FSR1* Is Essential for Virulence and Female Fertility in *Fusarium verticillioides* and *F. graminearum

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Fusarium verticillioides (teleomorph *Gibberella moniliformis*) and *F. graminearum* (teleomorph *G. zeae*) are well known to cause devastating diseases on cereal crops. Despite their importance, our understanding of the molecular mechanisms involved in these host-pathogen interactions is limited. The *FSR1* locus in *F. verticillioides* was identified by screening REMI mutants for loss of virulence in maize stalk rot inoculation studies. *FSR1* encodes an 823-codon open reading frame interrupted by two introns. The Fsr1 protein shares 60% sequence identity with the *Sordaria macrospora* Pro11, a multimodular protein with four putative protein-protein binding domains (caveolin-binding domain, coiled-coil structure, calmodulin-binding motif, and seven-WD40 repeats), which plays a regulatory role in cell differentiation and ascocarp development. Our data demonstrate that *FSR1* is essential for female fertility and virulence in *F. verticillioides*. Significantly, targeted disruption of the *FSR1* ortholog in *F. graminearum* (*FgFSR1*) reduced virulence on barley and deterred perithecia formation. Cross-complementation experiments demonstrated that the gene function is conserved in the two *Fusarium* species. *FSR1* is expressed constitutively, and we hypothesize that Fsr1 regulates virulence by acting as a scaffold for a signal transduction pathway. A survey of available genome databases indicates Fsr1 homologs are present in a number of filamentous fungi and animal systems but not in budding yeast or plants. A maximum likelihood analysis of this gene family reveals well-supported monophyletic clades associated with fungi and animals.

Additional keywords: mating, pathogenicity, protein interaction, scab, striatin.

A complex of pathogens, primarily fungi such as *Fusarium verticillioides*, *F. graminearum*, *Macrophomina phaseolina*, and *Colletotrichum graminicola* cause stalk rots in maize. It is generally perceived that, when plants experience abiotic stress, particularly at the end of the growing season, pathogens take advantage of the weak host and colonize the vulnerable stalk tissues (Dodd 1980; Michaelson 1957; White 1999). Despite

the variety of pathogens implicated in this maize disease, we postulate that these ‘rot’ pathogens possess specific genetic mechanisms essential for virulence. In a number of fungal pathogen-host interactions, regulation of fungal cellular functions, particularly morphogenesis and virulence, is well characterized (Lengeler et al. 2000). Some signal transduction pathways involved include G protein, cAMP/PKA, and mitogen activated protein (MAP) kinase signal cascades. In contrast, little is known about the specific signaling mechanisms controlling host recognition and virulence in maize stalk rot pathogens. Identification of such mechanisms will open new avenues for understanding this complex disease.

We selected the *F. verticillioides*-maize system as a model to study the molecular basis of pathogenesis in rot fungal pathogens (Jurgenson et al. 2002; Klittich and Leslie 1988; Shim and Woloshuk 2001). Members of the genus *Fusarium* are commonly found in nature and agroecosystems as soil saprophytes as well as plant pathogens of cereal grains and other crops. A select number of *Fusarium* species are particularly well known for their ability to cause devastating diseases of plants. *F. verticillioides* (Sacc.) Nirenberg (synonym *F. moniliforme* Sheldon, teleomorph *Gibberella moniliformis* Wineland) is one of the fungi that causes ear rot and stalk rot of maize, prevalent diseases in U.S. maize-growing regions (White 1999). The fungus also produces mycotoxins, most notably fumonisins, in infested corn. *F. graminearum* Schwabe (teleomorph *G. zeae* [Schweinitz] Petch) is the causal agent of head blight (scab) of wheat and barley, and it produces a wide variety of mycotoxins, most notably the trichothecenes and zearalenone, which are major food safety issues worldwide. *F. oxysporum* and *F. solani* (teleomorph *Nectria haematococca*) are also well-characterized plant pathogens.

We isolated a loss-of-virulence mutant (LD2) from a pool of *F. verticillioides* REMI mutants. Subsequent characterization of LD2 led to the identification of a fungal locus essential for virulence in the *Fusarium*-maize interaction, *FSR1*. Conceptual translation of the *FSR1* revealed a putative multimodular protein with four domains associated with protein-protein interactions, a caveolin-binding domain, a coiled-coil structure, a calmodulin-binding motif, and WD40 repeats. Here, we describe the role of *FSR1* in fungal virulence and sexual fertility in *F. verticillioides* and *F. graminearum*. We demonstrate that *FSR1* function is conserved in *F. graminearum*, playing a direct role in pathogenesis and sexual reproduction in that homothallic fungus. Also, we survey available genome sequences to determine the species distribution and degree of conservation for this gene.

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Nucleotide sequence data for *FSR1* is available in the GenBank database under accession number AY862192.

RESULTS

Phenotype of *F. verticillioides* mutant LD2.

Mutant strain LD2 was identified from the library of *F. verticillioides* REMI mutants in W. B. Shim's laboratory at Texas A&M University (TAMU). On potato dextrose agar (PDA), colonies of LD2 produced fewer aerial mycelia, exhibited increased reddish-brown pigmentation, and displayed slower radial growth. Differences in radial growth were evident when fungal strains were grown on both PDA (wild type 16.3 ± 0.29 mm to LD2 10.5 ± 0.5 mm) and V-8 agar (wild type 23.7 ± 0.58 mm to LD2 15.7 ± 0.29 mm). However, there was no difference in hyphal branching, conidiophore development, or microconidia production for the colonies on PDA, corn leaf agar, and KCl agar (data not shown). Wild type and LD2 produced comparable fungal mass in potato dextrose broth (PDB) (wild type 46.0 ± 3.2 to LD2 43.0 ± 7.0 mg/ml), Myro broth (wild type 32.0 ± 10.5 to LD2 35.0 ± 16.9 mg/ml), and Czepak-Dox broth (wild type 51.6 ± 6.3 to LD2 47.7 ± 8.6 mg/ml). Finally, it is important to note that both wild-type and the LD2 mutant strains produced high levels of fumonisin B1 (>100 mg/g) when grown on cracked-corn medium.

