Proteomic identification of potential target proteins regulated by the SCF^{Fbp1}-mediated proteolysis pathway in *Fusarium oxysporum*

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SUMMARY

F-box proteins function in the recruitment of proteins for SCF ubiquitination and proteasome degradation. Here, we studied the role of Fbp1, a nonessential F-box protein of the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*. The $\Delta fbp1$ mutant showed a significant delay in the production of wilt symptoms on tomato plants and was impaired in invasive growth on cellophane membranes and on living plant tissue. To search for target proteins recruited by Fbp1, a combination of sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) was used to compare proteins in mycelia of the wild-type and $\Delta fbp1$ mutant. The proteomic approach identified 41 proteins differing significantly in abundance between the two strains, 17 of which were more abundant in the $\Delta fbp1$ mutant, suggesting a possible regulation by proteasome degradation. Interestingly, several of the identified proteins were related to vesicle trafficking. Microscopic analysis revealed an impairment of the $\Delta fbp1$ strain in directional growth and in the structure of the Spitzenkörper, suggesting a role of Fbp1 in hyphal orientation. Our results indicate that Fbp1 regulates protein turnover and pathogenicity in *F. oxysporum*.

INTRODUCTION

Fusarium oxysporum is a ubiquitous soil-borne ascomycete, which causes vascular wilt disease on more than 100 plant species, provoking severe losses in important crops, such as banana, cotton, melon and tomato (Gordon and Martyn, 1997). This fungus is also being recognized as an emerging human pathogen which poses a lethal threat to immune compromised individuals (Nucci and Anaissie, 2007). Its remarkably broad host range and the array of molecular tools available make *F. oxysporum* an attractive model for the study of the different aspects of fungal infection (Di Pietro *et al.*, 2003; Michiels and Rep, 2009).

In recent years, several genes governing the pathogenicity of *Fusarium* have been identified, including those involved in signal

transduction (Di Pietro *et al.*, 2001), nutrient signalling (Lopez-Berges *et al.*, 2010) and cell wall biosynthesis (Madrid *et al.*, 2003), among others. Moreover, F-box proteins, such as Frp1 (Duyvesteijn *et al.*, 2005) and Fbp1 (Han *et al.*, 2007), have been shown to function in the pathogenicity of *F. oxysporum* and *F. graminearum*, respectively. F-box proteins play a key role in protein turnover, an important regulatory mechanism of cellular function in eukaryotic cells. In addition to proteolytic degradation, other biological pathways that are regulated by ubiquitination include endocytosis and intracellular trafficking (Madhus, 2006; Mosesson and Yarden, 2006; Saksena *et al.*, 2007), histone and transcriptional regulation (Kodadek, 2010), autophagy (Kirkin *et al.*, 2009), DNA repair (Thomson and Guerra-Rebollo, 2010) and diverse cellular signalling (Chen and Sun, 2009; Haglund and Dikic, 2005; Kawadler and Yang, 2006).

Fungal F-box proteins have been reported to regulate distinct cellular functions (Jonkers and Rep, 2009). The GRR1 F-box protein of *Saccharomyces cerevisiae* is part of the SCF^{GRR1} E3 ligase which interacts with Skp1 via its F-box domain and with downstream targets via its leucine-rich repeat (LRR) domain. GRR1 was originally identified as a primary element responsive for glucose repression (Flick and Johnston, 1991), and was also found to control amino acid sensing (Bernard and Andre, 2001) and cell cycle regulation (Blondel *et al.*, 2000). In addition to *S. cerevisiae*, the role of F-box proteins in fungal development has been reported in other ascomycetes, such as Grr1 and Cdc4 in *Candida albicans* (Atir-Lande *et al.*, 2005; Butler *et al.*, 2006; Li *et al.*, 2006), GrrA in *Aspergillus nidulans* (Krappmann *et al.*, 2006), Fwd1 and Scorn2 in *Neurospora crassa* (He *et al.*, 2003; He and Liu, 2005; Kumar and Paietta, 1998), Pth1 in *Magnaporthe grisea* (Sweigard *et al.*, 1998), Fbp1 in *F. graminearum* (Han *et al.*, 2007) and Frp1 in *F. oxysporum* (Duyvesteijn *et al.*, 2005). Recently, a role of Fbp1 has been reported in the sexual reproduction and virulence of the basidiomycetous yeast pathogen *Cryptococcus neoformans* (Liu *et al.*, 2011). Interestingly, all F-box proteins tested so far in plant fungal pathogens (Pth1, Fbp1 and Frp1) are required for fungal pathogenicity. However, despite their importance in the regulation of fungal development and virulence, the role of F-box proteins in pathogenicity remains poorly understood.

In this study, we analysed the role of Fbp1 in the pathogenicity of *F. oxysporum* by construction of a $\Delta fbp1$ null mutant. We found that Fbp1 is essential for invasive growth and tomato plant

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infection. We carried out a proteomic approach to identify the target proteins of Fbp1 with a possible role in the pathogenicity process. In addition to proteins involved in metabolism and oxidation–reduction processes, some candidate proteins related to adhesion, cell signalling and vesicle trafficking were identified. This is the first proteomic approach revealing the candidate targets of Fbp1-mediated proteolysis in fungal plant pathogens.

