The *FRP1* F-box gene has different functions in sexuality, pathogenicity and metabolism in three fungal pathogens

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SUMMARY

Plant-pathogenic fungi employ a variety of infection strategies; as a result, fungi probably rely on different sets of proteins for successful infection. The F-box protein Frp1, only present in filamentous fungi belonging to the Sordariomycetes, Leotiomycetes and Dothideomycetes, is required for nonsugar carbon catabolism and pathogenicity in the root-infecting fungus *Fusarium oxysporum*. To assess the role of Frp1 in other plant-pathogenic fungi, *FRP1* deletion mutants were generated in *Fusarium graminearum* and *Botrytis cinerea*, and their phenotypes were analysed. Deletion of *FgFRP1* in *F. graminearum* led to impaired infection of barley roots, but not of aerial plant parts. Deletion of *BcFRP1* in *B. cinerea* did not show any effect on pathogenicity. Sexual reproduction, however, was impaired in both *F. graminearum* and *B. cinerea* *FRP1* deletion mutants. The mutants of all three fungi displayed different phenotypes when grown on an array of carbon sources. The *F. oxysporum* and *B. cinerea* deletion mutants showed opposite growth phenotypes on sugar and nonsugar carbon sources. Replacement of *FoFRP1* in *F. oxysporum* with the *B. cinerea* *BcFRP1* resulted in the restoration of pathogenicity, but also in a switch from impaired growth on nonsugar carbon sources to impaired growth on sugar carbon sources. This effect could be ascribed in part to the *B. cinerea* *BcFRP1* promoter sequence. In conclusion, the function of the F-box protein Frp1, despite its high sequence conservation, is not conserved between different fungi, leading to differential requirements for pathogenicity and carbon source utilization.

INTRODUCTION

Fungi associated with plants employ different strategies to colonize or infect host plants. In addition, they exhibit lifestyles ranging from saprotrophic to biotrophic or necrotrophic growth. As a result of this broad variety of strategies and lifestyles, the requirement for certain cellular processes and proteins involved in the infection mechanism probably varies between fungi.

The plant-pathogenic fungi *Fusarium oxysporum*, *Fusarium graminearum* and *Botrytis cinerea* are examples of fungi with different lifestyles and infection strategies. The soil-borne hemibiotrophic fungus *F. oxysporum* infects its specific host through the roots and frequently colonizes the xylem vessels, causing wilt disease (Di Pietro *et al.*, 2003; Gordon and Martyn, 1997; Michielse and Rep, 2009; Roncero, 2003). *Fusarium graminearum*, a putative hemibiotrophic fungus, is dispersed mainly as sexual ascospores and predominantly infects aerial plant parts, causing head blight or crown rot on cereal plants (Goswami and Kistler, 2004; Stephens *et al.*, 2008). *Botrytis cinerea* is a necrotrophic fungus with over 200 hosts, infecting field crops, ornamental flowers and harvested fruits and vegetables. *Botrytis cinerea* can enter plant tissue immediately after conidial germination via cuticle penetration through the formation of an appressorium-like structure (Choquer *et al.*, 2007; van Kan, 2006; Williamson *et al.*, 2007). Despite different infection strategies and distant taxonomy, plant-pathogenic fungi still share conserved genes that are important during infection, such as genes encoding proteins of conserved signalling pathways. For instance, deletion of one or more mitogen-activated protein (MAP) kinase or G-coupled protein genes shows an effect on pathogenicity and/or cell wall-degrading enzyme (CWDE) gene expression in many fungi, including *F. oxysporum*, *F. graminearum* and *B. cinerea* (Di Pietro *et al.*, 2001; Doehlemann *et al.*, 2006; Jain *et al.*, 2002, 2003, 2005; Jenczmionka *et al.*, 2003; Rui and Hahn, 2007; Schulze Gronover *et al.*, 2001; Segmüller *et al.*, 2007; Xu, 2000; Yu *et al.*, 2008; Zheng *et al.*, 2000).

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In addition to conserved genes fulfilling a role in pathogenicity, fungi also require genes for their specific pathogenic lifestyle and/or infection strategy. For instance, the melanin-deficient mutant *buf1* and the cyclic AMP-dependent protein kinase A mutant *cpkA* in *Magnaporthe grisea* (Sesma and Osbourn, 2004) and isocitrate lyase (*ICL1*) mutants in different plant-pathogenic fungi (Idnurm and Howlett, 2002; Jonkers *et al.*, 2009; Wang *et al.*, 2003) are deficient in leaf infection, but still invade roots. Conversely, *FOW1*, a gene required for root infection in *F. oxysporum*, is not required for appressorium formation and leaf penetration in *M. grisea* (Inoue *et al.*, 2002; Sesma and Osbourn, 2004).

Frp1 is a fungal protein required for pathogenicity in *F. oxysporum* (Duyvesteijn *et al.*, 2005; Jonkers *et al.*, 2009). Frp1 belongs to the family of F-box proteins that commonly facilitate the degradation of target proteins via ubiquitination through a Skp, Cullin, F-box protein (SCF)-containing complex (Jonkers and Rep, 2009a; Willems *et al.*, 2004). Fungal F-box proteins have been shown to be involved in the control of the cell division cycle, glucose sensing and mitochondrial connectivity (reviewed by Jonkers and Rep, 2009a). In *Neurospora crassa*, the F-box protein Fwd1 has been shown to be required for degradation of the circadian clock protein FREQUENCY (FRQ) via ubiquitination through the SCF complex (He and Liu, 2005).

The *FoFRP1* deletion mutant shows impaired root colonization and penetration defects, which are related to growth impairment on a broad spectrum of nonsugar carbon sources, such as organic acids, amino acids and cell wall polymers. The *FoFRP1* deletion mutant also shows impaired expression of CWDE genes and other carbon catabolite repression (CCR) genes (Jonkers and Rep, 2009b; Jonkers *et al.*, 2009). Mutation of *CRE1*, a gene controlling CCR gene expression, in the *FoFRP1* deletion mutant restored the above-described impairments (Jonkers and Rep, 2009b).

At present, it is unknown whether Frp1 homologues in other fungi also regulate CCR and carbon source assimilation, and whether these processes are required for pathogenicity in fungi with a different infection strategy and lifestyle. Therefore, the *F. graminearum* and *B. cinerea* orthologues of the F-box protein Frp1 were studied to reveal whether they have similar functions as in *F. oxysporum*. In addition, functional conservation of *FRP1* was assessed by cross-species' complementation.

RESULTS

Conservation of *FRP1* in filamentous fungi

To examine the conservation of *FRP1* in the fungal kingdom, we searched for Frp1 homologues in available fungal sequences at the Broad Institute (<http://www.broadinstitute.org>), the DOE

Joint Genome Institute (<http://genome.jgi-psf.org>) and the *Podospora anserina* Genome Project (<http://podospora.igmors.u-psud.fr>) using the BLASTP algorithm (Altschul *et al.*, 1997). Using a cut-off score of 1×10^{-10} , single Frp1 homologues were found in fungi from classes Sordariomycetes, Leotiomycetes and Dothideomycetes, all belonging to the Ascomycetes. The only putative Frp1 homologue belonging to the Eurotiomycetes retrieved using the BLAST search, CIMG_02198 from *Coccidioides immitis*, had a score (1×10^{-6}) below the cut-off and was therefore not included.

A CLUSTALW alignment of predicted Frp1 proteins that were retrieved from the various databases was used to create a phylogenetic tree (Fig. 1A). This tree is based on amino acids 175–278 of the alignment, which includes and extends beyond the F-box domain (Fig. S1, see Supporting Information). The putative Frp1 homologues from the Dothideomycetes are substantially shorter and less similar than those in the Sordariomycetes and Leotiomycetes; for example, the *S. nodorum* Frp1 homologue is 338 amino acids long, in contrast with the 527 amino acids of *F. oxysporum*.

