The transmembrane protein Sho1 cooperates with the mucin Msb2 to regulate invasive growth and plant infection in *Fusarium oxysporum*

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SUMMARY

In the vascular wilt pathogen *Fusarium oxysporum*, the mitogen-activated protein kinase (MAPK) Fmk1 is essential for plant infection. The mucin-like membrane protein Msb2 regulates a subset of Fmk1-dependent functions. Here, we examined the role of the tetraspan transmembrane protein Sho1 as an additional regulator of the Fmk1 pathway and determined its genetic interaction with Msb2. Targeted $\Delta sho1$ mutants were generated in wild-type and $\Delta msb2$ backgrounds to test possible interactions between the two genes. The mutants were examined for hyphal growth under different stress conditions, phosphorylation of the MAPK Fmk1 and an array of Fmk1-dependent virulence functions. Similar to Msb2, Sho1 was required for the activation of Fmk1 phosphorylation, as well as Fmk1-dependent gene expression and invasive growth functions, including extracellular pectinolytic activity, cellophane penetration, plant tissue colonization and virulence on tomato plants. $\Delta sho1$ mutants were hypersensitive to the cell wall-perturbing compound Calcofluor White, and this phenotype was exacerbated in the $\Delta msb2 \Delta sho1$ double mutant. These results highlight that Sho1 and Msb2 have partially overlapping functions upstream of the Fmk1 MAPK cascade, to promote invasive growth and plant infection, as well as cell wall integrity, in *F. oxysporum*.

Keywords: fungal plant pathogen, *Fusarium oxysporum*, invasive growth, mitogen-activated protein kinase, virulence.

INTRODUCTION

Plant-pathogenic fungi have evolved mechanisms to invade their hosts, overcome plant defences and colonize plant tissues, thereby causing disease. The soil-borne pathogen *Fusarium oxysporum* causes vascular wilt in a wide range of economically important crops, such as banana, cotton, canola, melon and tomato (Dean *et al.*, 2012), and is also an opportunistic pathogen of humans (Nucci and Anaissie, 2007). In order to initiate plant infection and

propagate within the host, *F. oxysporum* must sense and respond to a range of physical and chemical cues, such as surface hydrophobicity, nutrient status and plant signals. It is thought that these stimuli are perceived by cell surface receptors, which engage in signalling through downstream pathways, giving rise to adaptive cellular responses.

The pathogenicity mitogen-activated protein kinase (MAPK) cascade is required for infection in all plant pathogens studied to date (Turra *et al.*, 2014). This MAPK is orthologous to Fus3 and Kss1 from *Saccharomyces cerevisiae*, which regulate the pheromone response and the filamentous growth/invasive growth (FG/IG) pathways, respectively. The orthologous MAPK in *F. oxysporum*, Fmk1, is essential for pathogenicity on tomato plants (Di Pietro *et al.*, 2001). Fmk1 regulates infection-related processes, such as vegetative hyphal fusion, root adhesion, host penetration, pectinolytic activity and invasion of living plant tissue (Di Pietro *et al.*, 2001; Prados Rosales and Di Pietro, 2008). Moreover, it contributes to growth during nutrient limitation and cell wall stress, suggesting that it could play a role in cell wall remodelling (Di Pietro *et al.*, 2001; Perez-Nadales and Di Pietro, 2011; Prados Rosales and Di Pietro, 2008). Despite its critical role in virulence, the cellular mechanisms through which Fmk1 controls this wide array of pathogenicity functions are largely unknown. To date, two additional components of the Fmk1 pathway have been characterized in detail: the mucin-like transmembrane protein Msb2 and the downstream transcription factor Ste12 (Perez-Nadales and Di Pietro, 2011; Rispaill and Di Pietro, 2009). As both proteins are only required for a subset of Fmk1-dependent functions, additional components must contribute to the regulation of this MAPK cascade (Di Pietro *et al.*, 2001; Perez-Nadales and Di Pietro, 2011; Prados Rosales and Di Pietro, 2008; Turra *et al.*, 2014).

The tetraspan transmembrane protein Sho1 (synthetic high osmolarity sensitive) was characterized in detail in *S. cerevisiae*, where it functions together with Msb2 to regulate FG/IG and hyperosmotic stress responses (Cullen *et al.*, 2004; Maeda *et al.*, 1995; Tatebayashi *et al.*, 2006, 2007). During hyperosmotic shock, Sho1 activates the HOG (high osmolarity glycerol) MAPK pathway through one of its two upstream branches. The transmembrane domains of Sho1 are important for the reception of the osmostress

