The coiled-coil protein-binding motif in *Fusarium verticillioides* Fsr1 is essential for maize stalk rot virulence

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Fusarium verticillioides (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland) is one of the key pathogens of maize stalk rot disease. However, a clear understanding of stalk rot pathogenesis is still lacking. Previously, we identified the *F. verticillioides* *FSR1* gene, which plays a key role in fungal virulence and sexual mating. The predicted Fsr1 protein contains multiple protein-binding domains, namely a caveolin-binding domain, a coiled-coil structure, and a calmodulin-binding motif at the N terminus and a WD40 repeat domain at the C terminus. Fsr1 shares significant similarity to a family of striatin proteins that play a critical role in cellular mechanisms that regulate a variety of developmental processes. Significantly, *FSR1* function is conserved in *Fusarium graminearum*, where it also plays a direct role in pathogenesis. In this study, our goal was to determine the motif(s) in Fsr1 that are directly associated with fungal virulence. We complemented the *FSR1* knockout ($\Delta fsr1$) strain with mutated versions of the *FSR1* gene, and determined that the Fsr1 C-terminal WD40 repeat domain is dispensable for vegetative growth and maize stalk rot virulence. We also examined the potential link between *FSR1*-mediated virulence and cell wall-degrading enzyme (α -amylase, pectinase and cellulase) activities. Further characterization of the N-terminal region revealed that the coiled-coil structure is essential for virulence in *F. verticillioides*. The coiled-coil domain is involved in a variety of protein–protein interactions in eukaryotic systems, and thus we hypothesize that the interaction between Fsr1 and the putative Fsr1-binding protein triggers downstream gene signalling that is associated with *F. verticillioides* virulence.

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INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland) is an aggressive pathogen of maize, causing stalk rot and ear rot (White, 1999). The fungus is also known to produce a group of carcinogenic mycotoxins called fumonisins in infested ears (Desjardins *et al.*, 2002). While ear rot research has received considerable attention due to the impact of fumonisins in food and feeds, stalk rot research has lapsed due to the lack of an adequate model system to study the mechanism of virulence in stalk rot-causing fungi. Recently, our group has isolated and characterized a gene in *F. verticillioides*, designated *FSR1*, that is critical for maize stalk rot virulence and fertility (Shim *et al.*, 2006). Significantly, disruption of the *FSR1* orthologue in *Fusarium graminearum*, *FgFSR1*, results in loss of virulence on barley and maize, suggesting that the *FSR1*-mediated

virulence mechanism is conserved in *F. graminearum* (Shim *et al.*, 2006). *FSR1* is predicted to encode a multimodular protein, Fsr1, with four functional domains that are known to be involved in protein–protein interactions. The Fsr1 protein shares significant similarity to a family of animal proteins, e.g. striatin and zinedin, that are critical for a variety of developmental processes and cellular mechanisms. The Fsr1-mediated virulence in *F. verticillioides* provides a new avenue to explore a novel signalling mechanism that controls virulence mechanisms in maize stalk rot pathogens (Shim *et al.*, 2006).

The four putative functional domains in Fsr1 include a caveolin-binding domain, a coiled-coil structure, and a calmodulin-binding domain at the N terminus and a WD40 repeat domain at the C terminus (Fig. 1a). From the studies performed in mammalian and *Drosophila* systems (Castets *et al.*, 1996; Moreno *et al.*, 2000), these functional domains are suggested to play critical roles in signalling pathways by mediating protein–protein interactions. The mammalian homologues striatin and zinedin are suggested to be involved in Ca^{2+} -dependent signalling in cells of the central nervous system, and function as scaffolding proteins linking

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Abbreviation: CWDE, cell wall-degrading enzyme.

Fig. 1. *F. verticillioides* *FSR1* locus molecular manipulation. (a) Schematic diagram of the *FSR1* locus in *F. verticillioides* wild-type (WT) and $\Delta fsr1$ strains. The nucleotide sequences that correspond to the caveolin-binding domain, coiled-coil structure, calmodulin-binding motif and WD40 repeat motif are represented by black, white and shaded boxes, and shaded/numbered ovals, respectively. The dotted line indicates a deleted gene region. Double arrows (NT probe and CT probe) show the gene regions used as the probes for Southern and Northern analyses. *HPH*, hygromycin phosphotransferase gene. (b) The *FSR1*-complementation vector (FCV) (Shim *et al.*, 2006) was used as a template to construct *fsr1* Δ NT and *fsr1* Δ CT, and *fsr1* Δ NT and *fsr1* Δ CT illustrate complementation constructs used to test the functional roles of the C-terminus and N-terminus regions, respectively, in *F. verticillioides* growth and stalk rot virulence. *GEN*, geneticin-resistance gene. Arrows depict the primers used to amplify the specific DNA fragments: 1=M13F, 2=b4-RV, 3=a-FW, 4=FSR1-U4, 5=d-RV2, and 6=WD40-FW. (c) Southern blot analysis of the selected transformed strains. Fungal genomic DNA was digested with *Bam*HI, and the blot was hybridized with 32 P-labelled DNA probes for NT and CT (shown in Fig. 1a). The position of molecular size markers (kb) is indicated between the panels. Anticipated bands for *fsr1* Δ CT and *fsr1* Δ NT constructs when hybridized with the NT and CT probes, respectively, are indicated with arrows. (d) Northern blot analysis of the transformants probed with NT (upper panel) and CT (middle panel) 32 P-labelled DNA fragments to determine the expression of the *fsr1* Δ CT and *fsr1* Δ NT constructs, respectively. An ethidium bromide-stained agarose gel (lower panel) confirms uniformity of loading (rRNA) in each lane.