RESULTS

The *fbp1* gene encodes an F-box protein conserved among fungi that is important for invasive growth and pathogenicity of *F. oxysporum*

The genome of *F. oxysporum* f. sp. *lycopersici* contains 53 predicted F-box proteins, as revealed by a search of the genome database. *FOXG_04438* encodes the orthologue of the *F. graminearum* *fbp1* gene (Han *et al.*, 2007). The deduced protein is 743 amino acids in length with the genomic sequence interrupted by three putative introns. Like yeast GRR1 and the orthologues in other fungi, Fbp1 contains a putative F-box domain and 12 tandem copies of a 26-amino-acid sequence, the LRR domain (Fig. S1, see Supporting Information). The predicted protein sequence of Fbp1 presents high similarity (87% amino acid identity) to *F. graminearum* Fbp1. The identity level increases to nearly 99% when the conserved F-box and LRR domains are compared.

We analysed *fbp1* gene expression during different growth conditions using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). *fbp1* was constitutively expressed in the wild-type strain grown in potato dextrose broth (PDB) liquid medium (Fig. 1A). This result is consistent with those reported in *F. graminearum* (Han *et al.*, 2007). We also tested the effect of the carbon source on the expression of the *fbp1* gene. Our qRT-PCR results showed a lower expression level in glucose than in galactose (Fig. 1B).

The biological role of Fbp1 in *F. oxysporum* was explored by the generation of a $\Delta fbp1$ deletion allele by replacing the *fbp1* open reading frame (ORF) with the hygromycin resistance cassette (Fig. S2A, see Supporting Information). PCR and Southern blot analysis (Fig. S2B,C) established that two independent mutants, named $\Delta fbp1\#1$ and $\Delta fbp1\#3$, lacked a functional copy of the *fbp1* gene.

To confirm that the phenotype of the $\Delta fbp1$ mutant was indeed caused by a loss of *fbp1* function, a 5-kb fragment encompassing the complete *F. oxysporum* *fbp1* gene was introduced into the $\Delta fbp1$ strain by co-transformation with the phleomycin resistance marker. Three phleomycin resistance transformants were analysed by PCR with gene-specific primers and found to have integrated an intact copy of the *fbp1* gene into the genome (Fig. S2D).

Invasive growth, a virulence-related process with analogies to agar invasion in yeast, was analysed using the cellophane

Fig. 1 Expression of *fbp1* under different culture conditions. (A) Transcript levels of the *fbp1* gene measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA was isolated from mycelia obtained after the indicated times of growth in potato dextrose broth (PDB) medium. (B) Transcript levels of *fbp1* under induction (galactose medium) or repression (glucose medium) conditions measured by qRT-PCR analysis. Relative expression levels represent the mean cycle threshold values normalized to actin gene expression levels and relative to the expression values at 72 h (A) or in galactose medium (B) set to unity. Bars represent standard errors calculated from three independent experiments with three replicates each.

penetration assay (Prados Rosales and Di Pietro, 2008). In contrast with the wild-type, the $\Delta fbp1$ mutant was unable to penetrate cellophane membranes (Fig. 2A), suggesting that Fbp1 is important for invasive growth. Cellophane penetration was restored in the two independent complemented strains. To determine whether Fbp1 is required for the invasive growth of *F. oxysporum* on living host plant tissue, tomato fruits were inoculated by the injection of a microconidial suspension of the different strains into the fruit tissue (Di Pietro *et al.*, 2001). Figure 2B shows the fruit after 4 days of incubation: although the $\Delta fbp1$ mutant was impaired in invasive growth, the wild-type and complemented strains colonized and rotted the fruit tissue surrounding the site of inoculation.

We next asked whether the absence of Fbp1 affected the ability to infect tomato plants. Roots of tomato seedlings were dip inoculated with microconidia of the wild-type, $\Delta fbp1$ mutants and *fbp1* complemented strains (Di Pietro and Roncero, 1998). Plants inoculated with the wild-type showed a progressive increase in vascular wilt symptoms, and most were dead 30 days after inoculation (Fig. 2C). Two independent $\Delta fbp1$ mutants showed reduced virulence, but still produced a low level of wilt symptoms. Virulence was restored in the two complemented strains.

Two-dimensional gel electrophoresis protein profile of the wild-type and the $\Delta fbp1$ mutant

Our next goal was to investigate the potential proteins targeted by the SCF^{Fbp1}-mediated proteolysis pathway. Our starting hypothesis was that, when Fbp1 is defective, the protein targets are not

Fig. 2 Fbp1 is required for invasive growth and contributes to virulence on tomato plants. (A) Penetration of cellophane membranes. Fungal colonies were grown for 4 days on a plate with minimal medium covered by a cellophane membrane (before); the cellophane with the colony was removed and plates were incubated for an additional day (after). (B) Invasive growth of *Fusarium oxysporum* strains on tomato fruits at the inoculation site after 4 days of incubation at 100% relative humidity and 28 °C. (C) Incidence of *Fusarium* wilt on tomato plants inoculated with the indicated strains. The severity of disease symptoms was recorded at the indicated times after inoculation using an index ranging from 1 (healthy plant) to 5 (dead plant). The data shown are from three representative experiments. Error bars represent the standard error (SE) calculated from 10 plants. wt, wild type.

degraded by the 26S proteasome and thus should exhibit higher abundance in the $\Delta fbp1$ mutant strain than in the wild-type. Total cellular protein extracts were obtained from germlings grown under the conditions in which $\Delta fbp1$ was unable to penetrate cellophane membranes. Six replicate gels were run per strain and stained with SYPRO RUBY (Fig. 3). Image analysis with PD-QUEST

software showed significant changes in the expression of 80 proteins, all of which could be identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) [see Experimental Procedures and Table S1 (Supporting Information)]. The proteins were re-annotated by BLAST searches against the *Fusarium* genome database, leading to 46 hits corresponding to 41 unique protein accessions (see Table S2, see Supporting Information). A comparison of the proteomes of the wild-type and the $\Delta fbp1$ strain revealed 17 protein spots that were always more abundant in the $\Delta fbp1$ mutant than in the wild-type strain in different batches of protein samples, whereas 24 protein spots were more abundant in the wild-type strain (Table S2).

