

FsFKS1, the 1,3- β -Glucan Synthase from the Caspofungin-Resistant Fungus *Fusarium solani*

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The cell wall, a mesh of carbohydrates and proteins, shapes and protects the fungal cell. The enzyme responsible for the synthesis of one of the main components of the fungal wall, 1,3- β -glucan synthase, is targeted by the antifungal caspofungin acetate (CFA). Clinical isolates of *Candida albicans* and *Aspergillus fumigatus* are much more sensitive to CFA than clinical isolates of *Fusarium* species. To better understand CFA resistance in *Fusarium* species, we cloned and sequenced FsFKS1, which encodes the *Fusarium solani* f. sp. *pisi* β (1,3)-D-glucan synthase, used RNA interference to reduce its expression and complemented deletion of the essential *fks* gene of the CFA-sensitive fungus *A. fumigatus* with FsFKS1. Reduction of the FsFKS1 message in *F. solani* f. sp. *pisi* reduced spore viability and caused lysis of spores and hyphae, consistent with cell wall defects. Compensating for the loss of *A. fumigatus fks1* with FsFKS1 caused only a modest increase in the tolerance of *A. fumigatus* for CFA. Our results suggest that FsFKS1 is required for the proper construction of *F. solani* cell walls and that the resistance of *F. solani* to CFA is at best only partially due to resistance of the FsFKS1 enzyme to this antifungal agent.

Fungal infections are a frequent cause of death among immunocompromised patients, and the increasing number of immunosuppressed patients has spurred development of new antifungals (30). Because the cell wall protects the fungus and because humans lack many cell wall components, the fungal cell wall is an attractive drug target. Caspofungin acetate (CFA), an inhibitor of the cell wall biosynthetic enzyme β (1,3)-D-glucan synthase, is effective against the major human pathogens *Candida albicans* and *Aspergillus fumigatus* (2, 23). However, some less common fungal pathogens of humans, including *Fusarium* spp., are resistant to clinically relevant levels of CFA (24). Fusariosis is increasing among immunocompromised patients, with *Fusarium solani* identified as the causative agent in roughly half the cases (10, 12). More than 15 species of *Fusarium* have now been reported to cause human and animal infections, with *F. solani* being the most frequent agent (11). The resistance of *Fusarium* species to CFA is puzzling, since β (1,3)-glucan is thought to be an essential component of all fungal cell walls. It is not clear whether caspofungin resistance is a property of the *Fusarium* 1,3- β -glucan synthase or of some other factor such as transport or modification of the drug by the fungus. *Fusarium* species are commonly found in soil and as pathogens of plants. *F. solani* is the designation for the anamorphic (asexual) form of the pathogen. We used a telomorphic (sexual) strain of *F. solani* f. sp. *pisi* (telomorph: *Nectria hematococca* mating population VI) for our studies of 1,3- β -glucan synthase. *N. hematococca* 156-30-6 was first isolated as a plant pathogen and has not been shown to cause disease in humans or mouse models; however, we chose to use it for our studies because it has a well-defined sexual cycle and many molecular tools are available for its

manipulation (4, 32). For simplicity we will refer to the strain by the asexual designation *F. solani* f. sp. *pisi* isolate 156-30-6.

The β (1,3)-D-glucan synthase genes were identified in *Saccharomyces cerevisiae* by complementation of a mutation conferring hypersensitivity to the immunosuppressant FK506 (*fks1-1*) (7, 21). In *S. cerevisiae*, β (1,3)-glucan is made by one of two synthase complexes containing either Fks1p or Fks2p (the catalytic subunit) and Rho1p (regulator) (5, 7). The two catalytic subunits have different roles but can partially substitute for each other. Disruption of both *FKS1* and *FKS2* is lethal (18, 28, 29). In *C. albicans*, there are also two *FKS* genes (6). In *Schizosaccharomyces pombe*, there are four *FKS* genes, although it is not yet clear which of them is responsible for glucan synthase activity (13). Single-copy *FKS* genes have also been identified in *Neurospora crassa* (27), *Aspergillus nidulans* (14), *Aspergillus fumigatus* (1), *Cryptococcus neoformans* (33), *Paracoccidioides brasiliensis* (22) and *Yarrowia lipolytica* (15). All fungal *FKS* enzymes so far examined have highly conserved amino acid sequences, and single-copy *FKS* genes are essential. To better understand CFA resistance in *Fusarium* spp., we cloned and sequenced the *F. solani* f. sp. *pisi* gene encoding the 1,3- β -glucan synthase (FsFKS1), used RNA interference (RNAi) to reduce its expression, and complemented the deletion of the essential *fks* gene of the CFA-sensitive fungus *A. fumigatus* with FsFKS1.