To determine the effect of the LD2 mutation on virulence of *F. verticillioides*, a stalk-rot assay was performed on 8-week-old B73 maize stalks. The wild-type strain successfully colonized the mature maize stalk and caused severe deterioration of stalk tissues. In contrast, the LD2 mutant failed to cause symptoms beyond the inoculation site (Fig. 1A). Microscopic examination of inoculated stalks revealed vigorous growth for the wild type, whereas LD2 caused little rot and failed to pene-

trate and grow in the maize stalk (Fig. 1B). From this in planta assay, we concluded that LD2 is not capable of colonizing maize stalk tissue and causing rot.

Genetic and molecular characterization of *FSR1* in *F. verticillioides*.

Southern analysis confirmed that LD2 contains a single vector insertion (data not shown). To establish that this single vector insertion is responsible for the mutant phenotype, we performed random ascospore analysis. LD2 (hygromycin B-resistant [Hyg^r]; Fsr1⁻) served as the male strain in crosses to female *F. verticillioides* 7598 (hygromycin B-sensitive [Hyg^s]; Fsr1⁺). Analysis of 216 random ascospores on PDA plates resulted in 115 Hyg^s Fsr1⁺ progeny to 101 Hyg^r Fsr1⁻ (here the Fsr1⁻ phenotype refers to reduced growth rate). Chi-square analysis indicated that this pattern of segregation was not significantly different from 1:1 ($P > 0.95$), as is expected for a single genetic locus. Subsequently, we randomly selected four single-spore isolates from each F1 progeny pool and performed stalk-rot assays. The Hyg^s Fsr1⁺ isolates caused rot, whereas the Hyg^r Fsr1⁻ isolates did not cause visible disease symptoms (data not shown). Thus, the Southern analysis and genetic data suggested that a single gene mutation due to the vector insertion is responsible for loss of virulence in the LD2 mutant. We designated this locus *Fusarium* stalk rot 1, *FSR1*.

We rescued and sequenced 350 bp of DNA flanking the vector insertion site in LD2. The sequence of this fragment did not correspond to any sequence in the GenBank database. However, sequence data obtained from the upstream region (approximately 3,000 bp) revealed that the vector insertion site (R) was located 151 nucleotides downstream of an open reading frame (ORF) (Fig. 2A). The ORF sequence is homologous to a gene encoding Pro11, a protein recently discovered and characterized in *Sordaria macrospora* (Pöggeler and Kück 2004). Southern and sequence analyses of the mutated *fsr1* locus in LD2 suggested that a portion of the ORF was deleted during vector insertion (Fig. 2B). Sequence analysis of this region in the wild type revealed a putative 2,568-bp gene interrupted by two introns. Conceptual translation resulted in an 823-amino acid polypeptide with 60% identity to Pro11. Pro11, a putative 845-amino acid protein, plays an important role in *S. macrospora* cell differentiation, particularly ascocarp development (Pöggeler and Kück 2004). Interestingly, the successful sexual cross with LD2 suggests that *FSR1* is not critical for male fertility in *F. verticillioides*. However, since *F. verticillioides* is heterothallic and perithecial development proceeds through the female, we crossed LD2 as a female to an opposite mating type wild-type strain, 7598 (Chulze et al. 2000). In this cross, perithecia were absent, which demonstrated that *FSR1* is necessary for female fertility and perithecial development.

To complement LD2, we constructed two complementation vectors, containing the geneticin (*GEN*) marker *FSR1* or the *F. graminearum* *FSR1* ortholog (FG01665.1) (designated *FgFSR1*) ORF and approximately 800 bp of the 5' untranslated region (UTR) DNA of the respective genes (Fig. 2C). The genomic DNA sequence of the *FgFSR1* ORFs (2,514 bp) shares greater than 85% identity with *FSR1*, and the putative 837-amino acid FgFsr1 protein (EAA68366) shares over 90% identity with Fsr1. The vectors were independently introduced into the mutant genome by polyethylene glycol-mediated fungal protoplast transformation. From the complementation tests, we recovered geneticin-resistant transformants that grew at wild-type rates on 0.2× PDA plates (Fig. 2D and E). Polymerase chain reaction (PCR) and Southern analysis showed that these transformants carried at least one copy of the complementation vector in the genome (data not shown). Pathogenicity assays on B73 maize stalks showed that virulence was restored in the

Fig. 1. *Fusarium verticillioides* and *Fusarium* stalk rot. **A**, Eight-week-old maize stalks (B73) were inoculated with water control (CT), wild-type *F. verticillioides* (WT), and an LD2 mutant strain (LD2) and were incubated for 7 days at 25°C. Maize stalks were split longitudinally to assay the amount of stalk rot. Two independent repetitions are shown. **B**, WT- and LD2-infected maize stalks were observed under the microscope to assess the severity of the rot and fungal colonization.

complemented strains (Fig. 2D and E). Restoration of growth and virulence in *FSR1*-complemented strains further demonstrates that the mutant phenotype of LD2 was due to loss of *fsr1* function.