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signal via the mucin sensors Hkr1 and Msb2, whereas its cytoplasmic SH3 domain contributes to the assembly of a protein complex with the PAK-type protein kinase Ste20, the scaffold protein Ste50 and the MAPKKK Ste11, as well as the MAPKK Pbs2, resulting in phosphorylation of the MAPK Hog1 (Maeda *et al.*, 1995; Tanaka *et al.*, 2014; Tatebayashi *et al.*, 2006, 2007; Yang *et al.*, 2009). In addition, Sho1 and Msb2 signal upstream of the FG/IG MAPK Kss1, and loss of Sho1 can be rescued by the overexpression of Msb2 or when the FG/IG pathway is activated by defects in protein glycosylation (Cullen *et al.*, 2004; Yang *et al.*, 2009). In the human pathogen *Candida albicans*, Sho1 and Msb2 also function coordinately to regulate cell wall biogenesis via the Kss1 MAPK orthologue Cek1 in response to cell wall-damaging compounds, such as Congo Red (CR), Calcofluor White (CFW), echinocandins, zymolyase or tunicamycin (Roman *et al.*, 2005, 2009). Deletion of *sho1* in *Aspergillus fumigatus* negatively affects adaptation to oxidative stress, germination and hyphal growth (Ma *et al.*, 2008). In the aerial plant pathogens *Ustilago maydis* and *Magnaporthe oryzae*, Sho1 and Msb2 have distinct, but overlapping, functions in the recognition of plant surface signals and in the activation of the Kss1 MAPK pathway, appressorium formation and virulence (Lanver *et al.*, 2010; Liu *et al.*, 2011). In this study, we examined the role of *F. oxysporum* Sho1 in signalling through the Fmk1 MAPK cascade, its contribution to the stress response and virulence, as well as its genetic interaction with Msb2.

RESULTS

Fusarium oxysporum Sho1 encodes a predicted tetraspan transmembrane protein with a short cytoplasmic region containing an SH3 domain

The *F. oxysporum* *sho1* gene has been identified previously through BLASTP search of the complete genome database of *F. oxysporum* (Rispaill *et al.*, 2009). The gene (accession no. *FOXG_06120*) has two introns and encodes a predicted 311-amino-acid protein homologous to Sho1 proteins from *S. cerevisiae* (28.0% identity), *C. albicans* (27.3% identity), *U. maydis* (29.2% identity), *Ashbya gossypii* (30.6% identity), *A. fumigatus* (54.2% identity), *M. oryzae* (62.3% identity), *Neurospora crassa* (55.5% identity) and *Fusarium graminearum* (93.6% identity). SMART analysis (<http://smart.embl-heidelberg.de/>) revealed a conserved domain architecture between Sho1 from *F. oxysporum* and orthologues from these fungi [Figs 1A and S1 (see Supporting Information)], including four predicted transmembrane domains near the N-terminus (amino acids 22–44, 64–83, 88–110 and 123–145, respectively), a linker domain and an SH3 domain at the C-terminus (amino acids 254–310). Sequence conservation was highest in the transmembrane regions. A phylogram of the predicted Sho1 proteins further reflects the close phylogenetic relationship among Sho1 orthologues in filamentous fungi (Fig. 1B).

Fig. 1 *Fusarium oxysporum* Sho1 is a structural orthologue of the *Saccharomyces cerevisiae* Sho1 protein. (A) Graphic representation of *F. oxysporum* Sho1 protein with the four predicted transmembrane domains. The C-terminal SH3 domain is highlighted in black; aa, amino acids. (B) Phylogram of predicted Sho1 proteins from ascomycetes. Percentage bootstrap values obtained from 1000 replicates are indicated at the nodes. Scale bar indicates the relative length of each branch. Ag, *Ashbya gossypii*; Af, *Aspergillus fumigatus*; Ca, *Candida albicans*; Fg, *Fusarium graminearum*; Fo, *F. oxysporum*; Mg, *Magnaporthe oryzae*; Nc, *Neurospora crassa*; Sc, *S. cerevisiae*.

Sho1 contributes to hyphal growth on solid surfaces under conditions of nitrogen limitation and cell wall stress

To examine the biological function of Sho1 in *F. oxysporum* and to analyse the possible genetic interactions with the Msb2 mucin, $\Delta sho1$ mutants were generated in the wild-type (wt) and $\Delta msb2$ background by targeted gene replacement with the hygromycin or phleomycin resistance cassette, respectively. Polymerase chain reaction (PCR) analysis with primers flanking the region of interest detected homologous replacement of the *sho1* locus in two transformants from the wt background ($\Delta sho1\#8$ and $\Delta sho1\#10$, 5.6-kb PCR amplicon) and in one transformant from the $\Delta msb2$ background ($\Delta msb2 \Delta sho1$, 5.1-kb amplicon) (Fig. S2A,B, see Supporting Information). A complemented strain ($\Delta sho1 + sho1$) was obtained by homologous reinsertion of the wt $\Delta sho1$ allele in the $\Delta sho1$ mutant (3.2-kb amplicon) (Fig. S2A,B). PCR amplification with a primer pair designed to amplify the *sho1* locus only in the

Fig. 2 Sho1 regulates hyphal growth under conditions of nutrient limitation. (A) Colony phenotype of the indicated strains grown on YPD (0.3% yeast extract, 1% peptone and 2% glucose), minimal medium (MM) and MM supplemented with 1% casaminoacids (MM+CA). Plates were spot inoculated with 10^5 microconidia per strain, incubated for 3 days at 28 °C and scanned. Bar, 1 cm. (B) Colony diameter on the indicated media was measured at day 3 post-inoculation and plotted relative to the wild-type (WT) strain (100%). Error bars represent standard deviations calculated from five independent plates. Values with the same letter are not significantly different according to Mann–Whitney test ($P \leq 0.05$).

case of homologous replacement with the resistance cassette yielded a 2.8-kb band in the $\Delta sho1$ single and double mutants, but not in the wt and complemented $\Delta sho1 + sho1$ strain (Fig. S2C).