MATERIALS AND METHODS

Strains, media, and growth conditions. *F. solani* f. sp. *pisi* isolate 156-30-6 (*Nectria hematococca* MPVI, 156-30-6, ATCC 204495), purchased from the Fungal Genetics Stock Center, was grown and maintained on potato dextrose agar medium at room temperature (25°C). For the RNAi study, *F. solani* f. sp. *pisi* transformants were cultured in minimal medium (140 mM NaNO₃, 14 mM KCl, 4 mM MgSO₄, 19m M KH₂PO₄, 20m M K₂HPO₄ [pH 7.5 with NaOH]) supplemented with 100 mg/ml hygromycin B and either 0.1 M glycerol and 0.3 M ethanol for induction or 2% glucose and 0.5% yeast extract for repression. *Aspergillus fumigatus* AF293.1 is a clinical isolate provided by Gregory May (M.D. Anderson Health Science Center, Texas). It was grown and maintained on

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Mag medium (2% malt extract, 2% dextrose, and 0.2% peptone with 1 ml vitamin mixture) containing 10 mM uracil and 5 mM uridine with or without 1.6% agar. The vitamin mixture contains 0.01 g biotin, 0.01 g pyridoxine HCl, 0.01 g thiamine HCl, 0.01 g riboflavin, 0.01 g *p*-aminobenzoic acid, and 0.01 g nicotinic acid (or niacin) per 100 ml. *A. fumigatus* transformants were selected on complete medium (2.5% glucose, 0.5% yeast extract, and 1.5% agar with 2 ml vitamin mixture) with 10 mM uracil, 5 mM uridine, and 150 mg/ml hygromycin B. *Escherichia coli* XL1-Blue and TOP 10 cells (Stratagene) were used for cloning.

PCR and screening of genomic library. To amplify FKS probes, PCRs were performed using the same degenerate oligonucleotide primers used to clone *FKS1* from *Yarrowia lipolytica* (15): FGS1, 5'-GGK TTY CAR MGR GAY WSY ATG MGM AAY ATG-3'; FGS3, 5'-CGG GGG AAG AAC TCG GTC TCA AAG G-3'; FGSD, 5'-GAY YTN CCN TWY TAY TGY ATN GGN TTY AAR-3'; and FGSR, 5'-RAA RTC NYY CCA NGC RAA YTG RTG NGG RTT-3' (R is A or G; Y is C or T; N is A, C, G, or T; and D is A, G, or T). PCR mixtures contained 10 ng of *F. solani* f. sp. *pisi* genomic DNA, 10 ng of each primer (FGS1 plus FGS3 and FGSD plus FGSR), 0.2 mM deoxynucleoside triphosphates, 1.5 mM magnesium acetate, 20 mM Tricine (pH 8.8), 85 mM potassium acetate, 8% glycerol, 2% dimethyl sulfoxide, and 2.5 U of *Taq* polymerase in a reaction volume of 50 μ l. PCR was carried out at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. PCR products were analyzed in a 0.8% agarose gel. PCR products of the expected size (1 to 2 kb) were isolated from the gel by using a gel extraction kit (QIAGEN), cloned into pGEM-Easy vector (Promega), and sequenced using the T7 sequencing primer. Probes were labeled with [α -³²P]dCTP by using RediprimeII (Amersham Pharmacia Biotech) and used to screen a cosmid library of *N. hemato-cocca* strain 156-30-6 (4). Hybridizations were performed using standard methods (26). Sequencing of the entire cosmid clone containing the *FKS1* gene was performed using the GPSTM-1 genome priming system (New England Biolabs). To obtain the full-length sequence of *FKS1*, rapid amplification of cDNA ends (RACE) was performed using the GeneRacer kit (Invitrogen). For 5' RACE, primer 5'-AAA GAA GCG CCT GGT TGG GGG TC-3' was used as a reverse primer in combination with the GeneRacer 5' primer provided in the kit according to the manufacturer's instructions. For 3' RACE, 5'-CAT TCT ATG CTG TTC TGG CTT CGT CCT-3' was used as a forward primer with the GeneRacer 3' primer.

DNA manipulations. Standard molecular biology techniques were used throughout for DNA manipulation and transformation in *E. coli* (26). To delete *FsFKS1*, the *hph* cassette was flanked by 1 kb upstream and downstream of *FsFKS1*, which was amplified by PCR using the cosmid clone 16C3 as a template. To construct the RNAi vector (pYH47), pTMH 78.1 (19) was modified. pRMH 78.1 was digested with EcoRI and SacI to remove 1 kb of the *gpd* promoter, followed by ligation with the *alcA* promoter flanked by EcoRI and SacI. The rest of the *gpd* promoter was removed by SacI and NcoI digestion, and a 500-bp fragment of *FsFKS1* (positions +2279 to +2778) was inserted right before 260 bp of green fluorescent protein linker. Finally, the same fragment was ligated into the BamHI-HindIII sites to introduce the same fragment of *FsFKS1* after the green fluorescent protein linker as an inverted repeat.

Fungal transformations. pYH47 was introduced into *F. solani* 156-30-6 by transformation of protoplasts as described previously (31) to produce RNAi transformants. *A. fumigatus* AF293.1 was transformed according to the method described by May (16) with several modifications. Germinated conidia from a 50-ml overnight culture were harvested and digested with 125 mg β -D-glucanase G (InterSpex) and 85 mg β -glucuronidase (Sigma) to generate protoplasts. Transformants were selected on minimal medium supplemented with uridine/uracil containing 300 μ g/ml of hygromycin for 3 days at 37°C.