F. verticillioides *FSR1* expression in synthetic media and in planta.

FSR1 expression in wild type and LD2 were studied by quantitative real-time (QRT)-PCR. We selected the *F. verticillioides* β -tubulin gene (*TUB2*) as an expression standard, given that it is constitutively expressed under a variety of culture conditions (Shim and Woloshuk 2001). The expression level of *FSR1* in cultures was calculated by the comparative cycle threshold (Ct) method with *TUB2* as the endogenous reference for normalization (Pirttilä et al. 2004). We anticipated that *FSR1* is expressed at elevated levels during pathogenesis in planta, since *FSR1* is required for virulence and host-tissue colonization. However, no significant difference in *FSR1* transcription levels was observed when the wild type was grown in PDB ($2^{-\Delta\Delta Ct} = 1.0$ [0.8 to 1.3]), Myro broth ($2^{-\Delta\Delta Ct} = 0.98$ [0.6 to 1.7]), or maize stalk ($2^{-\Delta\Delta Ct} = 0.91$ [0.7 to 1.2]) ($P < 0.05$). The relative expression of each gene was calculated as $2^{-\Delta\Delta Ct}$, and the range of expression for each gene is $2^{-\Delta\Delta Ct-2} - 2^{-\Delta\Delta Ct+2}$, where S is the standard deviation of the $\Delta\Delta Ct$ values (Pirttilä et al. 2004). As expected, *FSR1* expression was not detected in LD2 cultures (potato dextrose broth [PDB] and Myro broth); LD2 *FSR1* gene expression could not be tested in maize stalks (in planta) because the mutant failed to colonize live stalk tis-

sue. To determine whether this consistent *FSR1* expression pattern in a variety of cultures means the gene is constitutively expressed, we measured *FSR1* transcript levels over time. Specifically, we measured transcription of *FSR1* in the wild-type strain grown in Myro broth for 4, 6, and 8 days. Transcript levels were not significantly different across these timepoints (data not shown) ($P < 0.05$), suggesting that *FSR1* is a constitutively expressed gene.

Targeted gene disruption and virulence assay in *F. graminearum*.

As indicated previously, *F. graminearum* *FSR1* ortholog *FgFSR1* shares significant homology to *FSR1* at both nucleotide (>85% identity) and amino acid (>90% identity) levels. Furthermore, *FgFSR1* complemented LD2 strain and restored growth and virulence, prompting us to test the hypothesis that the gene function is conserved in *F. graminearum*. We deleted *FgFSR1* from *F. graminearum* Z03639, completely replacing the ORF with the *GEN* marker (Fig. 3A and B). Targeted gene disruption of *FgFSR1* was confirmed by Southern analysis, which showed a transformant carrying an 8-kb hybridizing band instead of the 2.6-kb band found in the wild-type strain (Fig. 3C). Deletion of *FgFSR1* demonstrated that *FSR1* function is essential for virulence and sexual fertility in *F. graminearum*. Similar to LD2, the knock-out mutant (SJF01) grew at a normal rate in liquid medium but grew more slowly on PDA while producing a higher level of carmine red pigment, aurofusarin (Fig. 3D) (Malz et al. 2005). To determine the effect of *Fgfsr1*

Fig. 2. *Fusarium verticillioides* *FSR1* gene. **A**, Schematic depiction of *FSR1* locus in the wild-type genome. The disruption vector and insertion sites are denoted with the letters R and L. **B**, Schematic depiction of disrupted *fsr1* locus in the LD2 genome. *HPH* = hygromycin phosphotransferase. **C**, Complementation vector constructed by fusing *FSR1* or *FgFSR1* to *GEN* by joint-polymerase chain reaction strategy. 1 = primer M13F, 2 = primer M13R, 3 = *FSR1*-D4t or *FgSTR*-F1t, and 4 = primer *FSR1*-U4 or *FgSTREcoR1*-R1. **D**, Wild-type *F. verticillioides*, LD2, and *FgFSR1*-complemented LD2 strains grown on 0.2x potato dextrose agar (PDA). The virulence assay was performed on B73 corn stalk. **E**, Wild-type *F. verticillioides*, LD2, and *FSR1*-complemented LD2 strains grown on 0.2x PDA. The virulence assay was performed on B73 corn stalk.

gene knock-out mutation on virulence, we spray-inoculated wild-type and SJF01 strains onto the heads of barley plants. The wild type caused typical head blight symptoms just 1 week after inoculation, whereas SJF01 was significantly reduced in virulence (Fig. 3E). When grown on carrot agar, SJF01 failed to produce perithecia, suggesting that the mutant lost sexual fertility (data not shown).

We complemented SJF01 by introducing the *FSR1* ORF and the *FgFSR1* ORF, independently, into the mutant genome. *Hyg^r* transformants were analyzed for functional *FSR1* and *FgFSR1* genes by PCR with gene-specific primers FSR1-D4t and FSR1-U4 and FgSTR-F1t and FgSTREcoR1-R1, respectively, and by Southern analysis (data not shown). Both genes were able to restore the wild-type phenotype, including growth rate (Fig. 3F), virulence on young maize ears (Fig. 3G), and fertility (data not shown). We concluded that *FgFSR1* is required for virulence and sexual fertility in *F. graminearum* and that *FSR1*/*FgFSR1* function is conserved in the two plant pathogenic *Fusarium* species.