The sequence conservation, including the *F. oxysporum*, *F. graminearum* and *B. cinerea* Frp1 homologues, is unequally distributed over the protein. In particular, the regions near the N-terminus (amino acids 38–175) and after the central domain (amino acids 425–495) are weakly conserved, whereas the regions in the central domain (amino acids 175–425) and the C-terminus (amino acids 495–595) show greater conservation (Fig. 1B).

Generation of *F. graminearum* and *B. cinerea* *FRP1* deletion mutants

To investigate the function of Frp1 in fungi other than *F. oxysporum*, deletions of *FRP1* were made in *F. graminearum* and *B. cinerea*, a phylogenetically and phenotypically more distant fungus. The entire *FgFRP1* open reading frame (ORF) (*FGSG_01326*) was replaced in *F. graminearum* strain Z03643 by a geneticin resistance cassette (Han *et al.*, 2007) (Fig. S2A, see Supporting Information). Correct homologous recombination in two independent mutants was confirmed by Southern analysis (Fig. S2B). Vegetative growth of the mutants was similar to that of the wild-type, but conidiation was reduced in the mutants when grown on CMC medium (see Experimental details). The addition of 1% glucose to the medium restored conidiation to the wild-type level (data not shown).

In *B. cinerea*, the *BcFRP1* ORF (*BC1G_09967*) was replaced in strain B05.10 by a nourseothricin resistance cassette (Fig. S3A, see Supporting Information). Correct homologous recombination in two independent mutants was confirmed by polymerase chain reaction (PCR) (Fig. S3C) and Southern analysis (Fig. S3E). In addition, one deletion mutant was

Fig. 1 Occurrence of Frp1 homologues in various fungi. (A) A phylogenetic tree was created with MegAlign software using amino acids 175–278 from the middle domain of the Frp1 homologues, which includes the F-box domain. The fungal classes to which the respective fungi belong are given next to the tree. Frp1 homologues of the following organisms were used in this alignment: Ab, *Alternaria brassicicola* (AB01633.1); Bc, *Botrytis cinerea*; Cg, *Cheatomium globosum* (CHGG_00736.1); Fg, *Fusarium graminearum* (FGSG_01326.3); Fo, *Fusarium oxysporum* (FOXG_00058.2); Fs, *Fusarium solani* (Nectria haematococca) protein ID 103049; Fv, *Fusarium verticillioides* (FVEG_01458.3); Hj, *Hypocrea jecorina* (*Trichoderma reesei*) protein ID 120583; Mg, *Magnaporthe grisea* (MGG_06351); Nc, *Neurospora crassa* (NCU09899); Pa, *Podospora anserina* (Pa_1-12760); Ptr, *Pyrenophora tritici-repentis* (PTRG_0644.1); Sn, *Stagonospora nodorum* (SNOG_14560.1); Ss, *Sclerotinia sclerotiorum* (SS1G_14401). For *B. cinerea* Frp1, an alternative gene model was used. (B) An alignment of the *F. oxysporum*, *F. graminearum* and *B. cinerea* Frp1 proteins was created using MacVector software. The F-box domain is underlined.

complemented with the *BcFRP1* locus (Fig. S3B), resulting in replacement of the nourseothricin resistance cassette by the *BcFRP1* locus and the hygromycin resistance cassette. Again, homologous recombination was verified by PCR (Fig. S3D) and Southern analysis (Fig. S3E). The vegetative growth and conidiation of these mutants were similar to those of the wild-type (data not shown).

FRP1 is not required for natural infection in *F. graminearum* or *B. cinerea*

As the *F. oxysporum* $\Delta frp1$ mutant displays a loss of pathogenicity, the *F. graminearum* and *B. cinerea* $\Delta frp1$ mutants were also assessed for their ability to cause disease on their natural hosts. Wild-type *F. graminearum*, two $\Delta Fgfrp1$ mutants and an

Fig. 2 The $\Delta frp1$ mutant strains of *Fusarium graminearum* and *Botrytis cinerea* are not affected in the infection of commonly infected plant organs. (A) *Fusarium graminearum* wild-type (WT), ectopic mutant and two $\Delta Fgfrp1$ mutant strains were assessed in a wheat infection assay. Bars indicate the total amount of infected spikes after 2 weeks. (B) *Botrytis cinerea* WT and two $\Delta Bcfrp1$ mutants were assessed on primary leaves of bean plants. Photographs were taken 72 h post-inoculation (hpi). (C) *Botrytis cinerea* WT and mutant $\Delta Bcfrp1$ #4 were assessed in leaf and fruit infection assays. Bars indicate the lesion diameter.

ectopic transformant were point inoculated in wheat ears. Two weeks after inoculation, the total number of infected spikes on each ear was counted. The two independent $\Delta Fgfrp1$ mutants caused disease similar to the wild-type and the ectopic transformant (Fig. 2A), indicating that *FgFRP1* is dispensable for ear infection.

The wild-type and two $\Delta Bcfrp1$ mutants of *B. cinerea* were inoculated on the primary leaves of bean plants (Fig. 2B). In addition, one deletion mutant was extensively tested on various plants and fruits, such as detached leaves of tobacco and tomato plants, apple, pepper and tomato fruits. In none of these assays a significant difference in lesion size or appearance was observed between the wild-type and $\Delta Bcfrp1$ mutant strains (Fig. 2C), indicating that, also in *B. cinerea*, *BcFRP1* is not required for pathogenicity.

FgFRP1* is required for root infection by *F. graminearum

Although the common infection route of *F. graminearum* on cereals is believed to be by the dispersion of spores on cereal ears, infections of stems, seeds and roots have also been reported (Lysøe *et al.*, 2006; Stephens *et al.*, 2008). Therefore, the

Fig. 3 *Fusarium graminearum* $\Delta Fgfrp1$ mutants show impaired infection of barley roots. *Fusarium graminearum* wild-type (WT), an ectopic transformant and two $\Delta Fgfrp1$ strains were assessed in a barley root infection assay. Bars indicate the percentage of roots containing lesions. The two $\Delta Fgfrp1$ mutant strains show a significantly smaller number of diseased roots when compared with the wild-type.

ability of the *F. graminearum* $\Delta Fgfrp1$ mutant to cause disease on barley roots was assessed. Barley seeds were incubated with a spore suspension of the wild-type, the ectopic mutant or the *F. graminearum* $\Delta Fgfrp1$ mutants and, after 2 weeks of germination, lesions on the roots and fungal growth within the roots were determined. The roots developed from seeds infected with the *F. graminearum* $\Delta Fgfrp1$ mutants showed less brown spots and lesions compared with roots developed from seeds infected with the wild-type and ectopic mutant (Fig. 3). When seeds and attached roots were surface sterilized, cut into pieces of approximately 5 mm and placed on agar plates, mycelial outgrowth from all root pieces (~5.5 cm in total) could be observed from wild-type infected roots. The outgrowth of the $\Delta Fgfrp1$ mutant from infected roots was restricted to the seed and the first ~1 cm of the root (Table 1). This suggests that the *F. graminearum* $\Delta Fgfrp1$ mutant, like the *F. oxysporum* $\Delta Fofrp1$ mutant, has a reduced ability to colonize and grow inside roots, resulting in a reduced pathogenicity phenotype.