For the introduction of *FsFKS1* into *A. fumigatus*, the cosmid clone 16C3, which contained the entire 5.3-kb *FsFKS1* gene within its 28-kb insert, was used. The hygromycin-resistant transformants were collected for further characterization. The pF4 vector used for the disruption of the endogenous *A. fumigatus* *fsk* gene was the gift of Anne Beauvis and Jean-Paul Latge (Institut Pasteur). It contains a 2.3-kb KpnI internal fragment of *fsk* and two fungal markers: *hph*, which confers hygromycin resistance, and *pyrG*, which confers uridine/uracil prototrophy (9).

Southern and Northern analyses. For Southern analysis, 40 μ g genomic DNA of *F. solani* or *A. fumigatus* was digested with restriction enzymes, and the resulting fragments were separated by electrophoresis in 0.8% agarose gels, transferred to MagnaGraph nylon filters (MSI), and hybridized by standard methods. For Northern analysis, 30- μ g aliquots of total RNA were mixed with 0.5 μ g ethidium bromide, 3.2 μ l formaldehyde, 3 μ l 10 \times MOPS buffer (0.2 M 3-*N*-morpholinopropanesulfonic acid [MOPS], 50 mM sodium acetate [pH 7], and 10 mM EDTA), and 3 μ l staining dye in a 30- μ l volume, heated at 75°C for

TABLE 1. In vitro activity of CFA on fungal isolates

Organism (no. of isolates tested)	MIC ₉₀ ^a (mg/liter)		Reference
	Geometric mean	Range	
<i>F. solani</i> f. sp. <i>pisi</i> (1)	40.2	34.9–45.4	This study
<i>F. solani</i> (5)	59.46	50–>100	17
<i>F. oxysporum</i> (5)	75.78	50–>100	17
<i>H. capsulatum</i> (20)	16.6	8–32	14a
<i>C. neoformans</i> (10)	>16	16–>16	8a
<i>A. fumigatus</i> (13)	2.15	0.5–>16	24
<i>C. albicans</i> (40)	0.5	0.25–>0.50	23

^a Concentration of caspofungin causing 90% growth inhibition compared to a no-drug culture. MICs were obtained from turbidity measured at 24 h postinoculation. Results are based on five repetitions.

5 min, and electrophoresed in 1% agarose gels containing 5.4 ml formaldehyde in 1 \times MOPS buffer. RNA was blotted onto MagnaGraph nylon filters (MSI) and hybridized by standard methods. Radiolabeled signal was detected by exposing X-ray film (Kodak) at –80°C for 1 to 3 days. The 17S rRNA gene visualized by ethidium bromide was used as an internal control to ensure equivalent loading.

Caspofungin assay. The sensitivity of fungal strains to caspofungin was measured as previously described (25). Caspofungin stock solutions in distilled water were prepared at 10 times the strength of the final concentrations. The fungal strains were inoculated onto agar plates and incubated at room temperature for 5 days. The spores were collected by flooding the plate with distilled water, pipetting or scraping the surface with a spreader, and then withdrawing the resulting suspension with sterile pipette tips. The number of spores in suspension was counted using a hemacytometer and adjusted to 3 \times 10⁷ spores/ml. Working spore suspensions were made by adding 0.1 ml of spore suspension in potato dextrose broth to 0.8 ml potato dextrose broth and mixed with 0.1 ml of caspofungin stock. For the growth control, 0.9 ml of spore suspension was mixed with 0.1 ml of drug-free medium. All tubes were incubated without agitation at 30°C for 72 h as described by Pfaller et al. (24). The turbidity was measured at 580 nm every 24 h for the determination of *F. solani* f. sp. *pisi* isolate 156-30-6 caspofungin sensitivity and at 24 h postinoculation for the *A. fumigatus* *FsFKS1* transformants. Each experiment was repeated four or five times. MIC₉₀ values of *A. fumigatus* transformants bearing *FsFKS1* were assessed using SAS (version 7.0) to test whether increased resistance of *A. fumigatus* transformants is statistically significant. *P* values were calculated from *T* distributions for individual transformants and wild-type *A. fumigatus* AF 293.1 (degree of freedom, 2; tail, 2). The MIC₉₀ of each transformant was compared to those of all other transformants. All *P* values were statistically significant (*P* < 0.05).

Microscopy. All microscopic observation was performed using a Zeiss Axio-plan microscope and digital camera.

Nucleotide sequence accession number. The *FsFKS1* sequence has been deposited in GenBank (accession number DQ351540).

RESULTS

***F. solani* f. sp. *pisi* 156-30-6 is resistant to caspofungin.** To determine whether the plant pathogenic isolate *F. solani* f. sp. *pisi* 156-30-6 shows the same resistance to caspofungin as clinical *F. solani* isolates, spore suspensions were grown in medium containing various concentrations of caspofungin and incubated at 30°C. Based on culture turbidity at 580 nm at 24 h postinoculation, caspofungin at 40 mg/liter inhibited 90% of *F. solani* f. sp. *pisi* 156-30-6 growth (Table 1). This MIC₉₀ is 20-fold to 80-fold higher than MIC₉₀ values previously reported for *C. albicans* and *A. fumigatus*, respectively, and 53.0% to 67.6% of the values previously reported for *Fusarium oxysporum* and *F. solani* clinical isolates (17, 23, 24).