The $\Delta sho1$ and $\Delta msb2 \Delta sho1$ mutants showed significantly slower colony growth than the wt on solid minimal medium (MM) containing the non-preferred nitrogen source nitrate (NaNO_3), similar to the $\Delta msb2$ and $\Delta fmk1$ mutants (Fig. 2). Colony growth of the mutants was partially rescued on MM containing casaminoacids and fully recovered on nutrient-rich medium (YPD: 0.3% yeast extract, 1% peptone and 2% glucose). The $\Delta sho1$ single mutant resembled the $\Delta msb2$ mutant in the increased

Fig. 3 Sho1 and Msb2 have additive roles in the Calcofluor White (CFW)-induced cell wall stress response. (A) Colony phenotype of the indicated strains grown on potato dextrose agar (PDA) supplemented with 200 $\mu\text{g}/\text{mL}$ CFW or 600 $\mu\text{g}/\text{mL}$ Congo Red (CR). Plates were spot inoculated with the indicated amount of microconidia, incubated for 3 days at 28 °C and scanned. Bar, 1 cm. (B) Colony diameter of the indicated media was measured at day 3 post-inoculation and plotted relative to the wild-type strain (100%). Error bars represent standard deviations calculated from five independent plates. Values with the same letter are not significantly different according to Mann–Whitney test ($P \leq 0.05$).

sensitivity to the cell wall targeting compounds CR and CFW (Fig. 3). The $\Delta msb2 \Delta sho1$ double mutant was significantly more sensitive to CFW than either of the single mutants; however, this additive effect was not observed on CR. No significant differences between strains were detected in the sensitivity to menadione (oxidative stress), sorbitol or sodium chloride (osmotic and salt stress) (Fig. S3, see Supporting Information). We conclude that *F. oxysporum* Sho1, similar to Msb2, has specific functions in vegetative hyphal growth under conditions of nitrogen limitation and cell wall stress. These results also indicate that the two genes interact to regulate cell integrity in the presence of CFW.

Fig. 4 Sho1 contributes to phosphorylation of the mitogen-activated protein kinase (MAPK) Fmk1. Strains were germinated in liquid potato dextrose broth (PDB) for 15 h (0'), transferred to minimal medium (MM) agar plates and incubated at 28 °C for the indicated time periods. Total protein extracts were submitted to immunoblot analysis with anti-phospho-p44/42 MAPK (Fmk1-P), anti-Fus3 MAPK (Fmk1) and anti- α -tubulin antibodies.

Sho1 promotes phosphorylation of the pathogenicity MAPK Fmk1

Previous studies have revealed that the transfer of *F. oxysporum* from submerged culture to solid medium triggers a rapid and transient increase in Fmk1 phosphorylation levels which is dependent on Msb2 (Perez-Nadales and Di Pietro, 2011). Here, we found that, like Msb2, Sho1 is required for the transient induction of Fmk1 phosphorylation (Fig. 4). Fmk1 phosphorylation in the Δ *msb2*, Δ *sho1* and Δ *msb2* Δ *sho1* mutants was also reduced in submerged culture with respect to the wt. Thus, Sho1 and Msb2 both promote MAPK phosphorylation on a solid surface and contribute to the maintenance of basal Fmk1 phosphorylation levels under submerged culture growth conditions.

Sho1 controls the expression of Fmk1-regulated genes

Real-time quantitative PCR analysis revealed significantly increased transcript levels of the *msb2* gene in a Δ *sho1* mutant (Fig. 5). However, consistent with previous findings (Perez-Nadales and Di Pietro, 2011), *msb2* expression was decreased in the Δ *fmk1* mutant. The levels of *sho1* transcript were slightly reduced in Δ *fmk1* but normal in the Δ *msb2* mutant. These results suggest that *sho1*, like *msb2*, is a transcriptional target of the Fmk1 MAPK.

We have found previously that, during growth on solid medium, *msb2* and *fmk1* interact genetically to regulate the expression of two target genes: *fpr1*, encoding a secreted protein with a sperm-coating protein/pathogenesis-related-1 (SCP-PR-1) like domain, and *chsV*, encoding a class V chitin synthase (Madrid *et al.*, 2003; Perez-Nadales and Di Pietro, 2011; Prados-Rosales *et al.*, 2012). Here, we found that *fpr1* transcript levels were strongly reduced in all mutant strains analysed (Fig. 5), although significantly less so in the Δ *sho1* mutant than in the Δ *msb2* and Δ *msb2* Δ *sho1* mutants. However, *chsV* expression was similarly reduced in the Δ *sho1*, Δ *msb2* and Δ *fmk1* mutants, but decreased further in the Δ *msb2* Δ *sho1* double mutant.

We next examined the expression of three additional cell wall biosynthesis genes: *chs3*, encoding a structural (class III)

chitin synthase (Martin-Urdiroz *et al.*, 2008); *fks1*, encoding an essential β -1,3-glucan synthase; and *gas1*, encoding a β -1,3-glucanosyltransferase required for plant infection (Caracul *et al.*, 2005). Among these, *gas1* and *fks1* were significantly down-regulated in the Δ *msb2*, Δ *sho1* and Δ *fmk1* mutants (Fig. 5). Similar to *chsV*, *gas1* was further down-regulated in the Δ *msb2* Δ *sho1* double mutant compared with the respective single mutants. Overall, these results indicate that Sho1 and Msb2 have common and partially overlapping functions in promoting the expression of Fmk1-regulated cell wall-related genes, such as *chsV*, *fks1* and *gas1*.