We next assigned the proteins to functional categories according to their involvement in biological processes. The classification was based on gene ontology (GO) using the information from the UniProt-GO Annotation database (<http://www.ebi.ac.uk/GOA>) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) protein interaction pathways (<http://www.genome.jp/kegg/pathway.html>) to assign the differentially expressed proteins to metabolic pathways.

The classification of proteins by biological process showed 11 different groups. More than 50% of the proteins fell into two categories: (i) oxidation–reduction processes (29%) and (ii) metabolic processes (24%) (Fig. 4A). Interestingly, the majority of the proteins up-regulated in the wild-type strain fell into these two groups. By contrast, proteins up-regulated in the $\Delta fbp1$ mutant were involved in proteolysis, protein folding, response to stress, cellular component organization, cellular processes and translation (Fig. 4B).

Classification of proteins accumulating at a higher level in the $\Delta fbp1$ mutant than in the wild-type strain

Among the protein spots identified, two were involved in vesicular trafficking: (i) the coatamer subunit epsilon, Sec28 (FOXG_05228), a component of the retrograde pathway belonging to the COPI coat proteins, which are responsible for the biogenesis and sorting of vesicles from the Golgi apparatus and their delivery to the endoplasmic reticulum via retrograde transport (Cai *et al.*, 2007; Cosson *et al.*, 1996; Spang, 2002); and (ii) FOXG_00146, corresponding to a 14-3-3 protein orthologue to *S. cerevisiae* BMH2. 14-3-3 proteins are dimeric proteins abundant in the cytosol, which interact with a broad array of cellular proteins controlling different cellular processes, including vesicle trafficking (Yuan *et al.*, 2003).

The proteome of the $\Delta fbp1$ mutant also showed two up-regulated chaperones: FOXG_00893 (orthologue to *S. cerevisiae* SSB1) and FOXG_04346, a member of the Hsp90 family of chaperones (Peisker *et al.*, 2010). The Hsp90 chaperone is involved in the endoplasmic reticulum-associated degradation (ERAD)

Fig. 3 Two-dimensional images of proteins extracted from the wild-type and the $\Delta fbp1$ mutant strains of *Fusarium oxysporum*. Two-dimensional gel analysis of proteins extracted from mycelia grown for 8 h on minimal medium (MM) plates, separated by two-dimensional polyacrylamide gel electrophoresis, and detected by Sypro Ruby staining. Some differentially expressed proteins are indicated in the bottom panels. Bars corresponding to the spot intensity are represented for six different gels.

process which starts with the recognition of a misfolded protein substrate, followed by substrate ubiquitination, and retrotranslocation from the endoplasmic reticulum for degradation by the 26S proteasome (Ciechanover, 2005).

With regard to pathogenicity, one of the most abundant proteins in the $\Delta fbp1$ mutant, FOXG_07877, corresponds to a putative adhesin from the HAD superfamily of hydrolases. In the human pathogen *Paracoccidioides brasiliensis*, a 32-kDa adhesion protein has been shown to play an important role in adherence and pathogenicity (Gonzalez *et al.*, 2008).

Another differentially regulated protein with a putative function in pathogenicity was FOXG_12759, orthologue to the cobalamine-independent fungal methionine synthase MET6, essential for complete virulence in the human pathogen *Cr. neoformans* (Pascon *et al.*, 2004).

Both eukaryotic and prokaryotic organisms generate reactive oxygen species (ROS) as by-products of their metabolism, mainly through aerobic respiration. One of the mechanisms that supplies reducing power is the thioredoxin and glutathione redox system. The FOXG_10111 protein corresponds to a peroxiredoxin, an important class of cellular antioxidants identified in a wide range of organisms (Rhee *et al.*, 2005). FOXG_09187 is an orthologue of ZTA1, a NADPH-dependent quinone reductase in *S. cerevisiae*. A protein complex between a quinone reductase and the 20S proteasome has been shown recently to stabilize transcription factors by acting as an inhibitor of their proteasomal degradation (Sollner and Macheroux, 2009). Finally, we also identified an alcohol dehydrogenase 2 (FOXG_12790) and an aldo-keto-reductase

(FOXG_09876), involved in glycolysis and gluconeogenesis, both of which have been implicated in the oxidative stress response (Aguirre *et al.*, 2005).

Proteins with decreased levels in the $\Delta fbp1$ mutant

The largest group of proteins down-regulated in the $\Delta fbp1$ strain corresponded to proteins involved in oxidation–reduction and metabolic processes (Fig. 4B). Among these were eight oxidoreductases: thioredoxin reductase (FOXG_00831), uricase (FOXG_11511), a mitochondrial precursor of D-lactate dehydrogenase 2 (FOXG_02132) and several unannotated proteins that were identified as belonging to different oxidoreductase families. A phenol 2-monooxygenase (FOXG_17384) was also overexpressed in the wild-type strain. Phenol 2-monooxygenase is a hydrolase involved in the degradation of phenolic compounds. It is known that a large number of plant antifungal secondary metabolites are phenolic compounds whose detoxification facilitates host colonization by fungi (Michiels *et al.*, 2012).