***F. solani* f. sp. *pisi* contains a 1,3- β -glucan synthase (FKS) ortholog.** All glucan synthases have highly homologous catalytic domains of 818 amino acids. Previously, degenerate primers were used to clone the 5' and central regions of the

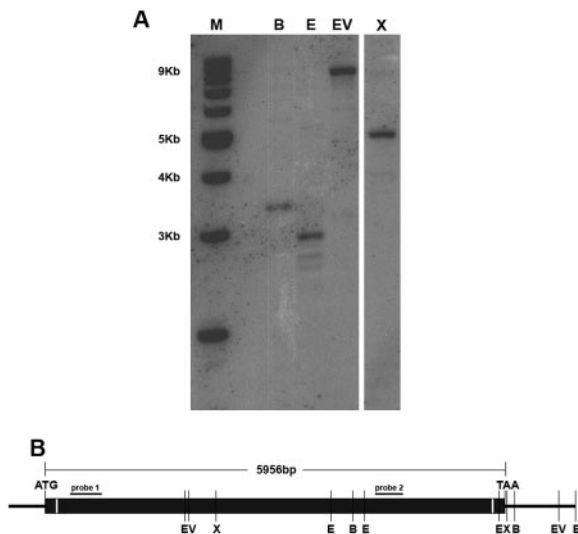


FIG. 1. *F. solani* f. sp. *pisi* FKS1 genomic region. (A) Southern hybridization of *F. solani* f. sp. *pisi* 156-30-6 genomic DNA with conserved FsFKS1 probe 2. *F. solani* f. sp. *pisi* 156-30-6 DNA was digested with BamHI (B), EcoRI (E), EcoRV (EV), and XhoI (X); separated along with 1-kb ladder (M) on a 0.8% agarose gel; and probed with radiolabeled FsFKS1 probe 2. (B) Restriction map of the chromosome region containing the FsFKS1 gene. PCR probes used for screening library are numbered 1 and 2. White boxes after ATG and before TAA indicate introns.

catalytic domain of the 1,3- β -glucan synthase (FKS1) from *Yarrowia lipolytica* (15). We used the same degenerate primers to amplify *F. solani* f. sp. *pisi* 156-30-6 genomic DNA. The resulting 2.0-kb and 1.3-kb amplicons were radiolabeled and used to probe 4,300 colonies from an *F. solani* genomic library (4). Among the seven clones that strongly hybridized to both probes, the clone (16C3) that displayed the largest insert based on restriction mapping and Southern hybridization was selected (data not shown). The entire 16C3 cosmid was sequenced using transposon sequencing. BLAST analysis of the 28-kb insert identified a 5,350-bp 1,3- β -glucan synthase domain in the middle of the insert. To identify the translational start and stop codons, 5' and 3' RACE was performed on RNA isolated from *F. solani* f. sp. *pisi* 156-30-6. The results of the RACE experiments revealed an ATG codon (759 bp upstream of the predicted 1,3- β -glucan synthase domain), a translation stop codon (bp +5911), and two short introns (50 to 55 bp), one near the ATG codon (+177 to +231) and one upstream of the translation termination codon (+5533 to +5583) (Fig. 1B). Since the rest of the sequence predicts a protein that aligns with other FKS genes with no reading frame shifts, these two are likely the only introns. The sequence encodes a predicted protein of 1,935 amino acids with a molecular mass of 220.77 kDa and a pI of 8.36. A search using the NCBI conserved-domain database returned only 818 amino acids of a 1,3- β -glucan synthase domain. The sequence displayed a high degree of similarity (75% to 88%) to the FKS homologs from *N. crassa* (ncu06871.1), *P. brasiliensis* (AF148715), *A. fumigatus* (AAB58492), and *S. cerevisiae* (U08459). We designated the sequence from *F. solani* f. sp. *pisi* FsFKS1. *F. solani* f. sp. *pisi* DNA probed with radiolabeled FsFKS1 at low stringency showed a single strong band and several much weaker bands

(Fig. 1A). These results suggest that there is at least one FKS gene in *F. solani* f. sp. *pisi*.

The predicted FKS1 protein from *F. solani* f. sp. *pisi* is highly similar to 1,3- β -glucan synthases from other fungi. However, the previously identified 8-amino-acid echinocandin binding domain (5) is modified in *F. solani* f. sp. *pisi*. All CFA-sensitive FKS proteins so far identified contain a Phe at the start of this domain, while in *F. solani* f. sp. *pisi* there is a Tyr (position 682).