Sho1 is responsible for a subset of Fmk1-dependent virulence functions

We next investigated the role of Sho1 in vegetative hyphal fusion, a developmental process that can be macroscopically monitored in submerged culture by the presence of interconnected mycelial networks (Fig. 6A, left). In contrast with the wt, the Δ *fmk1* mutant is defective in hyphal fusion and the formation of hyphal aggregates (Prados Rosales and Di Pietro, 2008). However, this function is not dependent on Msb2 (Perez-Nadales and Di Pietro, 2011). We found that Δ *sho1* and Δ *msb2* Δ *sho1* mutants produced hyphal aggregates to a similar extent to the wt and Δ *msb2* strains, indicating that Sho1 is also dispensable for vegetative hyphal fusion. Microscopic analysis confirmed the presence of hyphal fusion events in the Δ *sho1* and Δ *msb2* Δ *sho1* mutants, similar to the Δ *msb2* strain (results not shown). It has been suggested previously that impaired hyphal fusion in the Δ *fmk1* mutant may be one of the causes of its failure to adhere efficiently to tomato roots (Prados Rosales and Di Pietro, 2008). Consistent with this idea, the Δ *sho1* and Δ *msb2* Δ *sho1* mutants still showed robust root adhesion, similar to Δ *msb2* (Fig. 6A, right).

Both Fmk1 and Msb2 are required for several functions associated with IG on plant tissue. First, Δ *fmk1* and Δ *msb2* mutants exhibit reduced extracellular pectinolytic activity during growth on plates containing polygalacturonic acid, as shown by a lack of clear halo production, with Δ *fmk1* showing the most extreme

Fig. 5 Sho1 and Msb2 promote the expression of Fmk1-regulated cell wall remodelling genes. mRNA abundance of the indicated genes was measured 6 h after transfer of the strains to minimal medium (MM) plates using quantitative real-time polymerase chain reaction (PCR). Relative expression levels represent mean ΔCt values normalized to actin gene expression levels and relative to expression values in the wild-type strain. Bars represent standard errors calculated from three biological replicates. Values with the same letter are not significantly different according to Mann–Whitney test ($P \leq 0.05$).

phenotype (Delgado-Jarana *et al.*, 2005; Perez-Nadales and Di Pietro, 2011). $\Delta sho1$ and $\Delta msb2 \Delta sho1$ also display a reduced pectinolytic activity, similar to the $\Delta msb2$ mutant (Fig. 6B, left). Second, $\Delta fmk1$ is defective in the penetration of cellophane membranes, whereas the $\Delta msb2$ strain is significantly reduced (Lopez-Berges *et al.*, 2010a; Perez-Nadales and Di Pietro, 2011; Prados Rosales and Di Pietro, 2008). We found that the $\Delta sho1$ mutant also shows a reduced ability to penetrate cellophane, although less pronounced than in the $\Delta msb2$ and $\Delta msb2 \Delta sho1$ mutants (Fig. 6B, right). Third, $\Delta fmk1$ fails to invade and colonize living fruit tissue, whereas $\Delta msb2$ shows reduced invasion (Di Pietro *et al.*, 2001; Perez-Nadales and Di Pietro, 2011; Rispail and Di Pietro, 2009). Both $\Delta sho1$ and $\Delta msb2 \Delta sho1$ also show a reduced ability to invade tomato fruits and apple slices, similar to the $\Delta msb2$ mutant, the difference being more marked on tomato than on apple (Fig. 7). All IG defects of the $\Delta sho1$ mutant were fully restored in the complemented strain. We conclude that Sho1 plays an important role in MAPK-controlled IG of *F. oxysporum*.

Sho1 is necessary for full virulence of *F. oxysporum* on tomato plants

Tomato plants inoculated with the wt showed a progressive increase in vascular wilt symptoms, and most plants were dead 20 days after inoculation (Fig. 8). As reported previously (Di Pietro *et al.*, 2001), the $\Delta fmk1$ mutant failed to cause any disease symptoms, whereas the $\Delta msb2$ mutant was strongly attenuated. The $\Delta sho1$ strain also showed significantly reduced virulence, although disease progression was slightly faster than in the $\Delta msb2$ or $\Delta msb2 \Delta sho1$ mutant. Complementation with the wt allele fully restored the virulence of the $\Delta sho1$ mutant.

Msb2 and Sho1 are dispensable for infection in the animal model *Galleria mellonella*

We also examined the role of Sho1 and Msb2 in the virulence of *F. oxysporum* in a non-vertebrate animal infection model, the greater wax moth *Galleria mellonella* (Navarro-Velasco *et al.*,

Fig. 6 Sho1 regulates a subset of Fmk1-controlled functions related to invasive growth. (A) Sho1 is not required for mycelial aggregation (Aggreg.) and root adhesion. Left: hyphal aggregates forming 36 h after conidial germination in potato dextrose broth (PDB) diluted 1 : 50 with water and supplemented with 20 mM glutamic acid. Cultures were vortexed to dissociate weakly adhered hyphae and observed in a binocular microscope. Right: roots of tomato seedlings were immersed for 36 h in a microconidial suspensions, washed by vigorous shaking in water and observed in a binocular microscope. Adhering mycelium is visible as a white mass covering the roots. Bar, 1 cm. (B) Sho1 contributes to pectinolytic activity and penetration of cellophane membranes. Extracellular pectinolytic activity on plates containing polygalacturonic acid (PGA) was visualized as a contrasting halo underneath the fungal colony (degrad., degradation). Cellophane penetration was determined by growing colonies for 4 days on MM plates covered with a cellophane membrane (Before). The cellophane with the fungal colony was removed and plates were incubated for an additional day to visualize hyphal growth on the plate (After). Bar, 1 cm.