Primary metabolism proteins constituted the bulk of proteins highly expressed in the wild-type, including enolase (FOXG_00035), involved in glycolysis and gluconeogenesis, and aconitase (FOXG_03713), required for the tricarboxylic acid (TCA) cycle, as well as enzymes involved in amino acid metabolism, such as aspartate-semialdehyde dehydrogenase (FOXG_03301), which functions in the common pathway for methionine and threonine biosynthesis, O-acetylhomoserine (FOXG_15240), dedicated to the cysteine and methionine biosynthesis pathway, and glutamine

Fig. 4 Functional classification of differentially expressed proteins. (A) The pie chart depicts the distribution (%) of differentially expressed proteins by functional category families (biological process) estimated from the gene ontology (GO) data in Table S2. (B) Classification of up- and down-regulated proteins in the $\Delta fbp1$ mutant according to biological process.

synthetase (FOXG_05182), a key enzyme in the pathway of glutamine biosynthesis from glutamate and ammonia.

Another important metabolic enzyme overexpressed in the wild-type was the *N*-acetylglucosamine (GlcNAc) transferase Gnt1 (FOXG_12436). This enzyme plays a role in *S. cerevisiae* in the transfer of GlcNAc to the N-linked glycans attached to nascent glycoproteins, as they are translocated to the endoplasmic reticulum (Yoko-O *et al.*, 2003).

The synthesis of vitamins B6 and B1 was also regulated by Fbp1. The $\Delta fbp1$ strain showed a lower intensity of the protein spot corresponding to Pdx1 (FOXG_08652), the orthologue of *S. cerevisiae* SNZ1 functioning in the biosynthesis of vitamin B6. Similarly, two components of the biosynthesis of vitamin B1 (thiamine), the precursor hydroxymethylpyrimidine (FOXG_11035) and

the thiazole intermediate (FOXG_10428), were down-regulated in the $\Delta fbp1$ mutant.

Among the spots overrepresented in the wild-type strain, two corresponded to proteins involved in vacuole fusion and protein transport. The first is FOXG_08026, identified as an orthologue of SEC17, a t-SNARE protein required for vesicular transport between the endoplasmic reticulum and the Golgi (Haas and Wickner, 1996). The second is the enolase (FOXG_00035). It has been shown that ENO1 and ENO2 are necessary to induce the homotypic vacuole fusion in *S. cerevisiae* (Decker and Wickner, 2006).

Fbp1 contributes to growth on solid surfaces under nutrient-limiting conditions

Because carbon and nitrogen metabolism pathways appeared to be attenuated in the $\Delta fbp1$ strain, we asked whether this down-regulation would result in poor nutrient assimilation in this mutant. Indeed, the $\Delta fbp1$ mutant showed reduced hyphal growth on all carbon and nitrogen sources analysed (Fig. 5B,C). The radial growth rate of the $\Delta fbp1$ mutant was reduced in all cases relative to the wild-type, except on glutamine. As glutamine is a preferred source of nitrogen, this result is in line with the findings in our proteomic analysis, suggesting that the assimilation of glutamine is not impaired in the mutant. Moreover, the $\Delta fbp1$ mutant grew similarly to the wild-type strain on complete medium, such as potato dextrose agar (PDA) (Fig. 5A). Collectively, these results suggest that $\Delta fbp1$ is impaired in nutrient assimilation.

Fbp1 is involved in directional growth on solid medium

Hyphal orientation, an essential aspect of polarized growth in fungi, is determined by the continuous flow of secretion vesicles from the hyphal cell body to the hyphal tip, resulting in cell wall and membrane extension (Gow *et al.*, 2002). One of the characteristics of filamentous fungi is the presence of the Spitzenkörper (Spk), a highly dynamic vesicle supply centre adjacent to the site of polarized cell extension (Harris *et al.*, 2005). As mentioned previously, some differentially expressed proteins in our proteomic analysis are involved in vesicle trafficking. In order to determine whether Fbp1 contributes to hyphal orientation in colony development, we compared the hyphal growth pattern of the $\Delta fbp1$ mutant and the wild-type on solid medium. In the mutant strain, leader hyphae displayed a zig-zag growth phenotype relative to straight hyphae in the wild-type and complemented strains, compatible with a defect in directional growth (Fig. 6A,B). This meandering phenotype was quantified by counting wavy leader hyphae in independent growing colonies. We obtained 100% of wavy hyphae from a population of about 200 mutant hyphae. In contrast, all of the analysed leader hyphae in the wild-type and complemented strains displayed the straight phenotype. We next

Fig. 5 Effect of *fbp1* deletion on carbon and nitrogen utilization in *Fusarium oxysporum*. Growth of the indicated strains on potato dextrose agar (PDA) (A), on minimal medium with different carbon sources (B) and on minimal medium with different nitrogen sources (C) (see Experimental procedures). CA, casaminoacids; wt, wild-type. Bars, 5 mm.

questioned whether the growing tips had a defined Spk. For this purpose, we stained growing hyphae with FM4-64, which has been used in several fungi to stain Spk (Fischer-Parton *et al.*, 2000; Penalva, 2005). Spk appeared to be well defined in the growing tips of the wild-type and complemented strains, whereas abundant and delocalized staining was observed in the $\Delta fbp1$ mutant (Fig. 6C).