FRP1* is required for sexual reproduction in *F. graminearum* and *B. cinerea

As sexual reproduction of *F. oxysporum* remains to be established under laboratory conditions and has not been observed in nature, the role of Frp1 during this process has not been evaluated previously. However, both *F. graminearum* and *B. cinerea* are capable of a sexual life cycle, and the role of Frp1 during the sexual life cycle was assessed in these two fungi. As a homothallic fungus, *F. graminearum* can develop perithecia and ascospores without the need for a mating partner. When placed on carrot agar plates, which induces the sexual reproduction process in the wild-type, the *F. graminearum* $\Delta Fgfrp1$ mutant

Table 1 Fungal outgrowth of roots infected with wild-type or $\Delta Fgfrp1$ mutants.

Strain	Root length displaying fungal outgrowth (cm)
Wild type PH-1	5.5 $\pm$ 0.5
$\Delta Fgfrp1$ #10	1 $\pm$ 0.5
$\Delta Fgfrp1$ #14	1 $\pm$ 0.5
Control (water)	0

proved to be sterile (Fig. 4A). This suggests that Frp1 is required for sexual reproduction in *F. graminearum*.

Botrytis cinerea is a heterothallic fungus with a biallelic mating system. This requires for sexual reproduction: the fertilization of sclerotia (melanized mycelial mass structures) of one isolate (defined as 'female') by microconidia (defined as 'male') of the opposite mating type (Faretra *et al.*, 1988). The *B. cinerea* $\Delta Bcfrp1$ mutant is able to produce sclerotia, although with a delay of approximately 5 days when compared with the wild-type (Fig. 4B, top left). Moreover, sclerotia of the $\Delta Bcfrp1$ mutant show a slightly different appearance relative to the wild-type recipient strain B05.10; they are smaller and have an intensely black colour and a rough-edged surface. By contrast, sclerotia of the wild-type are typically larger, have a smoother surface and a shiny, silvery colour (Fig. 4B, bottom left). When the $\Delta Bcfrp1$ mutant (generated in strain B05.10 of MAT1-1 identity) was used as a male in a cross with wild-type strain SAS405 (MAT1-2 identity), acting as female, it resulted in the production of apothecia of normal appearance, similar to those obtained in crosses of the wild-type strain B05.10 with SAS405 (Fig. 4B, top right, and Table 2). However, when sclerotia of the $\Delta Bcfrp1$ mutant were fertilized with microconidia of SAS405, no apothecia were produced, whereas the fertilization of sclerotia of B05.10 with microconidia of SAS405 resulted in the formation of apothecia of normal appearance. The sclerotia of the $\Delta Bcfrp1$ mutant formed a thick gelatinous polysaccharide layer, and mycelium grew into the air (Fig. 4B, bottom right, and Table 2) and occasionally formed conidiophores with macroconidia (data not shown). This peculiar phenotype has not been observed in other transformants or mutants analysed to date (Rui and Hahn, 2007; Segmüller *et al.*, 2008; J. van Kan, unpublished data). We conclude that the *B. cinerea* $\Delta Bcfrp1$ mutant is female sterile.

Deletion of *FRP1* results in a unique carbon source utilization phenotype for each of the examined fungi

The *F. oxysporum* $\Delta Fofrp1$ mutant shows significant growth reduction on a broad variety of carbon sources, including root exudate and cell wall components, which we correlated with a loss of pathogenicity (Jonkers *et al.*, 2009). As the deletion of *FRP1* in the two other fungi does not result in a loss of pathogenicity on aerial plant parts, it raises the question whether

Fig. 4 The $\Delta frp1$ mutants from *Fusarium graminearum* and *Botrytis cinerea* are sterile. (A) The $\Delta Fgfrp1$ mutant from *F. graminearum* does not produce perithecia when placed on carrot agar, whereas the wild-type (WT) strain does. (B) *Botrytis cinerea* WT and $\Delta Bcfrp1$ mutant strains are able to produce sclerotia that are morphologically slightly different (left panels). Apothecia are produced when wild-type sclerotia are fertilized with microconidia of *B. cinerea* wild-type or $\Delta Bcfrp1$ mutant strain (male, top right panel). Sclerotia of the wild-type *B. cinerea* strain can produce apothecia after fertilization with microconidia from a wild-type strain, whereas sclerotia of $\Delta Bcfrp1$ mutant strain are female sterile after fertilization with microconidia from a wild-type strain (female, bottom right panel). Note the slimy appearance of the mutant sclerotia, which produce copious amounts of extracellular polysaccharide.

$\Delta frp1$ mutants of the other two fungi display the same growth phenotype as the *F. oxysporum* $\Delta Fofrp1$ mutant. To investigate this, the three fungal species (wild-type strains and the corresponding $\Delta frp1$ mutants) were analysed in a BiologFF Microtiter plate™ assay. This method allows simultaneous growth assessment on 95 different carbon sources (Khalil and Alsanius, 2009).

The growth behaviour of the *F. oxysporum* $\Delta Fofrp1$ mutant largely corresponds to the growth phenotype observed earlier on agar plates (Jonkers *et al.*, 2009). Strong growth reduction is seen on carboxylic acids and amino acids and on a few polysugars and sugar acids. Enhanced growth is observed on two cycle polysaccharides, two methylglycosides and two sugar alcohols (Fig. 5 and Figs S4–S9, see Supporting Information). The *F. graminearum* $\Delta Fgfrp1$ mutant is reduced in growth on several mono- and polysugars and on a few amino acids and carboxylic acids. Enhanced growth is observed on two sugar alcohols and two polysugars (Fig. 5 and Figs S4–S9). In general, deletion of *FgFRP1* in *F. graminearum* leads to a less severe growth phenotype on various carbon sources than the deletion of *FoFRP1* in *F. oxysporum*. In contrast, the *B. cinerea* $\Delta Bcfrp1$ mutants show no growth reduction on any carbon source, but enhanced growth on many, mainly mono-, di- and trisaccharides, two sugar

Table 2 *Botrytis cinerea* crosses and apothecia formation with wild-type strains SAS405 and B05.10 and with SAS405 and the $\Delta Bcfrp1$ mutants as male and female.

Cross no.	No. of biological replicates	Sclerotia (♀)	Microconidia (♂)	Formation of apothecia
1	5	SAS405	B05.10	+
2	1	SAS405	—	—
3	3	B05.10	SAS405	+
4	5	$\Delta frp1\#4$	SAS405	—
6	1	$\Delta frp1\#4$	—	—
7	6	SAS405	$\Delta frp1-4-1$	+

alcohols and two glucosides (Fig. 5 and Figs S4–S9). Growth of the complemented $\Delta Bcfrp1$ mutant on the above-mentioned carbon sources was indistinguishable from that of the wild-type (data not shown). Both *F. graminearum* and *B. cinerea* $\Delta frp1$ strains grow normally on agar plates containing ethanol or cell wall components (data not shown). This contrasts with the growth impairment on these carbon sources seen with the *F. oxysporum* $\Delta Fofrp1$ mutant (Jonkers *et al.*, 2009). We conclude that the function of Frp1 in growth on nonsugar carbon sources is not conserved in *F. graminearum* and *B. cinerea*.

