The F-box protein Fbp1 functions in the invasive growth and cell wall integrity mitogen-activated protein kinase (MAPK) pathways in *Fusarium oxysporum*

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SUMMARY

F-box proteins determine substrate specificity of the ubiquitin–proteasome system. Previous work has demonstrated that the F-box protein Fbp1, a component of the SCF^{Fbp1} E3 ligase complex, is essential for invasive growth and virulence of the fungal plant pathogen *Fusarium oxysporum*. Here, we show that, in addition to invasive growth, Fbp1 also contributes to vegetative hyphal fusion and fungal adhesion to tomato roots. All of these functions have been shown previously to require the mitogen-activated protein kinase (MAPK) Fmk1. We found that Fbp1 is required for full phosphorylation of Fmk1, indicating that Fbp1 regulates virulence and invasive growth via the Fmk1 pathway. Moreover, the $\Delta fbp1$ mutant is hypersensitive to sodium dodecylsulfate (SDS) and calcofluor white (CFW) and shows reduced phosphorylation levels of the cell wall integrity MAPK Mpk1 after SDS treatment. Collectively, these results suggest that Fbp1 contributes to both the invasive growth and cell wall integrity MAPK pathways of *F. oxysporum*.

Keywords: F-box, *Fusarium oxysporum*, MAPK pathways, virulence.

INTRODUCTION

Phosphorylation and ubiquitination, two of the most common post-translational modifications in eukaryotic proteomes, are essential to virtually every cellular process (Swaney *et al.*, 2013). Phosphorylation is the primary mechanism for the regulation of cell signalling, whereas ubiquitination regulates protein degradation. There is evidence for crosstalk between these two post-translational modifications, as phosphorylation can promote or inhibit ubiquitination, which, in turn, regulates proteasomal degradation, processing or intracellular trafficking of membrane proteins (Hunter, 2007).

Protein kinases catalyse reversible phosphorylation on a large proportion of cellular proteins, modifying their activity and regu-

lating a variety of cellular processes (Cohen, 2000). Protein kinases are key players in fungal pathogenicity on plants (Rispaill *et al.*, 2009; Turra *et al.*, 2014). Mitogen-activated protein kinases (MAPKs) are a prominent group of protein kinases, which function at the end of a three-component module conserved from yeasts to humans, consisting of three kinases that establish a sequential activation pathway by means of phosphorylation events (Qi and Elion, 2005). Most fungal pathogens contain three MAPKs that are orthologues of the *Saccharomyces cerevisiae* Fus3/Kss1, Slt2/Mpk1 and Hog1 MAPKs, and function in separate signalling cascades to regulate infection-related morphogenesis, cell wall integrity (CWI) and the high-osmolarity stress response, respectively (Hamel *et al.*, 2012; Rispaill *et al.*, 2009; Zhao *et al.*, 2007).

The MAPK orthologue to yeast Fus3/Kss1 represents one of the most conserved pathogenicity mechanisms in fungi, and was first reported in the rice blast fungus *Magnaporthe oryzae* (Xu and Hamer, 1996). Kss1 orthologues are required for infection in all plant pathogens tested to date (Turra *et al.*, 2014). The Kss1 orthologue in *Fusarium oxysporum*, Fmk1, is essential for infection on tomato plants and regulates multiple virulence-related functions, such as adhesion to tomato roots, root penetration, secretion of pectinolytic enzymes and vegetative hyphal fusion (Delgado-Jarana *et al.*, 2005; Di Pietro *et al.*, 2001; Prados Rosales and Di Pietro, 2008). In addition to Fmk1, other conserved components of this MAPK pathway have been identified in *F. oxysporum*, including the mucin-like transmembrane protein Msb2, the tetraspan protein Sho1 and the homeodomain transcription factor Ste12 (Perez-Nadales and Di Pietro, 2011, 2014; Rispaill and Di Pietro, 2009).

Two additional MAPK pathways, the high-osmolarity glycerol (HOG) and CWI pathways, are important for the response to osmotic, oxidative and cell wall stress (Nikolaou *et al.*, 2009). The CWI MAPK pathway is well conserved in fungi, although the biological functions may vary among different species (Yun *et al.*, 2014). The role of Mpk1 orthologues has been determined in a number of fungal pathogens (Turra *et al.*, 2014).

