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

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Chapter III

The F-box protein arsenal of four *Fusarium* species

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Abstract

The availability of genome sequences of related organisms allows a comprehensive comparison of genes present in the genomes of such organisms. Such a comparison may help to develop models of gene and genome evolution, to identify candidate genes underlying common traits as well as traits unique to particular species and to find leads to control disease caused by these fungi. Through data mining in the available genome sequences of four *Fusarium* species, the arsenals of F-box proteins were obtained. To date, very little is known about F-box proteins from filamentous fungi. It appears that filamentous fungi such as the four *Fusarium* species contain much larger numbers of F-box proteins than yeast: 60-94 in *Fusarium* species compared to 21 in yeast. This suggests that F-box proteins contribute to the regulation of the more complex and developmental and metabolic processes occurring in filamentous fungi.

A set of six F-box proteins is conserved throughout the fungal kingdom, including ascomycetes and basidiomycetes, and forms the core family of fungal F-box proteins. On the other end of the spectrum, we found F-box proteins that are exclusively present in the four *Fusarium* or even in one species only, suggesting that genus- and species-specific roles for F-box proteins have emerged during evolution. Many, but to date still a minority of the F-box proteins contain additional recognizable amino acid sequence motifs N- and C-terminal of the F-box domain. Newly recognized fungal protein domain combinations include an F-box with an ankyrin repeat, a JmjC domain or a cyclic nucleotide binding domain.

The F-box protein arsenal of *F. graminearum* was also studied regarding the expression patterns of the encoding genes during growth on various media and during infection of barley, as retrieved from literature. This revealed that some genes encoding F-box proteins are exclusively expressed *in planta* on or media lacking either a carbon or nitrogen source, which may suggest a link with pathogenicity.
Introduction

With genome sequences of many fungi becoming available, it is now possible to compare the genomic blueprints of different fungal species. The *Fusarium* genus contains phylogenetically related, yet phenotypically distinct pathogenic species, four of which have been sequenced: *F. graminearum*, *F. verticillioides*, *F. oxysporum* and *F. solani*. Genome comparison revealed greatly expanded lineage-specific (LS) regions in *F. oxysporum*, including four entire chromosomes. Genes encoded in these regions are enriched for functions related to host-pathogen interactions, including known effectors, enzymes targeting plant substrates or processes, and genes involved in lipid signal transduction. In this chapter, genome comparison is focused on genes encoding F-box proteins from the four sequenced *Fusarium* species.

The F-box protein is a protein containing an F-box domain of about 40 amino acids that mediates interaction with Skp1, a subunit of SCF complexes. Proteins containing an F-box or F-box-like domain can be found in species ranging from viruses to humans. To date, only a small fraction of all genes predicted to encode F-box proteins has been studied. From these studies it has become clear that F-box proteins fulfill multiple roles, usually resulting from their involvement in ubiquitination of specific target proteins. The structure of an F-box protein is besides determined by its F-box domain, also defined by the occurrence of another recognizable domain C-terminal of the F-box domain, which is involved in recognition of target proteins. To date, two genes encoding F-box proteins in plant pathogenic *Fusarium* species have been found to play a role in virulence: *FBP1* (*GRR1*) in *F. graminearum* (Han et al., 2007) and *FRP1* in *F. oxysporum* (Duyvesteijn et al., 2005). Here, the characteristics of predicted F-box proteins of the four *Fusarium* species are summarized, the conservation of these proteins within the *Fusarium* genus and other fungi is assessed and the occurrence of genes encoding F-box proteins on LS regions is investigated.

Results

In the *Fusarium* databases, a total of 300 genes were found that encode proteins containing an F-box domain: 78 in *F. graminearum*, 68 in *F. oxysporum*, 60 in *F. verticillioides* and 94 in *F. solani*. In Table 1, a total number of 77 different orthologs are listed. They are categorized according to the type of C-terminal motifs, their conservation across different fungal species and occurrence in the four *Fusarium* species.