signalling and eukaryotic endocytosis (Castets *et al.*, 1996; Moreno *et al.*, 2000). While homologues of Fsr1 can be found in filamentous fungi, their functional role is not fully understood. Pro11 in the homothallic ascomycete *Sordaria macrospora* was the first striatin orthologue identified and characterized in filamentous fungi. Pro11 plays a critical role in cell differentiation, particularly fruiting body development (Pöggeler & Kück, 2004). A mutation in the *pro11* gene results in loss of fertility and increased aerial hyphae, giving the fungus a cotton ball-like appearance. Complementation with a full-length version of the *PRO11* gene completely restores the wild-type phenotype. Interestingly, a truncated version containing the coding region for the N-terminal part results only in partial complementation; the colony morphology is not restored, although the formation of fertile fruiting bodies is restored, although at a reduced frequency. These results led the investigators to conclude that while the N terminus of Pro11 is sufficient to complement fertility, the complete gene is necessary for full function. Pöggeler &

Kück (2004) concluded that the C-terminal region of Pro11, which contains WD40 repeats, is essential for fungal differentiation and protoperithecia development in *S. macrospora*. However, the role of these motifs in Fsr1 function associated with *F. verticillioides* development and virulence is not clearly understood.

The Fsr1 protein harbours four putative protein-binding motifs that are known to mediate protein–protein interactions. In particular, a caveolin-binding motif, a coiled-coil structure and a WD40 repeat motif share significant amino acid identity to corresponding motifs in other well-defined animal and fungal homologues (Shim *et al.*, 2006). The Fsr1 calmodulin-binding motif shares low similarity to striatin and Pro11. Our hypothesis is that one of these four motifs is directly responsible for *F. verticillioides* maize stalk rot virulence. Here, we test our hypothesis through a series of motif-deletion studies and arrive at the conclusion that the coiled-coil motif is critical for Fsr1-mediated virulence in *F. verticillioides*. We also investigate the roles of these

putative motifs in the expression of selected cell wall-degrading enzymes (CWDEs) in *F. verticillioides*.

METHODS

Fungal strains, media and culture conditions. *F. verticillioides* 7600 (M3125; Fungal Genetics Stock Center) was used as the wild-type strain in this study. *F. verticillioides* 7598 (Fungal Genetic Stock Center) was used as a female wild-type strain in sexual crosses. The *FSR1* disruption-mutant strain $\Delta fsr1$ has been described previously (Shim *et al.*, 2006). The fungal strains were grown on V8 agar (Shim & Woloshuk, 2001) at 25 °C for inoculum preparation and routine maintenance. Colony growth rate, morphology and pigment production were compared on potato dextrose agar (PDA; Difco). To study expression of genes that encode CWDEs, we prepared 'crude stalk juice' by pulverizing 8-week-old maize stalks in a blender, adjusting the final concentration to 10 % (w/v) stalk in water, followed by autoclaving. Fungal conidia produced on 7-day-old V8 agar plates were collected with deionized water and adjusted to 1×10^6 conidia ml⁻¹. The conidia suspension (1 ml) was inoculated into 100 ml crude stalk juice broth and incubated on a rotary shaker (150 r.p.m.) at 25 °C for 7 days.

Generation of *Fsr1* motif-deletion mutants. To investigate the functional role of *Fsr1* N-terminus and C-terminus regions, we first assembled two complementation constructs, one with a complete deletion of the N terminus (*fsr1*ΔNT) and the other with a complete deletion of the C terminus (*fsr1*ΔCT), and introduced each to the $\Delta fsr1$ protoplasts (Fig. 1). Both constructs contained the native promoter of *FSR1* and were fused to a geneticin-resistance (*GEN*) marker (Flaherty