Introduction of the *BcFRP1* gene in the *F. oxysporum* *FRP1* deletion mutant results in partial growth restoration

A different molecular function of Frp1 between the two *Fusarium* species and *Botrytis* might explain the observed differences in carbon source utilization and pathogenicity. To test this, we introduced *FgFRP1* or *BcFRP1* in the *F. oxysporum* $\Delta Fofrp1$ mutant. The *F. oxysporum* $\Delta Fofrp1$ mutant was transformed not only with the *BcFRP1* gene driven by its native promoter sequence, but also with the *BcFRP1* gene driven by the *FoFRP1* promoter sequence. *Fusarium oxysporum* wild-type, $\Delta Fofrp1$ and the different transformants were tested in the microtitre assay mentioned earlier to assess growth on different carbon sources. Full growth restoration was observed for the *F. oxysporum* $\Delta Fofrp1$ mutant transformed with *FoFRP1* or *FgFRP1*. In addition, the transformants with the *BcFRP1* gene controlled by the *FoFRP1* promoter showed normal growth on all carbon sources tested (Fig. 6A). However, the strain with the *BcFRP1* gene behind its native promoter showed a different phenotype. Growth restoration was observed on organic acids (2-keto-D-gluconic acid, α -keto-glutaric acid, L-malic acid, quinic acid, D-saccharic acid and succinic acid) and amino acids (L-alanine, L-aspartic acid, L-glutamic acid, L-serine, L-threonine), but lack of full restoration or even growth reduction was observed on several sugar carbon sources, including the monosaccharides L-arabinose, D-fructose, D-galactose, α -D-glucose, L-rhamnose and D-xylose and on α -D-lactose and glycogen (Fig. 6A). This growth reduction, however, was not seen with all sugar carbon sources, as growth enhancement was observed on sucrose and raffinose. These experiments suggest that Frp1 of *B. cinerea* and *F. graminearum* are functional homologues of *F. oxysporum*

Frp1, but that the promoter sequence of the *BcFRP1* gene affects the ability of this protein to restore a wild-type growth phenotype in *F. oxysporum* $\Delta Fofrp1$. When the relative transcript levels of the two different *BcFRP1* versions were compared with each other by quantitative reverse transcription-polymerase chain reaction (RT-PCR) using the constitutively expressed *FEM1* gene (Schoffemeer *et al.*, 2001) as reference, the level of *BcFRP1* driven by the *FoFRP1* promoter was about five to ten times higher than the level of *BcFRP1* driven by its native promoter (Fig. 6B). This suggests that a low *BcFRP1* transcript level limits growth on simple sugars, in contrast with either no transcript or higher transcript levels.

Both *FgFRP1* and *BcFRP1* can complement the pathogenicity defect of the *F. oxysporum* $\Delta Fofrp1$ mutant

To determine whether the *FRP1* genes from the different fungal species can restore pathogenicity to the *F. oxysporum* $\Delta Fofrp1$ strain, the *F. oxysporum* wild-type, $\Delta Fofrp1$ strain and the transformants containing *FgFRP1* and *BcFRP1* genes controlled by their native promoters were tested in a bioassay. All strains tested were pathogenic to the same level as the wild-type (Fig. 7), confirming that the *FgFRP1* and *BcFRP1* genes are functional homologues of *FoFRP1*. Interestingly, the *F. oxysporum* transformant harbouring *BcFRP1* controlled by its native promoter is fully pathogenic, in spite of its reduced growth on several sugars.

DISCUSSION

Most F-box proteins play a crucial role in the ubiquitination and degradation of proteins that have become redundant. They act by recruiting target proteins to an SCF complex where they are marked with ubiquitin for proteasomal degradation (Willems *et al.*, 2004). However, some F-box proteins, including Frp1 from *F. oxysporum*, seem not to comply with this hypothesis (Jonkers and Rep, 2009a). Although the molecular function of Frp1 remains to be discovered, this work sheds new light on the biological function of Frp1 in different fungi. Frp1 conservation is restricted to filamentous fungi from the classes Sordariomycetes, Leotiomycetes and Dothideomycetes, with homologues from the latter class showing much less conservation. That this specific

Fig. 5 Growth of the *Fusarium oxysporum*, *Fusarium graminearum* and *Botrytis cinerea* $\Delta frp1$ mutants on various carbon sources. Growth is indicated by the absorbance at 600 nm (OD_{600}) of: (A) *F. oxysporum* wild-type (WT), $\Delta Fofrp1$ and $\Delta Fofrp1$ complemented with *FRP1*; (B) *F. graminearum* and two independent $\Delta Fgfrp1$ mutants; and (C) *B. cinerea* and two independent $\Delta Bcfrp1$ mutants.

Fig. 6 Growth of the *Fusarium oxysporum* Δ Fofrp1 mutant is restored by transformation with *FgFRP1*, but not with *BcFRP1*, controlled by its native promoter. (A) Growth indicated by the absorbance at 600 nm (OD₆₀₀) of: *F. oxysporum* wild-type (WT), Δ Fofrp1 and transformants bearing the *FgFRP1* gene, the *BcFRP1* gene or *BcFRP1* driven by the *FoFRP1* promoter (pFoFRP1-*BcFRP1*) on various carbon sources. (B) Relative transcript levels of *BcFRP1* in transformants bearing the *BcFRP1* gene or *BcFRP1* driven by the *FoFRP1* promoter (pFoFRP1-*BcFRP1*). *FEM1* was used as reference to calculate the relative expression levels using the Δ C_t method.

F-box protein is not conserved in Eurotiomycetes could be caused by gene loss. Fungi have many F-box proteins and it cannot be ruled out that a functional counterpart of Frp1 is present in Eurotiomycetes. The fact that over 50 F-box proteins, many of unknown function, can be found in a single fungal genome underlines the complexity of processes requiring F-box proteins.

In this study, we compared the role of Frp1 among three plant-pathogenic fungi in pathogenicity, carbon source assimila-

tion and sexual reproduction. For this purpose, in addition to the Δ Fofrp1 mutant of the root pathogen *F. oxysporum*, deletions of *FRP1* in the putative hemibiotrophic wheat ear pathogen *F. graminearum* (Sordariomycete) and the necrotrophic broad host range pathogen *B. cinerea* (Leotiomycete) were created, followed by extensive comparisons of the deletion phenotypes. As observed earlier in *F. oxysporum*, the deletion of *FgFRP1* in *F. graminearum* resulted in impaired infection of roots. This reduction in pathogenicity was not observed on wheat ears.

Fig. 7 The *FgFRP1* or *BcFRP1* genes restore virulence of the *Fusarium oxysporum* Δ *Fofrp1* mutant. Bars indicate the disease index scores of tomato plants infected by *F. oxysporum* wild-type (WT), Δ *Fofrp1*, four transformants bearing the *FgFRP1* gene and two transformants bearing the *BcFRP1* gene.

Deletion of *BcFRP1* in *B. cinerea* had no effect on its ability to cause disease on the various plant leaves and fruits tested. These differences could be related to different requirements of metabolic pathways in roots and aerial plant parts, or, for *B. cinerea*, a different role of Frp1 in the regulation of metabolism.

In *F. oxysporum*, Frp1 is required for growth on a broad array of nonsugar carbon sources, such as polysaccharides, and for the expression of CWDE genes (Jonkers *et al.*, 2009), qualitative traits which are likely to be required for root infection. Cross-species' complementation of the *Fofrp1* mutant with the *BcFRP1* gene restores pathogenicity and growth on nonsugar carbon sources, such as amino acids and carboxylic acids. However, growth on monosaccharides is not restored and, on some sugar sources, growth is even repressed. This indicates that a loss of pathogenicity in the *Fofrp1* mutant is not a result of inefficient growth on the monosaccharides L-arabinose, L-rhamnose or D-xylose. In addition, growth on the monosaccharides D-fructose, D-galactose and α -D-glucose is apparently not a prerequisite of plant pathogenicity. In contrast with the *F. oxysporum* Δ *Fofrp1* mutant, the *F. graminearum* Δ *Fgfrp1* mutant grows normally on agar plates containing various nonsugar carbon sources and polysaccharides. This suggests that the *F. graminearum* Δ *Fgfrp1* mutant secretes sufficient amounts of CWDEs and uses the degradation products to generate energy. However, the expression of the CWDE gene xylanase 4 (*XYL4*) in the *F. graminearum* Δ *Fgfrp1* mutant grown on oat spelt xylan is undetectable, whereas in the wild-type it is observed (Fig. S10, see Supporting Information). This suggests that the *F. graminearum* Δ *Fgfrp1* mutant also shows altered CWDE gene expression, possibly leading to compromised root infection, but not to reduced growth on agar plates and cereal ear infection.