Many fungal F-box proteins may harbor recognizable motifs in the C-terminal part of the protein, which are mostly involved in protein-protein interactions and recruitment of substrates for ubiquitination. In Table 1, the predicted F-box proteins are characterized according to the presence of such domains. Besides the well-described WD40 (Neer et al., 1994) (#1-9) and LRR (Kajava, 1998) motifs (#10-15) and the SEC10 domain (Lipschutz et al., 2003) (#23) also fungal F-box proteins with novel domain combinations are found. These include F-box/ankyrin (#16-22), a F-box/JmjC (#24) and a F-box/transmembrane combination (#25). The ankyrin repeat is in several proteins involved in protein-protein interactions and is a domain consisting of repeats of about 33 amino acids forming a helix-loop-helix structure with a beta-hairpin/loop region projecting out from the helices at a 90° angle (Mosavi et al., 2002, Gorina & Pavletich, 1996). The JmjC domain has been identified in the jumonji family of transcription factors, which are predicted to be metallo-enzymes that adopt the cupin fold. The cupin fold is a flattened β-barrel structure containing two sheets of five antiparallel β strands that form the walls of a zinc binding cleft (PROSITE documentation PDOC51183) (Clissold & Ponting, 2001). Interestingly, the orthologs of yeast Cdc4 in *F. oxysporum* and *F. verticillioides* also contain an N-terminal myosin domain (Rayment et al., 1993).
Table 1: F-box proteins in four *Fusarium* species

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An identical protein is number 56797, \( ^a \) = homolog in S. cerevisiae, \( ^b \) = homolog in N. crassa, \( ^c \) = homolog in S. pombe, \( ^d \) = homolog in F. oxysporum, \( ^e \) = additional N-terminal myosin domain, \( ^f \) = additional N-terminal TPR domain, \( ^g \) = additional N-terminal cyclic nucleotide-binding domain.

Conservation of F-box proteins was examined in the following fungal species belonging to seven fungal classes (A-G) of the phyla Ascomycetes (A-E) and Basidiomycetes (F and G). Conservation is indicated by black cells.

A) Sordariomycetes: 1) Neurospora crassa, 2) Cheatomium globosum, 3) Magnaporthe grisea
B) Dothideomycetes: 4) Stagnospora nodorum
C) Eurotiomycetes: 5) Aspergillus species, 6) Coccidioides immitis
D) Saccharomycetaceae: 7) Saccharomyces cerevisiae
E) Schizosaccharomycetales: 8) Schizosaccharomyces pombe
F) Agaricomycota: 9) Cryptococcus species
G) Ustilaginomycotina: 10) Ustilago maydis
The *Fusarium* orthologs of yeast Dia2, like Dia2 itself, contain an N-terminal TPR domain (Sikorski *et al.*, 1990) and in the Pof10 orthologs in *Fusarium* ubiquitin interacting motifs (UIMs) (Hofmann & Falquet, 2001) are present, unlike in Pof10 itself. The combination of a F-box domain and a transmembrane domain is highly unusual, and may suggest that this F-box protein plays a role at a membrane. One predicted F-box protein contains a cyclic nucleotide-binding domain at the N-terminus. These domains can act as a receptor for cyclic nucleotides, like the prokaryotic catabolite gene activator, a cAMP receptor protein (Busby & Ebright, 1999). Remarkably, one predicted F-box protein (#26), contains two F-box domains. In Figure 1, the architecture of examples of F-box proteins containing the above mentioned recognizable motifs N- or/and C-terminal of the F-box is shown.

In total, 26 F-box proteins in *Fusarium* contain at least one additional recognizable domain, of which 19 are found in all four *Fusarium* species as well as other fungal species. Six out of the 21 budding yeast F-box proteins (Chapter II) encoded by *CDC4*, *MET30*, *GRR1*, *DIA2*, *RCY1* and *HRT3*, have putative orthologs in all four *Fusarium* species, and these six proteins are conserved throughout the fungal kingdom including the Basidiomycetes. These proteins, therefore, represent the core fungal F-box proteins set (protein # 1, 2, 10, 11, 23 and 27 in Table 1). In addition, two other characterized fungal F-box proteins are found in all four *Fusarium* species, encoded by *POF10* from *S. pombe* (# 9) and *FWD1* from *N. crassa* (# 3).

