Growth of the indicated strains on minimal medium (MM) (A) or YPD (3% yeast extract, 1% peptone, 2% glucose and 1.5% agar) plates (B), before and after staining with 4-nitroblue tetrazolium chloride (NBT). Images below the plates show enlargements of the colony margins. Bar, 1 cm. (C) Quantitative analysis of the colony area (cm^2) after 5 days of growth at 28 °C was performed using MultiGauge software. Data were analysed and plotted with the program GraphPad Prism version 5. Error bars represent standard deviations of colony areas calculated from four independent replicates. Values with the same letter are not significantly different according to Dunnett's multiple comparison test ($P \leq 0.05$).

Fig. S3 Hog1 activity is dispensable for vegetative hyphal fusion in *F. oxysporum*. (A–C) Fifty microlitres of a 7×10^7 mL^{-1} conidial suspension were spread on top of a plate containing 4 mL of solid minimal medium (MM) supplemented with 25 mm $NaNO_3$. Where indicated, a 5-μL drop of NaCl (0, 0.4, 0.8, 1.2 M), the specific p38 mitogen-activated protein kinase (MAPK) inhibitor SB202190 (30 μM) or dimethylsulfoxide (DMSO) was added on top of the medium, and the plates were incubated for 14 h at 28 °C. The number of germ tubes involved in vegetative hyphal fusion was scored and expressed as a percentage of fusing hyphae over the total number of counted hyphae. (A) Percentage of vegetative hyphal fusion of the indicated strains. (B, C) Percentage of vegetative hyphal fusion in the wild-type (wt) strain was scored in the area in which NaCl (B), SB202190 or DMSO (control) (C) had been previously added. Experiments were performed three times with similar results. Statistical analysis was conducted using t-test; values with the same letter are not significantly different.

Fig. S4 Quantification of fruit tissue necrotic area and halo production on polygalacturonic acid medium. (A) The graph shows the mean relative intensity of the necrotic area produced by mutant strains normalized to that of the wild-type (wt) strain. The average pixel intensity of necrotic areas was subtracted from the average pixel intensity of the control (water-inoculated area) from the same apple slice. Error bars indicate standard deviation; $n = 4$ biological replicates. (B) Extracellular pectinolytic activity was quantified by subtracting the area of the colony from the area of the clear halo. The graph shows the mean area of clear halo produced by mutants normalized to that of wt. Data are from one representative experiment. Experiments were performed three times with similar results. Data were analysed and plotted with the program GraphPad Prism version 5. Values with the same letter are not significantly different according to Dunnett's multiple comparison test ($P \leq 0.05$).

Table S1 *Fusarium oxysporum* strains used in this study.

Table S2 List of primers used in this study.

Table S3 Statistical significance (P values) of *Solanum lycopersicum* survival curves after inoculation with the *Fusarium oxysporum* wild-type strain or different gene knockout mutants. NS, not statistically significant.

Table S4 Statistical significance (P values) of *Galleria mellonella* survival curves after inoculation with the *Fusarium oxysporum* wild-type strain or different gene knockout mutants. NS, not statistically significant.