A decrease in FsFKS1 mRNA reduces germination and causes cell lysis. To address the function of FsFKS1, we attempted disruption of the gene by homologous integration. *F. solani* f. sp. *pisi* was transformed with a hygromycin cassette flanked by 1 kb upstream and downstream of the FsFKS1 coding region. PCR analysis of 150 hygromycin-resistant transformants showed no homologous integration of the hygromycin cassette at the FsFKS1 gene locus (data not shown), suggesting that FsFKS1 is essential. Therefore, we attempted to silence the FsFKS1 gene by using an inducible RNAi construct. A vector previously developed for RNAi in *A. nidulans*, pTMH78.1 (19), was modified by the addition of the *hph* gene and the *alcA* promoter, which is induced by 0.3 M ethanol and/or 0.1 M glycerol and repressed by 2% glucose (35). The 500-bp FsFKS1 conserved region (positions +2279 to +2778) was introduced as an inverted repeat under the regulation of the *alcA* promoter, yielding vector pYH47 (20). pYH47 was transformed into *F. solani* f. sp. *pisi* 156-30-6, and 20 hygromycin-resistant colonies that were indistinguishable from the wild type on solid repression medium were selected for further analysis. When the spores from these transformants were inoculated onto solid *alcA* induction medium, the transformants did not display an obvious phenotype. However, when spores from the RNAi transformants were inoculated into liquid *alcA* induction medium, the germination rates ranged from 0 to 10% ($n > 300$) (Table 2). The germination rates of spores from wild-type *F. solani* f. sp. *pisi* in liquid induction or repression medium and of spores from RNAi transformants in liquid repression medium were >98% ($n > 300$). Microscopic observation of the RNAi transformants in *alcA* induction medium showed lysis of most ungerminated spores and the release of intracellular contents (Fig. 2 and data not shown). Interestingly, in lysed spores, cell debris was always visible at the poles where the initial germ tubes emerge (Fig. 2B). In the few spores that did not lyse, germ tubes were approximately 3 times wider than in the wild type, and some cell compartments were swollen (Fig. 2C). These results suggest that a reduction of 1,3- β -glucan in transformants weakens the cell walls of spores and young germlings, especially in areas of new growth.

To determine the function of FsFKS1 in later hyphal growth, RNAi transformants were cultured in repression medium for 48 h and transferred to induction medium for 8 h. The wild-type strain continued normal hyphal growth (Fig. 2D). However, the RNAi transformants displayed swelling, lysis, and release of intracellular contents at about half of their hyphal tips and branch points ($n = 100$) (Fig. 2E and F). These results suggest that the reduction of 1,3- β -glucan weakens the cell walls of mature hyphae, especially in areas of new growth.

In individual FsFKS1/RNAi transformants, the severity of germination inhibition in spores correlated with the severity of swelling and lysis in hyphae (Table 2). To determine if the

TABLE 2. Phenotypes of *FsFKSI*/RNAi transformants on *alcA* induction medium

Strain	% Germination (<i>n</i> > 300 spores)	Hyphal phenotype ^a (abundance of swollen hyphae)
156-30-6 (wild type)	>98	Normal
<i>FsFKSI</i> /RNAi 7801 (empty vector)	>98	Normal
<i>FsFKSI</i> /RNAi 1	1.0	Severe
<i>FsFKSI</i> /RNAi 2	1.9	Severe
<i>FsFKSI</i> /RNAi 3	0	Severe
<i>FsFKSI</i> /RNAi 4	7.7	Moderate
<i>FsFKSI</i> /RNAi 5	7.3	Moderate
<i>FsFKSI</i> /RNAi 6	7.5	Moderate
<i>FsFKSI</i> /RNAi 7	5.9	Moderate
<i>FsFKSI</i> /RNAi 8	9.9	Moderate
<i>FsFKSI</i> /RNAi 9	1.9	Severe
<i>FsFKSI</i> /RNAi 10	9.4	Moderate
<i>FsFKSI</i> /RNAi 11	8.2	Moderate
<i>FsFKSI</i> /RNAi 12	10.0	Moderate
<i>FsFKSI</i> /RNAi 13	9.7	Moderate
<i>FsFKSI</i> /RNAi 14	3.2	Severe
<i>FsFKSI</i> /RNAi 15	9.9	Moderate
<i>FsFKSI</i> /RNAi 16	9.8	Moderate
<i>FsFKSI</i> /RNAi 17	6.2	Moderate
<i>FsFKSI</i> /RNAi 18	1.6	Severe
<i>FsFKSI</i> /RNAi 19	0.0	Severe
<i>FsFKSI</i> /RNAi 20	9.7	Moderate

^a Normal, no lysis of hyphae; moderate, lysis in ~20% of hyphae; severe, lysis in ~50% of hyphae.

range of phenotypes in transformants was a reflection of the *FsFKSI* transcript level, Northern analysis was performed. More *FsFKSI* transcript was present in the transformants with less severe phenotypes (Fig. 3, lanes 2 and 3) than in those with more severe phenotypes (Fig. 3, lanes 4 and 5). These results in combination with the inducibility of the aberrant morphology suggest that the mutant phenotypes are a result of reduction in *FsFKSI* transcript levels rather than disruption of some other gene by the RNAi vector.