For six of the conserved F-box proteins mentioned above, one or more targets have been identified (Chapter II), and the possible conservation of these targets was investigated by similarity searches (Table 2). Not all targets of the conserved F-box proteins turned out to be conserved in the *Fusarium* species. Of the known targets of Cdc4, only homologs of Clb6, Cdc6 and Gcn4 are found in *Fusarium* species. No homologs were found of Sic1, Swi5, Cbl6, Far1, Tec1, Ctf13, Hac1 and Rcn1. Of the known targets of Grr1, only Cln1/2, Ime2 and Hof1 are found in *Fusarium* species, except for a homolog of Hof1 in *F. solani*, which was not found. Of the other Grr1 targets, Gic2, Mth1/Std1, Gis4 and Mks1, no homologs were found in *Fusarium*. Although Met30 is conserved in *Fusarium*, no homology to the Met30 target Met4 from yeast is found suggesting less conservation between yeast and the filamentous fungi for this protein. Instead, proteins are present in *Fusarium* with homology to Cys3, the target of SconC (Met30 homolog from *N. crassa*). Rcy1 recycles Kex2 and Snc1, and homologs are present in *Fusarium* of both these proteins. Dia2 targets Tec1, which, as mentioned above, has no *Fusarium* homologs and for Pof10 and Hrt3, no targets have been identified. Frequency (Frq), the Fwd1 target in *N. crassa*, is a transcription factor regulating the circadian clock in fungi. Homologs of Frq are found in all four *Fusarium* species. In *F. solani*, two homologs are present, and as many as ten homologs are present in *F. oxysporum*. Interestingly, six homologs in *F. oxysporum* are present on the lineage-specific chromosomes 3 and 15 (Table 2, underlined).

That the F-box protein targets described above are present in *Fusarium* species suggests that the function of the respective F-box proteins may be (partly) conserved. On the other hand, differentiation is likely given the absence of some known targets and the possibility of additional ones. Targeted gene inactivation and other studies are necessary to assess the conservation of their function in *Fusarium*. The homolog of Grr1 in *F. graminearum*, Fbp1, proved to have different functions compared to budding yeast Grr1 (Han *et al.*, 2007) and also Frp1 has probably a different function in other fungi. This was shown when the gene deletion mutant phenotypes of FRP1 in *F. graminearum* and *B. cinerea* were compared to the one in *F. oxysporum* (see general discussion, Chapter VII).

Fifteen F-box proteins without an additional recognizable domain, including Frp1, are present in all four *Fusarium* species and are also found in other fungal species (#27-42). Three additional F-box proteins without an additional domain are conserved in two or three of the four *Fusarium* species and are also found in other fungal species (#47-49).
Remarkably, the conservation of these proteins in other fungal species is not always in accordance with overall species phylogeny. From the species phylogeny, it is expected that conserved genes are first found in Sordariomycetes, to which *Fusarium* belongs. However, this is not the case for each gene; some are only present in Dothideomycetes and/or Eurotiomycetes (#21, 31, 49, 64, 66 and 69-71). When genes are present in Sordariomycetes other than *Fusarium*, some are only found in one or two out of the three species examined (#14, 15, 25, 32-34, 37, 38, 42, 48, 65 and 68). Nineteen *Fusarium* F-box proteins are not present in any other sequenced fungal species and can thus be called *Fusarium*-specific F-box proteins. Six of these contain an additional domain like ankyrin repeats (#17-20) or a second F-box domain (#26). Six (#17, 18, and 43-46) are conserved in all four *Fusarium* species, and ten (#19, 26 and 50-57) are present in two or three of *Fusarium* species. Some F-box proteins only exist in a single *Fusarium* species, which suggests a role in a species-specific lifestyle. Four of such proteins are found in *F. graminearum* (#58-61), three in *F. oxysporum* (#20, 62 and 63), none in *F. verticillioides* and seven in *F. solani* (#21, 22 and 72-77). One of the three genes specific for *F. oxysporum*, encoding an F-box/ankyrin combination (#20), is located on lineage-specific (and pathogenicity-associated) chromosome 3.

In Table 3, eight groups of homologous genes encoding F-box proteins within *Fusarium* species are listed. The occurrence of these groups suggests multiple gene duplications for some types of F-box proteins. Phylogeny of the genes for these proteins could determine whether duplication has occurred before or after species divergence.