The major differences between the Δ *frp1* mutants of the two *Fusarium* spp. and *B. cinerea* relative to the wild-type strains

concern carbon catabolism. The *B. cinerea* Δ *Bcfrp1* mutant shows enhanced growth on specific simple sugars, whereas the *F. oxysporum* Δ *Fofrp1* mutant shows reduced growth on nonsugar carbon sources. This suggests that, in *B. cinerea*, Frp1 suppresses growth on simple sugar sources, whereas, in *F. oxysporum*, Frp1 is required to activate growth on nonsugar sources. During the infection of leaves and fruits, however, no faster spread or disease development was observed. Apparently, the suppression of growth on simple sugars by Frp1 has no role during the infection process in *B. cinerea*, and the relevance of this suppression remains to be elucidated.

Based on the cross-species' complementation experiments, where the *B. cinerea* and *Fusarium* *FRP1* genes were introduced into the *F. oxysporum* Δ *Fofrp1* mutant strain, it seems that *B. cinerea* Frp1 functions differently in carbon source utilization in *F. oxysporum* than Frp1 from the *Fusarium* species. This difference could be attributed in part to the promoter sequence. Usage of the native *B. cinerea* promoter led to lower transcript levels and reduced growth on simple sugars when compared with the usage of the *F. oxysporum* promoter. How these lower transcript levels influence the activity and levels of the protein is unknown. As reported earlier, the promoter sequence of *FoFRP1* contains an upstream ORF that starts 163 bp upstream of ATG and is 33 bp long (Duyvesteijn *et al.*, 2005). We found evidence that, in *F. oxysporum*, the upstream ORF suppresses the expression levels of *FoFRP1* (W. Jonkers and M. Rep, unpublished data). In the upstream region of the *BcFRP1* gene, a possible upstream ORF is also present (W. Jonkers, personal observations), which starts 175 bp upstream of ATG and is 96 bp long. Whether this upstream ORF also affects the expression of *BcFRP1* in these different complemented strains is unknown.

Considering the above, it seems that the molecular function of Frp1 is conserved in the different fungi, but is used by each of the fungi in a different manner. Whether an interplay between Frp1 and Cre1, as seen in *F. oxysporum* (Jonkers and Rep, 2009b), is also present in *F. graminearum* and *B. cinerea* remains to be elucidated, and, if there is an interplay, whether it contributes to the phenotypes observed. Another conserved protein that plays a role in CCR and pathogenicity, but is used differently in different fungi, is the protein kinase Snf1. In yeast, this kinase is known to phosphorylate the Cre1 counterpart Mig1 (Treitel *et al.*, 1998). A role of Snf1 in regulating Cre1 in filamentous fungi has still not been established (Cziferszky *et al.*, 2003). Snf1 is required for pathogenicity in different plant-pathogenic fungi but deletion of the *SNF1* gene results in different phenotypes in each fungus (Lee *et al.*, 2009a; Ospina-Giraldo *et al.*, 2003; Tonukari *et al.*, 2000; Yi *et al.*, 2008). Both the *F. oxysporum* and *F. graminearum* Δ *snf1* mutants show reduced expression of CWDE genes, but they differ in their growth phenotypes on sugars. Both mutants show reduced growth on galactose, trehalose and xylan, but only the *F. oxysporum* Δ *snf1* mutant shows

reduced growth on arabinose, fructose and xylose. This suggests that, in these two fungi, and similar to Frp1, Snf1 is required in common as well as different metabolic pathways. The *B. cinerea* *SNF1* deletion mutant shows a more severe phenotype; it grows poorly on all carbon sources, including glucose. In fact, the growth phenotype of this mutant on any carbon source is comparable with growth on water agar: it only produces very thin aerial mycelium. This indicates that *SNF1* in *B. cinerea* is required for basal growth and/or carbon source sensing, and not specifically for growth on alternative carbon sources or the expression of CWDE genes (J. Schumacher and B. Tudzynski, University of Münster, Münster, Germany, personal communication). It appears, therefore, that CCR is regulated differently in *B. cinerea* compared with other fungi, although the proteins involved are similar and, in the case of Frp1, have been proven to be functional homologues of each other.

This work also attributes a novel function to Frp1; it is required for sexual reproduction in *F. graminearum* and *B. cinerea*. How Frp1 regulates the sexual cycle in *F. graminearum* is not known. Using gene expression profiling, *FgFRP1* was found to be down-regulated in a *mat1-2* mutant in *F. graminearum*, suggesting that *FgFRP1* itself might be controlled by mating-type locus genes (Lee *et al.*, 2006). Alternatively, the function of FgFrp1 in carbon assimilation may explain its requirement in the sexual cycle. Such a relationship has been shown for the $\Delta snf1$ and glyoxylate and methylcitrate cycle mutants $\Delta icl1$ and $\Delta mcl1$ in *F. graminearum* (Lee *et al.*, 2009a, b). The genes deleted in the last two mutants are required for growth on C2 carbon sources (*ICL1*) or propionate (*MCL1*). The double-deletion mutant ($\Delta icl1/\Delta mcl1$) shows impaired sexual reproduction.

The *B. cinerea* *BcFRP1* deletion mutant displayed a specific impairment in female fertility, but not in male fertility. To our knowledge, this is the first description of a *B. cinerea* mutant that is capable of producing sclerotia, but fails to develop apothecia. Some previously described mutations in *B. cinerea* also result in female sterility, but, in these cases, the mutants are entirely defective in the production of sclerotia (Rui and Hahn, 2007; Segmüller *et al.*, 2008) and are therefore, by definition, female sterile. It is unclear whether the mating defect in the *B. cinerea* *BcFRP1* deletion mutant occurs during fertilization of the sclerotia by microconidia of the mating partner, or during plasmogamy. The mechanism underlying the defect in sexual reproduction in the *B. cinerea* *BcFRP1* deletion mutant also remains to be resolved. It is possible that Frp1 is required to target specific proteins to an SCF complex during sexual reproduction. The identification of Frp1 target proteins in *F. graminearum* and *B. cinerea* might clarify its role during sexual reproduction.

In conclusion, Frp1, a conserved F-box protein present in some classes of filamentous fungi, plays a role in carbon assimilation and sexual reproduction in different fungal species. The protein itself can be exchanged between the different fungi investi-

gated; however, it is used in each in a specific manner, resulting in different carbon source assimilation phenotypes. This divergence of function may be correlated with differences in fungal lifestyle and infection strategies.