et al., 2003). *FSR1*-complementation vector (FCV) (Shim *et al.*, 2006) was used as a template to construct *fsr1*ΔNT and *fsr1*ΔCT. A double-joint PCR strategy was used to generate all complementation constructs (Shim *et al.*, 2006; Yu *et al.*, 2004). All primers used in this study are listed in Table 1. Primers used for construction of *fsr1*ΔNT and *fsr1*ΔCT are as follows: for the 5' flanking region of *fsr1*ΔCT, M13F and b4-RV; for the 3' flanking region of *fsr1*ΔCT, a-FW and *FSR1*-U4; for the 5' flanking region of *fsr1*ΔNT, M13F and d-RV2; and for the 3' flanking region of *fsr1*ΔNT, WD40-FW and *FSR1*-U4. Subsequently, *fsr1*ΔCTΔCav and *fsr1*ΔCTΔCC complementation constructs were created by amplifying DNA fragments using *fsr1*ΔCT construct as the template. For construction of *fsr1*ΔCTΔCav and *fsr1*ΔCTΔCC, the following primers were used: for the 5' flanking region of *fsr1*ΔCTΔCav, M13F and h-RV; for the 3' flanking region of *fsr1*ΔCTΔCav, g-FW and FVLM2F1; for the 5' flanking region of *fsr1*ΔCTΔCC, M13F and j-RV; and for the 3' flanking region of *fsr1*ΔCTΔCC, i-FW and FVLM2F1. The final (nested) PCR round was performed using the primers GeneF and FVLM2F1. The final PCR product sizes of *fsr1*ΔNT, *fsr1*ΔCT, *fsr1*ΔCTΔCav and *fsr1*ΔCTΔCC were 5.1, 4.8, 3.9 and 3.8 kb, respectively. The PCR products were used directly for fungal transformation. All PCR amplifications associated with complementation constructs were performed with the Expand Long Template PCR system (Roche) following the manufacturer's suggested protocol. *Fusarium* transformations were performed as described previously (Shim *et al.*, 2006). Briefly, protoplasts were prepared and the transformation construct (10 μg), *fsr1*ΔNT, *fsr1*ΔCT, *fsr1*ΔCTΔCav or *fsr1*ΔCTΔCC, was added to the protoplasts (100 μl). Transformants were regenerated in *Fusarium* regeneration agar medium [1 M sucrose, 0.02 % (w/v) yeast extract (Difco), 1 % (w/v) agar] containing hygromycin B (100 μg ml⁻¹; Calbiochem) and

Table 1. Primers used in this study

The underlined sequences were added to the 5' ends of the primers to facilitate fusion PCR.

Primer	Sequence (5'–3')
M13F	TTGTAAAACGACGGCCAGTGA
GeneF	TGAACAAGATGGATTGCACGCAGG
a-FW	<u>CAAAAAGCTGAGAGTGGTAATTTCTAATGGAGATAGATTCTTTAC</u>
b4-RV	<u>GTAAGAATCTATCTCCATTAACTCGGAGTCCAAATCTGAGCTT</u>
WD40-FW	AAGCTCAGATTTGGACTCCGAGGTCACCTC
d-RV2	<u>CCTCGGAGTCCAAATCTGAGCTTTTCGCATGACGCCCTGGAGGGTG</u>
FSR1-U4	AGGATAACAGCAGTAGATGGCAGC
FVLM2F1	AAATGCCGGGTATCGACGAGTAGT
g-FW	<u>ACCCTCCAGGGCGTCATGCGACGCGATAGGAACTCGTGGGAGATT</u>
h-RV	<u>AATCTCCCACGAGTTCTATCGCGTCGCATGACGCCCTGGAGGGT</u>
i-FW	<u>CATGAGCGGATAGGAACTCGACGGCTCAGCTTCAGGCTCGGGAG</u>
j-RV	<u>CTCCCGAGCCTGAAGCTGAGCCGTCGAGTTCCTATCGCGCTCATG</u>
FSR1-U3	GTGACTCGTCACTTCTTGCGTGC
FSR1-D2	AATGGGACCTATGATGCAGGGCAA
FSR1-U1	GCACGCAAGAAGTGACGAGTCAC
CoilBD-FW	TGGGAGATTGAGAAGCAGGAG
CoilBD-RV	GCTCCTTAACCTTTCGCTCCAG
FSR-1186-FW	CTACCTTGGAGGATATGCCAGTG
FSR-BD-RV	GAAATTACCACTCTCAGCTTTTGTG
Fv-amylase-F	GTATCCGCATCGATACAGTCAA
Fv-amylase-R	CCCAAGTCTTGTTGTTCTTTC
FvPG-F	TCATCGTTGTCAAGAAAGACCA
FvPG-R	GTTACAATCTGCGAGTTCAAG
Fv-cellulase-F	GCTGTAACGAGCTCGACATC
Fv-cellulase-R	CTGCACCTTGACGATGTTCTT

geneticin (250 µg ml⁻¹; Research Products International). After 5–7 days of incubation at 25 °C, drug-resistant colonies were selected for further study.

Nucleic acid manipulations, Southern and Northern blot analyses, and PCR conditions. Bacterial plasmids were isolated with the Wizard miniprep DNA purification system (Promega). Fungal genomic DNA was isolated from the frozen mycelium as previously described (Shim & Woloshuk, 2001). Total RNA samples were isolated with Trizol reagent (Invitrogen) by following the manufacturer's suggested protocol. Standard molecular biology techniques, including Southern and Northern analyses, were performed as described by Sambrook & Russell (2001). Restriction enzyme-digested (*Bam*HI or *Eco*RI) fungal genomic DNA (7 µg) was subjected to electrophoresis in a 1.0% (w/v) agarose gel and transferred onto a Hybond-N nylon membrane (Amersham) for Southern analysis. For Northern hybridization, total RNA (15 µg) was separated by electrophoresis on a 1.2% (v/v) formaldehyde agarose gel and blotted onto a Hybond-N nylon membrane (Amersham).