In addition to phosphorylation, protein ubiquitination has emerged as an important regulator of cell signalling and pathogenicity in fungi. During this tightly regulated process, ubiquitin becomes attached to lysine residues of target proteins by a

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three-step process involving an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase (Hershko and Ciechanover, 1992). Substrate specificity is controlled by E3, which is frequently recruited by the phosphorylation of target proteins. The Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase is a multiprotein complex that utilizes a variety of F-box proteins to identify phosphorylated substrates (Cardozo and Pagano, 2004). The F-box protein binds directly to the substrate and therefore defines the substrate specificity of the SCF complex. The SCF E3 ligase complex and its F-box protein Grr1 have been characterized extensively in *S. cerevisiae* (Li and Johnston, 1997). Grr1 homologues have also been characterized in other fungi, such as *Candida albicans* (Grr1) (Butler *et al.*, 2006), *Aspergillus nidulans* (GrrA) (Krappmann *et al.*, 2006), *Gibberella zeae* (Fbp1) (Han *et al.*, 2007) and *Cryptococcus neoformans* (Liu *et al.*, 2011), but, in most cases, their substrates remain to be identified. A recent study has revealed that inositol phosphosphingolipid-phospholipase C1 (Isc1) is a substrate of Fbp1 in *C. neoformans* (Liu and Xue, 2014).

We have shown previously that the Grr1 orthologue Fbp1 is required for plant infection by the vascular wilt fungus *F. oxysporum*, a soil-borne pathogen that attacks a wide range of agriculturally important crops (Miguel-Rojas and Hera, 2013). Fbp1 is thought to be a component of the SCF complex based on: (i) functional complementation of the *S. cerevisiae* *grr1* mutant with the orthologous genes from *F. graminearum* and *C. neoformans* (Han *et al.*, 2007; Liu *et al.*, 2011; Miguel-Rojas and Hera, 2013); and (ii) the accumulation in the $\Delta fbp1$ mutant of the G2/M cyclin Clb2, a known target of Grr1 (C. Miguel-Rojas *et al.*, unpublished results).

In addition to pathogenicity, Fbp1 is required for invasive growth on cellophane membranes and on tomato fruit tissue (Miguel-Rojas and Hera, 2013). These functions have been shown previously to depend on the Fmk1 MAPK pathway in *F. oxysporum* (Di Pietro *et al.*, 2001; Perez-Nadales and Di Pietro, 2011; Rispaill and Di Pietro, 2009). This suggests that Fbp1 may promote invasive growth by controlling the levels of one or several Fmk1 pathway components. Here, we investigated the role of Fbp1 in two MAPK signalling pathways regulating invasive growth and CWI. Our results suggest that Fbp1 regulates each pathway upstream of the MAPKs, as deduced by phenotypical analysis and the MAPK phosphorylation pattern in the mutant strain.

RESULTS

Fbp1 is required for efficient hyphal fusion and root adhesion

Vegetative hyphal fusion can be monitored macroscopically in submerged cultures by the presence of interconnected hyphal

Fig. 1 Fbp1 is required for hyphal fusion and root adhesion. (A) The indicated strains were grown in potato dextrose broth (PDB) diluted 1 : 50 with water and supplemented with 20 mM of glutamic acid. Top panel: hyphal aggregates forming 36 h after conidial germination. Cultures were vortexed to dissociate weakly adhered hyphae and observed in a binocular microscope. Bottom panel: germ tubes formed after 12 h at 28 °C and 170 rpm. Events of germ tube fusion were determined microscopically with a Leica DMR (40 $\times$) using the Nomarsky technique. (B) Root adhesion assay. Roots of tomato seedlings were immersed for 24 h in microconidial suspensions of the indicated strains in PDB diluted 1 : 50 with water and supplemented with 20 mM of glutamic acid. They were then washed by vigorous shaking in water and observed in a binocular microscope. Adhering fungal mycelium is visible as a white mass covering the roots. wt, wild-type.

aggregates or, microscopically, as fusion bridges formed between hyphae (Prados Rosales and Di Pietro 2008). In contrast with the wild-type strain, $\Delta fbp1$ mutants were defective in germ tube fusion and hyphal fusion and aggregation (Fig. 1A). The complemented strain recovered the ability of both hyphal fusion and aggregation. The efficiency of hyphal fusion was further analysed by determining the frequency of heterokaryon formation between different nitrate non-utilizing mutants (*nit1* and *nitM*) obtained in the wild-type and $\Delta fbp1$ backgrounds (Prados Rosales and Di Pietro, 2008). A reduction of 95% was observed in the frequency of heterokaryon formation in $\Delta fbp1$ compared with the wild-type background (Fig. S1 and Table S1, see Supporting Information).