DISCUSSION

Fbp1 governs invasive growth and virulence in *F. oxysporum*

In this work, we have shown that Fbp1 is essential for invasive growth and virulence in *F. oxysporum*. *Fusarium oxysporum* Fbp1 shares sequence and structural similarities with Grr1 in *C. albicans* and Fbp1 in *F. graminearum* and *Cr. neoformans* (Fig. S1). As several orthologues of GRR1 in other plant-pathogenic fungi have also been reported to play a role in fungal pathogenicity, the involvement of F-box proteins in virulence appears to be conserved in fungal pathogens.

The $\Delta fbp1$ mutants were impaired in invasive growth on cellophane membranes and on tomato fruit tissue. Both phenotypes have been related to the Fmk1 pathogenicity mitogen-activated protein kinase (MAPK) pathway in *F. oxysporum*. Three different key components of the Fmk1 MAPK cascade, the Fmk1 protein (MAPK), the transcription factor Ste12 and the mucin-like transmembrane protein Msb2, are essential to invasive growth and to the complete virulence of *F. oxysporum* f. sp. *lycopersici* (Di Pietro *et al.*, 2001; Perez-Nadales and Di Pietro, 2011; Rispail and Di Pietro, 2009). Here, we show that the $\Delta fbp1$ mutant shares the invasive growth phenotype with the $\Delta fmk1$, $\Delta ste12$ and $\Delta msb2$ knockout mutants, suggesting that Fbp1 may promote invasive growth by controlling the levels of one or several of the Fmk1 MAPK pathway components. The link between MAPK signalling and protein degradation to control signalling specificity, which defines growth versus differentiation, has been shown previously in *S. cerevisiae* (Bao *et al.*, 2004; Chou *et al.*, 2004, 2006, 2008; Molina *et al.*, 2010). Future work is needed to determine the possible involvement of Fbp1 in the Fmk1 MAPK signalling cascade of *F. oxysporum*.

Potential targets of SCF^{Fbp1} involved in primary metabolism and oxidation–reduction

Fbp1 has been reported to be a versatile F-box protein participating in the formation of the SCF complex, which controls regulatory processes for major traits in *Gibberella zeae*, including sexual reproduction and virulence (Han *et al.*, 2007). Several lines of evidence suggest that Fbp1 also participates in the development and virulence of *Cr. neoformans* (Liu *et al.*, 2011). Nevertheless,

Fig. 6 Effect of *fbp1* deletion on directional hyphal growth of *Fusarium oxysporum*. (A) Growth phenotypes at the edge of the fungal colony (a–i). Scale bar, 1 mm. (B) Optical images showing meandering growth in a leader hypha of the $\Delta fbp1$ strain. Scale bar, 10 μm . (C) FM4-64 staining reveals that the $\Delta fbp1$ mutant is defective in the formation of the Spitzenkörper. White arrow indicates Spitzenkörper. Scale bar, 10 μm . DIC, differential interference contrast image; wt, wild-type.

nothing is known so far about the proteins targeted by the SCF^{Fbp1} complex for proteasomal degradation.

We reasoned that the proteins involved in invasive growth should be differentially expressed in the $\Delta fbp1$ mutant strain. As mentioned previously, invasive growth resembles root adhesion in being associated with virulence functions of *F. oxysporum* (Prados-Rosales *et al.*, 2009). Interestingly, 19 of the 41 proteins differentially expressed in the present work were also found in a previous proteomic study which identified 174 expressed proteins from *F. oxysporum* cell walls under adhesion-inducing conditions, including those with a known biochemical function in primary metabolism, such as enolase and pyruvate decarboxylase PdcA (Prados-Rosales *et al.*, 2009).

The 41 differentially expressed proteins detected here are involved in a plethora of biological processes, most related to the oxidation–reduction process and primary metabolism (Fig. 4A, Table S2). It is known that GRR1 (Spielewoy *et al.*, 2004) plays a role in both glucose and amino acid signalling by targeting Yck1/2-phosphorylated pathway components for ubiquitination by SCF^{GRR1}. In a proteomic screen developed by Benanti *et al.* (2007), seven new metabolic targets of SCF^{GRR1} were identified, including two regulators of glycolysis. These results demonstrated that the glycolytic–gluconeogenic switch was regulated by targets of

SCF^{GRR1}. We identified three proteins involved in this pathway, two up-regulated in $\Delta fbp1$ (Pdc1 and Adh2) and one up-regulated in the wild-type strain (Eno1). Some of the up-regulated proteins in the $\Delta grr1$ mutant belong to coordinately transcribed groups, such as the regulators of methionine biosynthesis, suggesting that some are transcriptionally regulated (Benanti *et al.*, 2007). In our work, two enzymes involved in the biosynthesis of methionine were identified [MET6 (FOX_12795) and MET17 (FOXG_15240)], which were up- and down-regulated, respectively, in $\Delta fbp1$. Although the expression of these proteins was transcriptionally regulated, the regulators may be SCF^{Fbp1} controlled and targeted to degradation. Fbp1 regulates primary metabolism, as deduced by the results of proteomic analysis, but also by phenotypic growth assays (Fig. 5). The reduced radial colony growth observed on different nutrient sources suggests that certain metabolic pathways are affected in the $\Delta fbp1$ mutant strain.

The second major class of differentially expressed proteins was related to the oxidation–reduction process. ROS play a major role in pathogen–plant interactions. Recognition of a pathogen by the plant rapidly triggers the oxidative burst, which is necessary for further defence reactions. Protection against oxidative stress is also required for normal fungal development, because a transient

increase in ROS levels acts as an internal signal for developmental progression (Aguirre *et al.*, 2005). The role of the up-regulated oxido-reductases identified in the wild-type proteome might thus be related to the reduced virulence observed in the mutant strain.