The DNA probes were ³²P-labelled with a Prime-It II random primer labelling kit (Stratagene). All primers used in this study are listed in Table 1. For probes NT, CT, CC and DS, primer pairs FSR-D2 + FSR-U1, WD40-FW + FSR-U3, CoilBD-FW + CoilBD-RV and FSR-1186-FW + FSR-BD-RV, respectively, were used to amplify the DNA fragments from the *F. verticillioides* genome. *F. verticillioides* AMY1 (GenBank DQ143884) has been isolated and characterized by Bluhm & Woloshuk (2005). The sequence of the pectinase (*PTN*) and cellulase (*CEL*) genes were obtained from the *Fusarium* Group Database housed at the Broad Institute of the Massachusetts Institute of Technology (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html) using *Gibberella fujikuroi* pectinase (GenBank Q017181) and *Fusarium oxysporum* cellulase (GenBank P46237) as query. To amplify specific probes for AMY1, *PTN* and *CEL*, primer pairs Fv-amylase-F + Fv-amylase-R, FvPG-F + FvPG-R and Fv-cellulase-F + Fv-cellulase-R were used, respectively.

Virulence assay. The stalk rot assays were performed as described previously (Shim *et al.*, 2006). Briefly, 8-week-old B73 maize stalks (near pollinating stage) were wounded with a sterile tooth pick and infected with fungal strains. Agar blocks for inoculation were prepared from wild-type, Δ *fsr1*, *fsr1*ΔCT5, *fsr1*ΔNT2, *fsr1*ΔCTΔCav9, *fsr1*ΔCTΔCC3 and Δ *amy1* strains grown on V8 agar plates (at 25 °C for 7 days) with a cork borer (1 cm in diameter) and placed on wound sites. Infected maize plants were incubated in a growth chamber with controlled temperature (25 °C), relative humidity (70%) and light cycle (14 h light/10 h dark). Plants were incubated for 10 days, and stalks were split longitudinally to inspect the extent of rot.

Hydrolysis of starch. The ability of all mutants to degrade starch was determined by culturing them on starch agar plates [0.3 M NaNO₃, 6 mM K₂HPO₄, 4 mM MgSO₄, 7 mM KCl, 66 µM FeSO₄, 2% (w/v) starch, 2% (w/v) agar]. After anaerobic incubation at 25 °C for 3 days, the plates were flooded with an iodine solution [0.5% (v/v) iodine, 5% (v/v) potassium iodide], as described by Bluhm & Woloshuk (2005).

RESULTS

Constructing *FSR1* N terminal-truncated (*fsr1*ΔNT) and C terminal-truncated (*fsr1*ΔCT) *F. verticillioides* strains

Based on *in silico* analysis of the four putative protein-binding domains in the Fsr1 protein, i.e. a caveolin-binding

motif, a coiled-coil structure, and a calmodulin-binding motif at the N terminus and a WD40 repeat domain at the C terminus (Shim *et al.*, 2006), it was conceivable that any one, or a combination of two or more, of the four motifs could play a key role in *F. verticillioides* virulence (Fig. 1a). After *in silico* analysis and literature review (Ganem *et al.*, 2004; Pöggeler & Kück, 2004; Shim *et al.*, 2006; Smith *et al.*, 1999), we hypothesized that the C terminus of Fsr1, containing the WD40 repeats motif, is directly associated with maize stalk rot virulence. To test our hypothesis, we first complemented the *FSR1* knockout strain (Δ *fsr1*) with mutated versions of the *FSR1* gene (Fig. 1b). Two complementation constructs, one with a complete deletion of the N-terminus region (*fsr1*ΔNT) and the other with a complete deletion of the C-terminus region (*fsr1*ΔCT), were developed by the double-joint PCR strategy (Yu *et al.*, 2004) (Fig. 1b). Both constructs contained the native promoter of *FSR1* and were fused to a geneticin-resistance gene (*GEN*) as the selectable marker. The two complementation constructs were independently introduced into Δ *fsr1* protoplasts, and the geneticin-resistant colonies were recovered as described previously (Shim *et al.*, 2006). We selected three *fsr1*ΔNT-complemented transformants (*fsr1*ΔNT1, *fsr1*ΔNT2 and *fsr1*ΔNT5) and three *fsr1*ΔCT-complemented transformants (*fsr1*ΔCT5, *fsr1*ΔCT7 and *fsr1*ΔCT10) for further molecular characterization. Southern analysis showed that the NT and CT probes hybridized to the wild-type genomic DNA but did not hybridize to the Δ *fsr1* genomic DNA (Fig. 1c), which confirms that the complementation constructs were properly introduced into the transformants. Notably, *fsr1*ΔCT5 and *fsr1*ΔNT2 transformants were the only strains that harboured a single copy of *fsr1*ΔCT and *fsr1*ΔNT constructs, respectively (Fig. 1c). Subsequently, we performed Northern analyses to verify that the *fsr1*ΔNT and *fsr1*ΔCT constructs in the transformants were properly expressed. The anticipated 2.8 kb transcript was detected in the wild-type strain but not in Δ *fsr1* strain when probed with NT and CT (Fig. 1d). The *fsr1*ΔCT5 and *fsr1*ΔCT10 strains revealed a high level of *fsr1*ΔCT expression when the blot was hybridized with the NT probe (Fig. 1d). A high level of *fsr1*ΔNT expression was observed in *fsr1*ΔNT1, *fsr1*ΔNT2 and *fsr1*ΔNT5 transformants when the blot was hybridized with the CT probe (Fig. 1d). We concluded that *fsr1*ΔNT and *fsr1*ΔCT constructs successfully integrated in the Δ *fsr1* genome and were properly expressed, particularly in single-insertion transformants *fsr1*ΔCT5 and *fsr1*ΔNT2.