***FsFKSI* can replace *A. fumigatus fks*.** We attempted to determine whether reducing the expression of *FsFKSI* in RNAi transformants altered *F. solani* f. sp. *pisi* resistance to CFA by measuring the MIC₉₀ values for these strains, but they lysed almost completely during 24 h of incubation in induction me-

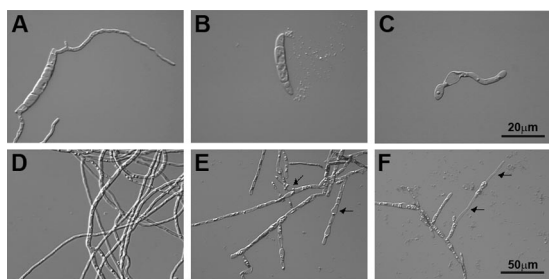


FIG. 2. Spores, germ tubes, and hyphal morphology of wild-type and *FsFKSI*/RNAi transformants. (A and D) Wild type; (B, C, E, and F) *FsFKSI* RNAi transformants. (A to C) Spores were inoculated into liquid induction medium and incubated for 18 h. (D to F) Cultures were grown in liquid repression medium for 48 h and transferred to induction medium for 24 h. Arrows indicate swollen cells and empty compartments.

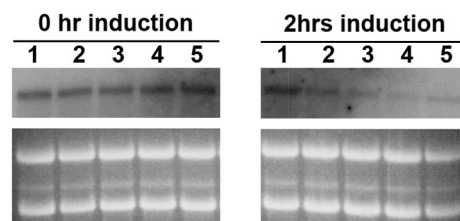


FIG. 3. Northern analysis of *FsFKSI* in wild-type and *FsFKSI*/RNAi transformants. Cultures were grown in liquid repression medium for 48 h and transferred to liquid induction medium. Lanes: 1, wild-type strain with empty vector; 2 and 3, RNAi transformants showing moderate phenotypes (*FsFKSI*/RNAi 4 and *FsFKSI*/RNAi 5, respectively); 4 and 5, transformants showing severe phenotypes (*FsFKSI*/RNAi 3 and *FsFKSI*/RNAi 9, respectively).

dium without CFA, making the drug inhibition measurements impossible. As an alternate approach to determine the contribution of *FsFKSI* to the CFA resistance of *F. solani* f. sp. *pisi*, we transformed the 16C3 cosmid into a CFA-sensitive fungus, *A. fumigatus* AF293.1. After 8 h of incubation on complete medium containing hygromycin, transformants were overlaid with complete medium top agar containing caspofungin. Thirty hours after overlay, *A. fumigatus* transformed with empty vector had not grown through the top agar (Fig. 4A, panel 1), while *A. fumigatus* transformed with *FsFKSI* had grown through the top agar and conidiated (Fig. 4A, panels 2 to 6).

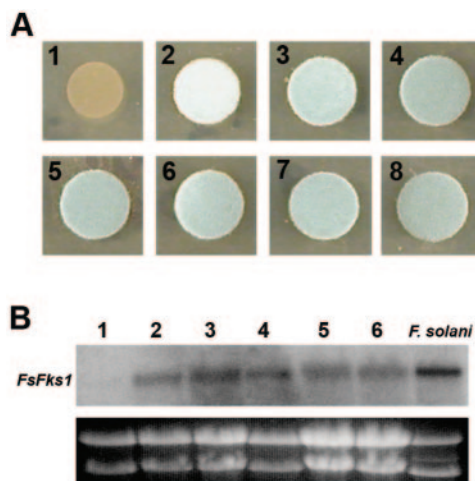


FIG. 4. *A. fumigatus* wild-type strain and *FsFKSI* transformants on caspofungin-containing medium. (A) Colony phenotype and (B) Northern hybridization of *FsFKSI* in wild-type *A. fumigatus* and *FsFKSI* transformants. (A) Wild-type *A. fumigatus* and *FsFKSI* transformants were inoculated in medium containing hygromycin as a selection marker. After colonies were visible, plates were overlaid with agar containing caspofungin. 1, *A. fumigatus* containing empty vector pAN7-1; 2 to 6, *A. fumigatus* transformed with *FsFKSI*, 2, *A. fumigatus*/*FsFKSI*-12; 3, *A. fumigatus*/*FsFKSI*-16; 4, *A. fumigatus*/*FsFKSI*-18; 5, *A. fumigatus*/*FsFKSI*-19; 6, *A. fumigatus*/*FsFKSI*-20; 7, *A. fumigatus*/*FsFKSI*-pF4 ectopic integrant 13 (*A. fumigatus*/*FsFKSI*-18 in which an *Af**fks* gene disruption construct was ectopically integrated); 8, *A. fumigatus*/*FsFKSI*-*Af**fks*1 disruptant 29 (*A. fumigatus*/*FsFKSI*-18 in which the *Af**fks* gene is disrupted); 7 and 8, colonies incubated on minimal medium containing hygromycin and caspofungin. (B) Lanes 1 to 6, as in panel A. The lower panel shows total RNA stained with ethidium bromide.

