The role of the F-Box protein Frp1 in pathogenicity of fusarium oxysporum

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Frp1 is an F-box protein that functions independently from binding to Skp1

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Abstract

The F-box protein Frp1 is required for growth on non-sugar carbon sources and for pathogenicity of the fungus *Fusarium oxysporum* at the early steps of the infection process. How exactly the Frp1 protein is involved in these processes is still unknown. In general, F-box proteins recruit other proteins to an SCF complex where these proteins are ubiquitinated and thereby marked for proteasomal degradation. To be able to recruit target proteins to an SCF complex, binding of the F-box protein to the SCF component Skp1 is crucial. We show here that Frp1 is able to fulfill its role in pathogenicity even when binding to Skp1 is compromised by one or two point mutations in the F-box domain. This suggests that Frp1 does not primarily function by targeted protein turnover via an SCF complex. Besides the F-box domain, some F-box proteins harbor C-terminal protein-protein interaction domains, like LRR and WD40 motifs. The C-terminus of Frp1 does not harbor a recognizable domain and with yeast two-hybrid no interacting partners were identified excepts a probably artifactual interaction with a 14-3-3 protein from tomato. Altogether these results suggest that Frp1 does not target other proteins for degradation but affects plant infection in another, still unknown way.
Introduction

F-box proteins can assemble into an SCF-complex where they recruit other proteins for ubiquitination, commonly leading to proteasomal degradation (Cardozo & Pagano, 2004, Craig & Tyers, 1999, Bai et al., 1996, Patton et al., 1998, Zhou & Howley, 1998). F-box proteins and SCF complexes are typically eukaryotic, but F-box proteins are also found in pathogenic bacteria and viruses that secrete these proteins into their hosts where they interfere with endogenous SCF-complexes (Lechner et al., 2006, Bortolamiol et al., 2007, Pazhouhandeh et al., 2006, Sonnberg et al., 2008, Sperling et al., 2008, van Buuren et al., 2008, Angot et al., 2006, Schrammeijer et al., 2001). The F-box hypothesis states that F-box proteins recruit target proteins via their variable C-terminal domain to an SCF, in which they bind Skp1 via the N-terminal F-box domain. The interaction between an F-box protein and Skp1 is of major importance since it brings the target protein into the proximity of the ubiquitination machinery. Using crystallography, the molecular details of such an interaction were clarified with human Skp1 and the F-box protein Skp2 (Schulman et al., 2000). This revealed that the interaction involves two sites. One is conserved in all F-box proteins and forms the core interface, consisting of three α-helices of Skp1 and three of Skp2. The second site is not conserved between different F-box proteins and involves one α-helix of Skp1 and the first LRR of Skp2. Fifteen amino acids of the 40-50 amino acid long F-box domain of Skp2 contribute to the interaction and are conserved among different F-box proteins. Mutation of five amino acids among these 15 of Skp2 resulted in loss of function when mutated individually. This suggests that these amino acids contribute to the interaction between Skp1 and Skp2 and, because of their conservation, also between Skp1 and other F-box proteins.

In fungi, some F-box proteins do not require interaction with Skp1 to fulfill their function (Chapter II), although most F-box proteins from budding and fission yeast bind Skp1 and (presumably) assemble into an SCF complex (Kus et al., 2004, Lehmann A., 2004, Seol et al., 2001). However, the biological relevance of this assembly is not certain for all F-box proteins (Tafforeau et al., 2006, Sakaguchi et al., 2008). Some F-box proteins do not even assemble into an SCF complex and bind Skp1 only for functioning as a mini-complex alone or with other proteins (Russell et al., 1999, Galan et al., 2001) or even interact through their F-box, with other proteins different from Skp1 (Ribar et al., 2007) (see Chapter II for a review).

Besides binding to Skp1 via the N-terminal F-box domain, the interaction of an F-box protein to other proteins via motifs in its C-terminus is essential to recruit target proteins to the SCF complex. These domains in the C-terminus thereby determines the specificity of the F-box protein and in a substantial number of F-box proteins, this domain contains recognizable protein-protein interaction motifs. With genome sequences of many organisms becoming available, the known arsenal of F-box proteins increases and a wide range of different interaction domains has been found in C-terminal parts of F-box proteins. In budding yeast, WD40 and LRR domains are most common (Willems et al., 1999, Willems et al., 2004) and in viruses, for example, ankyrin (Mosavi et al., 2002) motifs have been found (Sonnberg et al., 2008, Sperling et al., 2008). Besides protein-protein interaction domains, also other motifs, like helicases and lectin-binding motifs (Sakaguchi et al., 2008, Lannoo et al., 2008, Yoshida, 2007, Yoshida et al., 2003) can be present. In Arabidopsis 568 (Kuroda et al., 2002) or 694 (Gagne et al., 2002) F-box proteins and in rice 687 (Jain et al., 2007) F-box proteins have been found with between 16 and 18 different domain types, respectively. One type of such a domain that is highly represented among these plant F-box proteins are kelch repeats (Adams et al., 2000, Andrade et al., 2001), which seem to be specific to plant F-box proteins. Of 33 identified mammalian F-box proteins five contain WD40 domains, twelve LRR domains, three a newly identified F-box
associated domain (FBA) and one a cyclin box (Cenciarelli et al., 1999, Winston et al., 1999).