Two groups only consist of F-box proteins from *F. graminearum* and *F. solani* and one group only of F-box proteins from *F. graminearum*, *F. oxysporum* and *F. solani*. To examine whether the identified F-box genes are transcribed, we searched for transcriptional data available for these genes. For *F. graminearum* genes, their expression patterns in planta and/or on medium lacking nitrogen (N) or carbon (C) are available. In Table 1 and 3, detection of transcripts for the respective proteins is indicated with a “y” (“n” meaning no transcripts). One *F. graminearum* gene encoding an F-box protein (#8) is only transcribed when the fungus grows in planta or when it is grown in medium lacking a C- or N-source. This gene encodes a conserved protein that contains WD40 domains and could therefore be involved in target ubiquitination. Ten other F-box genes are only transcribed when *F. graminearum* is grown in medium lacking a C- or N-source, including four (#11, 16, 25, 46) from Table 1 and
six (02128 group #1, 11252, group #2, 03362 and 08770 group #4, 11173 group #5 and 04444 group #6) from Table 3. It has been reported that some pathogenicity genes in pathogenic fungi are induced only in planta or during growth in medium lacking a N-source (Van den Ackerveken et al., 1994, van der Does et al., 2008, Stephenson et al., 2000, Talbot et al., 1993). These F-box proteins may therefore be more likely involved in pathogenicity. These include an F-box/WD40 protein (#8), the homolog of Dia2 (#11), an F-box/ankyrin protein (#16), an F-box/transmembrane protein (#23) as well as two Fusarium specific F-box proteins (#41, Table 1 and 04444 group #6, Table 3).

**Table 3:** Groups of similar F-box proteins in four *Fusarium* species.

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*F. graminearum* gene # 11252 from group 2 contains a predicted signal peptide for secretion in its predicted protein ([http://www.cbs.dtu.dk/services/SignalP-3.0/](http://www.cbs.dtu.dk/services/SignalP-3.0/)). This protein may be misannotated because the probable orthologs do not contain a signal peptide (some F-box proteins may be secreted like those found in plant-pathogenic bacteria that interfere with a host’s SCF-complex (Schrammeijer et al., 2001, Angot et al., 2006), but until now, no eukaryotic examples of secreted F-box proteins are known).

Conservation of F-box proteins was examined in the following fungal species belonging to seven fungal classes (A-C) of the phyla Ascomycetes. Conservation is indicated by black cells.

A) Sordariomycetes: 1) *Neurospora crassa*, 2) *Cheatomium globosum*, 3) *Magnaporthe grisea*
B) Dothideomycetes: 4) *Stagnospora nodorum*
C) Eurotiomycetes: 5) *Aspergillus* species, 6) *Coccidioides immitis*
The predicted proteomes of four *Fusarium* species harbor large arsenals of F-box proteins, ranging from 60- to 95 proteins. These arsenals are about five times bigger than in yeast, and most F-box proteins are conserved within filamentous fungi. Four F-box proteins from filamentous fungi have been characterized, the homologs of Grr1 and Met30 and two specific filamentous fungi ones, Fwd1 and Frp1. That is about 1-2% of the number of F-box proteins in *Fusarium*, a small number compared to budding yeast of which more than 60% of the F-box proteins has been characterized to some extent.

In the analysis presented here, a new class of protein-protein interacting domain, the ankyrin repeat, was found in eukaryotic F-box proteins. This type of F-box protein was previously only found in F-box proteins from viruses (Sonnberg *et al.*, 2008, Sperling *et al.*, 2008, van Buuren *et al.*, 2008). Other protein-protein interaction domains found C-terminally in F-box proteins are WD40 domains (nine of which five were newly identified) and LRR domains (six...
of which four were newly identified). These 22 F-box proteins from *Fusarium* containing additional protein-protein interacting domains probably comply with the F-box hypothesis (e.g. recruiting target proteins to an SCF complex for ubiquitination).

