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 (Δfsr1) 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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Abbreviation: CWDE, cell wall-degrading enzyme.
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 Δ*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’ of 8-week-old maize stalks, which was left to drain for 24 h before being used for RNA extraction. Fungal conidia produced on 7-day-old V8 agar plates were collected and were molded in a 0.01% (w/v) solution of sodium carbonate in water, followed by autoclaving. Fungal conidia produced on 7-day-old V8 agar plates were collected and were molded in a 0.01% (w/v) solution of sodium carbonate in water, followed by autoclaving. A 1 mg/ml suspension (1 ml) was inoculated into 100 ml crude stalk juice broth containing 1% (w/v) yeast extract (Difco) 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 assembles two complementation constructs, one with a complete functional role of Fsr1 N-terminus and C-terminus regions, we first assembles two complementation constructs, one with a complete deletion of the N terminus (*fsr1ΔN*) and the other with a complete deletion of the C terminus (*fsr1ΔCT*), and introduced each to the *F. verticillioides* 7598 (Fungal Genetic Stock Center) was used as the wild-type strain in sexual crosses. 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’ of 8-week-old maize stalks, which was left to drain for 24 h before being used for RNA extraction. Fungal conidia produced on 7-day-old V8 agar plates were collected and were molded in a 0.01% (w/v) solution of sodium carbonate in water, followed by autoclaving. Fungal conidia produced on 7-day-old V8 agar plates were collected and were molded in a 0.01% (w/v) solution of sodium carbonate in water, followed by autoclaving. A 1 mg/ml suspension (1 ml) was inoculated into 100 ml crude stalk juice broth containing 1% (w/v) yeast extract (Difco) and incubated on a rotary shaker (150 r.p.m.) at 25 °C for 7 days.

**Table 1. Primers used in this study**

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

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<th>Primer</th>
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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) 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 (BamHI or EcoRI) 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 and CT, the native promoters of the wild-type strain (Shim & Woloshuk, 2005). The sequence of the pectinase (Pn) 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, PnT 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 toothpick and infected with fungal strains. Agar blocks for inoculation were prepared from wild-type, Δfsr1, Δfsr1ΔCT5, Δfsr1ΔNT2, Δfsr1ΔCTA Cav9, Δfsr1ΔCTA C3 and Δamy1 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 (fsr1ΔNT) and C terminal-truncated (fsr1ΔCT) 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 & Ruck, 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 (Δfsr1) with mutated versions of the FSR1 gene (Fig. 1b). Two complementation constructs, one with a complete deletion of the N-terminus region (fsr1ΔNT) and the other with a complete deletion of the C-terminus region (fsr1ΔCT), 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 Δfsr1 protoplasts, and the geneticin-resistant colonies were recovered as described previously (Shim et al., 2006). We selected three fsr1ΔNT-complemented transformants (fsr1ΔNT1, fsr1ΔNT2 and fsr1ΔNT3) and three fsr1ΔCT-complemented transformants (fsr1ΔCT5, fsr1ΔCT7 and fsr1ΔCT10) for further molecular characterization. Southern analysis confirmed that the NT and CT probes hybridized to the wild-type genomic DNA but did not hybridize to the Δfsr1 genomic DNA (Fig. 1c), which confirms that the complementation constructs were properly introduced into the transformants. Notably, fsr1ΔCT5 and fsr1ΔNT2 transformants were the only strains that harboured a single copy of fsr1ΔACT and fsr1ΔNT constructs, respectively (Fig. 1c). Subsequently, we performed Northern analyses to verify that the fsr1ΔNT and fsr1ΔCT constructs in the transformants were properly expressed. The anticipated 2.8 kb transcript was detected in the wild-type strain but not in Δfsr1 strain when probed with NT and CT (Fig. 1d). The fsr1ΔCT5 and fsr1ΔCT10 strains revealed a high level of fsr1ΔCT expression when the blot was hybridized with the NT probe (Fig. 1d). A high level of fsr1ΔNT expression was observed in fsr1ΔNT1, fsr1ΔNT2 and fsr1ΔNT5 transformants when the blot was hybridized with the CT probe (Fig. 1d). We concluded that fsr1ΔNT and fsr1ΔCT constructs successfully integrated in the Δfsr1 genome and were properly expressed, particularly in single-insertion transformants fsr1ΔCT5 and fsr1ΔNT2.

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 Δfsr1 strain produced fewer aerial mycelia. The Δfsr1 strain also exhibited increased reddish-brown pigmentation, and displayed slower radial growth (Fig. 2a).
Interestingly, growth rates of fsr1ΔCT5 and fsr1ΔCT10 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 fsr1ΔCT7 strain, however, was not restored (Fig. 2a), and this may be due to construct damage during transformation (Fig. 1c). On the other hand, fsr1ΔNT1, fsr1ΔNT2 and fsr1ΔNT5 mutants all mimicked the Δfsr1 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 fsr1ΔCT5 and fsr1ΔNT2 strains for a maize stalk rot assay. Maize plants were inoculated with wild-type, Δfsr1, fsr1ΔNT2 and fsr1ΔCT5 strains as described previously (Shim et al., 2006). After 10 days of incubation, the positive control (wild-type strain) and the negative control (Δfsr1 strain) infection assays yielded anticipated rot and no-rot results, respectively (Fig. 2b). The fsr1ΔCT5 strain showed vigorous growth and colonization of maize stalks, resulting in severe rot, whereas the fsr1ΔNT2 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 fumonisins 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 Δfsr1 and fsr1ΔNT2 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 fsr1ΔCT5 strains were able to digest starch, it was clear that Δfsr1 and fsr1ΔNT2 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 fsr1ΔNT2 strain is avirulent when inoculated on maize stalks. We were curious to test whether downregulation of AMY1 in the fsr1ΔNT2 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 Δamy1 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 Δamy1 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.
The phenotype of the fsr1ΔCTΔCav9 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, fsr1ΔCTΔCC3 grew more slowly on PDA, while producing a higher level of carmine red pigment, similar to Δfsr1. Also, virulence was not restored in fsr1ΔCTΔCC3 (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. verticillioides.

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. verticillioides maize stalk rot virulence.
We examined the role of individual domains in fungal virulence by generating a series of motif-deletion mutations in FSR1 (fsr1ΔNT, 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 fsr1ΔNT2 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. verticillioides 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),

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**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 indicate deleted gene regions. The fsr1ΔCT construct was used as a template to generate fsr1ΔCTΔCav and fsr1ΔCTΔCC constructs. Black arrows (CC and DS) show the probe sites for Southern and Northern blots. GEN, genetin resistance. Arrows depict the primers used to amplify the specific DNA fragments: 1=M13F, 2=h-RV, 3=g-FW, 4=FVM2F1, 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 fsr1ΔCTΔCav9 and fsr1ΔCTΔCC3 strains. Strains were grown on a 0.2% C190 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.
suggested 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[Fb1] 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, 1996; 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, celluloses of phytopathogenic bacteria may be directly responsible for pathogen aggressiveness, since cellulase gene disruption in Pseudomonas solanacearum, Xanthomonas campestris pv campestris, E. carotovora and Erwina 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 Δαamyl 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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