DISCUSSION

Characterization of *FSR1* demonstrated that this gene is essential for maize stalk rot virulence and opened a new avenue for understanding a novel virulence mechanism in *Fusarium* species. To date, the only other fungal gene known to influence maize stalk rot virulence is *CPR1* in *Colletotrichum graminicola* (Thon et al. 2002). However, *CPR1* encodes a putative signal peptidase that is thought to play a role in secretion of enzymes necessary for sustaining necrotrophic colonization in planta. The fact that LD2 can be complemented by introduction of *FgFSR1* gene suggested that the gene function is conserved in *F. graminearum*. This functional conservation was

verified by *FgFSR1* gene deletion and subsequent complementation with *FSR1*; *F. graminearum* SJF01 lost its ability to cause scab on barley (Fig. 3E) while the virulence was fully restored with introduction of *FSR1* gene (Fig. 3G). Our study attributes a novel role for this gene family as a virulence gene. While we found a loss of virulence in the mutants from both *F. verticillioides* and *F. graminearum*, an important question for future study will be whether this function is required for virulence in all pathogenic filamentous fungi.

The sexual cross and random ascospore analysis with *F. verticillioides* demonstrated that the *fsr1* mutant retains male fertility, however, it cannot function as a female parent. Similarly, disruption of *FgFSR1* resulted in loss of fertility and perithecial development in *F. graminearum*, a homothallic fungus like *S. macrospora*. Complementation of SJF01 (*Afgfsr1* strain) with functional *FSR1* and *FgFSR1* genes restored development of perithecia and viable ascospore production in *F. graminearum*. The *S. macrospora pro11* mutant forms normal ascogonia and protoperithecia but is unable to develop mature ascocarps. Thus, *Fsr1/Pro11* appears to be critical for mating in filamentous fungi, perhaps specifically controlling the development of female fertility. Thus, despite the fact that the two *Fusarium* species evolved distinct mating strategies (i.e., heterothallic vs. homothallic), the mechanism of regulating virulence and fertility via *FSR1* is conserved.

Single genes homologous to *Fsr1* (presumed orthologs) are present in all filamentous fungal genome sequence databases queried: *F. graminearum* (90% aa identity), *M. grisea* (60% aa identity), *Neurospora crassa* (58% aa identity), and *Aspergillus nidulans* (44% aa identity). In animal genomes there is at least one protein homologous to *Fsr1*, in some cases, more than one homolog is present (e.g., striatin, SG2NA, and zine-din in humans and mouse). It is important to note, however,

Fig. 3. Characterization of the *FgFSR1* locus in *Fusarium graminearum*. **A**, Schematic depiction of the *FgFSR1* locus in the wild-type genome. X = *XhoI* restriction sites. **B**, Schematic depiction of the deleted *Afgfsr1* locus in the SJF01 genome. The homologous recombination event resulted in a replacement of the *FgFSR1* open reading frame with the geneticin gene (*GEN*). 5' flanking DNA ($\leftrightarrow$) was used as a probe for Southern analysis. **C**, Southern analysis of wild type and *FgFSR1* knock-out transformants. A blot containing genomic DNA of wild type and transformants, digested with *XhoI*, was hybridized with the probe. Lane M, DNA marker; lane 1, wild-type *F. graminearum*; and lane 2, SJF01. **D**, Wild-type *F. graminearum* (WT) and SJF01 grown on potato dextrose agar. **E**, Wild-type *F. graminearum* (WT) and SJF01 virulence assay performed on barley. **F**, Mycelial growth of wild-type (WT), SJF01, *FSR1*-complemented SJF01 (FSR1-C), and *FgFSR1*-complemented SJF01 (FgFSR1-C) *F. graminearum* strains on V-8 agar medium. **G**, Young corn-ear virulence assay with wild-type, SJF01, FSR1-C, and FgFSR1-C *F. graminearum* strains.

that Fsr1 homologs (or striatin homologs) are not ubiquitous in eukaryotes. Available genome sequences for yeasts and plants do not harbor members of this gene family (Pöggeler and Kück 2004). The maximum likelihood analysis revealed strong support for independent lineages for fungal and animal gene family members (Fig. 4). This is consistent with a single gene in a common ancestor to animals and fungi diverging over time. Subsequently, within the fungal clade, the gene was apparently lost in the ancestor of yeasts. Since it is well established that fungi are more closely related to animals than plants (Baldauf and Palmer 1993), there is no need to invoke a loss in the ancestor to the plants. In animals, the lineages are consistent with two subsequent gene duplication events, giving rise to the striatin, the SG2NA, and the zinedin protein paralogs (Fig. 4). Interestingly, despite the divergence between the fungal and animal lineages in this tree, the mouse striatin gene complemented the *pro11* mutation in *S. macrospora* (Pöggeler and Kück 2004). Thus, it seems reasonable to conclude that the signaling mechanism in Fsr1/Pro11- and striatin-mediated pathways in fungi and animals are conserved.