In budding yeast, 11 out of 21 F-box proteins contain no recognizable motif in their C-terminus (Chapter II). One of these is Mdm30, which controls mitochondrial morphology and growth on non-glucose carbon sources and does recruit three identified targets to an SCF complex (Cohen et al., 2008, Muratani et al., 2005, Ota et al., 2008). Many F-box proteins from Arabidopsis and Rice also lack recognizable motifs in their C-terminal domains (Kuroda et al., 2002) and also most F-box proteins found in Fusarium species do not contain a recognizable motif in their C-terminus (Chapter III). Despite the example of Mdm30, the function of such F-box proteins may be different from recruiting proteins to an SCF. Furthermore, even for many F-box proteins that do carry a protein-protein interaction domain or other additional motif, no target or function has yet been identified.

Once an F-box protein is identified and considered to recruit proteins to an SCF, the next step is to find these interacting proteins. Various strategies have been employed to find F-box protein targets or other interacting proteins. One of these is yeast two-hybrid (YTH) screening (Drury et al., 1997, Kishi et al., 2008, Thomas et al., 1995). In such screens, large libraries of coding regions encoding prey proteins can be screened to find interacting proteins. Another method is affinity purification, making use of protein tags (e.g. with GFP (Baranes-Bacher et al., 2008)), which enables the isolation of interacting proteins using beads incubated with cell lysate. This can also be conducted on a large-scale (Ho et al., 2002). In addition, global protein stability profiling (Yen & Elledge, 2008) and proteomic screens (Benanti et al., 2007) have been performed to identify interacting proteins. The latter three methods are available for budding yeast but their application to other organisms is limited due to less developed molecular tools. Finally, mass spectrometry methods have been performed for target identification like stable isotope labeling in cell culture followed by parallel affinity purification and mass spectrometry (SILAC-PAP-MS) (Ota et al., 2008). Conversely, when a protein under study proves to have a short half-life or suddenly disappears upon a cellular switch, mutant screening to identify a gene that regulates its turnover may reveal an F-box protein (Jin et al., 2005).

This chapter reports a study on the interaction between the F-box protein Frp1 and Skp1 from Fusarium oxysporum f. sp. lycopersici (Fol) as well as screens conducted to find potential targets and/or other interacting proteins of Frp1. Previously, it was shown that Frp1 binds Skp1 in a YTH assay (Duyvesteijn et al., 2005). This interaction was further studied by affinity purification and with the use of point mutations in the F-box domain that reduce or completely prevent binding to Skp1. We demonstrate that binding to Skp1 is not necessary for Frp1 to function, suggesting that Frp1 does not primarily function by recruiting proteins to an SCF complex. We conducted YTH screens to identify potential binding partners of Frp1. In addition, tagged versions of Frp1 were produced in E. coli and used to find interacting proteins in Fol cell lysates. Despite these efforts no other interacting proteins of Frp1 were found besides Skp1 and a tomato Ftt (14-3-3) protein.

Results

The F-box domain mediates interaction between Frp1 and Skp1

In a previous study it was shown that Frp1 is able to bind Skp1 in an YTH assay. Interaction was demonstrated in two directions, with Frp1 fused to the Gal4-binding domain and Skp1 fused to the Gal4-activation domain and vice versa (Duyvesteijn et al., 2005). None of the four constructs showed auto-activation. To confirm that the interaction between Frp1 and Skp1 is a classical F-box/Skp1 interaction, a point mutation was created in the F-box domain replacing a highly conserved leucine with a serine residue. This mutant version of FRP1 was called FRP1L126S. During construction of this mutant, one clone was obtained that carried a second point mutation, resulting in a change of a glutamine residue into
leucine in the F-box domain. This version was called FRP1<sub>Q120L,L126S</sub>. In addition, the first 171 codons of the FRP1 open reading frame including the F-box domain were deleted a third mutant version, called FRP1-C (Figure 2A).

![Figure 1: The interaction between Frp1 and Skp1 required the F-box domain of Frp1.](image)

In this YTH assay, a specific interaction between full length Frp1 and Skp1 is observed (upper row) and a decreased interaction between Frp1<sub>L126S</sub>, Frp1<sub>Q120L,L126S</sub> or Frp1-C and Skp1 (three lower rows). (-WL = selection for the presence of both plasmids, -HWL = selection for weak interaction, -AWL = selection for strong interaction).

To determine whether the interaction between Frp1 and Skp1 is mediated by the F-box domain, the four different FRP1 versions were cloned into YTH vector pAS2-1 fused to the coding region of the Gal4-binding domain. SKP1 was cloned into YTH vector pACT2 fused to the coding region of the Gal4-activation domain and each combination of pACT2-SKP1 and pAS2-1-FRP1 was transformed to yeast. Interaction between Frp1 and Skp1 was implied by growth of the transformed yeast on the selection plates. Using wild type FRP1, growth was seen on –HWL plates, selecting for low stringent interactions, and to a small degree on -AWL, selecting for stringent interactions. This confirmed interaction between full length Frp1 and Skp1 (Figure 1, upper row). Less growth on –HWL and absence of growth on –AWL plates was observed with mutant FRP1<sub>L126S</sub> (Figure 1, second row). Very little growth on –HWL plates, and no growth on –AWL plates was observed with mutants FRP1<sub>Q120L,L126S</sub> while with FRP1-C (Figure 1, two lower rows) no growth on either –HWL or –AWL plates was observed. The various constructs of Frp1 did not show any auto-activation when transformed together with empty vectors, nor did Skp1. These results confirm that the interaction between Frp1 and Skp1 is a classical F-box/Skp1 interaction, i.e. mediated by the F-box domain.