Fbp1 contributes to directional hyphal growth and may participate in the ubiquitination of proteins involved in vesicle trafficking

Approximately half of the proteins more abundant in the $\Delta fbp1$ mutant are involved in a variety of cellular processes that are susceptible to ubiquitination: transport, proteolysis, response to stress and cellular component organization. Classically, polyubiquitination is a signal that directs proteins to the proteasome, where ubiquitin is recycled and the protein is degraded (Hershko and Ciechanover, 1992). Nevertheless, ubiquitination also affects nonproteasomal functions, such as endocytosis (Hicke and Riezman, 1996; Kolling and Hollenberg, 1994; Mukhopadhyay and Riezman, 2007), where monoubiquitination is sufficient as an endocytic internalization signal (Galan and Haguenauer-Tsapis, 1997; Terrell *et al.*, 1998). In yeasts and other fungi, endocytosis and intracellular transport events are important for cellular functions, including polarity establishment, hyphal growth and virulence (Atkinson *et al.*, 2002; Dou *et al.*, 2011; Read and Kalkman, 2003; Shaw *et al.*, 2011; Wang and Shen, 2011).

Intracellular transport implies the transfer of material between organelles, which is mediated by carrier vesicles that continually bud from one membrane and fuse with another. Vesicle budding is mediated by protein coats that cycle on and off membranes: COPI (coat protein complex I) and COPII (coat protein complex II) mediate vesicle transport in the early secretory pathway (Cai *et al.*, 2007). The final steps in vesicle-mediated membrane trafficking are the tethering and fusion of the vesicle with its target membrane. Fusion is thought to occur by the pairing of SNAREs, a family of membrane proteins (Chen and Scheller, 2001). COPI vesicles mediate the retrograde transport from the Golgi to the endoplasmic reticulum, as well as intra-Golgi transport. We have shown here that one of the up-regulated proteins in the $\Delta fbp1$ mutant is Sec28, the orthologue of the human ϵ -COP subunit. A recent model for the SEC28 trafficking pathway in yeast suggests that UBC1 (ubiquitin ligase 1) controls the concentration of SEC28 at the vacuolar membrane (Brown *et al.*, 2008). We further found that the t-SNARE protein Sec17 was up-regulated in the wild-type strain. It has been shown previously in yeast that SEC28 and SEC17 are involved in vesicle formation and membrane fusion, respectively. We consider that Fbp1 could be implicated in these processes in *F. oxysporum*. FM4-64 staining of Spk indicates that secretory carriers reaching this structure recycle endocytosed membrane to the apex, implicating this recycling in apical extension (Fischer-Parton *et al.*, 2000). Recent studies on the molecular machinery regulating hyphal orientation have shown that muta-

tions that affect these processes result in hyphae that have either abnormal meandering trajectories or attenuated tropic responses (Brand and Gow, 2009). Mutation in two cell-end marker proteins, TeaA and TeaC, of *A. nidulans* resulted in a zig-zag hyphal phenotype (Higashitsuji *et al.*, 2009), underscoring the importance of these proteins in hyphal orientation. We found that Fbp1 is important for correct Spk formation and directional growth, as suggested by the zig-zag pattern of $\Delta fbp1$ hyphae (Fig. 6).

Our proteomic approach revealed a range of *F. oxysporum* proteins whose orthologues were shown to be ubiquitinated in *S. cerevisiae* (Table S3, see Supporting Information). Interestingly, among the proteins up-regulated in the $\Delta fbp1$ mutant, only one, Dbp5, has not been shown previously to be ubiquitinated in yeast (Venancio *et al.*, 2009; Ziv *et al.*, 2011). Metabolic proteins (Eno1) and heat shock proteins (Ssb) have also been shown to be ubiquitinated in a proteomic study in *C. albicans* (Leach *et al.*, 2011). Moreover a number of spots identified in our work correspond to proteins that have been co-purified with the 20S proteasome in *Trichoderma reesei* (Grinyer *et al.*, 2007; Kautto *et al.*, 2009). Among these are the unfolded protein response (UPR) proteins, such as chaperones, and proteasome-interacting proteins (PiPs), including 14-3-3-like protein, thioredoxin and actin. The t-SNARE protein SEC17 is also associated with the 20S proteasome (Kautto *et al.*, 2009).

It remains to be demonstrated whether the identified *F. oxysporum* proteins are indeed ubiquitinated and regulated by the proteasome. However, our results are consistent with the hypothesis that proteins detected as differentially expressed in our proteomic approach are regulated by Fbp1 and thus susceptible as potential targets of ubiquitination and proteasome degradation by the SCF^{Fbp1} system.

EXPERIMENTAL PROCEDURES

Fungal isolates and culture conditions

Fusarium oxysporum f. sp. *lycopersici* race 2 wild-type 4287 (FGSC 9935) was used in all experiments. All fungal strains were stored as microconidial suspensions at -80°C with 30% glycerol. For the extraction of DNA, microconidia production and fungal development, cultures were grown in liquid PDB at 28°C with shaking at 170 rpm (Di Pietro and Roncero, 1998). For protein extraction, 5×10^8 freshly obtained microconidia from each strain were inoculated into 50 mL of PDB. After 12 h of incubation at 28°C and 170 rpm, mycelia were harvested, washed three times in sterile water, resuspended in 4 mL of sterile water and transferred onto two minimal medium (MM) (Puhalla, 1985) agar plates. The plates were incubated for 8 h at 28°C , and the mycelia were harvested, frozen in liquid nitrogen and stored at -80°C . For colony growth analysis, drops of water with 2×10^4 microconidia were spotted onto PDA plates (Difco, Detroit, MI, USA) or MM agar containing the indicated carbon or nitrogen sources. The plates were incubated for 4 days at 28°C and scanned. All experiments included three replicate plates and were performed three times with similar results.