The C-terminal region of Fsr1, which contains a WD40 repeat domain, is dispensable for maize stalk rot virulence

We observed a difference in growth rate and morphology when the mutants were grown on PDA plates. The colonies of the wild-type strain produced aerial mycelia, whereas the colonies of the Δ *fsr1* strain produced fewer aerial mycelia. The Δ *fsr1* strain also exhibited increased reddish-brown pigmentation, and displayed slower radial growth (Fig. 2a).

Fig. 2. Growth and virulence assay. (a) Phenotypic comparison of wild-type (WT) and $\Delta fsr1$ against $fsr1\Delta CT$ ($\Delta CT5$, $\Delta CT7$ and $\Delta CT10$; left panel) and $fsr1\Delta NT$ ($\Delta NT1$, $\Delta NT2$ and $\Delta NT5$; right panel) transformants. All strains were grown on 0.2× PDA plates for 4 days. (b) Virulence assay performed on B73 corn stalks. Maize stalks inoculated with WT, $\Delta fsr1$, $fsr1\Delta CT5$ and $fsr1\Delta NT2$, and a water-inoculated negative control, were photographed at 10 days post-inoculation.

Interestingly, growth rates of $fsr1\Delta CT5$ and $fsr1\Delta CT10$ recovered to be similar to that of the wild-type progenitor, which is consistent with the *FSR1* gene expression level we observed in the Northern blot (Fig. 1d). Growth in $fsr1\Delta CT7$ strain, however, was not restored (Fig. 2a), and this may be due to construct damage during transformation (Fig. 1c). On the other hand, $fsr1\Delta NT1$, $fsr1\Delta NT2$ and $fsr1\Delta NT5$ mutants all mimicked the $\Delta fsr1$ phenotype with low growth rates and increased reddish-brown pigmentation (Fig. 2a), regardless of the *FSR1* gene expression levels (Fig. 1d). Based on molecular and phenotypic characterization, we selected the $fsr1\Delta CT5$ and $fsr1\Delta NT2$ strains for a maize stalk rot assay. Maize plants were inoculated with wild-type, $\Delta fsr1$, $fsr1\Delta NT2$ and $fsr1\Delta CT5$ strains as described previously (Shim *et al.*, 2006). After 10 days of incubation, the positive control (wild-type strain) and the negative control ($\Delta fsr1$ strain) infection assays yielded anticipated rot and no-rot results, respectively (Fig. 2b). The $fsr1\Delta CT5$ strain showed vigorous growth and colonization of maize stalks, resulting in severe rot, whereas the $fsr1\Delta NT2$ mutant caused little rot and failed to penetrate and grow into maize stalks (Fig. 2b). These results clearly demonstrated that the C-terminal region of the Fsr1 protein, which contains a WD40 repeat domain, is dispensable for virulence, and suggest that a motif(s) critical for regulating maize stalk rot virulence in *F. verticillioides* is present in the N terminus of the Fsr1 protein.

Impact of Fsr1 on selected CWDEs in *F. verticillioides*

It is generally acknowledged that many plant-pathogenic fungi can produce a variety of CWDEs that are involved in plant pathogenesis (Lalaoui *et al.*, 2000; Lehtinen, 1993). These enzymes are particularly important for so-called ‘rot’ pathogens when attacking their hosts by breaking down host tissue (Lehtinen, 1993). Consequently, we examined whether mutations in *FSR1* affect the expression of genes that encode CWDEs in *F. verticillioides*. We selected pectinase (*PTN*) and cellulase (*CEL*, endo-1,4- β -glucanase) as the key CWDEs, and investigated the transcript level of

these genes in the wild-type and *FSR1* mutant strains. We also included α -amylase 1 (*AMY1*), a key enzyme that is responsible for starch metabolism in fungi, in the study. Importantly, Bluhm & Woloshuk (2005) have shown that *AMY1* is directly associated with fusarium kernel rot and fumonisin B₁ production in *F. verticillioides*.