To confirm these results in another way, the different FRP1 versions were cloned in frame with GST (encoding glutathione S-transferase) in the E. coli expression vector pGEX-KG (Guan & Dixon, 1991) (Figure 2A). This is a bacterial expression vector containing the GST ORF behind an inducible promoter of the lac operon. With isopropyl β-D-thiogalactopyranoside (IPTG) as inducer, high levels of GST-fusion proteins can be obtained. In the same plasmid, downstream of the GST-fusions and separated by a ribosome binding sequence, SKP1 with a C-terminal HA-epitope (hemagglutinin) was inserted (Figure 2A). This resulted in transcription of a polysistronic mRNA from which both cistrons are translated and both proteins being produced in comparable amounts. The four different GST-Frp1 versions and potential protein complexes were purified from induced E. coli cultures using glutathione beads. The GST-Frp1 protein complexes were separated on an SDS-PAGE gel, which was subsequently stained with coomassie brilliant blue (cbb) (Figure 1B, left panel). The arrows in Figure 2B indicate the full-length versions of the fusion proteins GST-Frp1, GST-FRP1<sub>L126S</sub>, GST-FRP1<sub>Q120L,L126S</sub> and GST-FRP1-C, which correspond to 90 kDa for the three Frp1 proteins containing the F-box domain and 60 kDa for the shorter version, Frp1-C. A duplicate gel was blotted and treated with anti-HA...
antibodies to visualize co-immunoprecipitated Skp1-HA (Figure 2B, right panel). Lane 1 of both the cbb-stained gel and the blot shows that Skp1-HA was co-precipitated with full-length GST-Frp1. This was also observed, albeit in a lesser extent, for GST-Frp1L126S in the second lane. No co-precipitation of Skp1-HA was observed with GST-Frp1Q120L,L126S (lane 3) or with GST-Frp1-C (lane 4). That interaction of Skp1 to Frp1 is not influenced by the HA-tag on Skp1 was demonstrated by using the same pGEX-KG vector with FRP1 and untagged SKP1 and the same vector with FRP1 or FRP1-C without SKP1. Using high amounts of protein from E. coli cultures harboring these plasmids, a specific band of about 20 kDa was present in the lane with full-length GST-Frp1 purified on glutathione beads (Figure 2C, lane 1). No such band was seen with vectors only containing FRP1 or FRP1-C and no SKP1 (Figure 2C lane 2 and 3). Together, these experiments demonstrate that the F-box domain of Frp1 mediates interaction with Skp1 and that a single point mutation (L→S) in the F-box domain impairs interaction with Skp1 while a double point mutation (Q→L,L→S) abolishes interaction.

**Figure 2**: The interaction between Frp1 and Skp1 can be demonstrated using affinity purification.

**A**) In the pGEX-KG vector, different gene fusions of FRP1 to a GST-tag were used (FRP1, FRP1L126S, FRP1Q120L,L126S and FRP1-C) together with HA-tagged SKP1.

**B**) A coomassie brilliant blue (cbb) stained gel and Western blot demonstrate the purification of GST-Frp1 complexes containing HA-tagged Skp1. The cbb gel shows the GST-Frp1 fusion proteins and Western blot analysis using anti-HA antibodies shows the presence of Skp1-HA in precipitates of GST-Frp1 (lane 1). A weaker interaction was observed between Frp1L126S and Skp1 (Lane 2), and no interaction with Frp1Q120L,L126S or Frp1-C and Skp1 (lane 3 and 4, respectively).

**C**) Interaction between Frp1 and Skp1 was also observed without HA-tagging of Skp1. A specific 20 kDa band corresponding to the size of Skp1 appears on a cbb stained gel loaded with affinity purified GST-Frp1 sample (lane 1). This band was not observed in lane 2 and 3, which contained affinity purified GST-Frp1 and GST-Frp1-C, respectively, produced from the pGEX-KG vector without a SKP1 cistron. M = marker.
Point mutations in the F-box domain of Frp1 do not cause loss of pathogenicity

To investigate whether binding to Skp1 is required for the biological function of Frp1, constructs of FRP1 for transformation of Fol were made that encode the mutated and truncated versions of Frp1 described above. The different FRP1 versions behind their own promoter were re-introduced into the Δfrp1 strain. Remarkably, both FRP1L126S and FRP1Q120L,L126S could restore the growth defects of the Δfrp1 mutant on ethanol containing plates (data not shown). Furthermore, a bioassay using the same strains revealed that FRP1L126S and FRP1Q120L,L126S could largely restore pathogenicity, in contrast to FRP1-C, which did not. This suggests that the N-terminal part including the F-box is essential for function Frp1 with regard to growth and pathogenicity, but interaction with Skp1 is not. Although the weight of the tomato plants inoculated with the different strains does not show much difference in appearance (Figure 3A), the disease index of the inoculated plants does (Figure 3B). Compared to the mock inoculated plants (water) or plants inoculated with a strains carrying FRP1-C, an higher disease index was obtained with strains carrying the point mutation versions of FRP1. This disease index is comparable to that of plants inoculated with a strain carrying full length FRP1. Taken together, these results suggest that binding of Frp1 to Skp1 is not required for F. oxysporum to cause disease in tomato plants.