TABLE 3. In vitro activity of CFA on *A. fumigatus*/FsFKS1 transformants

Strain	MIC ₉₀ ^a (mg/liter)	
	Geometric mean	Range
<i>A. fumigatus</i> AF293.1	0.54	0.49–0.58
<i>A. fumigatus</i> /FsFKS1-12	1.52	1.48–1.55
<i>A. fumigatus</i> /FsFKS1-18	2.21	2.07–2.33
<i>A. fumigatus</i> /FsFKS1-19	1.21	1.05–1.29
<i>A. fumigatus</i> /FsFKS1-20	0.95	0.89–1.04
<i>A. fumigatus</i> /FsFKS1-pF4 ectopic integrant 13	1.72	1.59–1.79
<i>A. fumigatus</i> /FsFKS1-Affks1 disruptant 29	2.00	1.93–2.01

^a Concentration of CFA causing 90% growth inhibition compared to a no-drug culture. MICs were obtained from turbidity measured at 24 h postinoculation. Results are based on four repetitions.

All *A. fumigatus* transformants carrying FsFKS1 consistently had two- to fourfold-higher MIC₉₀ values with CFA than the wild type (Table 3). Because the 40-kb FsFKS1 circular cosmid 16C3 was used to transform *A. fumigatus* and because the cosmid integration site was unknown, copy number analysis by the usual PCR or Southern strategies was not possible. However, Northern analysis showed that the FsFKS1 transcript was present in all *A. fumigatus* transformants at much lower levels than seen in *F. solani* f. sp. *pisi* (Fig. 4B).

To determine whether the *F. solani* f. sp. *pisi* FKS1 gene could substitute for the *A. fumigatus* *fks* gene, we exploited the pF4 vector, which was previously developed to disrupt *fks* in *A. fumigatus*. Using pF4, Firon et al. (9) disrupted *fks* in an *A. fumigatus* diploid by homologous recombination and through breakdown of the diploid showed that *fks* is an essential gene in *A. fumigatus*. We transformed an *A. fumigatus* haploid strain carrying FsFKS1 (*A. fumigatus*/FsFKS1-18) with pF4. PCR analysis revealed one transformant (*A. fumigatus*/FsFKS1-Affks disruptant 29) that produced the predicted 2.6-kb fragment amplified from the integration of pF4 at the *fks* locus (Fig. 5). Our ability to delete the essential *fks* gene shows that FsFKS1 functionally complements *fks* in *A. fumigatus*. *A. fumigatus*/FsFKS1-Affks disruptant 29 overlaid by caspofungin-containing top agar grew through the top agar and conidiated (Fig. 4A, panel 8) and showed a small decrease in the CFA MIC₉₀ relative to that of *A. fumigatus*/FsFKS1-18 (Table 3).

These results show that the 1,3- β -glucan synthase from *F. solani* f. sp. *pisi* can substitute for the 1,3- β -glucan synthase from *A. fumigatus* and that at best it is only slightly more resistant to CFA.

DISCUSSION

There is at least one FKS isoform in *F. solani* f. sp. *pisi*. One possible explanation for the increased resistance of *F. solani* to CFA might be the presence of multiple FKS isoforms. We detected a single strong band in low-stringency hybridizations of *F. solani* DNA probed with radiolabeled FsFKS1, though several much weaker bands are also visible (Fig. 1A). All four PCR products amplified from *F. solani* by using fully degenerate FKS primers had identical sequences. Several filamentous fungi with complete genome sequences, including *N. crassa*, *A. nidulans*, *A. fumigatus*, and *C. neoformans*, have a single FKS

gene. A search of the genome of *Fusarium graminearum*, the only *Fusarium* species whose whole genome has been sequenced, revealed a single FKS gene. Taken together, these data suggest that FsFKS1 is the only FKS gene in *F. solani* f. sp. *pisi*, though we cannot rule out the possibility that other FKS genes might be present.

FKS1 from *F. solani* f. sp. *pisi* is similar to FKS in CFA-sensitive fungi. Another possible explanation for the increased resistance of *F. solani* f. sp. *pisi* to CFA might be changes in key amino acid residues. The predicted FKS1 protein from *F. solani* f. sp. *pisi* is highly similar (75 to 88%) to 1,3- β -glucan synthases from other fungi in deduced amino acid sequence, predicted secondary structure, and intron positions (5). However, within a previously identified 8-amino-acid echinocandin binding domain (5, 8), Tyr₆₈₂ is a Phe in the equivalent position in the CFA-sensitive fungi *S. cerevisiae*, *C. albicans*, and *A. fumigatus*. However, this conservative Tyr-for-Phe substitution seems unlikely to be the source of CFA resistance of *F. solani* f. sp. *pisi*. *C. neoformans* and *Histoplasma capsulatum*, both of which are relatively resistant to CFA, have Phe at this position. In addition, genome searches show that the FKS1 ortholog in *F. graminearum* also contains Tyr in this position, raising the possibility that this is a genus-specific substitution.