Impaired hyphal fusion has been suggested previously to be a probable cause of the lack of hyphal adhesion of the $\Delta fmk1$ mutant to tomato roots (Prados Rosales and Di Pietro 2008). The phenotypic association between hyphal fusion and root adhesion was also observed for the $\Delta fbp1$ mutant. The robust root adhesion

observed in the wild-type and complemented strain was nearly abolished in the $\Delta fbp1$ mutant (Fig. 1B).

Fbp1 contributes to the maintenance of the surface hydrophobicity of aerial mycelium

Among the key factors mediating fungal adhesion is hyphal surface hydrophobicity (Singleton *et al.*, 2005). To test the effect of the $\Delta fbp1$ mutation on the surface hydrophobicity, drops of water were placed on top of the colonies of the different strains (Fig. 2A). The drops on the colony of the wild-type strain remained completely intact, whereas those on the $\Delta fbp1$ mutant were rapidly soaked into the colony. Thus, like Fmk1 (Perez-Nadales and Di Pietro, 2011), Fbp1 is required for full hydrophobicity of aerial mycelia. To further investigate the role of Fbp1 in the regulation of surface hydrophobicity, we used quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to determine the transcript levels of the hydrophobin

genes *hyd1*, *hyd3*, *hyd4* and *hyd5* (Ruiz-Roldan *et al.*, 2008). The expression of *hyd3* and *hyd4* genes was similar in wild-type and mutant strains (data not shown). Nevertheless, the expression of *hyd1* and *hyd5* was attenuated in the $\Delta fbp1$ mutant compared with the wild-type (Fig. 2B). These results suggest a role of Fbp1 in surface hydrophobicity via transcriptional regulation of the hydrophobins Hyd1 and Hyd5.

Fbp1 regulates invasive growth through the Fmk1 MAPK cascade

The virulence-related phenotypes of the $\Delta fbp1$ mutant have been reported previously in mutants lacking the MAPK Fmk1 (Di Pietro *et al.*, 2001; Prados Rosales and Di Pietro, 2008) or other components of this signalling pathway, such as Msb2, Sho1 and Ste12 (Di Pietro *et al.*, 2001; Perez-Nadales and Di Pietro, 2011, 2014). To test whether Fbp1 functions upstream of Fmk1, we compared the phosphorylation levels of Fmk1 in the wild-type and mutant strains. Immunoblot analysis with α -phospho-p44/42 MAPK antibody detected a rapid increase in Fmk1 phosphorylation in the wild-type strain on transfer from submerged culture to solid minimal medium (MM) (Fig. 3A). In contrast, the $\Delta fbp1$ mutant failed to show an increase in Fmk1 phosphorylation, whereas the phosphorylation level was restored in the complemented strain. Thus, Fbp1 is required for transient phosphorylation of the Fmk1 MAPK on contact with a solid surface.

To further investigate the role of Fbp1 as an upstream component of Fmk1, we examined the expression of *fpr1*, a gene encoding a secreted protein with an SCP-PR-1-like (cysteine-rich secretory proteins and pathogenesis-related 1 proteins) domain, whose expression has been shown previously to be controlled by Fmk1 (Prados-Rosales *et al.*, 2012). Transcript levels of *fpr1* were reduced in the $\Delta fbp1$ strain (Fig. 3B), as shown previously in the $\Delta fmk1$ and $\Delta msb2$ mutants (Perez-Nadales and Di Pietro, 2011; Prados-Rosales *et al.*, 2012).

Production of pectinolytic enzymes is not affected in the $\Delta fbp1$ mutant

We next investigated whether Fbp1 mediates the Fmk1-dependent production of secreted pectinolytic enzymes. For this point, we analysed the production of a clear halo on plates containing polygalacturonic acid (PGA) (Di Pietro *et al.*, 2001). The $\Delta fmk1$ mutant showed a strongly reduced clear halo production compared with the wild-type strain (Fig. 4A). By contrast, $\Delta fbp1$ strains produced clear halos of a size similar to those of the wild-type. In addition, the transcript levels of three polygalacturonase genes and one pectate lyase gene under inducing conditions (submerged culture with 1% pectin or 1% PGA, respectively; Di Pietro *et al.*, 2001) did not differ significantly between the wild-type and the $\Delta fbp1$ mutants. Taken together, our results from the phenotypic

Fig. 2 Fbp1 is involved in colony hydrophobicity. (A) The indicated strains were grown on minimal medium (MM) plates with 3% sucrose for 6 days at 28 °C. Twenty microlitres of water stained with bromophenol blue were applied on the colony surface (at room temperature) and images were taken. During this time, the drop remains intact on hydrophobic colonies, but is entirely soaked up by hydrophilic colonies. (B) Germlings formed after 15 h at 28 °C in potato dextrose broth (PDB) were twice washed and transferred onto MM agar plates for 8 h. Transcript levels of hydrophobins *hyd1* and *hyd5* were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Relative expression levels represent the mean cycle threshold values normalized to actin gene expression levels and relative to the expression values in the wild-type. Bars represent standard errors calculated from three independent experiments with three replicates each. wt, wild-type.