EXPERIMENTAL PROCEDURES

Biomaterials and plate assays

Fusarium oxysporum f.sp. *lycopersici* race 2 isolate 007 (Mes *et al.*, 1999), the *F. oxysporum* $\Delta Fofrp1$ strain (Duyvesteijn *et al.*, 2005), *Fusarium graminearum* isolate Z03643 and *F. graminearum* $\Delta Fgfrp1$ strains (Han *et al.*, 2007) and *Botrytis cinerea* strain B05.10 (Quidde *et al.*, 1999) were used in this study. Spores from *F. oxysporum* strains were harvested after 5 days of growth in NO₃ medium [5 mM KNO₃, 0.17% yeast nitrogen base (YNB) without amino acids or ammonia (Difco, Le Pont de Claix, France) and 3% sucrose] after 5 days of shaking on a rotary shaker at 150 rpm and 25 °C. Spores from *F. graminearum* strains were harvested after 7 days of growth in CMC medium (15 g/L carboxymethylcellulose, 1 g/L yeast extract, 0.5 g/L MgSO₄·7H₂O, 1 g/L NH₄NO₃, 1 g/L KH₂PO₄ and, for the *F. graminearum* $\Delta Fgfrp1$ strains, 1% w/v glucose) on a rotary shaker at 150 rpm and 25 °C. *Botrytis cinerea* spores were harvested from 10- to 14-day-old PDAB (potato dextrose agar supplemented with 10% w/v hackled bean leaves) plates and resuspended in H₂O for the BiologFF assay or Gamborg's B5 medium (Duchefa, Haarlem, The Netherlands) supplemented with 10 mM glucose and 10 mM KH₂PO₄/K₂HPO₄, pH 6.4, for the plant infection assays.

Fusarium oxysporum plate assays were performed on agar plates containing NO₃ medium, 1.5% bacto agar and 1% w/v variable carbon source instead of sucrose. *Fusarium graminearum* plate assays were performed on DFM (Yoder and Christianson, 1998) agar plates containing 1% w/v variable carbon source instead of glucose. Plates were inoculated with 2×10^4 spores on the centre of the plate, followed by 1 week of incubation at 25 °C.

Self-fertilization tests in *F. graminearum* were performed by growing mycelia of strains on carrot agar for 5–7 days. Strains were mock fertilized as described previously (Leslie and Sumnerell, 2006). For the *B. cinerea* outcrossing tests, the heterothallic strain carrying the *mat1-2* deletion (Lee *et al.*, 2003) was used as female and $\Delta Bcfrp1$ was used as male, so that all ascospore progeny resulted from heterozygous crosses. Female mycelia on plates were fertilized with a suspension of conidia from the male strains grown on CMC medium. Ascospores and asci in perithecia were observed after incubation for 10–14 days following fertilization.

The *B. cinerea* plate assays were performed on Gamborg B5 agar plates containing 1% w/v variable carbon source. Plates

were inoculated with mycelial plugs. Sclerotia formation was analysed by inoculating CM agar plates (Pontecorvo *et al.*, 1953) with a mycelial plug, followed by incubation in the dark for 2 weeks at room temperature. Sexual crosses of *B. cinerea* were performed as described previously (Farettra *et al.*, 1988; Van der Vlugt-Bergmans *et al.*, 1993).

For the BiologFF assay (Biolog FF plates™), each well was filled with 10^4 spores in 150 µL. Time point zero was taken after 2 h on a spectrophotometer at 600 nm, when the lyophilized carbon sources were dissolved. Further measurements were made in duplicate at time points 24, 48, 72 and 96 h after inoculation.

Plant infections

The tomato cultivar C32, which is generally susceptible to all *F. oxysporum* f.sp. *lycopersici* races, was used for inoculations. Ten-day-old seedlings were uprooted, dipped into a 10^7 spore/mL suspension and potted in soil. The biomass and disease index were quantified as described by Mes *et al.* (1999) 3 weeks after inoculation.

Point inoculation of wheat cultivar Bobwhite was performed as described previously (Seong *et al.*, 2005). Barley seeds of cultivar 'Tippel' (Boerengoed, Wilsum, the Netherlands) were inoculated by incubating the sterile seeds for 1 h in a shaking macroconidia spore suspension, and subsequently potted in vermiculite (Lysøe *et al.*, 2006). After 2 weeks of growth, the roots were assessed for brown lesions.

Intact leaves of French bean (*Phaseolus vulgaris*), tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*) and fruits of apple (*Malus domestica* 'granny smith'), red bell pepper (*Cap-sicum annuum*) and tomato were inoculated with droplets (7–10 µL) of a *B. cinerea* conidial suspension (2×10^5 spores/mL). For a reproducible infection, the fruits were wounded with a needle and the droplets were placed on the wound. The infected leaves and fruits were incubated in a plastic propagator box at 20 °C under natural illumination.

Deletion and complementation constructs

For the targeted gene deletion of *FgFRP1* in *F. graminearum*, a construct carrying a geneticin resistance gene cassette, flanked by the 5' and 3' flanking regions of *FgFRP1*, was amplified by a double-joint PCR method (Yu *et al.*, 2004) with two sets of PCR primers, 1 + 2 and 3 + 4 (Table 3), respectively. The primers 2 and 3 (Table 3) carry sequence tails that overlap with the 5' and 3' ends of the gene, respectively. The geneticin resistance gene was controlled by the *Aspergillus nidulans* trpC promoter and terminator, and the gene fragment was amplified from the vector pII99 (Namiki *et al.*, 2001) with the primers 5 and 6 (Table 3). Three amplicons (5' flanking, 3' flanking and gene) were mixed and used as a template for the second round of PCR, which was followed by the final round of PCR using the new nested primer pair 7 and 8 (Table 3). The final product was transformed into the wild-type strain GZ03643 to generate the *FgFRP1* deletion mutant $\Delta Fgfrp1$.

Primer no.	Sequence (5'–3')
1	GAAAGCTGCGGAAGAAACGGGTAA
2	CCTCAATATCATCTTCTGTCGATTGGTGTGACAAGACAAG
3	GCACAGGTACACTTGTGTTAGAGAACGAGAACCTTGCTTCGCTT
4	GGAACATGCGCCCTTGCTTACTC
5	CGACAGAAGATGATATTGAAGG
6	CTCTAAACAAGTGACCTGTG
7	GCATCATTCCTTGCATCA
8	TTGCGGTGGTGTGTATG
9	ATAGTTTAGCGGCCGCGATTGAGTCCGGCTGTATCG
10	GCTCTAGATTATTTACCTGCGGGCTACG
11	CCCAAGCTTAGAGAGGCAGGGAAGGTTT
12	CCGCTCGAGGAAGAGACGGGAGCGAGAC
13	ATAAGAATGCGCCGCGAAGAGACGGGAGCGAGAC
14	CCCAAGCTTGATTGAGTCCGGCTGTATCG
15	AAAGGGGCCCAACGTCAC
16	TTGGATCCTCGCTTTGATGCTAGAAT
17	AAAAGTGCAGCATGGAACCACTTCATCTTACT
18	AAAAAGCTTACCCCCAATCAATATAAAC
19	AAATCTAGATGTCCTCCACAGCTGC
20	AAAAGTGCAGGGCGTGAAGAGAAAAAC
21	GTCATCGAGAGTTACGGTTC
22	ACTAGTCTGGACATAGATAGAA
23	TCAGTGGCTCATTGGGTGCTCTAA
24	TGTGTCTGGGTGTCTCGACAAT
25	AAGCCTTACACCATCCGCTACTCT
26	ACCAGCCTTGTCTGGTATCTTGAA

Table 3 Primers used in this study.

To generate the *B. cinerea* *BcFRP1* disruption construct, pBcFRP1KO, PCR was performed with primer combinations 9 and 10 (Table 3), in order to amplify a 929-bp *NotI*–*XbaI* fragment corresponding to the upstream region, and primers 11 and 12 (Table 3), in order to amplify a 939-bp *HindIII*–*XhoI* fragment corresponding to the downstream region, using genomic DNA as a template. The fragments were sequentially cloned into pNR1 (Malonek *et al.*, 2004), resulting in pBcFRP1KO. The *BcFRP1* complementation construct, pBcFRP1com, was generated by cloning a 3914-bp *HindIII*–*NotI* PCR fragment containing the *BcFRP1* ORF, including 929-bp upstream and 939-bp downstream sequences obtained with primers 13 and 14 (Table 3), into pOliHP (Rolke *et al.*, 2004), resulting in pBcFRP1com.