Fsr1 and Pro11 share a number of structural features with a recently described novel class of proteins in the striatin family (Castets et al. 2000). Members of this family, such as striatin (Bartoli et al. 1998; Castets et al. 1996), zinedin (Castets et al. 2000), SG2NA (Moreno et al. 2000), and CKA (Chen et al. 2002), contain four putative protein-protein interaction motifs, a caveolin-binding domain, a coiled-coil structure, a putative calmodulin-binding motif in the N-terminus, and putative WD40-repeat domain in the C-terminus (Fig. 5A). 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). Caveolin is a major structural protein of caveolae, vesicular invaginations of the plasma membrane, and it forms a scaffold with a variety of signaling molecules to generate 'preassembled signaling complexes' (Okamoto et al. 1998; Smart et al. 1999). A conserved caveolin-binding motif, which is required for direct interaction with caveolin and the localization of signaling molecules to membranes, may also functionally regulate the activation state of caveolae-associated signaling molecules (Okamoto et al. 1998). It remains to be tested whether Fsr1 is localized and regulated by a similar mechanism in *F. verticillioides* cells. However, our effort to identify a fungal 'caveolin homolog' in public databases was unsuccessful, which raises the possibility that the Fsr1 caveolin-binding motif may interact with a different type of molecule in fungal cells. The coiled-coil structure, a bundle of α -helices that are wound into a superhelix, is a common structural motif mediating protein-protein interactions (Lupas 1996). The coiled-coil prediction algorithm of MultiCoil indicates that the coiled-coil structure region in Fsr1 has a >70% probability of forming a dimer (Wolf et al. 1997). However, coiled-coil interacting molecules of mammalian striatin homologs have not been identified. Ca^{2+} - and calmodulin-dependent signaling pathways in fungi have been shown to play a critical role in virulence, morphogenesis, and stress response via distinct mechanisms in each fungus (Kraus and Heitman 2003).

The members of the striatin family are involved in Ca^{2+} -dependent signaling in cells of the mammalian central nervous system, acting as scaffolds, as signaling proteins, or as catalytic subunits of protein phosphatase 2A (Bartoli et al. 1998; Castets et al. 1996; Moreno et al. 2000). These proteins also are suggested to function as scaffolding proteins that link signaling and eukaryotic endocytosis (Pöggeler and Kück 2004). Additionally, in *Drosophila melanogaster*, the CKA (connector of kinase to AP-1) protein regulates the Jun N-terminal kinase signal transduction pathway, which in turn, controls epithelial

sheet movement that occurs during dorsal closure (Chen et al. 2002). Fsr1, like the members of the striatin family, contains four putative functional domains that are suggested to carry out unique cellular functions (Fig. 3A), a caveolin-binding domain (aa 39 to 48) (Fig. 5B), a coiled-coil structural motif (aa 54 to 96) (Fig. 5C), a calmodulin-binding domain (aa 131 to 148), and WD40-repeat domain (aa 388 to 823) (Fig. 5D). Fsr1 and Pro11 share 100, 79, and 74% amino acid identity in the caveolin-binding domain, coiled-coil structure, and the WD40-repeat domain, respectively. However, the calmodulin-binding motif in Fsr1 contains a relatively low similarity to striatin (<35%).

Characterization of protein-binding motifs in Fsr1 may provide additional understanding of how this protein mediates sexual fertility and virulence in *Fusarium* species. Pöggeler and Kück (2004) showed that, while full-length *PRO11* gene completely restored the wild-type phenotype, the truncated version of the complementation vector, which contained the coding region for N-terminal region of Pro11, only partially restored perithecia production. The N-terminal region of Pro11 with a frame-shift mutation failed to complement the mutant. Significantly, the partially complemented strains produced fewer perithecia and ascospores, and hyphal morphology was not restored to the wild-type form. Therefore, it seems reasonable to suggest that the N-terminus and the C-terminus of

Fig. 4. Maximum likelihood tree for the gene family harboring Fsr1. The sequences used in this analysis are *Fusarium verticillioides* Fsr1 (FvFsr1, AY862192), *Sordaria macrospora* Pro11 (SmPro11, AJ564211), a *F. graminearum* homolog (FgFsr1, EAA68366.1), a *Magnaporthe grisea* homolog (MgHmg, EAA53083), an *Aspergillus nidulans* homolog (AnHmg, EAA59693), a *Neurospora crassa* homolog (NcHmg, XP_330858), a *Ustilago maydis* homolog (UmHmg, EAK84819), a *Cryptococcus neoformans* var. *neoformans* B-3501A homolog (CnHmg, EAL23411), *Homo sapiens* striatin (HsStr, NP_003153), *H. sapiens* zinedin (HsZin, AAH34604), *H. sapiens* SG2NA (HsSG2NA, NP_055389), *Mus musculus* striatin (MmStr, NP_035630), *M. musculus* zinedin (MmZin, NP_598550), *M. musculus* SG2NA (MmSG2NA, Q9ERG2), a *Xenopus laevis* striatin homolog (XiStr, AAH81006), and *Drosophila melanogaster* CKA (DmCka, AF364305).

Pro11 are required for perithecia development and hyphal morphogenesis, respectively, in *S. macrospora*. In *F. verticillioides* and *F. graminearum*, it remains to be tested whether similar functions are conserved in Fsr1 N-terminus and C-terminus. In addition to perithecia and hyphae development, functional domains responsible for virulence can be tested and identified in *Fusarium* species.

Studies have shown that signaling pathways, e.g., G proteins, cAMP/PKA, and MAP kinase pathways, work closely together to regulate sexual fertility, development, and virulence in fungi (Bölker 1998; Lengeler et al. 2000). Particularly, coregulation of sexual mating and virulence has been described in a number of fungal systems, e.g., *Cryptococcus neoformans*, *F. graminearum*, and *Cochliobolus heterostrophus* (Ganem et al. 2004; Jenczmionka et al. 2003; Wang et al. 2000). However, it is important to recognize that additional signaling components that interact with these known signaling pathways may be present in fungi. For example, one hypothesis is that Fsr1 and its putative binding partners serve in upstream pathways. Further experiments are in progress to determine whether G proteins and MAP kinase signaling pathways interact with the *FSR1* pathway and regulate virulence and female fertility in *Fusarium* species.