Fig. 3 Fbp1 contributes to the phosphorylation of the Fmk1 mitogen-activated protein kinase (MAPK). (A) Total protein extracts from the indicated strains, cultured for 15 h in potato dextrose broth (PDB) and transferred onto minimal medium (MM) plates for the indicated time periods (min), were subjected to immunoblot analysis with anti-phospho-p44/42 MAPK antibody (α -P-erk) which only detects the phosphorylated form of Fmk1, and anti-FUS3 to detect total protein. A monoclonal α -tubulin antibody was used as loading control. (B) Germlings formed after 15 h at 28 °C in PDB were twice washed and transferred onto MM agar plates for 8 h. Transcript levels of *fpr1* were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Relative expression levels represent the mean cycle threshold values normalized to actin gene expression levels and relative to the expression values in the wild-type. Bars represent standard errors calculated from three independent experiments with three replicates each. wt, wild-type.

Fig. 4 Pectinolytic gene expression is not regulated by Fbp1. (A) Production of extracellular polygalacturonase activity was determined on plates containing polygalacturonic acid (PGA). Enzyme activity is visualized as a contrasting halo surrounding the fungal colony. (B) The indicated strains were grown for 15 h in potato dextrose broth (PDB), three times washed and transferred to minimal medium (MM) supplemented with 1% pectin or 1% PGA. Expression levels were measured by reverse transcription-polymerase chain reaction (RT-PCR). wt, wild-type.

analysis indicate that, among the different Fmk1 outputs, Fbp1 regulates principally those related to invasive growth and adhesion.

Fbp1 contributes to the membrane and cell wall stress response through the MAPK Mpk1

In *S. cerevisiae*, components of the CWI MAPK pathway are regulated by ubiquitination (Molina *et al.*, 2010). Indeed, the *C. neoformans* SCF^{Fbp1} complex has been shown to be involved in the response to cell wall-damaging compounds, such as sodium dodecylsulfate (SDS), calcofluor white (CFW) and Congo Red (CR) (Liu *et al.*, 2011; Wang *et al.*, 2011). SDS disrupts the plasma membrane, whereas CFW and CR specifically bind to chitin and β -1,3-glucan of the cell wall (Elorza *et al.*, 1983; Wood and Fulcher, 1983). We found that the growth of the Δ *fbp1* mutant in comparison with the wild-type was severely affected by SDS, highly reduced by CFW, but not by CR. The addition of 1.25 M sorbitol partially rescued the growth defect on CFW but, as expected, not on SDS, where the growth defect was more pronounced (Fig. 5A).

Increased sensitivity to CFW in Δ *fbp1* suggests a possible deregulation in chitin synthesis. Thus, we studied the expression of three genes encoding different chitin synthases of *F. oxysporum*. Chs2 is a class II chitin synthase important for resistance to detergents and for virulence (Martin-Udiroz *et al.*, 2004); Chs3 belongs to class III and is thought to be essential based on the failure to obtain knockout mutants (Martin-Udiroz *et al.*, 2004); ChsV is a Class V chitin synthase essential for virulence (Madrid *et al.*,

Fig. 5 Fbp1 plays an important role in cell integrity. (A) The colony phenotypes of the indicated strains grown on yeast peptone glucose agar (YPDA) supplemented with 100 $\mu\text{g/mL}$ Congo Red (CR), 50 $\mu\text{g/mL}$ calcofluor white (CFW) or 0.0125% sodium dodecylsulfate (SDS) in the absence or presence of 1.25 M sorbitol. Plates were spot inoculated, incubated for 44 h (YPDA, CR, SDS) or 72 h (CFW) at 28 °C and scanned. (B) RNA was isolated from mycelia obtained after 15 h of growth in potato dextrose broth (PDB) medium. The transcript levels of three chitin synthase genes (*chs2*, *chs3* and *chsV*) were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Relative expression levels represent the mean cycle threshold values normalized to actin gene expression levels and relative to the expression values in the wild-type. Bars represent standard errors calculated from three independent experiments with three replicates each. (C) Total protein extracts from the indicated strains cultured for 15 h in PDB, supplemented with SDS 0.013% and incubated for another 15, 30 or 60 min. Samples were subjected to immunoblot analysis with anti-phospho-p44/42 MAPK antibody (α -P-erk), which only detects the phosphorylated form of Mpk1, and anti-Mpk1 to detect total protein. A monoclonal α -tubulin antibody was used as loading control. wt, wild-type.