The product of FsFKS1 is important for cell wall synthesis. Another possible explanation for the increased resistance of *F. solani* to CFA might be that FsFKS1 plays a different role or

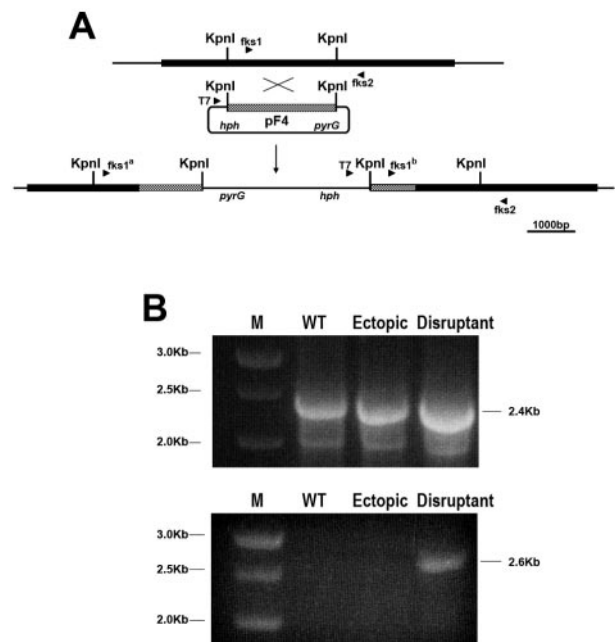


FIG. 5. *Affks* genomic region. (A) Diagram and (B) PCR analysis of *Affks* genomic region in wild type and in gene disruptant. (A) The KpnI fragment of *Affks* (stippled box) was ligated into a vector carrying selectable markers to produce pF4. *fks1*^a and *fks1*^b are identical primers annealing to endogenous *Affks* (*fks1*^a) or to the identical sequence derived from pF4 (*fks1*^b). (B) Upper panel, 2.4-kb PCR products amplified with *fks1* and *fks2* primers. Lower panel, 2.6-kb PCR products generated by T7 and primer *fks1*^b, indicating disruption of the *Affks* gene. M, 1-kb marker; WT, *A. fumigatus* AF293.1; Ectopic, *A. fumigatus*/FsFKS1-pF4 ectopic integrant 13; Disruptant, *A. fumigatus*/FsFKS1-Affks1 disruptant 29.

that its product, 1,3- β -glucan, is less important in the wall of this fungus. Many attempts to delete *FsFKS1* by homologous integration of a marker were unsuccessful, suggesting that it might be an essential gene, as it is in *A. fumigatus* (9). We were able to recover *FsFKS1* knockdown strains by using RNAi with the inducible *alcA* promoter (35). This is the first reported use of the *alcA* promoter in *Fusarium* spp., though it has been used in other filamentous fungi (34, 35). When induced, *FsFKS1* knockdown strains showed swelling of hyphae, lysis of spores and hyphae, and a dramatic decrease in spore germination rates (Fig. 2; Table 2). The severity of phenotypes correlated with decreased *FsFKS1* mRNA levels (Fig. 3). Swollen cells were found mostly at hyphal tips or branches, where cell wall is newly synthesized. The emergence of germ tubes from spores also requires cell wall synthesis. In lysed hyphae and spores, cell contents often appeared to leak from these areas of new cell wall growth. Similar results have been reported for *A. fumigatus* (2). Caspofungin-treated *A. fumigatus* germlings showed more lysis at tips and branch points. Similarly, caspofungin-treated *C. albicans* cells lysed largely at the growing tips of buds (3). The apparent requirement for functional *FsFKS1*, the phenotypes of the *FsFKS1* knockdown strains, and the similarity of those phenotypes to CFA-induced changes in other fungi all argue that the product of *FsFKS1* is important for cell wall synthesis in *F. solani* f. sp. *pisi*, as it is in CFA-sensitive fungi.

***FsFKS1* makes *A. fumigatus* slightly more resistant to CFA.**

The increased resistance of *F. solani* f. sp. *pisi* to CFA could be derived from properties of the *FsFKS1* enzyme or from other factors, such as efflux of the drug or detoxification by the fungus. To distinguish between the inherent properties of *FsFKS1* and other factors, we transformed CFA-sensitive *A. fumigatus* AF293.1 with *FsFKS1*. All transformants assayed showed an improved ability to grow through CFA-containing top agar and a slight increase in MIC₉₀, even when the endogenous *A. fumigatus fks* was deleted (Fig. 4; Table 3). Though we were unable to determine the precise number of *FsFKS1* copies present, Northern blotting experiments showed that the *A. fumigatus* transformants had relatively low levels of *FsFKS1* expression and that the expression level did not directly correlate with the CFA resistance level (Fig. 4B).

The modestly increased MIC₉₀ of CFA for *A. fumigatus* carrying *FsFKS1* is 20- to 40-fold lower than the MIC₉₀ for the wild-type *F. solani* f. sp. *pisi*. Our results suggest, therefore, that the innate properties of *FsFKS1* are not sufficient to account for the high resistance of *F. solani* f. sp. *pisi* to CFA. While this paper was in revision, the *Nectria hematococca* genome was made public (<http://genome.jgi-psf.org/Necha1/Necha1.home.html>). In addition to *FsFKS1*, BLAST searches identified a second FKS with 63% identity to *FsFKS1* and 95% identity to *C. albicans* GSK2. Though it is not clear whether this second *FKS* gene is transcribed, since no corresponding expressed sequence tag was found, a second *FKS* could potentially be responsible for the increased CFA resistance of *F. solani* f. sp. *pisi*. Other possible explanations for the resistance to CFA include low intracellular drug accumulation, factors such as melanin, or differences in cell wall repair via the cell wall integrity pathway. Future work will address these possibilities.

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