2011). As reported previously, injection of microconidia of the wt strain into the haemocoel of *G. mellonella* resulted in rapid killing of the larvae (Fig. S4, see Supporting Information). $\Delta msb2$, $\Delta sho1$ and $\Delta msb2 \Delta sho1$ showed no significant differences in killing efficiency compared with the wt strain.

DISCUSSION

In fungal plant pathogens, contact with the host surface and perception of host signals trigger infection-related development, exemplified by the differentiation of dome-shaped appressoria on the leaf surface by the rice blast fungus *M. oryzae* (Wilson and Talbot, 2009). In the non-appressorium-forming pathogen *F. oxysporum*, the presence of the host roots induces differentiation of infection hyphae that can penetrate the root directly (Bishop and Cooper, 1983; Lagopodi *et al.*, 2002; Perez-Nadales and Di Pietro, 2011; Rodriguez-Galvez and Mendgen, 1995). The MAPK Fmk1 is essential for these early infection processes (Di Pietro *et al.*, 2001; Perez-Nadales and Di Pietro, 2011). Previous studies have estab-

lished that Msb2 contributes to Fmk1 phosphorylation and Fmk1-dependent virulence functions, but have also suggested the presence of additional sensors feeding into the pathway (Di Pietro *et al.*, 2001; Perez-Nadales and Di Pietro, 2011). Here, we provide several lines of evidence suggesting that Sho1 and Msb2 function together upstream of Fmk1. First, both Sho1 and Msb2 are necessary for full phosphorylation of Fmk1. Second, Sho1 and Msb2 contribute to the up-regulation of Fmk1-regulated genes encoding different enzymes involved in cell wall biogenesis. Third, Msb2 and Sho1 contribute in an additive manner to the cell wall stress response. Fourth, Sho1 and Msb2 promote Fmk1-dependent IG functions and pathogenicity on tomato plants.

Role of Sho1 and Msb2 in the cell wall stress response

Changes in the fungal cell wall occur during normal hyphal growth, but are dramatically enhanced in response to external insults, such as those encountered during fungus–host interactions (Brown *et al.*, 2014). In the human pathogens *C. albicans*

Fig. 7 Sho1 promotes invasive growth on living fruit tissue. Tomato or apple fruits were inoculated with 5×10^4 microconidia of the indicated strains, incubated at 28 °C for 4 days and photographed.

and *A. fumigatus*, Sho1 and Msb2 serve as sensors of cell wall stress (Boisnard *et al.*, 2008; Ma *et al.*, 2008; Norice *et al.*, 2007; Perez-Nadales and Di Pietro, 2011; Roman *et al.*, 2005, 2009). We have shown previously that Msb2 promotes the resistance of *F. oxysporum* to CFW and CR, which bind to glucan and chitin components in the cell wall (Maeda and Ishida, 1967; Ram and Klis, 2006), and that this role is partially independent of the MAPK Fmk1 (Perez-Nadales and Di Pietro, 2011). Here, we found that Sho1 also contributes to the cell wall stress response. Genetic evidence suggests that Sho1 and Msb2 have both distinct and overlapping functions in mediating resistance to CFW and CR. A distinct phenotypic response to CFW and CR has been reported previously in cell wall mutants of *Aspergillus nidulans* (Hill *et al.*, 2006) and may be explained by differences in the affinity of these compounds to β -1,3-glucan (Wood, 1980).

Fig. 8 Sho1 is required for virulence of *Fusarium oxysporum* on tomato plants. Incidence of *Fusarium* wilt on tomato plants (cv. Monica) inoculated with the indicated strains. Uninoculated plants were used as controls. The severity of disease symptoms was recorded using an index ranging from '1' (healthy plant) to '5' (dead plant). Error bars represent standard errors calculated from 15 plants.

Sho1, Msb2 and Fmk1 were required for the up-regulation of three genes involved in cell wall biogenesis, *chsV*, *fks1* and *gas1*, on transfer to a solid surface. This finding further supports a role of these two membrane proteins and of the Fmk1 pathway in cell wall remodelling. The finding that Sho1 and Msb2 also promote Fmk1 phosphorylation in submerged culture suggests a function for this MAPK in actively growing hyphae that may be related to cell wall biogenesis. Fmk1 orthologues have been implicated in cell wall biogenesis in several fungi. In *S. cerevisiae*, the FG/IG MAPK pathway contributes to the maintenance of cell wall integrity in vegetative yeast cells (Cullen *et al.*, 2000; Lee and Elion, 1999). In *C. albicans*, the Cek1 pathway is activated under conditions that require active cell growth and/or cell wall remodelling (Roman *et al.*, 2005, 2009; Román and Pla, 2013). Sho1 is also required for pseudohyphal growth and cell wall biogenesis in the opportunistic yeast *Candida lusitanae* (Boisnard *et al.*, 2008). Our results favour a cooperative role of Msb2 and Sho1 in the cell wall integrity of *F. oxysporum*, because the expression of *chsV* and *gas1* was more severely reduced in a $\Delta msb2 \Delta sho1$ double mutant than in the corresponding single mutants. The finding that the decrease in *chsV* transcript levels in the $\Delta msb2 \Delta sho1$ mutant was more drastic than in the $\Delta fmk1$ mutant, together with previous genetic evidence (Perez-Nadales and Di Pietro, 2011), suggests that Sho1 and Msb2 regulate additional downstream signalling pathways distinct from Fmk1. In *S. cerevisiae*, elements of the HOG MAPK pathway, including Sho1 and Msb2, have been implicated in the response to zymolyase-induced cell wall damage through the Mpk1 cell wall integrity pathway (Bermejo *et al.*,

2008). Additional signalling pathways may also contribute to cell wall reorganization. For instance, the protein kinase A isoform Tpk1 is specifically required for hyphal formation of *C. albicans* in solid but not liquid media (Bockmuhl *et al.*, 2001).