2003). The expression of *chs3* and *chsV* was increased in the $\Delta fbp1$ mutant compared with the wild-type strain (Fig. 5B). We also tested the sensitivity of the $\Delta fbp1$ mutant to hyperosmotic (1.2 M of sorbitol or 1.5 M of NaCl) or oxidative (menadione or caffeine) stress, and found no significant differences from the wild-type strain (Fig. S2, see Supporting Information). Thus, Fbp1 functions in the response of *F. oxysporum* to membrane and cell wall stress.

We analysed the phosphorylation pattern of the CWI MAPK Mpk1 in mycelia grown in the presence of 0.013% SDS, using Western blot analysis with the α -phospho-p44/42 MAPK antibody (Perez-Nadales and Di Pietro, 2011). The level of Mpk1 phosphorylation was reduced in the $\Delta fbp1$ mutant relative to the wild-type and complemented strain (Fig. 5C). These results suggest that Fbp1 regulates the phosphorylation of Mpk1 after SDS treatment as a CWI/membrane stressor.

DISCUSSION

Fbp1 functions in the Fmk1 MAPK pathway

Given that Fbp1 is essential for virulence in *F. oxysporum*, our interest was directed towards understanding the cellular pathways associated with Fbp1 function. Previous work has suggested that Fbp1 may promote invasive growth by controlling the stability of one or several components of the Fmk1 invasive growth MAPK pathway (Miguel-Rojas and Hera, 2013). We found that, in addition to invasive growth, vegetative hyphal fusion and root adhesion are also significantly reduced in the $\Delta fbp1$ strain. Adhesion to the host tissue is a key step during fungal infection, which provides the base for fungal proliferation, tissue invasion and dissemination (Naglik *et al.*, 2003; Prados-Rosales *et al.*, 2006). Numerous fungal species respond to contact with a surface by undergoing differentiation (Kumamoto, 2008). In *F. oxysporum*, the presence of the host roots induces adhesion and differentiation of infection hyphae that directly penetrate the root (Perez-Nadales and Di Pietro, 2011). These processes are directed by the Fmk1 MAPK pathway (Delgado-Jarana *et al.*, 2005; Di Pietro *et al.*, 2001). This is further evidenced by a rapid increase in Fmk1 phosphorylation level on contact with a solid surface (Perez-Nadales and Di Pietro, 2011). We found that the phosphorylation level of Fmk1 is reduced in the absence of Fbp1, suggesting that this F-box protein functions upstream of Fmk1. In line with this idea, Fbp1 was required for the high expression of *fpr1*, a gene regulated by the Fmk1 MAPK pathway. Upstream, MAPKK Ste7, MAPKKK Ste11 and the adaptor protein Ste50 are also essential for the differentiation of penetration structures and plant infection in plant pathogens (Turra *et al.*, 2014). We analysed the *F. oxysporum* orthologues of Ste7, Ste11 and Ste50 for the presence of a PEST domain (<http://emboss.bioinformatics.nl/cgi-bin/>

emboss/pepfind). PEST domains are regions rich in proline, glutamate, serine and tyrosine that target proteins for rapid degradation (Rechsteiner and Rogers, 1996). Interestingly, all three upstream components contain a PEST domain, indicating that they are potential targets of Fbp1. In the yeast mating pathway, ubiquitination has been shown to contribute to the attenuation of MAPK signalling, as some pathway components appear to be degraded in an SCF-dependent manner (Cappell *et al.*, 2010; Garrenton *et al.*, 2009).

Nevertheless, our results suggest a model in which Fbp1 promotes Fmk1 signalling by targeting a negative regulator of Fmk1. Interestingly, two protein phosphatase 2Cs (PP2Cs), Ptc1 and Ptc3, are involved in the regulation of stress tolerance and virulence in *Botrytis cinerea* (Yang *et al.*, 2013). It has been suggested that the CWI and HOG pathways are negatively regulated by the PP2C FgPtc3 in *F. graminearum* (Yun *et al.*, 2014).