F. verticillioides strains were grown in 10 % crushed maize-stalk liquid medium, and fungal mass was harvested for total RNA extraction. Subsequently, we performed Northern analysis to investigate the impact of different *FSR1* mutations on the transcript levels of *AMY1*, *PTN* and *CEL*. Interestingly, no *PTN* expression was detected in any strain tested, including the wild-type strain, whereas high *CEL* expression was observed in all strains (Fig. 3a). Strikingly, we observed a lower *AMY1* expression in $\Delta fsr1$ and $fsr1\Delta NT2$ mutants than in the wild-type strain, suggesting that the N-terminal region of the Fsr1 protein is necessary for proper *F. verticillioides* *AMY1* gene expression (Fig. 3a). Furthermore, to test whether down-regulation of *AMY1* is directly associated with enzyme activity, we performed an amylase activity assay on all four strains. While wild-type and $fsr1\Delta CT5$ strains were able to digest starch, it was clear that $\Delta fsr1$ and $fsr1\Delta NT2$ were restricted in the ability to utilize starch as the sole carbon source (Fig. 3b). Therefore, we concluded that a complete Fsr1 N terminus is important for proper α -amylase activity in *F. verticillioides*.

From our earlier result, we showed that the $fsr1\Delta NT2$ strain is avirulent when inoculated on maize stalks. We were curious to test whether downregulation of *AMY1* in the $fsr1\Delta NT2$ strain (Fig. 3a, b) is directly associated with loss of virulence. To test this hypothesis, we inoculated mature maize stalks with the wild-type and the $\Delta amy1$ mutant strain, which is completely inhibited in its ability to digest starch (Bluhm & Woloshuk, 2005), and observed the rot severity after 1 week of incubation. When maize stalks were split longitudinally, we determined that the $\Delta amy1$ mutant was indistinguishable from the wild-type strain in its ability to cause maize stalk rot (Fig. 3c). These *in vitro* results suggest that *FSR1*-mediated maize stalk rot virulence in *F. verticillioides* could be independent of CWDE activities.

Fig. 3. Analysis of cell wall-degrading enzymes *in vitro*, produced during infection by *F. verticilliioides* mutants. (a) Northern analyses of α -amylase 1 (*AMY1*), pectinase (*PTN*) and cellulase (*CEL*) genes. The wild-type (WT), $\Delta fsr1$, $\Delta fsr1\Delta CT5$ and $\Delta fsr1\Delta NT2$ strains were grown in crude stalk juice broth for 10 days. Total RNA samples (15 μ g) were subjected to electrophoresis in a 1.2% denaturing agarose gel. The gel was stained with ethidium bromide to confirm uniformity of loading (*rRNA*). The 32 P-labelled *AMY1*, *PTN* and *CEL* probes for the Northern analysis were generated via PCR. (b) Production of α -amylase by *F. verticilliioides* WT, $\Delta fsr1$, $\Delta fsr1\Delta CT5$ and $\Delta fsr1\Delta NT2$ strains. Fungi were grown for 3 days on starch agar plates and then stained with an iodine solution; the clear zone around the growth indicates hydrolysis of starch. (c) Virulence assays performed on B73 corn stalks. Maize stalks inoculated with WT and $\Delta amy1$ strains were photographed at 10 days post-inoculation.

The Fsr1 coiled-coil protein-binding motif is required for maize stalk rot virulence and proper growth in *F. verticilliioides*

Our results suggest that a functional motif(s) present in the N terminus of the Fsr1 protein is essential for virulence (Fig. 2). Of the three potential protein-binding motifs present in the N terminus, the caveolin-binding domain and coiled-coil structure are highly conserved, whereas the calmodulin-binding motif shares low similarity to other fungal orthologues (Shim *et al.*, 2006). Therefore, we constructed $\Delta fsr1\Delta CT\Delta Cav$ and $\Delta fsr1\Delta CT\Delta CC$, in which the DNA regions corresponding to the caveolin-binding motif and coiled-coil structure, respectively, were deleted from the $\Delta fsr1\Delta CT$ construct (Fig. 4a). The $\Delta fsr1$ protoplasts were transformed with these constructs, and after screening for geneticin-resistant transformants, we selected $\Delta fsr1\Delta CT\Delta Cav9$ and $\Delta fsr1\Delta CT\Delta CC3$ strains for further characterization.

Southern analysis indicated that $\Delta fsr1\Delta CT\Delta Cav9$ has a single-copy $\Delta fsr1\Delta CT\Delta Cav$ construct in the genome (Fig. 4b). Furthermore, when the blot was hybridized to the CC probe, a smaller-size transcript (1.3 kb) was observed in the mutant (Fig. 4a), which is indicative of caveolin-binding motif deletion. Insertion of the $\Delta fsr1\Delta CT\Delta CC$ construct into the $\Delta fsr1$ strain was also confirmed by Southern analysis (Fig. 4b). We subsequently determined by Northern analysis that the truncated gene (1.2 kb) is expressed in the transformant $\Delta fsr1\Delta CT\Delta CC3$ (Fig. 4b). In addition, we also verified via quantitative real-time PCR that no coiled-coil motif expression could be detected in $\Delta fsr1\Delta CT\Delta CC3$ (data not shown).