Figure 3: Loss of Skp1 binding does not cause loss of pathogenicity
A) Bioassay with tomato plants inoculated with Δfrp1 strains complemented with full length FRP1 (positive control), FRP1L126S, FRP1Q120L,L126S or FRP1-C. Only the FRP1-C containing strain does not cause disease symptoms (upper right panel). One of the two strains carrying the FRP1Q120L,L126S constructs show reduced disease symptoms (lower left panel) when compared to the another strain carrying the FRP1L126S construct (upper left panel) or the full-length (lower right panel).
B) Disease index and weight scores of the tomato plants inoculated with the different FRP1 constructs show full or partial restoration of disease symptoms by FRP1 full length, FRP1L126S or FRP1Q120L,L126S but FRP1-C does not restore disease symptoms. (0 = healthy plant, 1 = thickening of hypocotyls but no vessel browning, 2 = or two brown vessels, more than four true leaves, 3 = three or more brown vessels, more than four true leaves, 4 = four or less true leaves.)
N-terminally GFP-tagged versions of Frp1 are unstable, regardless of binding to Skp1
An explanation for the restoration of carbon source utilization and pathogenicity by Frp1 versions that do not interact with Skp1 is that Frp1 does not function by targeting other proteins for ubiquitination. Binding of Frp1 to Skp1 could instead be required for ubiquitination and degradation of Frp1 itself. To investigate this possibility, a GFP tag was N-terminally linked to different versions of FRP1, FRP1Q120L,L126S and FRP1-C driven by the native FRP1 promoter. These contracts were transformed to Fol in such way that the fused genes were expressed ectopically or in locus (knock-in). As a control for expression from the FRP1 promoter, a strain that contains GFP driven by the FRP1 promoter in locus was used. This strain is used to monitor GFP expression using a wild type and a mutated leader. With wild type leader, GFP expression was observed as expected, but in lower amounts than with a mutated leader (See General discussion). However, we were unable to visualize any of the GFP-Frp1 fusion proteins microscopically or on western blot using antibodies against GFP (data not shown). That these version of FRP1 were functional and transcribed was shown by the ability of GFP-FRP1Q120L,L126S to replace native FRP1, without altering the phenotype (data not shown). We conclude that the GFP-tagged Frp1 constructs are highly unstable regardless of Skp1 binding.

No additional interactors of Frp1 were found using YTH
Since Frp1 does not strictly require Skp1 binding to function or to be (presumably) degraded, the recruitment of target proteins to an SCF complex by Frp1 seems unlikely. Nevertheless, Frp1 could still interact with other proteins and the identification of such proteins might give clues to the biochemical function of Frp1. We therefore decided to search for interactors using the YTH system. Screens were conducted with yeast transformants grown on different carbon sources. Conventional selection plates contain glucose as carbon source, but since Frp1 is required for growth on alternative carbon sources, we reasoned that the interaction of Frp1 with other proteins might depend on the carbon source even in yeast which is an ascomycete like Fol. Post-translational modifications such as phosphorylation of Frp1 itself and/or interacting partner might be required for binding and may depend on the available carbon source. Therefore selection was performed on minimal plates containing sucrose, glycerol or ethanol besides glucose. First, an interaction library made with cDNA from Fol-infected tomato plants (de la Fuente van Bentem et al., 2005) was used to screen for Frp1 interactors. The contribution of fungal cDNA clones in this library is ~5%. With this library, about 1.75 million clones were screened and one specific interactor was found: a Ftt (14-3-3) protein from tomato, isoform 9 (accession P93214). A strong and specific interaction of this YTH clone was demonstrated with the C-terminal part of Frp1, which was used as bait when the Ftt clone was isolated, and a weaker, but still specific interaction was found with full length Frp1 (Figure 4A). Nevertheless, we consider it unlikely that this interaction is biologically relevant since during the interaction of Fol and tomato, Frp1 and Ftt9 are both expected to act intracellularly in the two different organisms. Also, Ftt proteins are known to bind a very large number of proteins (Schoonheim et al., 2007, Alexander & Morris, 2006, Fuller et al., 2006, Kakiuchi et al., 2007), suggesting that interaction with Frp1 may be artifactual in the sense that it is a result from the general ability of Ftt proteins to interact with many different proteins. Nevertheless, it could be that the interaction of Frp1 to a Ftt protein is conserved across kingdoms and that Frp1 is able to bind a Ftt protein from Fol itself, even though such proteins were not found in the YTH screen. To test this for this possibility, the two FTT genes from Fol, FTT1 (FOXG_01979) and FTT2 (FOXG_00146), were cloned and constructs for YTH were made. Although a strong interaction was observed between Frp1-C and Ftt isoform 1, suggesting a specific interaction, the YTH unfortunately turned out not to be reliable, mostly due to growth problems of some of the control strains (Figure 4B). This possibility, therefore, remains to be resolved.
Figure 4: YTH interactions between Frp1 or Frp1-C and Ftt proteins.
A) YTH interactions with the different Frp1 and tomato Ftt9 constructs as bait in the pAS2-1 vector (b) and as prey in the pACT2 vector (p). The strongest interaction is seen with Frp1-C as bait and Ftt9 as prey and the weakest interaction with Ftt9 as bait and Frp1 as prey. Ftt9 together with empty vectors show the background growth.
B) YTH interaction is observed with Fol Ftt1 as bait (b) and Frp1 as prey (p), but not in the opposite direction. A much stronger interaction is seen with Ftt1 as bait and Frp1-C as prey, which is also only present in this direction. The controls of Ftt1 with empty vectors, on the other hand, showed lack of growth. Interaction between Ftt2 and Frp1 or Frp1-C is also apparent in the same direction as with Frp1 and Ftt1, but the controls of Ftt2 with empty pACT2 show auto-activation. Frp1 and Frp1-C do not show auto-activation when transformed together with empty vectors (data not show). (-WL = selection for the presence of both plasmids, -HWL = selection for weak interaction, -AWL = selection for strong interaction)