The pRD803 plasmid containing *HPH* and *BLE* resistance cassettes (Duyvesteijn *et al.*, 2005) was used to create the plasmid containing the *F. graminearum* *FgFRP1* gene pRD803FgFRP1AB. *Fusarium graminearum* *FgFRP1*, including a 1347-bp upstream and 516-bp downstream region, was amplified using primers 15 and 16 (Table 3) containing *Apal* and *Bam*HI sites in their linker, respectively. The amplified *F. graminearum* *FgFRP1* locus was cloned into the pRD803 plasmid previously linearized by *Apal* and *Bam*HI.

The pRD803 plasmid was also used to create the plasmid pRD803BcFRP1PH containing the *B. cinerea* *BcFRP1* gene, including 759-bp upstream and 939-bp downstream sequences. A *Bgl*II/*Hind*III *BcFRP1* fragment isolated from pBcFRP1com was cloned into a *Bam*HI and *Hind*III linearized pRD803 plasmid. To obtain the final plasmid pRW1pBcFRP1PH, *BcFRP1* was digested from pRD803BcFRP1PH using *Mlu*I and *Hind*III restriction sites, and cloned into plasmid pRW1p containing a *BLE* resistance cassette (Houterman *et al.*, 2009), which had been linearized previously by *Mlu*I and *Hind*III.

To create the vector containing the *BcFRP1* ORF preceded by the *FoFRP1* promoter sequence, a three-point ligation strategy was used. The ORF of *BcFRP1* was amplified with primers 17 and 18 (Table 3) containing *Pst*I and *Hind*III linkers, respectively. The promoter sequence was amplified with primers 19 and 20 (Table 3) containing *Xba*I and *Pst*I linkers, respectively. The two PCR fragments were cloned into vector pRW1p, which was linearized using restriction sites *Xba*I and *Hind*III, thereby creating pRW1ppFoBcFRP1XH.

Fungal transformations

Agrobacterium-mediated transformation was performed as described previously (Takken *et al.*, 2004). Putative transformants were transferred to Czapek Dox agar (CDA, Oxoid, Basingstoke, Hampshire, England) plates equilibrated with 100 mM Tris-HCl, pH 8.0, containing 100 µg/mL zeocin, 100 µg/mL hygromycin and 200 µM cefotaxim (Duchefa). After 4 days, when the colonies appeared, spores from putative transformants were suspended in

10 µL of sterile water and spread onto potato dextrose agar (PDA, Difco) plates containing 100 µg/mL zeocin, 100 µg/mL hygromycin and 200 µM cefotaxim. Single-spore colonies were punched out from the plates by ringcaps (Hirschmann, Eberstadt, Germany) and placed on fresh PDA plates. Genomic DNA was obtained from mycelium by extraction with 200 µL of glass beads, 300 µL of phenol–chloroform and 400 µL of TE buffer, pH 8.0, per 1 cm² of mycelium. The presence of the different complemented *FRP1* genes in the transformants was verified by PCR, and at least two correct individual transformants were used.

To perform protoplast transformation of *F. graminearum*, inoculation of mycelial blocks of the wild-type strain GZ03643 was started in CMC liquid medium (Cappellini and Peterson, 1965) and grown at 25 °C for 3 days. Fungal conidia (1×10^6) produced in CMC were inoculated into 50 mL of YPG (3 g yeast extract, 10 g peptone and 20 g glucose per litre) liquid medium and grown at 25 °C for 12 h. Mycelia harvested by filtration were incubated in 80 mL of 1 M NH₄Cl containing Driselase (10 mg/mL; InterSpex Products, Inc., Foster City, CA, USA) to generate protoplasts. Approximately 5 µg of fusion PCR product obtained by double-joint PCR was added directly, together with 1.2 mL of 60% (w/v) polyethyleneglycol (PEG) ($M_w = 3350$), to protoplast suspensions. Fungal transformants carrying the geneticin resistance cassette were selected on regeneration medium (1 g yeast extract, 1 g casein enzymatic hydrolysate, 342 g sucrose and 15 g agar per litre) containing geneticin (75 µg/mL; Sigma-Aldrich, Zwijndrecht, The Netherlands). Homologous recombination at the *FgFRP1* locus was analysed by Southern analysis. For this, genomic DNA was digested with *Eco*RI and hybridized with an [α -³²P]dCTP-labelled probe corresponding to the downstream region of *FgFRP1*, as described by standard procedures (Sambrook and Russell, 2001).

Protoplast transformation of *B. cinerea* was performed as described previously (Schulze Gronover *et al.*, 2001). Fungal genomic DNA of the transformants was isolated as described previously (Cenis, 1992). Homologous recombination at the *BcFRP1* locus was analysed by PCR and Southern analysis. For the latter, genomic DNA was digested with *Pst*I and hybridized with a [α -³²P]dCTP-labelled probe corresponding to the upstream region of *BcFRP1*, as described previously (Segmuller *et al.*, 2007).

Northern blotting

Wild-type and a *F. graminearum* $\Delta Fgfrp1$ mutant were grown in 100 mL NO₃ medium containing 1% glucose for 5 days with shaking on a rotary shaker at 150 rpm and 25 °C; mycelium was harvested over two layers of miracloth (Calbiochem, Darmstadt, Germany), washed with sterile water and transferred to 100 mL NO₃ medium containing 1% xylan as a carbon source. After 2 days, the mycelium and spores were harvested, followed by RNA extraction and Northern blotting as described previously

(Jonkers *et al.*, 2009). Probes for *XYL4* were obtained by PCR using primers 21 and 22 (Table 3).

Quantitative RT-PCR analysis

RNA was isolated from two individual complemented strains containing the *BcFRP1* gene driven by its native promoter sequence, and from three individual strains containing the *BcFRP1* gene driven by the *FoFRP1* promoter sequence, growing on complete medium for 2 days. The subsequent cDNA obtained by RT-PCR was used as template for quantitative PCR (qPCR), which was performed in three replicates with a DyNamo™ SYBR® Green qPCR (Finnzymes, Woburn, MA, USA) using a DNA-Engine Peltier thermal cycler (Bio-Rad, Hercules, CA, USA) equipped with a Chromo4™ real-time PCR detector and MJ Opticon Monitor™ analysis software (Bio-Rad, Hercules, CA, USA). To quantify mRNA levels of *BcFRP1* and the constitutively expressed *FEM1* gene, primers 23 and 24, and 25 and 26, respectively, were used (Table 3).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Alignment of Frp1 protein domains used for phylogenetic tree construction. An alignment of the various Frp1 proteins was created using MacVector software. The alignment is based on the domain of the Frp1 proteins showing the highest homology, which includes and extends beyond the F-box domain (underlined).

Fig. S2 Analysis of transformants deleted for *FgFRP1* in *Fusarium graminearum*. A knockout construct containing a gene-tic resistance cassette was introduced into the wild-type strain.

(A) Schematic representation of the knockout strategy for *FgFRP1* drawn to scale. (B) Southern analysis was performed to verify correct homologous recombination at the *FgFRP1* locus in the *FgFRP1* disruptant. To this end, chromosomal DNA of the various mutants was digested with *EcoRI*, blotted and hybridized with the indicated probe corresponding to the *FgFRP1* terminator. The *FgFRP1* locus of the wild-type strain (W) is visible as a 5.8-kb fragment corresponding to the *FgFRP1* terminator region and to the 3' part of the *FgFRP1* open reading frame (ORF). In the ectopic mutant (1), an extra band corresponding to a fragment of 10.0 kb is visible and, in the *FgFRP1* disruption mutant (2), introduction of the gene replacement cassette by homologous recombination led to the expected replacement of the 5.8-kb fragment by a fragment of 10.0 kb.