The data presented in this study show that *FSR1* is critical for sexual mating and virulence in *F. verticillioides* and *F. graminearum*. Evidently, the *FSR1*-mediated virulence and

sexual mating pathway provides another opportunity to understand this complex mechanism in fungi. Our observations that *FSR1* is a constitutively expressed gene (Table 1) and that Fsr1 contains multiple protein-binding domains (Fig. 5) suggest that the *FSR1*-mediated pathway is regulated via protein-protein interactions. Studies performed in a number of putatively orthologous striatin-mammalian systems support this suggestion (Bartoli et al. 1998; Castets et al. 1996; Moreno et al. 2000). This mechanism of pathway regulation is not unprecedented in *Fusarium* gene signaling. A C-type cyclin gene *FCCI*, which plays a key role in signal transduction regulating secondary metabolism and fungal development, is also expressed constitutively in *F. verticillioides* (Shim and Woloshuk 2001) and was shown to regulate a downstream signaling pathway by forming a complex with a cyclin-dependent kinase *FCK1* (Woloshuk 2004). Thus, it seems reasonable to hypothesize that Fsr1 regulates signaling acting as a scaffold for signal transduction pathways and interacting with one or more kinases, phosphatases, or transcription factors (Castets et al. 2000; Chen et al. 2002; Pöggeler and Kück 2004). Candidates for the Fsr1-interacting proteins include those similar to phocein (Baillat et al. 2001; Pöggeler and Kück 2004), protein phosphatase 2A (Moreno et al. 2000), and MOB1 (Moreno et al. 2001). In addition to these candidates, further characterization of an Fsr1-mediated signaling pathway in *F. verticillioides* and *F. graminearum* may

Fig. 5. *Fusarium verticillioides* Fsr1 protein and the four protein-binding domains. **A**, Schematic representation of the putative 823-amino acid (aa) Fsr1 protein. The black box represents the putative caveolin-binding motif, the white box represents the putative coiled-coil motif, the shaded box represents the putative calmodulin-binding domain, and numbered and shaded ovals show the position of WD40 repeats. **B**, Amino-acid alignment of putative caveolin-binding motif in *F. verticillioides* (Fv) Fsr1 (aa 39 to 48), *Sordaria macrospora* (Sm) Pro11, *Homo sapiens* (Hs) zinedine, and *Mus musculus* (Mm) striatin. **C**, Amino-acid alignment of putative coiled-coil motif in Fv Fsr1 (aa 54 to 96), Sm Pro11, Hs zinedine, and Mm striatin. **D**, Amino-acid alignment of the C-terminus region of *F. verticillioides* Fsr1 (aa 388 to 823), which contains seven putative WD40 repeats, and *S. macrospora* Pro11. Amino acids that are identical in other homologs are indicated by white letters on a black background, and the similar residues are indicated by white letters on a gray background.

reveal novel molecules involved in virulence mechanisms that have not been identified in pathogenic fungi.

MATERIALS AND METHODS

Fungal strains, media, and conidiation and growth assay.

Fusarium verticillioides 7600 (Fungal Genetics Stock Center, University of Missouri-Kansas City, Kansas City, MO, U.S.A.) was used as the wild-type strain. *F. verticillioides* 7598 (Fungal Genetics Stock Center) was used as a female wild-type strain in sexual crosses. *F. graminearum* Z03639 was selected for the scab pathogenicity assay and molecular manipulations (Lee et al. 2003). Conidia were produced for inoculum by growing the fungus on PDA (Difco, Detroit) at 25°C. Colony morphology and pigment production were compared on PDA. Radial growth was determined by measuring colony diameters after 5 days of growth on PDA and V-8 agar (Shim and Woloshuk 2001). The formation of microconidia in chains on monophialides was examined on corn leaf agar and KCl agar (Nelson et al. 1983). To quantify conidiation, agar cylinders were excised with a cork borer (1-cm diameter) from a V-8 agar culture, were placed in a test tube with 1 ml of sterile water, and were vortexed to dislodge the conidia, which were counted with a hemacytometer.

Fungal transformation and random ascospore analysis.

Fusarium transformations were performed as described previously (Kim et al. 2005; Shim and Woloshuk 2001). Briefly, protoplasts were prepared by suspending mycelium (1 g wet weight) in an enzyme solution (20 ml) containing Driselase (2 mg/ml) (Sigma, St. Louis), β -glucuronidase (5,200 U/ml) (Sigma), 10 mM NaH_2PO_4 (pH 5.8), 20 M CaCl_2 , and 1.2 M KCl. A transformation vector (10 μg) with either hygromycin B phosphotransferase (*HPH*) gene or geneticin resistance gene (*GEN*) as the selectable marker was added to the protoplasts (100 μl) (Flaherty et al. 2003; Shim and Woloshuk 2001). Transformation was performed with the aid of 40% polyethylene glycol 4000 (Sigma), and the protoplasts were regenerated in *Fusarium* regeneration agar medium (1 M sucrose, 0.02% yeast extract [Difco], and 1% agar) containing hygromycin B (100 $\mu\text{g}/\text{ml}$) (Calbiochem, La Jolla, CA, U.S.A.) (Shim and Woloshuk 2001) or geneticin (250 $\mu\text{g}/\text{ml}$) (Research Products International Corp., Mt. Prospect, IL, U.S.A.). After 5 to 7 days of incubation at 25°C, drug-resistant colonies were selected for further study. The colony diameters were measured after 5 days of growth on PDA and V-8 agar at 25°C. A thin-layer chromatography assay of fumonisin B1 production was performed as previously described (Shim and Woloshuk 2001).