Nucleic acid manipulations and targeted gene knockout

Total RNA and genomic DNA were extracted from *F. oxysporum* mycelia following previously reported protocols (Chomczynski and Sacchi, 1987; Raeder and Broda, 1985). DNA was treated with appropriate restriction enzymes and subject to Southern hybridization analysis following the standard protocols (Di Pietro and Roncero, 1998) using the nonisotopic digoxigenin labelling kit (Roche Diagnostics, Basel, Switzerland). Real-time qRT-PCR analysis was performed as described previously (Lopez-Berges *et al.*, 2010) using iQ SYBR Green Supermix in an iCycler iQ real-time PCR System (both from Bio-Rad, Hercules, CA, USA). Gene-specific primers (Table S4, see Supporting Information) were designed to flank an intron. The relative levels of the RT-PCR products were determined using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). C_t values were normalized to the C_t value of the actin gene.

An *fbp1* mutant allele was generated by fusion PCR (Yang *et al.*, 2004). For targeted *fbp1* gene knockout, protoplasts of *F. oxysporum* 4287 strain were transformed and hygromycin-resistant transformants were analysed (see Fig. S1).

Light and fluorescence microscopy

To examine hyphal morphology, fungal strains were grown for 36 h on MM agarose (3%) plates and observed in a Leica DMR microscope (Heerbrugg, St. Gallen, Switzerland) or a Zeiss binocular microscope (Oberkochen, Germany). Photographs were recorded with a Leica DC 300F digital camera or an AxioCam ICc 1. The vital dye FM4-64 (Molecular Probes, Eugene, OR, USA; Invitrogen, Carlsbad, CA, USA) was used as a marker of the Spk in mature hyphae. The edge of the colony was cut and placed on a slide, and MM liquid (20 μ L) containing FM4-64 (4.5 μ M as working solution concentration) was placed over it. The slice was covered with a coverslip, and the samples were kept at room temperature in the dark for 15 min and then analysed.

Protein extraction, two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and gel analysis

Germlings from PDB cultures were obtained as described above, transferred onto MM agar plates and incubated at 28 °C for the indicated time periods. 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 dodecylsulphate (SDS), 1% Triton, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulphonylfluoride (PMSF), Protease inhibitor cocktail P8215, Sigma-Aldrich, St. Louis, MO, USA]. The samples were kept on ice for 30 min and the supernatant was harvested by centrifugation at 11 336 *g* for 10 min at 4 °C. Samples were washed using the methanol-chloroform method, as described previously (Wessel and Flugge, 1984). The pellet was dried and resuspended in 1 $\times$ CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate] buffer (7 M urea, 2 M thiourea and 4% w/v CHAPS). The protein concentration of the supernatant was determined with the Bio-Rad Bradford reagent using bovine serum albumin (BSA) as a standard. 2D PAGE was performed by vertical electrophoresis. Total fungal proteins (150 μ g per gel) were loaded onto immobilized pH gradient strips

(immobilized, pH 5–8, linear gradient) by overnight active rehydration with immobilized pH gradient (IPG) (17 cm, Bio-Rad). Isoelectric focusing (IEF) was performed using the Protean IEF Cell System (Bio-Rad) at 1000 V for 1 h, 4000 V for 1 h, 30 min ramping to 8000 V and maintenance at 8000 V until 50 000 V h. The strips were then incubated with equilibration buffer 1 [375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol and 2% dithiothreitol (DTT)] and buffer 2 [375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol, 2% DTT and 2.5% iodoacetamide] for 15 min each. Second dimension SDS-PAGE was run on 11% polyacrylamide gels (26 cm $\times$ 20 cm) using the Protean Plus Dodeca Cell (Bio-Rad Laboratories, Hercules, CA, USA). Separation was carried out at 8 °C for 30 min at 30 V and then at 60 V until the dye front reached the bottom of the gel. After electrophoresis, the gels were stained with Sypro Ruby (SYPRO® Ruby Protein Stains, Bio-Rad) stain according to the manufacturer's protocol. Gel images were obtained by a Molecular Imager FX Pro Plus Multi-Imager System (Bio-Rad) using a wavelength of 535 nm. Scans were acquired at 100 μ m resolution. The digitized images were analysed with PDQUEST software (version 8.0.1, Bio-Rad).

In-gel digestion and protein identification

The protein spots were automatically excised from the gels using an Investigator™ ProPic (Genomic Solutions, Huntingdon, Cambridgeshire, UK) station. The digestion protocol of Schevchenko *et al.* (1996) was employed, with minor variations, using a ProPrep II (Genomic Solutions) digestion station. Gel plugs were de-stained by incubation (twice for 30 min) with a solution containing 200 mM ammonium bicarbonate in 40% acetonitrile at 37 °C. They were then subjected to three consecutive dehydration–rehydration cycles with pure acetonitrile and 25 mM ammonium bicarbonate in 40% acetonitrile, respectively, and finally dried over 4 h at room temperature. Then, 20 μ L trypsin, at a concentration of 12.5 ng/ μ L in 25 mM ammonium bicarbonate, was added to the dry gel pieces and the digestion was performed at 37 °C for 12 h. The digestion was stopped and the peptides were extracted from the gel plugs by the addition of 1 μ L of 10% (v/v) trifluoroacetic acid (TFA) and incubation for 15 min. The peptide fragments from digested proteins were desalted and concentrated using μ C-18 ZipTip columns (Millipore, Billerica, MA, USA) and loaded directly onto the MALDI plate using α -cyanohydroxycinnamic acid as a matrix.