The phenotype of the $\Delta fsr1\Delta CT\Delta Cav9$ strain appeared much like that of the wild-type strain in all aspects of growth on PDA (Fig. 4c). When we inoculated the mutant strain on an 8-week-old maize stalk, severe rot symptoms developed after 1 week of incubation (Fig. 4c). In contrast, $\Delta fsr1\Delta CT\Delta CC3$ grew more slowly on PDA, while producing a higher level of carmine red pigment, similar to $\Delta fsr1$. Also, virulence was not restored in $\Delta fsr1\Delta CT\Delta CC3$ (Fig. 4c). From these observations, we demonstrated that the Fsr1 coiled-coil protein-binding motif, not the caveolin-binding domain, is required for maize stalk rot virulence and proper growth in *F. verticilliioides*.

DISCUSSION

Fsr1 shares high similarity to a unique group of animal proteins, e.g. striatin, zinedin and SG2GA (Shim *et al.*, 2006). The striatin gene is mainly expressed in neurons of the mammalian central nervous system, and the protein contains multiple functional domains involved in protein-protein interactions (Bartoli *et al.*, 1998; Castets *et al.*, 2000; Moreno *et al.*, 2000). It acts as a scaffolding protein, and is involved in transducing and trafficking signals in mammalian systems (Gaillard *et al.*, 2006). Notably, striatin is absent in plants and budding yeast (Benoist *et al.*, 2006); however, there are reports of a striatin orthologue in *Drosophila melanogaster* (Chen *et al.*, 2002) and the filamentous fungus *S. macrospora* (Pöggeler & Kück, 2004). A review of the literature and results from our previous study (Shim *et al.*, 2006) prompted us to further characterize Fsr1, mainly to identify the specific domain(s) in Fsr1 directly associated with *F. verticilliioides* maize stalk rot virulence.

Fig. 4. Functional characterization of Fsr1 N terminus motifs. (a) Schematic depiction of N-terminal domain complementation constructs. The nucleotide sequences that correspond to the caveolin-binding domain, coiled-coil structure and calmodulin-binding motif are represented as black, white and shaded boxes, respectively. Dotted lines indicates deleted gene regions. The *fsr1ΔCT* construct was used a template to generate *fsr1ΔCTΔCav* and *fsr1ΔCTΔCC* constructs. Black arrows (CC and DS) show the probe sites for Southern and Northern blots. *GEN*, geneticin resistance. Arrows depict the primers used to amplify the specific DNA fragments: 1=M13F, 2=h-RV, 3=g-FW, 4=FVLM2F1, 5=j-RV, and 6=i-FW. (b) Southern (left panel) and Northern (right panel) blot analysis of *fsr1ΔCTΔCav9* and *fsr1ΔCTΔCC3*. Fungal genomic DNA samples were digested with *EcoRI* and subjected to electrophoresis in 1 % agarose gel. Total RNA samples (15 μg) were subjected to electrophoresis in a 1.2 % denaturing agarose gel. The gel was stained with ethidium bromide to confirm uniformity of loading (rRNA; bottom-right panels). The blots were hybridized with ³²P-labelled CC and DS probes (shown in Fig. 4a), respectively. (c) Growth and virulence phenotypes of *fsr1ΔCTΔCav* and *fsr1ΔCTΔCC3* strains. Strains were grown on a 0.2× PDA plate, and the growth pattern was observed after 4 days of incubation. The virulence assay was performed on B73 corn stalks. The maize stalk rot assay of *fsr1ΔCTΔCC3* was photographed at 10 days post-inoculation.

We examined the role of individual domains in fungal virulence by generating a series of motif-deletion mutations in *FSR1* (*fsr1ΔANT*, *fsr1ΔCT*, *fsr1ΔCTΔCav* and *fsr1ΔCTΔCC*) (Figs 1b and 4a). Our initial hypothesis was that the WD40 repeat domain in Fsr1 is essential for stalk rot virulence. The WD40 repeats are found in a number of eukaryotic proteins involved in signal transduction, RNA processing, gene regulation, cell division, cytoskeleton assembly and protein degradation (Smith *et al.*, 1999). Typically, the WD repeat is a 40 to 60-residue sequence that contains a glycine-histidine (GH) dipeptide 11–24 residues from its N terminus and a tryptophan-aspartate (WD) dipeptide at its C terminus. This domain is predicted to form a propeller-like structure, creating a stable platform that enables the protein to coordinate interactions with other proteins and/or small ligands (Smith *et al.*, 1999). The best-characterized WD-repeat protein is the Gβ subunit of heterotrimeric G protein, which is a well-characterized component of eukaryotic transmembrane signalling pathways (Smith *et al.*, 1999). The Fsr1 WD40 repeat motif shares 74 % amino acid identity with the corresponding motif in *S. macrospora*

Pro11, and the key amino acids are highly conserved (Shim *et al.*, 2006). To test our hypothesis, we first divided the Fsr1 protein into two larger regions, the N-terminus and C-terminus regions, instead of individually targeting all four putative motifs. Strikingly, contrary to our expectation, the *fsr1ΔCT5* mutant fully recovered virulence when inoculated on maize stalks (Fig. 2b), whereas the *fsr1ΔANT2* mutants showed near complete loss of pathogenicity on maize plants (Fig. 2b). Thus, we formulated an alternative hypothesis that a functional motif(s) present in the N terminus of the Fsr1 protein holds the key to maize stalk rot virulence.