In *S. cerevisiae*, Sho1 functions primarily in one branch of the HOG pathway during adaptation to osmotic and oxidative stress (Bilsland *et al.*, 2004; Hohmann, 2002; Saito and Posas, 2012), where it acts as a scaffold during osmostress sensing between the membrane mucins Msb2 and Hkr1 and the downstream components Ste20, Ste50/Ste11 and MAPKK Pbs2 (Saito and Posas, 2012; Tanaka *et al.*, 2014; Tatebayashi *et al.*, 2006, 2007; Yamamoto *et al.*, 2010). However, *F. oxysporum* $\Delta sho1$, $\Delta msb2$ and $\Delta sho1 \Delta msb2$ mutants did not show increased sensitivity to high osmolarity or oxidative stress, suggesting that Sho1 and Msb2 are not required for activation of the HOG pathway (Perez-Nadales and Di Pietro, 2011; and this work). In *C. albicans*, Sho1 and Msb2 are also dispensable for Hog1 activation during osmotic or oxidative stress, although *sho1* mutants are slightly more sensitive to both types of insult (Cheetham *et al.*, 2007; Roman *et al.*, 2005, 2009). Sho1 was also required for oxidant adaptation in *A. fumigatus* (Ma *et al.*, 2008), which may be significant for a pathogen exposed to oxidant attack by macrophages or neutrophils. In *U. maydis*, Sho1 and Msb2 are dispensable for osmotic, oxidative and cell wall stress (Lanver *et al.*, 2010). Overall, these findings suggest an evolutionary specialization of Sho1 and Msb2 proteins in different fungal species.

Sho1 cooperates with Msb2 during IG and plant infection

As reported previously for Msb2 (Perez-Nadales and Di Pietro, 2011), loss of Sho1 recapitulates only part of the $\Delta fmk1$ phenotypes. For example, Sho1 is dispensable for hyphal fusion and adhesion to tomato roots, two Fmk1-dependent processes (Prados Rosales and Di Pietro, 2008). However, all known Fmk1-controlled functions linked to IG were affected in the $\Delta sho1$ mutants, at least to some extent, including extracellular pectinolytic activity, penetration of cellophane membranes and colonization of tomato or apple fruit tissue. Importantly, virulence on tomato plants was significantly reduced in $\Delta sho1$, $\Delta msb2$ and the double mutants, albeit less dramatically than in the $\Delta fmk1$ strain. This indicates that additional upstream components must contribute to the activation of the Fmk1 MAPK during plant infection. The finding that Sho1 and Msb2 are not required for virulence in *G. mellonella* is consistent with previous results showing that the Fmk1 MAPK is dispensable in this non-vertebrate infection model (Navarro-Velasco *et al.*, 2011).

The nature of the signals sensed by Sho1 and Msb2, which lead to the activation of the Fmk1 pathway during plant infection by *F. oxysporum*, is currently unknown. Results from the present study and from previous work (Perez-Nadales and Di Pietro, 2011)

suggest that these two membrane proteins could be directly involved in the sensing of the host surface or nutrient signals during early stages of infection. However, formal proof for this hypothesis requires additional experimentation. In *U. maydis*, differentiation of appressoria in response to a hydrophobic surface was strongly attenuated in $\Delta msb2 \Delta sho1$ mutants, whereas hydroxy fatty acid-induced differentiation was unaffected, suggesting that Sho1 and Msb2 are specifically involved in the perception of host surface hydrophobicity (Lanver *et al.*, 2010). In *M. oryzae*, Sho1 and Msb2 appear to have overlapping functions in recognizing different plant surface signals, leading to MAPK activation and appressorium formation. Although Msb2 is critical for the sensing of surface hydrophobicity and cutin monomers, Sho1 may play a more important role in the recognition of rice leaf waxes (Liu *et al.*, 2011). Sensing of environmental cues, such as nutritional stress or surface contact, may be particularly meaningful for soil-borne plant pathogens such as *F. oxysporum*, which face situations of nutrient limitation during both saprophytic and infectious growth stages in the soil.

Exactly how Sho1 and Msb2 promote hyphal invasion remains to be determined. In *S. cerevisiae*, Sho1 and Msb2 localize predominantly to areas of polarized growth (Cullen *et al.*, 2004; Tatebayashi *et al.*, 2007). During osmostress, Msb2 binds the GTPase Cdc42 and the scaffold protein Bem1, two key regulators of morphogenesis and cell polarity establishment (Cullen *et al.*, 2004; Tatebayashi *et al.*, 2007). In the filamentous pathogen *A. fumigatus*, Sho1 functions in actin cytoskeleton organization and exocytosis during early stages of hyphal growth (Yang *et al.*, 2011). Thus, the cooperative role of Sho1 and Msb2 in cytoskeleton reorganization and polarity establishment could be conserved in other fungi, including *F. oxysporum*, affecting fundamental processes of fungal pathogenesis, such as IG and infectious-related development.