Of 30 F-box proteins in the four *Fusarium* species no orthologs were found in any other sequenced species, and these therefore appear to be *Fusarium* specific and of these 30, some are species specific. *F. verticillioides* has no species-specific genes encoding F-box proteins in contrast to *F. graminearum* (4), *F. oxysporum* (3) and *F. solani* (7). One F-box protein from *F. oxysporum* (#20) containing several ankyrin repeats was found encoded on LS chromosome 3. A gene located on such LS chromosome may be involved in host (tomato)-specific virulence of this forma specialis. Of the seven unique *F. solani* F-box protein genes, one (#75) is located on dispensable chromosome fourteen.

Clearly, the large majorities of the F-box protein genes are located on conserved (vertically inherited) chromosomes and probably have basal functions within these pathogenic fungi. Still, some might be required for pathogenicity as was demonstrated for Fbp1 (Han et al., 2007) and Frp1 (Duyvesteijn et al., 2005). Targeted inactivation of genes encoding F-box proteins described here may yield clues to the function of these proteins and reveal whether their function is related to pathogenicity. Also, it remains to be shown for each predicted protein that it is a true F-box protein. This involves Skp1-binding and SCF assembly experiments, target protein identification screens and mutational studies.

**Methods**

The genomes of three Fusarium species (*F. graminearum* strain PH-1 (NRRL 31084), *F. verticillioides* strain 7600 (FRC M3125=NRRL 20956) and *F. oxysporum* f.sp. lycopersici strain 4287) were sequenced at the Broad institute and the genome databases of these fungi are available from the Broad website (Table 5). Additional information (i.e. gene annotation and transcriptional data) for *F. graminearum* is available from the MIPS *F. graminearum* Genome Database. The fourth *Fusarium* species (*F. solani*) was sequenced at the DOE Joint Genome Institute and the genome database of this fungus is available from their website.

To search for genes encoding F-box proteins, the F-box motif from the PFAM database and the HMM logo, PF00646 was used. With the HMM search program from Anabench, predicted F-box proteins present in the databases of the four species were identified. This search lists all genes encoding proteins containing an F-box domain supplied with a corresponding e-value. This value indicates the degree to which the domain fits the HMM model (lower value means better fit). A cutoff per-sequence (e-value) score of 10.0 results in 89 *F. graminearum*, 62 *F. verticillioides*, 77 *F. oxysporum* and 179 *F. solani* possible genes encoding F-box proteins (see supplementary data, Table S1). To verify the effectiveness and reliability of this search, the predicted budding yeast proteome was examined in a similar way using the protein database of budding yeast from the Broad institute website and information from the Saccharomyces Genome Database, SGD™. The results of this search were compared to results of searches with other database search engines from the SMART, PFAM and Superfam websites. Using a cut-off score of 10.0, our search found 17 of the 21 yeast F-box proteins (Willems et al., 2004). This score was higher than with the other search engines (Table 4). Our search did yield four false positive yeast proteins (these proteins did not contain a F-box domain as verified on the SGD website and earlier studies (Willems et al., 2004)). None of the searches identified four genuine yeast F-box proteins, which all bind Skp1, except for Amn1. That these F-box proteins were not found might be because these proteins contain a gap in the F-box sequence or an interspersed region (Table 4). Still, one protein containing an interspersed region of 18 amino acids in the F-box domain, Ydr306c, was found. To exclude false positives as much as possible from the Fusarium search results, proteins returned by the HMM search were considered as true F-box proteins only when 1) an F-box domain was indicated at the Broad or JGI institute website or 2) an F-box domain was also indicated at the SMART database. A second prerequisite was that at least one putative ortholog from another Fusarium species, if present, also contained an F-box domain. To assess this, for each gene F-box protein found, putative orthologs in the other three Fusarium species were searched using BLAST at the Broad and JGI websites (see above). Further, each F-box protein was examined for the presence of other domains using the SMART database and putative orthologs in other fungal species were searched using the NCBI Blast search engine from greengene at UML. Additionally, for the *F. graminearum* F-box proteins, the presence of an EST encoding the protein was examined. This was done by using Affymetrix GeneChip data that profiled fungal gene expression in vitro and in planta (Güldener et al., 2006).
Table 4: Comparison of different methods to identify F-box proteins in budding yeast

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Table 5: Websites consulted in this study

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