Fol sequences make up only about 5% of the YTH library derived from infected tomato plants (de la Fuente van Bentem et al., 2005). To screen for proteins of Fol that may not be represented in this library, a new cDNA library was made using RNA isolated from Fol grown on different carbon and nitrogen sources. A total number of 6 million clones were screened using different baits. With full length Frp1, 27 Skp1 clones were isolated confirming the effectiveness of this procedure and library. However, no other proteins were found to specifically interact with Frp1, neither with glucose containing selection plates nor with an alternative carbon sources. Also with Frp1-C or Frp1<sup>L126S</sup>, used to screen specifically for proteins excluding Skp1, no specific interaction was detected. This suggests that besides Skp1 no other protein stably interacts with Frp1.
Discussion

In this study we provide evidence that the F-box protein Frp1 appears not to comply with the F-box hypothesis. Interaction of Frp1 with Skp1 is mediated by the F-box domain but this interaction is not required for Frp1 function regarding growth on ethanol or pathogenicity towards tomato. Since Frp1 functions largely independently from binding to Skp1, in all likelihood no ubiquitination of an Frp1-target protein is required for its function in plant infection. Although disease was clearly observed with the strains carrying point mutations in \textit{FRP1}, which is not seen for the \textit{FRP1-C} strain full pathogenicity was not observed. It might therefore be that Skp1 binding is to some extent still required for Frp1 to fully function.

Taken the above observations into account, it is likely that Frp1 does not act as a classical F-box protein, and may instead act, for example, as a transcription factor or as a component of a transcriptional complex. There are other examples of F-box proteins containing a DNA-binding domain, and in Arabidopsis an F-box protein acts as a transcriptional co-factor (Chae et al., 2008). However, Frp1 does not contain a recognizable DNA-binding motif and no interaction with other DNA binding proteins was demonstrated. This leaves the molecular function of Frp1 elusive.

Since Frp1 can largely function without binding to Skp1, why then, would Frp1 (and its orthologs) have retained a functional F-box and bind Skp1 at all? Possibly, the interaction of Frp1 with Skp1 may be solely required for auto-ubiquitination of Frp1, thus fine-tuning the activity or protein level of Frp1. Many F-box proteins are regulated by ubiquitination even when no target is bound (Galan & Peter, 1999). When Frp1 protein levels would be regulated by auto-ubiquitination via Skp1, higher levels of the Frp1 versions bearing the point mutations or lacking the F-box domain would have been expected but this is not the case. We demonstrated that the apparently very low levels of GFP-Frp1 fusions in \textit{Fol} was not due to very low levels of expression from the \textit{FRP1} promoter because GFP driven by this promoter was readily visible. Still, we cannot exclude that the chimeric proteins consisting of GFP and Frp1 are extremely unstable and Frp1 is regulated by auto-ubiquitination. Another explanation, is that Frp1 is subjected to proteolysis via protein(s) different from SCF\textsubscript{Frp1}, for instance by the action of another F-box protein as was shown for Ctf13 (Kaplan et al., 1997). Ctf13 is degraded via Cdc4, and the interaction of Ctf13 to Skp1 is not required for protein turnover, but rather for correct kinetochore assembly.

Instability of Frp1 was also apparent when GST-tagged Frp1 versions made in \textit{E. coli} were used to search for interacting proteins in \textit{Fol} lysates. Despite several attempts to inhibit protease activity in the lysates, it turned out to be impossible to prevent rapid degradation of the Frp1 bait construct (data not shown). In addition, TAP-tagged (Puig et al., 2001) versions of Frp1, for which constructs were transformed into the \textit{\Delta frp1} mutant for the isolation of Frp1 interacting proteins, could not be detected on Western blots despite multiple attempts (data not shown). Taken together, Frp1, or at least the various protein fusion derivatives (with GFP, GST ad TAP) appears to be highly prone to proteolysis, preventing identification of Frp1 protein complexes.