Hydrophobins and colony hydrophobicity is regulated by Fbp1

In addition to vegetative hyphal fusion and root adhesion, Fbp1 is also involved in colony hydrophobicity. Similar to $\Delta msb2$ and $\Delta fmk1$ colonies (Perez-Nadales and Di Pietro, 2011), the $\Delta fbp1$ mutant colonies are hydrophilic. The expression of two hydrophobins, Hyd1 and Hyd5, was reduced in the $\Delta fbp1$ mutant. Hydrophobins are ubiquitous in filamentous fungi, function in the formation of aerial hyphae and dispersal of conidia (Elliot and Talbot, 2004), and are virulence factors in plant-pathogenic/entomopathogenic fungi (Bayry *et al.*, 2012). *Fusarium* species possess multiple hydrophobin genes, but, to date, their role in pathogenicity has not been determined. The class II hydrophobin FgHyd5p in *F. graminearum* has been shown to be selectively activated in hyphae growing in contact with a hydrophobic surface, suggesting that FgHyd5p may act as a mediator between the hydrophilic cell wall and a hydrophobic surface, such as a leaf, in order to support the establishment of fungal structures during infection (Minenko *et al.*, 2014).

Pectinolytic enzyme regulation is independent of Fbp1

The results from this study suggest that Fbp1 contributes to various but not all phenotypes regulated by Fmk1. One of the processes controlled by Fmk1 orthologues in fungal pathogens is the regulation of secreted cell wall-degrading enzymes (Di Pietro *et al.*, 2001; Jenczmionka and Schafer, 2005; Lev and Horwitz, 2003; Rispail and Di Pietro, 2009; Xu and Hamer, 1996). The production of these enzymes is thought to be important in the initial steps of plant infection. The expression of pectinolytic enzymes was not altered in the $\Delta fbp1$ strain, suggesting that the

contribution of Fbp1 to the Fmk1 pathogenic pathway is restricted to invasive growth and adhesion to a solid surface, similar to the results obtained in $\Delta msb2$ and $\Delta ste12$ mutants (Perez-Nadales and Di Pietro, 2011; Risipail and Di Pietro, 2009).

Fbp1 contributes to the cell wall stress response through the Mpk1 MAPK pathway

The effect of Fbp1 on the cell integrity of *F. oxysporum* was more evident on cell membranes, as $\Delta fbp1$ mutants were highly sensitive to SDS. The reduced growth of $\Delta fbp1$ on CFW was partially recovered when 1,2 M sorbitol was added to the medium. In *Aspergillus fumigatus* and *Ustilago maydis*, the *BCK1*, *MKK1* and *SLT2* mutants impaired in the cell wall were partially stabilized by amending the medium with 1 M of sorbitol (Carbo and Perez-Martin, 2010; Valiante *et al.*, 2008, 2009).

Comparable levels of resistance to SDS have been shown in $\Delta chs2$ (class II) and $\Delta chs7$ (related to class IV) chitin synthase mutants of *F. oxysporum* (Martin-Udiroz *et al.*, 2004). The significant increase in *chs3* and *chsV* expression levels in the $\Delta fbp1$ mutant is in line with the compensatory reaction observed in fungi in response to treatment with cell wall-perturbing agents (Rogg *et al.*, 2013).

In *C. neoformans*, Fbp1 mutants showed reduced growth on SDS, but not on CFW or CR (Liu *et al.*, 2011). The authors suggested that Fbp1 may target certain membrane proteins that could be important for membrane integrity. Moreover, casein kinase I (Cck1) could mediate the phosphorylation of Fbp1 substrates, as indicated by the shared phenotypes of *cck1* and *fbp1* mutants (Wang *et al.*, 2011). This also suggests the possible involvement of Cck1 in the Mpk1 CWI pathway.

The CWI MAPK cascade has been studied in great detail in *S. cerevisiae* (Levin, 2011). Recently, orthologues of SlT2 in *U. maydis*, *Ashbya gossypii* and *A. fumigatus* have been identified (Carbo and Perez-Martin, 2010; Lengeler *et al.*, 2013; Valiante *et al.*, 2009). In *F. graminearum*, a crosstalk between SlT2 and Hog MAPK cascades has been suggested, because the mutation of components from one pathway induces responses in the other, suggesting crosstalk between cell wall stress and osmotic or oxidative stress (Yun *et al.*, 2014).

We found evidence that Fbp1 positively regulates the activity of Mpk1 after SDS treatment, as indicated by the drastic reduction in Mpk1 phosphorylation level in the *fbp1* mutant. Thus, Fbp1 regulates the cell wall stress response, probably through Mpk1 phosphorylation.

Collectively, our results suggest a model in which Fbp1 promotes Fmk1 and Mpk1 MAPK signalling by negatively controlling (proteasome degradation) inhibitors of MAPK phosphorylation. Future work in progress will be necessary to further address this hypothesis and to identify the negative regulators.