Subsequent experiments determined that the coiled-coil motif in the Fsr1 N terminus is crucial for *F. verticillioide*s virulence (Fig. 4c). In addition to loss of virulence, deletion of the coiled-coil domain (*fsr1ΔCTΔCC3*) causes multiple aberrant phenotypes that include elevated red-pigment production and slower growth, suggesting that the coiled-coil domain may affect multiple downstream genes. Coiled-coil motifs are known to be responsible for many protein–protein interactions (Newman *et al.*, 2000),

suggesting an important functional role in signal transduction. Patharkar & Cushman (2006) have reported that ice plant (*Mesembryanthemum crystallinum*) McCap1, a coiled-coil protein, interacts with McCpk1 (*M. crystallinum* calcium-dependent protein kinase 1) and is responsible for changes in its localization from the plasma membrane to the nucleus following exposure to low humidity. In particular, regulation of virulence via protein–protein interactions is frequently observed in eukaryotic systems, including fungi. For example, Fbp1 (F-box protein 1) in *F. graminearum* participates in the formation of the SCF^{FBP1} complex that controls the ubiquitin-mediated degradation of proteins involved in sexual reproduction and virulence (Han *et al.*, 2007). Cui *et al.* (2002) have generated a variety of spontaneous mutations in *Botryotinia fuckeliana* that have resulted in dicarboximide-fungicide resistance and osmotic-sensitivity phenotypes. The resistant strains show a variety of single amino-acid differences in the putative coiled-coil domain in the *BOS1* gene (encoding osmosensing histidine kinase), suggesting that the fungicide may specifically interact with this region (Cui *et al.*, 2002). In addition to mediating function through protein–protein interactions, Gaillard *et al.* (2006) have shown that the coiled-coil domain in the striatin protein determines the localization of the protein in spines and controls the oligomerization of members of the striatin family. Our study demonstrates that the coiled-coil motif is a key regulatory domain that regulates fungal virulence in *F. verticillioides*, and opens up a new avenue to investigate the mechanism of fungal virulence signalling. With an understanding that the coiled-coil domain is an important motif in protein–protein interactions *in vivo* and that this interaction may perhaps trigger downstream gene signalling associated with *F. verticillioides* virulence, our future studies will aim to identify and characterize putative Fsr1 coiled-coil motif binding proteins.

Another interesting observation from this study was the perspective that the *FSR1*-mediated virulence signalling pathway in *F. verticillioides* may not be directly associated with production of CWDEs. Many plant-pathogenic fungi produce various CWDEs for successful infection of host plants. In particular, endo-polygalacturonase (pectinase) is considered one of the key CWDEs associated with plant pathogenesis. Pectinases in *Erwinia carotovora* (Lei *et al.*, 1985) and *Claviceps purpurea* (Oeser *et al.*, 2002) are essential for virulence. Furthermore, at least five pectinase genes are present in *Botrytis cinerea* (ten Have *et al.*, 1998) and *F. oxysporum* (Di Pietro & Roncero, 1998; Garcia-Maceira *et al.*, 2001), and have been shown to have functional roles in fungal virulence and host infection. However, it is interesting to note that in some fungal species, e.g. *Cochliobolus carbonum*, pectinase is not directly required for pathogenesis (Scott-Craig *et al.*, 1990). Cellulase, however, is unlikely to be an important enzyme directly associated with host attack, because extensive cellulose degradation typically occurs only late in infection, suggesting that it may not be the determining factor for

pathogen aggressiveness in the early stages of pathogenesis (Novo *et al.*, 2006; Walton, 1994). On the other hand, cellulases of phytopathogenic bacteria may be directly responsible for pathogen aggressiveness, since cellulase gene disruption in *Pseudomonas solanacearum*, *Xanthomonas campestris* pv *campestris*, *E. carotovora* and *Erwinia chrysanthemi* all result in loss of virulence (Bortoli-German *et al.*, 1994; Gough *et al.*, 1988; Mae *et al.*, 1995; Roberts *et al.*, 1988). Interestingly, our Northern blotting data suggested a possible link between *FSR1* and the α -amylase gene, but not between the selected cellulase and pectinase genes, during maize stalk rot development (Fig. 3a). α -Amylase is a key starch-utilizing enzyme in fungi, and in *F. verticillioides* the enzyme has been shown to play an important role in maize kernel colonization and concomitant production of the mycotoxin fumonisin B₁ (Bluhm & Woloshuk, 2005). Significantly, when we performed a maize stalk rot assay with the Δ *Amy1* mutant strain, we observed no difference in rot virulence when compared to the wild-type progenitor (Fig. 3c), demonstrating that reduced amylase activity does not negatively impact *F. verticillioides* maize stalk rot virulence. However, it is important to note that additional genes that encode CWDEs are present in the *F. verticillioides* genome. A *Fusarium* Group Database search revealed eight putative amylase genes, seven putative pectinase genes and three putative cellulase genes, and therefore we cannot rule out the possibility that *FSR1* controls other CWDE genes during stalk rot pathogenesis. Further *in planta* experiments are necessary to unambiguously resolve this host–pathogen interaction mechanism.

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