To find proteins that bind Frp1 using another approach, YTH screens were performed. Using such screens, proteins that interact with F-box proteins were successfully found in other studies (Tokarz et al., 2004, Kaiser et al., 2000). YTH also has its disadvantages. First, the YTH assay performed in the present study uses Gal4 activation and binding domain fusion proteins. However, in \textit{F. oxysporum}, an ascomycetous fungus like yeast, Frp1 is involved in growth on alternative carbon sources and since Gal4 is a transcription factor also required for growth on an alternative carbon source (galactose), this system might not be the most suitable for YTH screenings involving Frp1. Second, interaction of Frp1 with a potential target might depend on phosphorylation of the target, and it is not known whether yeast kinases are able to phosphorylate these targets under YTH.
conditions. Finally, interaction of some F-box proteins to their target might not be stable enough to detect with YTH. Despite these drawbacks of the Gal4 YTH system two cDNA libraries were screened which resulted -besides another confirmation of interaction with Skp1- in a probably artifactual interaction with a Ftt protein from tomato. Since it is possible that a protein interacting with Frp1 is not produced under the conditions used for isolation of RNA for the cDNA libraries, a genomic YTH library was constructed consisting of genomic DNA sequences of 500-3500 bp cloned into three YTH vectors. These vectors differ only in the reading frame at the insertion site, allowing ORFs present in inserts to be in frame behind the Gal4-activation domain in at least one of the three vectors (see supplementary data). Also with this library, from which 36 million clones were screened, no specific interactor was found. Using this library, at least Skp1 should have been found, but this was not the case, possibly due to impaired splicing of \textit{F. oxysporum} introns by yeast. Altogether, the YTH screens resulted only in the observation that Frp1 can bind a tomato isoform of Ftt and perhaps to endogenous Ftt1 (the latter experiment was not conclusive). To confirm whether Ftt1 is a true interactor, an affinity purification experiment with tagged Frp1 and Ftt1 should be carried out to clarify this interaction. That Ftt1 was not found in one of the YTH screens can be explained by the fact that Frp1 and Ftt1 only interact in YTH when \textit{FTT1} is in the bait vector and \textit{FRP1} in the prey vector, and not the other way around. Among eukaryotes Ftt proteins are involved in many processes, like DNA damage control and replication (Grandin & Charbonneau, 2008, Zannis-Hadjopoulos et al., 2008) membrane transport (Mrowiec & Schwappach, 2006), cell cycle regulation (Hermeking & Benzinger, 2006) and many others (Dougherty & Morrison, 2004, Darling et al., 2005, Paul & van Heusden, 2005). Also in budding yeast many processes and interactions involve Bmh1 and 2, the two Ftt proteins in this fungus (Bruckmann et al., 2007, Bruckmann et al., van Hemert et al., van Hemert et al.). Interestingly, interaction between an F-box protein and a Ftt protein has also been found with Grr1 (Tomas-Cobos et al., 2005), a F-box protein from yeast involved, among other things, in glucose sensing and mitochondrial retrograde signaling. Binding of Ftt to Grr1 might be necessary for the induction of \textit{HXT} genes by assisting Grr1 in binding to its targets. This hypothesis was supported by a proteomic screen, in which interaction of Ftt to Grr1 targets Gal4, Gic2, Gis4 and Hof1 was found (Kakiuchi et al., 2007). In the same study, interaction with Mig1 and Snf1 was demonstrated, suggesting a role of Ftt in glucose repression in yeast. Additionally, Msk1, normally degraded via Grr1, is rescued when bound to Ftt or to Rtg2 (Liu et al., 2005). The biological relevance, if any, of an interaction between Frp1 to a Ftt protein is not known. Like in other systems, a Ftt protein might function as a bridge between Frp1 and other proteins. Since Ftt proteins by themselves can bind many different proteins, screens using the Frp1/Ftt mini complex could be performed to find potential specific interactors and confirm the relevance of the Ftt1 interaction. In conclusion from this study it appears that the most important function of Frp1 does not appear to involve ubiquitination of targets. This means that Frp1 is likely an F-box protein that does not comply with the F-box hypothesis, leaving its molecular function to be elucidated.

**Methods**

\textit{Yeast two-hybrid and affinity purification bait constructions}

The full length \textit{FRP1} constructs in pAS2-1 and pACT2 (Clontech) were used from Duyvesteijn et al. 2005 (Duyvesteijn et al., 2005). The point mutation constructs were obtained via PCR on the original full length construct in the pAS2-1 vector using primers 1 and 2 (Table 1) and the obtained amplification product was cloned into the vector using the MCS primers 3 and 4 (Table 1) and the restriction sites Ncol and EcoRI. The \textit{FRP1-C} part was amplified using primer 5 and 6 (Table 1) containing the Ncol and EcoRI restriction sites in the linkers and ligated in to the pAS2-1 vector using the same restriction sites. Constructs could were constructed from this vector into pACT2 using Ncol and EcoRI restriction sites and into the pGEX-KG vector (GEllifesiences) containing the GST tag sequence using the Ncol and Sall restriction sites. In the same
pGEX vector, SKP1, containing a ribosome binding sequence in front of the ATG and with or without C-terminal HA-tag was introduced into the HindIII restriction site using primers 7, 8 and 9 (Table 1).