EXPERIMENTAL PROCEDURES

Fungal isolates and culture conditions

Fusarium oxysporum sp. *lycopersici* race 2 wild-type 4287 (race 2) was obtained from J. Tello, Universidad de Almería, Spain. The generation of the *F. oxysporum* $\Delta fbp1$ mutant has been described previously (Miguel-Rojas and Hera, 2013). *Fusarium oxysporum nit1* and *nitM* strains in the background $\Delta fbp1$ were generated by growing 2.5×10^6 fresh spores on potato dextrose agar (PDA) plates supplemented with 0.12 M KClO₃. After 15 days at 28 °C, the spontaneous mutants were analysed using different nitrogen sources: nitrate, nitrite, hypoxanthine and ammonium. All fungal strains were stored as microconidial suspensions at –80 °C with 30% glycerol. For microconidia production and fungal development studies, 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 the determination of heterokaryon formation, non-nitrate-utilizing strains were grown on MM (Puhalla, 1985). For protein and RNA extraction, 5×10^8 or 10^9 freshly obtained microconidia from each strain were inoculated into 50 or 200 mL of PDB, respectively. After 15 h of incubation at 28 °C and 170 rpm, each culture was treated as described below.

Growth tests

Assays for polygalacturonase production on PGA plates were performed as described previously (Di Pietro and Roncero, 1998). For the phenotypic analysis of colony growth, water droplets with serial dilutions (10^4 , 5×10^3 , 10^3 , 5×10^2 or 10^2) of freshly obtained microconidia were spotted onto agar plates with complete rich medium YPD (0.3% yeast extract, 1% peptone and 2% glucose) containing the indicated compounds. For cell stress assays, 100 µg/mL CR or 50 µg/mL CFW (Sigma-Aldrich, St. Louis, MO, USA) were added to 50 mM 2-(N-morpholino)ethanesulfonic acid (MES)-buffered YPD plates, pH 6.5 (Ram and Klis, 2006) with or without 1.25 M sorbitol; SDS (0.0125%) was added directly to YPD plates without MES buffer. For osmotic and oxidative stress assays, YPD plates were supplemented with 1.5 M NaCl, 1.2 M sorbitol, 5 mM caffeine or 20 µg/mL menadione. Plates were incubated at the indicated times at 28 °C before being photographed.

Nucleic acid manipulations, RT-PCR and real-time qPCR analyses

Total RNA and genomic DNA were extracted from *F. oxysporum* mycelia following previously reported protocols (Chomczynski and Sacchi, 1987; Raeder and Broda, 1985). The quality and quantity of extracted nucleic acids were determined by running aliquots in ethidium bromide-stained agarose gels and by spectrophotometric analysis in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Wilmington, DE, USA), respectively.

Total RNA was treated with DNase I (Roche, Mannheim, Germany). First-strand cDNA was synthesized with iScript Reverse Transcription Superscript following the instructions of the manufacturer (Bio-Rad, Hercules, CA, USA) using either 2 or 3 µg of total RNA. Gene-specific primers (Table S2, see Supporting Information) were designed to flank an intron, if possible. RT-PCR analysis was performed using Expand High Fidelity Taq-

polymerase (Roche, Mannheim, Germany), 50 ng of cDNA template and 300 mM of each gene-specific primer in a final reaction volume of 10 μ L. The following PCR programme was used for all reactions: an initial step of denaturation (5 min, 94 °C), followed by 30 cycles of 35 s at 94 °C, 35 s at 62 °C and 30 s at 72 °C. Real-time qPCR was performed using FastStar Essential DNA Green Master (Roche, Indianapolis, IN, USA) in a CFX-Connect Real-Time System (Bio-Rad, Barcelona, Spain). Transcript levels were calculated by comparative $\Delta\Delta C_t$ and normalized to actin (Livak and Schmittgen, 2001). Expression values are presented as values relative to the expression in the wild-type strain. All experiments for qPCR included three technical replicates and were performed three times with similar results.