Fig. S3 Analysis of transformants deleted for *BcFRP1* in *Botrytis cinerea*. A knockout construct containing a nourseothricin resistance cassette was introduced into the wild-type strain. (A) Schematic representation of the knockout strategy for *BcFRP1* drawn to scale. (B) Schematic representation of the complementation strategy drawn to scale. (C) Polymerase chain reaction (PCR) analysis was performed to verify homologous recombination and the absence of wild-type nuclei in the *Bcfrp1* deletion strains. (D) PCR analysis was performed to verify homologous recombination and the absence of nuclei lacking *BcFRP1* in the *BcFRP1* complementation strains. (E) Southern analysis was performed to verify correct homologous recombination at the *BcFRP1* locus in the *Bcfrp1* deletion and complementation mutants. To this end, chromosomal DNA of the various mutants was digested with *PstI*, blotted and hybridized with a probe corresponding to the *BcFRP1* upstream region. The *FRP1* locus of the wild-type strain (WT) is visible as a 7.4-kb fragment. In the two *BcFRP1* deletion mutants, introduction of the gene replacement cassette by homologous recombination led to the expected replacement of the 7.4-kb fragment by a fragment of 2.5 kb. Introduction of the *BcFRP1* complementation cassette by homologous recombination in the *Bcfrp1* deletion mutant led to the expected replacement of the 2.5-kb fragment by a fragment of 5.0 kb.

Fig. S4 Growth of the *Fusarium oxysporum*, *Fusarium graminearum* and *Botrytis cinerea* $\Delta frp1$ mutants on monosaccharides. Growth is indicated by the absorbance at 600 nm (OD_{600}) of: (A) *F. oxysporum* wild-type (WT), $\Delta Fofrp1$ and $\Delta Fofrp1$ complemented with *FRP1*; (B) *F. graminearum* and two independent $\Delta Fgfrp1$ mutants; and (C) *B. cinerea* and two independent $\Delta Bcfrp1$ mutants. (D) Schematic representation of the observed growth phenotypes of the various wild-type and corresponding mutant strains. Empty cell, similar growth of wild-type and $\Delta frp1$ mutants; –, $\Delta frp1$ mutant growth is reduced when compared with the wild-type; +, $\Delta frp1$ mutant growth is enhanced when compared with the wild-type.

Fig. S5 Growth of the *Fusarium oxysporum*, *Fusarium graminearum* and *Botrytis cinerea* $\Delta frp1$ mutants on di-, tri- or cyclic (poly)saccharides. Growth is indicated by the absorbance at 600 nm (OD_{600}) of: (A) *F. oxysporum* wild-type (WT), $\Delta Fofrp1$ and $\Delta Fofrp1$ complemented with *FRP1*; (B) *F. graminearum* and two independent $\Delta Fgfrp1$ mutants; and (C) *B. cinerea* and two

independent $\Delta Bcfrp1$ mutants. (D) Schematic representation of the observed growth phenotypes of the various wild-type and corresponding mutant strains. Empty cell, similar growth of wild-type and $\Delta frp1$ mutants; –, $\Delta frp1$ mutant growth is reduced when compared with the wild-type; +, $\Delta frp1$ mutant growth is enhanced when compared with the wild-type.

Fig. S6 Growth of the *Fusarium oxysporum*, *Fusarium graminearum* and *Botrytis cinerea* $\Delta frp1$ mutants on amino acids. Growth is indicated by the absorbance at 600 nm (OD_{600}) of: (A) *F. oxysporum* wild-type (WT), $\Delta Fofrp1$ and $\Delta Fofrp1$ complemented with *FRP1*; (B) *F. graminearum* and two independent $\Delta Fgfrp1$ mutants; and (C) *B. cinerea* and two independent $\Delta Bcfrp1$ mutants. (D) Schematic representation of the observed growth phenotypes of the various wild-type and corresponding mutant strains. Empty cell, similar growth of wild-type and $\Delta frp1$ mutants; –, $\Delta frp1$ mutant growth is reduced when compared with the wild-type; +, $\Delta frp1$ mutant growth is enhanced when compared with the wild-type.

Fig. S7 Growth of the *Fusarium oxysporum*, *Fusarium graminearum* and *Botrytis cinerea* $\Delta frp1$ mutants on carboxylic acids. Growth is indicated by the absorbance at 600 nm (OD_{600}) of: (A) *F. oxysporum* wild-type (WT), $\Delta Fofrp1$ and $\Delta Fofrp1$ complemented with *FRP1*; (B) *F. graminearum* and two independent $\Delta Fgfrp1$ mutants; and (C) *B. cinerea* and two independent $\Delta Bcfrp1$ mutants. (D) Schematic representation of the observed growth phenotypes of the various wild-type and corresponding mutant strains. Empty cell, similar growth of wild-type and $\Delta frp1$ mutants; –, $\Delta frp1$ mutant growth is reduced when compared with the wild-type; +, $\Delta frp1$ mutant growth is enhanced when compared with the wild-type.

Fig. S8 Growth of the *Fusarium oxysporum*, *Fusarium graminearum* and *Botrytis cinerea* $\Delta frp1$ mutants on sugar acids and sugar alcohols. Growth is indicated by the absorbance at 600 nm (OD_{600}) of: (A) *F. oxysporum* wild-type (WT), $\Delta Fofrp1$ and $\Delta Fofrp1$ complemented with *FRP1*; (B) *F. graminearum* and two independent $\Delta Fgfrp1$ mutants; and (C) *B. cinerea* and two independent $\Delta Bcfrp1$ mutants. (D) Schematic representation of the observed growth phenotypes of the various wild-type and corresponding mutant strains. Empty cell, similar growth of wild-type and $\Delta frp1$ mutants; –, $\Delta frp1$ mutant growth is reduced when compared with the wild-type; +, $\Delta frp1$ mutant growth is enhanced when compared with the wild-type.

Fig. S9 Growth of the *Fusarium oxysporum*, *Fusarium graminearum* and *Botrytis cinerea* $\Delta frp1$ mutants on phosphate sugar, polyamine, methylglycosides, benzyl alcohol, purines, pyrimidine and glucosides. Growth is indicated by the absorbance at 600 nm (OD_{600}) of: (A) *F. oxysporum* wild-type (WT), $\Delta Fofrp1$ and $\Delta Fofrp1$ complemented with *FRP1*; (B) *F. graminearum* and two independent $\Delta Fgfrp1$ mutants; and (C) *B. cinerea* and two independent $\Delta Bcfrp1$ mutants. (D) Schematic representation of the observed growth phenotypes of the various wild-type and corresponding mutant strains. Empty cell, similar growth of wild-type and $\Delta frp1$ mutants; –, $\Delta frp1$ mutant growth is reduced when compared with the wild-type; +, $\Delta frp1$ mutant growth is enhanced when compared with the wild-type.

Fig. S10 Expression of *XYL4* is undetectable in the *Fusarium graminearum* $\Delta Fgfrp1$ mutant. Using Northern analysis, the expression of the cell wall-degrading enzyme (CWDE) gene *XYL4* was assessed in *F. graminearum* wild-type (WT) and $\Delta Fgfrp1$ mutant during noninducing (glucose) and inducing (xylan) conditions.

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