F. verticillioides sexual crosses were performed as described by Klittich and Leslie (1988). Transformant LD2 served as the male strain in crosses with *F. verticillioides* 7598. After 21 days of incubation at 25°C with a 14-h light and 10-h dark cycle, ascospores were collected from 20 perithecia and were pooled. Hygromycin B-resistant and -sensitive isolates were tested for the growth rate phenotype on PDA, and a subset of four progeny from each F1 progeny pool were used to test virulence on B73 maize stalks. *F. graminearum* sexual cross was performed as described by Kim and associates (2005).

Virulence assay.

Maize line B73 used in the stalk rot assay was provided by M. Kolomiets (Department of Plant Pathology and Microbiology, TAMU). A method described by Thon and associates (2000) was modified to develop this in vivo assay. Briefly, stalks of 8-week-old B73 maize (near pollinating stage) were wounded with a sterile toothpick and were infected with fungal strains. Agar blocks for inoculation were prepared from

wild-type and *fsr1* mutant strains grown on V-8 agar plates (at 25°C for 7 days) with a cork borer (1 cm in diameter) and were placed on wound sites. Infected maize plants were incubated in a growth chamber with temperature (25°C), relative humidity (70%), and light cycle (14-h light and 10-h dark) controlled. Plants were incubated for 1 week, and stalks were split longitudinally to inspect the extent of rot. The stalk rot assay included three replicates (inoculations of three independent corn plants) for each fungal isolate.

The scab virulence assay was performed to determine the virulence in SJF01 (Kim et al. 2005). Briefly, conidia (1×10^5 per milliliter of sterile water) from wild-type and mutant *F. graminearum* strains grown on carrot agar for two weeks were sprayed onto the heads of barley plants at early anthesis. The plants were incubated in a growth chamber (25°C with 100% relative humidity) for two days before they were transferred to a greenhouse. Symptoms appeared one week after inoculation. Virulence of wild-type, SJF01, *FSR1*-complemented SJF01, and *FgFSR1*-complemented SJF01 strains was determined on young ears of corn as described previously (Han et al. 2004). Fungal mycelia and conidia were injected into a young corn ear with a toothpick, and the corn samples were incubated for one week.

Nucleic acid manipulations, PCR conditions, and vector constructions.

Bacterial plasmids were isolated with the Wizard miniprep DNA purification system (Promega, Madison, WI, U.S.A.). Fungal genomic DNA was isolated from mycelium grown in potato dextrose broth (Difco) as previously described (Shim and Woloshuk 2001). Total RNA samples were isolated with Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) by following the manufacturer's suggested protocol. Standard molecular techniques, including Southern and Northern analyses, were performed as described (Sambrook and Russell 2001). The DNA probes were ^{32}P -labeled with a Prime-It II random primer labeling kit (Stratagene, La Jolla, CA, U.S.A.). Primers used in this study are listed in Table 1.

Table 1. Primers used in this study

Primer Name	5' → 3'
IPF	TATCGCTGCTCCATTCACCA
IPR	GCCCTCGGTAGTATTGGGAAA
FSR-f	CGTAAACGACCACTGCCAAAAGAAC
FSR-fgt ^a	<u>GCACAGGTACACTTGTGTTAGAGAGCGTGAC</u> AAGCTTGATACACTGAC
FSR-rgt ^a	<u>CCTTCAATATCATCTTCTGTCGACGGATCGG</u> TATGGGAGAGATAGC
FSR-r	TTGATACGGAACCTGAAGCGACTAAAA
FGT	CTCTAAACAAGTGATACCTGT
RGT	CGACAGAAGATGATATTGAA
FSR-nf	CGCTGCTTCGGCCTCTTCCA
FSR-nr	AGCCGAGCTACGAGACCCTGAACT
FSR1-D4t ^b	<u>AATCATGGTCATAGCTGTTCTCTGATTCAC</u> TCGCTGCAAGGTTCCAC
FSR1-U4	AGGATAACAGCAGTAGATGGCAGC
FgSTR-F1t ^b	<u>AATCATGGTCATAGCTGTTCTCTGTTGGGATG</u> GAAGTCGAGGAATGGA
FgSTREcoR1-R1	GGCTGTGGCTTATTGACTTGACCA
M13F	TTGTAACAGCAGCGCCAGTGA
M13R ^b	<u>CAGGAAACAGCTATGACCATG</u>
FSR1-f	CATGGCTGGTTCAGGTGTCGA
FSR1-r	GGAAAGACTGGAAATGGCGTCAGG
TUB2-f	CAGCGTTCCTGAGTTGACCAACAG
TUB2-r	CTGGACGTTGCGCATCTGATCCTCG

^a The sequences underlined in primers FSR-fgt and FSR-rgt indicate complementary FGT and RGT primer sequences, respectively that promote hybridization during double-joint polymerase chain reaction amplification.

^b Underlined sequences are homologous to the sequences of the primer M13R to promote hybridization between PCR amplicons.