EXPERIMENTAL PROCEDURES

Fungal isolates, constructions of mutants and culture conditions

Fusarium oxysporum f. sp. *lycopersici* race 2 wt strain 4287 (FGSC 9935) was used in all experiments. The generation and molecular characterization of the *F. oxysporum* $\Delta fmk1$, $\Delta msb2$ and $\Delta msb2+msb2$ strains have been described previously (Di Pietro *et al.*, 2001; Perez-Nadales and Di Pietro, 2011). Standard molecular techniques and cloning procedures (Sambrook and Russell, 2001) were used to prepare *hph*(B):*sho1*:pGemT and *phleo*(B):*sho1*:pGemT constructs, as described in Methods S1 (see Supporting Information). The *sho1* deletion constructs were PCR amplified from the plasmids *hph*(B):*sho1*:pGemT and *phleo*(B):*sho1*:pGemT using the primers *sho1*-for1 and *sho1*-rev2, and employed to transform protoplasts of the *F. oxysporum* wt and $\Delta msb2$ strains, respectively. Hygromycin- and phleomycin-resistant transformants were selected and purified by monoconidial isolation as described previously (Di Pietro *et al.*,

2001). Gene deletion in the resulting $\Delta sho1$ and $\Delta msb2 \Delta sho1$ mutants was confirmed by PCR analysis. The $\Delta sho1 + sho1$ complemented strain was generated by co-transformation of a 3.2-kb DNA fragment encompassing the complete *sho1* gene, PCR amplified from genomic DNA with primers *sho1-for3* and *sho1-rev2*, and the phleomycin resistance marker. Primer sequences are listed in Table S1 (see Supporting Information).

All fungal strains were stored as microconidial suspensions at -80°C with 30% glycerol. For the extraction of DNA, microconidium production and fungal development, cultures were grown in liquid potato dextrose broth (PDB; Difco, Detroit, MI, USA) at 28°C with shaking at 170 rpm (Di Pietro and Roncero, 1998). For RNA expression analysis and the analysis of MAPK phosphorylation, 5×10^8 freshly obtained microconidia from each strain were inoculated into 200 mL of PDB and grown for 15 h at 28°C and 170 rpm. Mycelia were then harvested, washed twice with sterile MM without trace elements (Puhalla, 1985), resuspended in 10 mL of MM and transferred onto three MM agar plates, which were incubated at 28°C for the indicated times. Mycelia were harvested by filtration, frozen in liquid nitrogen and stored at -80°C .

Colony growth assays

For the phenotypic analysis of colony growth, drops of water with serial dilutions (2×10^5 , 2×10^4 and 2×10^3) of freshly obtained microconidia were spotted onto agar plates with complete rich medium (YPD), MM or MM supplemented with 10 g/L casaminoacids (Difco). For cell wall stress assays, 600 $\mu\text{g/mL}$ CR or 30 $\mu\text{g/mL}$ CFW (Sigma, Madrid, Spain) were added to YPD plates buffered to pH 7 with 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Ram and Klis, 2006). For osmotic and oxidative stress assays, YPD plates were supplemented with 0.4, 0.8 or 1 M NaCl and 10 $\mu\text{g/mL}$ menadione in ethanol, respectively. Plates were incubated at 28°C for 3 days before scanning and measurement of colony diameter. Data from three independent experiments, each with three replicates, were analysed with the software SPSS 15.0 for Windows® (LEAD Technologies, Inc., Charlotte, NC, USA). Kruskal–Wallis analysis of variance (ANOVA) and Mann–Whitney test were used to assess statistically relevant differences among strains at $P \leq 0.05$.

Nucleic acid manipulations and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Nucleic acid extraction and manipulations were performed according to standard protocols (Sambrook and Russell, 2001). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul *et al.*, 1990). Quantitative real-time RT-PCR was performed as described by Lopez-Berges *et al.* (2010b). Gene-specific primers, namely *act-2* and *act-q6*, *sho1-F4* and *sho1-F5*, *msb2ORF-s* and *msb2ORF-as*, *09795-for* and *09795-rev*, *gas-1* and *gas-5*, *ChsV-20* and *ChsV-36B*, and *Chs3-12* and *Chs3-18*, were used to amplify *actin*, *sho1*, *msb2*, *fpr1*, *gas1*, *chsV* and *chs3* transcripts, respectively (Table S1). Transcript levels were calculated by the comparative ΔC_t method and normalized to *act1*. Expression values are presented as values relative to the expression in the wt strain.

Protein purification and Western blot analysis

Fungal mycelia were harvested, frozen in liquid nitrogen, ground in a mortar and resuspended in protein extraction buffer [10% glycerol,

50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecylsulfate (SDS), 1% Triton, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonylfluoride (PMSF), protease inhibitor cocktail P8215 (Sigma), phosphatase inhibitors, 50 mM NaF, 50 mM β -glycerophosphate, 1 mM sodium orthovanadate and PhosSTOP phosphatase inhibitor cocktail tablets (Roche, Diagnostics, Barcelona, Spain)]. Samples were centrifuged to pellet cell debris and the protein concentration of the supernatant was determined with the Bio-Rad protein assay reagent (Bio-Rad, Madrid, Spain) using bovine serum albumin as a standard. One hundred micrograms of total protein were separated in 14% polyacrylamide gels using standard protocols (Sambrook and Russell, 2001). Membranes were blocked using 5% non-fat skimmed milk for 1 h. Phosphorylated Fmk1 was detected using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP®, rabbit monoclonal antibody (#4370), horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (#7074, Cell Signaling Technology, Beverly, MA) and LumiGLO® Reagent (#7003) (Cell Signaling Technology). Unphosphorylated Fmk1 was detected using Fus3 (γ N-19) (sc6772) and donkey anti-goat immunoglobulin G (IgG)-HRP (sc 2020) (Santa Cruz Technology, Santa Cruz, CA, USA) and ECL™ Select Western blotting detection reagent (GE Healthcare, Madrid, Spain).