Heterokaryon tests

As described previously in Prados Rosales and Di Pietro (2008), in order to determine heterokaryon formation between two strains carrying different nitrate auxotrophic markers, drops of microconidial suspensions from the respective strains were inoculated on MM at a distance of 1 cm (Puhalla, 1985). Heterokaryons exhibiting vigorous wild-type growth on nitrate were observed in the colony contact zone after approximately 4 days of incubation at 28 °C. To measure the abilities of the different strains to undergo vegetative hyphal fusions, quantitative heterokaryon tests were performed (Xiang *et al.*, 2002). Conidial suspensions (10^7 conidia) of a *nitM* or *nit1* tester strain in a wild-type background were mixed with 10^7 , 10^6 , 10^5 , 10^4 , 10^3 or 10^2 conidia of *nit1* or *nitM* mutants, respectively, obtained in the $\Delta fbp1$ background (Table S3, see Supporting Information). Conidial mixtures were plated on PDA, and the number of heterokaryotic colonies per plate was determined after 4 days of incubation at 28 °C. Conidial suspensions of the different mutants, in the absence of the tester strain, were used as controls to check for revertants. The viability of conidia was determined by plating 100 and 1000 conidia from each strain individually on PDA. Treatments were performed in triplicate, and experiments were performed at least three times with similar results.

Virulence-related assays

For adhesion assays, the roots of tomato seedlings were placed in Erlenmeyer flasks containing a suspension of microconidia in PDB diluted 1 : 50 with water and supplemented with 20 mM glutamic acid. They were incubated for the indicated times at 28 °C and 170 rpm. The adhesion of germlings to the root surface was observed using a Leica, Madrid, Spain binocular microscope at different times after inoculation. For macro- and microscopic analysis of vegetative hyphal fusion, fungal strains were grown at different times in PDB diluted 1 : 50 with water, supplemented with 20 mM of glutamic acid and observed using a Leica DMR microscope employing the Nomarsky technique or in a Leica binocular microscope. Photographs of all experiments were recorded with a Leica DC 300F digital camera. The experiments were performed at least three times with similar results.

Protein purification and Western blot analysis

For the analysis of the phosphorylation state of the Fmk1 MAPK, germlings from PDB were harvested, washed three times in sterile water,

resuspended in 4 mL of sterile water and transferred onto two MM agar plates without trace elements (Puhalla, 1985). The plates were incubated for 8 h at 28 °C. For the analysis of the phosphorylation state of the Mpk1 MAPK, germling cultures from PDB were supplemented with SDS (0.013%) and incubated for another 15, 30 or 60 min. Mycelia of both experiments were rapidly harvested, frozen in liquid nitrogen, ground in a mortar and resuspended in protein extraction buffer [10% glycerol, 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonylfluoride (PMSF), Protease inhibitor cocktail P8215 (Sigma-Aldrich), phosphatase inhibitor (50 mM NaF), 3 mM sodium pyrophosphate 50 mM β -glycerophosphate, 1 mM sodium orthovanadate and PhosSTOP Phosphatase Inhibitor Cocktail tablets (Roche, Mannheim, Germany)]. Measurements of protein extraction and concentration were performed as described previously (Perez-Nadales and Di Pietro, 2011). One hundred micrograms of total protein were loaded onto a 14% Tris-glycine gel using standard protocols (Sambrook *et al.*, 2001). Separated proteins were transferred to nitrocellulose membrane (0.45 μ m, Bio-Rad, Munich, Germany). Membranes were blocked using 5% nonfat skimmed milk for 1 h. p44/42 MAP kinases Fmk1 and Mpk1 were detected using the PhosphoPlus p44/p42 MAP kinase (Thr-202/Tyr-204) Antibody kit #9100 (Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer's instructions, with some modifications as reported previously (Perez-Nadales and Di Pietro, 2011). Monoclonal α -tubulin antibody was obtained from Santa Cruz Biotechnology (3H3087) (Dallas, TX, USA). Time-course phosphorylation experiments were performed three times independently with similar results.

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The authors declare that no conflict of interest exists.

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SUPPORTING INFORMATION

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Fig. S1 Fbp1 is required for vegetative complementation of nitrate utilization deficiency in *Fusarium oxysporum*. Non-nitrate-utilizing *nit1* mutants were inoculated 1 cm from a *nitM* mutant onto minimal medium (MM) with nitrate as the sole nitrogen source. Vigorous hyphal growth in the region of contact between the colonies denotes the presence of vegetative complementation through heterokaryon formation.

Fig. S2 Response of the $\Delta fbp1$ strain to osmotic and oxidative stress compounds. The colony phenotype of the indicated strains grown on yeast–peptone–glucose (YPD) supplemented with 1.5 M NaCl, 1.2 M sorbitol, 5 mM caffeine or 20 μ g/mL menadione. Plates were spot inoculated, incubated for 40 h (YPD, YPD + sorbitol and YPD + caffeine), 70 h (YPD + menadione) or 108 h (YPD + NaCl) at 28 °C and scanned.

Table S1 Hyphal fusion frequency.

Table S2 Primers used in this study.

Table S3 *Fusarium oxysporum* strains used in this study.