Yeast two-hybrid assay
Yeast transformation was performed using Lithium-Acetate and polyethylene glycol 3350 according to Gietz et al, 2002 (Gietz & Woods, 2002). Transformants were selected on the minimal medium agar plates (2% glucose, sucrose, glycerol or ethanol, 0.5% (NH4)2SO4, 0.17% YNB, 0.002% histidine, 0.01% leucine, 0.002% tryptophane, 0.002% adenine sulphate, 0.0033% lysine, 0.004% methionine and 0.002% uracil). To select for the bait vector, tryptophane was left out of the medium, and to select for the prey vector leucine was left out. To select for less stringent interactions, histidine was left out and to select for stringent interactions, adenine-sulphate was left out.

GST fusion protein production and purification
The vector pGEX-KG containing the GST-FRP1 and SKP1-HA constructs was used to produce the encoded proteins in BL21 E. coli cells. GST-Frp1 proteins were purified from the E. coli cell lysates and immobilized on glutathione sepharose 4B beads (GE healthcare) and visualized on SDS-PAGE gels using Coommassie Brilliant Blue staining. Co-purified Skp1-HA was visualized using Western blotting and an antibody against the HA-tag containing a peroxidase conjugant (High affinity anti-HA, Roche).

Library construction and usage
The cDNA library was prepared from RNA isolated from Fol4287 grown on ethanol (5 mM KNO3, 0.17% Yeast Nitrogen Base (YNB) without amino acids or ammonia (Difco) and 1% ethanol), glucose (5 mM KNO3, 0.17% YNB and 1% glucose) and NH4 medium (3% w/v sucrose, 0.66% w/v (NH4)2SO4 and 0.17% YNB) for 5 days at 25°C and 150 rpm. Fungal material was harvested over two layers of miracloth and lyophilized overnight. RNA was isolated from Fol grown on each carbon source using Trizol (Invitrogen) and equal amount of RNA was used for further treatment.

cDNA synthesis and ligation of linkers was performed using the protocol supplied by Clontech, Strategene cat. #200401. Small fragments (0.5 kb -1.5 kb) and large fragments (> 1.5 kb) were ligated separately into to the pACT2 vector (Invitrogen) using the EcoRI and NcoI restriction sites. Using electroporation 1 μl ligase mix was transformed to E. coli DH10B cells. The number of primary transformants was determined by plating and the rest of the transformation mix was added to 3 liters of T broth medium + ampiciline. The culture incubated overnight at 37°C and plasmid DNA was isolated from the cells using a Maxiprep kit (Quigen). Presence of inserts in the library was checked using colony PCR from multiple colonies grown on plates used for titer determination using MCS primers on the pACT2 vector, (number 9 and 10 in Table 1). The tomato-Fol interaction cDNA library was made previously from RNA isolated from Fol007-infected tomato packed in lambda phages (de la Fuente van Bentem et al., 2005). The lambda phages were converted to the pACT2 backbone using the “in vivo mass excision” protocol (Elledge et al., 1991) and DNA was isolated from E. coli strain BNN132 using the Birnboim method (Birnboim, 1983). The libraries were transformed to yeast carrying a bait vector as described above.

Plasmid rescue from yeast
To obtain clones encoding potential interactors the YTH plasmid was isolated from an overnight grown minimal medium culture lacking leucine and tryptophane. After centrifugation of the culture, the plasmid DNA was isolated using an extraction buffer consisting of 200 μl isoamyl alcohol:phenol:chlorophorm (50:1:24:25), 200 μl of 2% Triton X100, 1% SDS, 100 mM NaCl, 10 mM Tris pH8.0 and 1 mM EDTA and 300 mg acid washed beads. After vortexing and centrifugation steps, the waterphase was transferred to a new tube and supplemented with 1/10 volume 3M NaAc and 2 volumes of isopropanol. Precipitated DNA was collected by centrifugation, washed with 70% alcohol and redissolved in 20 μl water. 8μl was used for transformation to E. coli strain JF1754 (Schultz & Friesen, 1983) grown on M9 selection plates to select for colonies only harbouring the pACT2 plasmid (one liter of M9 medium consists of: 20 ml 10x M9 salts (1 liter 10X M9 salts = 60g Na2HPO4, 30g KH2PO4, 5g NaCl and 10g NH4Cl), 400 μl 1M MgSO4, 4 ml 20% glucose, 20 μl 1M CaCl2, 15g agar, plus amino acids and nucleic acids, except for leucine, added after autoclaving in the same manner used as yeast minimal plates). To screen directly for SKP1 clones among the obtained pACT2 plasmids, primer 12 and 13 (Table 1) were used. To sequence a non-SKP1 clone, primer 10 and 11 (Table 1) were used.