Virulence-related assays

Assays for polygalacturonase production on polygalacturonic acid plates (Di Pietro and Roncero, 1998), penetration of cellophane membranes (Prados Rosales and Di Pietro, 2008) and adhesion to tomato roots (Lopez-Berges *et al.*, 2010a) were performed as described previously.

Plant and animal infection assays

Tomato root inoculation assays were performed as described previously (Di Pietro and Roncero, 1998). Briefly, roots of 2-week-old tomato seedlings (cultivar Monika, seeds kindly provided by Syngenta Seeds, Almeria, Spain) were immersed in a suspension of 5×10^6 microconidia/mL for 30 min, planted in vermiculite and maintained in a growth chamber. Fifteen plants were used for each treatment. The severity of disease symptoms was recorded daily for 40 days using an index ranging from '1' (healthy plant) to '5' (dead plant). Assays for IG on tomato fruits (cultivar Daniela) and apple fruit slices (variety Golden Delicious) were performed as described previously (Di Pietro *et al.*, 2001; Lopez-Berges *et al.*, 2009). All plant infection experiments were performed at least three times with similar results. *Galleria mellonella* larvae infections were performed as described elsewhere (Navarro-Velasco *et al.*, 2011).

Bioinformatic analysis

For phylogenetic analysis, previously identified Sho1 protein sequences from different fungal genomes (Rispaill *et al.*, 2009) were aligned with CLUSTAL W (Thompson *et al.*, 1994) and inspected manually. A maximum likelihood tree was built from the alignment by PhyMLversion4.0 using both parsimony and distance analysis (neighbour joining, NJ) with 1000 bootstrap replicates. SMART (<http://smart.embl-heidelberg.de/>) and TOPO2 (<http://www.sacs.ucsf.edu/TOPO2/>) software programs were used for domain prediction of all proteins and graphical representation of the *F. oxysporum* Sho1 predicted domain architecture, respectively.

Accession numbers

Sequence data from this article can be found in the following databases with the accession numbers listed: GenBank/EMBL databases under the accession numbers: YER118C (*S. cerevisiae* Sho1), AGL286C (*A. gossypii* Sho1), Afu5g08420 (*A. fumigatus* Sho1), NCU08067 (*N. crassa* Sho1), MGG_09125.5 (*M. oryzae* Sho1), UM03156.1 (*U. maydis* Sho1); *Fusarium* Comparative Database of the Broad Institute under the accession numbers: FOXG_06120.2 (*F. oxysporum* Sho1), FGSG_09435.3 (*F. graminearum* Sho1); *Candida* Genome Database under the accession number: orf19.4772 (*C. albicans* Sho1).

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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. Sequence alignment of Sho1 fungal orthologues. Highly conserved residues are shaded in black; moderately conserved residues are shaded in grey. Cytoplasmic (CT), transmembrane (TM), extracellular (EC) and SH3 domains are highlighted in light yellow, orange, blue and green, respectively.

Fig. S2. Targeted disruption of the *Fusarium oxysporum* *sho1* gene. (A) Physical maps of the *sho1* locus and gene replacement constructs obtained by polymerase chain reaction (PCR) amplification from plasmids *hph(B)sho1:pGemt* and *phleo(B)sho1:pGemT* (Methods S1). The relative positions of the primers used for PCR analysis of the transformants are indicated. Scale bar, 500 base pairs. (B) PCR amplification of genomic DNA of the wild-type (wt) and indicated mutant strains using primers *sho1-for3* and *sho1-rev2* indicated in (A) to differentiate the wild-type PCR product from that corresponding to the Δ *sho1* allele. (C) PCR amplification of genomic DNA of the indicated strains from (B) using forward primer *sho1-for3* and reverse primer *gpdA-9* specific to the *gpdA* promoter to confirm homologous insertion of the disruption construct at the *sho1* locus.

Fig. S3. Sho1 is not required for hyphal growth under conditions of osmotic or oxidative stress. Colony phenotype of the indicated strains grown on YPD (0.3% yeast extract, 1% peptone and 2% glucose) and YPD supplemented with the indicated concentrations of NaCl, 1 M sorbitol and 5 mM H₂O₂. Plates were spot inoculated with 10⁴ microconidia, incubated for 2 days at 28 °C and scanned. Bar, 1 cm.

Fig. S4. Sho1 and Msb2 are dispensable for virulence on *Galleria mellonella*. Kaplan–Meier plots of survival of *G. mellonella* larvae after injection of 1.6×10^4 microconidia of the indicated *Fusarium oxysporum* strains into the haemocoel and incubation at 30 °C. Data shown are from one representative experiment. Experiments were performed three times with similar results.

Table S1. Primers used in this study.

Methods S1. Generation of Δ *sho1*, Δ *msb2* Δ *sho1* and Δ *sho1*+*sho1* strains.