MALDI-TOF mass spectrometry (MS) analyses were performed in a MALDI-TOF/TOF mass spectrometer 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). The instrument was operated in reflector mode, with an accelerating voltage of 20 kV and in the *m/z* range 800–4000. Spectra were internally calibrated with peptides from trypsin autolysis ($M + H = 842.509$, $M + H^+ = 2211.104$). The most abundant peptide ions were then subjected to fragmentation analysis (MS/MS), providing information that can be used to determine the peptide sequence.

A combined PMF search (MS plus MS/MS) was performed using GPS Explorer™ software v3.5 (Applied Biosystems) over the *F. oxysporum* genome database (http://www.broadinstitute.org/annotation/genome/fusarium_group/) employing the Mascot 2.0 search engine (Matrix Science Ltd., London, UK; <http://www.matrixscience.com>). The following parameters were set for searching: taxonomy restriction to fungus, a minimum of four peptide matches, a maximum of one missed cleavage with the selected trypsin enzyme, an MS/MS fragment tolerance of 0.2 Da, a pre-

cursor mass tolerance of 100 ppm, and cysteine carbamidomethylation and methionine oxidation as possible modifications. The confidence in the peptide mass fingerprinting matches was based on the score level and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum. Proteins with a statistically significant value ($P < 0.05$) were positively assigned identification after considering M_i and pI values. Hypothetical proteins with unknown function were annotated by BLAST search in the National Center for Biotechnology Information (NCBI) database. Protein identifications are documented in Table S2. The chromosome location of the genes encoding the identified proteins was determined by searching in the *F. oxysporum* database. The proteomic (two-dimensional electrophoresis and MALDI-TOF) analysis was carried out in the Universidad de Córdoba-Servicios Centrales de Apoyo a la Investigación proteomics facility, a member of the Carlos III Networked Proteomics Platform, ProteoRed-ISCIII.

Bioinformatics prediction

The *F. oxysporum* Fbp1 protein was identified by a BLASTP search in the *Fusarium* Comparative Database of the Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html) with the *F. graminearum* Fbp1 protein sequence. The identification of putative GRR1 orthologues from different fungi was carried out by a BLASTP search in the NCBI database (<http://www.ncbi.nlm.nih.gov/>). AlignX was used to generate the sequence alignment shown in Fig. S1. The protein domain predictions were made using the Prosite database (ExPASy; Swiss Institute of Bioinformatics, Lausanne, Switzerland), SMART (<http://smart.embl-heidelberg.de/>) and UniProtKB (<http://www.uniprot.org/>).

Accession numbers

The accession numbers are as follows: M59247 (*Saccharomyces cerevisiae* Grr1); CAD21405 (*Neurospora crassa* Grr1); DQ309327 (*Aspergillus nidulans* GrrA); XP722013 (*Candida albicans* Grr1); FOXG_04438 (*Fusarium oxysporum* Fbp1); FGSG_02095 (*Fusarium graminearum* Fbp1); and CNAG_05280.2 (*Cryptococcus neoformans* Fbp1).

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The authors declare that no conflict of interest exists.

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SUPPORTING INFORMATION

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

Fig. S1 The *fbp1* gene product of *Fusarium oxysporum* represents an F-box protein that is conserved in fungi. (A) Schematic illustration of the *F. oxysporum* Fbp1 protein. F-box, F-box domain; LLRs, leucine-rich repeats; aa, amino acids. The positions of the amino acid residues at the domain boundaries are indicated. (B, C) Amino acid sequence alignment of the region spanning the F-box (B) and LRR motifs (C) in *Saccharomyces cerevisiae* (ScGrr1), *Candida*

albicans (CaGrr1), *Cryptococcus neoformans* (CnFbp1), *Aspergillus nidulans* (AnGrrA), *Neurospora crassa* (NcGrr1), *Fusarium graminearum* (FgFbp1) and *Fusarium oxysporum* (FoFbp1). Positions in the primary structure of the respective proteins are indicated on the left. Highly conserved residues are shaded in black, moderately conserved residues are shaded in dark grey and slightly conserved residues are shaded in light grey.

Fig. S2 Targeted replacement of the *Fusarium oxysporum fbp1* gene. (A) Physical maps of the *fbp1* locus and the targeted gene disruption construct obtained by fusion polymerase chain reaction (PCR). The relative positions of the *Bam*HI restriction enzyme and the primers used for PCR are indicated. *hyg^r*, hygromycin resistance gene; *PgpdA*, *gpdA* promoter; *TrpC*, *trpC* terminator (both from *Aspergillus nidulans*). (B) PCR amplification of genomic DNA of four independent transformants, using primers *fbp1*-3 and *hph2* (Table S4). (C) Southern blot analysis. Genomic DNA of the wild-type strain and four 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 in (A). (D) PCR amplification of genomic DNA of the wild-type strain, $\Delta fbp1$ mutant and three independent complemented transformants, using primers *fbp1*-1 and *fbp1*-2 (Table S4).

Table S1 Data obtained for the identification of 80 spots with $P < 0.05$ in Student's *t*-test.

Table S2 Differentially expressed proteins in the $\Delta fbp1$ mutant and the wild-type strain identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS).

Table S3 Proteins identified in this work which have been analysed previously for ubiquitination.

Table S4 Primers used in this study.