Construction of FTT1 and FTT2 YTH vectors
The sequence of FTT1 (FOXG_01979, incomplete sequence) and FTT2 (FOXG_00146 – Ftt protein 6) from Fol were obtained from the Broad institute (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html). The full sequence of FTT1 was obtained upon sequencing (Supplementary data). Using primers 14-17 (Table 1) containing NcoI and BamHI restriction sites, the two genes were amplified from the Fol cDNA library eluted above and ligated into the pAS2-1 and pACT2 vector. Yeast two-hybrid assays were performed as described above.
Construction of GFP-FRP1 vectors

The plasmid pPK2HPHGFP.frp1 (Chapter VI) was used as starting vector to make the different constructs. In this plasmid the upstream and downstream flanks of FRP1 are present, allowing it to be used for gene replacements. First the plasmid was cut with ApaI and religated to remove GFP from the plasmid, creating pPK2HPH.frp1. The downstream sequence of FRP1 was amplified using primers 18 and 19, annealing at position 1240 pb and 2106 pb from the FRP1 start codon, respectively, using genomic DNA as template. The forward primer used to amplify the downstream sequence contained a mutation replacing the SpeI site at position 1249 (ACTAGT) with an NheI site (GCTAGC) without changing the amino acid sequence. The reverse primer contained both SpeI and SalI sites in the linker. The amplified downstream sequence was cut with NheI and SalI and ligated into the SpeI and SalI sites of the linker, creating the amplified GFP construct. Then the FRP1 and the amplified GFP constructs were inserted into the Xba-digested pPK2HPH.frp1 vector (three points ligation). This created the vectors pPK2HPH/GFP-FRP1.frp1, pPK2HPH/GFP-FRP1Q120L,L126S.frp1 and pPK2HPH/GFP-FRP1-C.frp1. For construction of the control vectors only containing GFP driven by the FRP1 promoter, see General discussion chapter.

Fungal transformations

Agrobacterium mediated transformation was performed as described (Takken et al., 2004). Putative transformants were transferred to Czapek Dox agar plates (CDA, Oxoid) containing 100 μg/ml Zeocin (Invivogen) and 200 μg/ml Cefotaxim (Duchefa). After four days, when colonies appeared, spores from putative transformants were transferred to sterile water and spread on potato dextrose agar plates (PDA, Difco) containing 100 μg/ml Zeocin. Singles spore colonies were punched out from the plates by ringcaps (Hirschmann) and placed on fresh PDA plates. Genomic DNA was obtained from mycelium by extraction with 200 μl glass beads, 300 μl phenol/chloroform (1:1) and 400 μl TE buffer pH8.0 per 1 cm² of mycelium. Correct gene replacement in the transformants was verified by PCR.

Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>Primer #</th>
<th>Primer sequence</th>
<th>Primer name</th>
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<tbody>
<tr>
<td>1</td>
<td>GTAATTCCGTGGGAGATCCTAGGAAAAAGCT</td>
<td>pm-F-boxFRP1 R</td>
</tr>
<tr>
<td>2</td>
<td>GCTTTTCCCTAGGATCTCCCAACGAAATTACA</td>
<td>pm-F-boxFRP1 F</td>
</tr>
<tr>
<td>3</td>
<td>ATCATCGGAGAGAGTAG</td>
<td>pAS2-1 mcs F</td>
</tr>
<tr>
<td>4</td>
<td>CATAGAAATTGCAGCCGG</td>
<td>pAS2-1 mcs R</td>
</tr>
<tr>
<td>5</td>
<td>AAACATGGGATCATATTCGGTTACGCTTACCTCA</td>
<td>Ncol FRP1-C F</td>
</tr>
<tr>
<td>6</td>
<td>AAAAGAATTTCTAAATCCGATCTGATCATCAC</td>
<td>EcoRI FRP1-C R</td>
</tr>
<tr>
<td>7</td>
<td>FAAAAAGCTTAGAAGAAACAGATTTACTATGTGAAATCTACCTATA</td>
<td>HindIII-RBS-SKP1 F</td>
</tr>
<tr>
<td>8</td>
<td>TTTAAATTTGCTGCTGAGTCGTCTAGCTCAGGATAACGATCTTACAGCCCAC</td>
<td>HindIII SKP1-TAA+ HA-tag R</td>
</tr>
<tr>
<td>9</td>
<td>TTTAGCTTACGATCTTACAGGGA</td>
<td>HindIII SKP1 R</td>
</tr>
<tr>
<td>10</td>
<td>TAATACCATCACAAAGGATG</td>
<td>pACT MCS F</td>
</tr>
<tr>
<td>11</td>
<td>CAACTGTCATCGTCGAC</td>
<td>pACT MCS R</td>
</tr>
<tr>
<td>12</td>
<td>AGGCCAGAGGATGATGATTTAC</td>
<td>SKP1 F</td>
</tr>
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<td>FTT2 Ncol F</td>
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<tr>
<td>19</td>
<td>AAAGGATGAGGATTTTATGGGAGGCGGCGGAGCA</td>
<td>GFP-stop+NcoI</td>
</tr>
<tr>
<td>20</td>
<td>AAAACCATGGGCCACCGAGCGTGAAA</td>
<td>GFP-stop+NcoI R</td>
</tr>
<tr>
<td>21</td>
<td>TTTCCATGGACTGGATCATCTCGGTCGATCG</td>
<td>GFP-stop+NcoI R</td>
</tr>
</tbody>
</table>

Plant infections

The tomato cultivar C32, which is susceptibility to all Fol races, was used for the inoculations. Ten-day-old seedlings with cut roots were dipped into a 10⁷ spore/ml suspension and potted in soil. Biomass and disease index was quantified three weeks after infection as described (Mes et al., 1999a). Disease index was scored by visual observation (0 = healthy plant, 1 = air roots and thickening of stem; no vessel browning, 2 = one or two brown vessels, more than four true leaves, 3 = three or more brown vessels, more than four true leaves, 4 = four or less true leaves.)
Acknowledgements
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