To rescue the DNA fragments flanking the transformation vector insertion site, we performed inverse PCR. The *fsr1* mutant genomic DNA was digested with *Cla*I restriction enzyme and was subsequently recircularized in a self-ligation reaction (100 ng DNA in a 20- μ l volume). The ligation mix was used as the template, and the PCR amplifications were performed in a 50- μ l reaction mixture with the *Taq* polymerase (Promega) in a DNA thermal cycler (Model 480, Perkin Elmer Cetus, Torrance, CA, U.S.A.). The primers for the inverse PCR were IPF and IPR. The reaction was carried out for 30 cycles of 45 s of denaturation at 94°C, 45 s annealing at 57°C, and 3 min extension at 72°C. The amplified DNA fragment (1.6 kb) was cloned with the pGEM T-Easy cloning system (Promega) and was sequenced. In addition, The Genome Walker kit (Clontech, Palo Alto, CA, U.S.A.) was used to obtain the upstream DNA sequence from the rescued DNA fragment.

The disruption vector for *FgFSR1* was constructed using the double-joint PCR strategy described previously (Kim et al. 2005; Yu et al. 2004). DNA fragments corresponding to regions 5' (1.3 kb) and 3' (1.2 kb) of the *FgFSR1* ORF were amplified from genomic DNA of *F. graminearum* with primer pairs FSR-f/FSR-fgt and FSR-rgt/FSR-r, respectively. A 1.8-kb *GEN* marker was amplified from the vector pII99 with primers FGT and RGT (Lee et al. 2003). Nested primers FSR-nf and FSR-nr were used to amplify the 4.3-kb amplicon carrying the *GEN* marker fused to the *FgFSR1* flanking regions.

Complementation vectors were constructed by amplifying *FSR1* (including the 730-bp 5' and 800-bp 3' UTR) and *FgFSR1* (including the 800-bp 5' and 700-bp 3' UTR) with primers FSR1-D4/FSR1-U4 and FgSTR-F1t/FgSTREcoR1-R1, respectively. *HPH* and *GEN* markers, both harbored in pBluescript, were amplified with primers M13F and M13R. Nested primers FvR4, Fgr4, and M13F (for both *HPH* and *GEN* amplicons) were used in four different combinations to joint PCR-amplify *FSR1::HPH* (5.7 kb), *FSR1::GEN* (6.1 kb), *FgFSR1::HPH* (5.5 kb), *FgFSR1::GEN* (5.9 kb) complementation vectors as described (Yu et al. 2004). PCR amplifications were performed with Long Expand polymerase (Roche, Indianapolis, IN, U.S.A.) following the manufacturer's suggested protocol.

QRT-PCR.

The QRT-PCR experiments were performed in a Cepheid Smart Cycler real-time PCR system (Sunnyvale, CA, U.S.A.). Total RNA for QRT-PCR analysis was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) from mycelia grown in PDB (Difco), Myro broth (Shim and Woloshuk 1999), and maize stalk tissue in planta. The PDB and Myro broth cultures were incubated for 1 week at 25°C on a rotary shaker (150 rpm) with a 14-h light and 10-h dark cycle. The inoculated maize stalk tissues were prepared as described earlier for the *Fusarium* stalk rot virulence assay. The total RNA samples obtained from the wild type-infected maize stalk tissue contain both fungal RNA and maize RNA, thus providing a relatively lower fungal RNA template for QRT-PCR reactions. Primers for *FSR1* (FSR1-f and FSR1-r) and *TUB2* (TUB2-f and TUB2-r) genes were designed to detect amplification from contaminating genomic DNA by having primers flanking an intron. For *FSR1* and *TUB2*, the cDNA amplicon is expected to be approximately 50 bp smaller than the genomic DNA amplicon, and we programmed the Cepheid Smart Cycler so as to differentiate the molecules by melting temperature. For amplification and quantification, we used the QuantiTech SYBR Green RT-PCR kit (Qiagen, Valencia, CA, U.S.A.) and followed the manufacturer's suggested protocol. Total RNA (200 ng) was used as template in a 25- μ l reaction. The cycling conditions were 30 min at 50°C (reverse transcription), 15 min at

95°C (PCR initial activation step), and 40 cycles of 15 s at 94°C, 30 s at 55°C, followed by 30 s at 72°C. Expression of genes was measured in triplicate. QRT-PCR results with Ct values greater than 35 were determined not detectable.

Phylogenetic analysis.

The amino-acid sequence for Fsr1 generated from the *F. verticillioides* was aligned with sequences available in GenBank (Fig. 4). The alignment was generated with T-Coffee (Notredame et al. 2000; Poiriot et al. 2004). Since the amino acids at the N-terminus of these proteins shared little homology prior to the caveolin binding motif (beginning at aa 39 FvFsr1), they were trimmed manually from the alignment. Similarly, regions represented only in a few proteins were eliminated. The tree is based on a total of 891 aa from 16 species. The maximum likelihood tree was constructed with the BLOSUM 62 model (Henikoff and Henikoff 1993) of amino-acid substitution, and 1,000 replications in Tree-Puzzle 5.1 (Strimmer and von Haeseler 1996). The tree was drawn with PAUP* 4.0b (Sinauer Associates, Sunderland, MA, U.S.A.) with the *Drosophila* sequence selected as the outgroup. Quartet puzzling support values were assigned to each internal branch. Branches showing support between 90 and 100% are considered highly reliable.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

National Center for Biotechnology Information (NCBI) GenBank website:
<http://www.ncbi.nlm.nih.gov>
 Broad Institute Fungal Genome Initiative website:
www.broad.mit.edu/annotation/fgi/
 MultiCoil program: multicoil.lcs.mit.edu/cgi-bin/multicoil