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Regulation of Fumonisin Biosynthesis in *Fusarium verticillioides* by a Zinc Binuclear Cluster-Type Gene, *ZFR1*†

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***Fusarium verticillioides*, a pathogen of maize, produces a class of mycotoxins called fumonisins in infected kernels. In this study, a candidate regulatory gene, *ZFR1*, was identified in an expressed sequence tag library enriched for transcripts expressed by *F. verticillioides* during fumonisin B_1 (FB_1) biosynthesis. *ZFR1* deletion mutants exhibited normal growth and development on maize kernels, but fumonisin production was reduced to less than 10% of that of the wild-type strain. *ZFR1* encodes a putative protein of 705 amino acids with sequence similarity to the Zn(II)2Cys6 binuclear cluster family that are regulators of both primary and secondary metabolism in fungi. Expression of *ZFR1* in colonized germ and degermed kernel tissues correlated with FB_1 levels. Overexpression of *ZFR1* in *zfr1* mutants restored FB_1 production to wild-type levels; however, FB_1 was not restored in an *fcc1* (*Fusarium* C-type cyclin) mutant by overexpression of *ZFR1*. The results of this study indicate that *ZFR1* is a positive regulator of FB_1 biosynthesis in *F. verticillioides* and suggest that *FCC1* is required for *ZFR1* function.**

Fusarium verticillioides (Sacc.) Nirenburg (teleomorph: *Gibberella moniliformis* Wineland) is a phytopathogenic filamentous fungus that infects maize kernels, where it produces a group of mycotoxins known as fumonisins (24). Of the 15 fumonisin analogs isolated and characterized, fumonisin B_1 (FB_1) typically is found at the highest levels and is toxicologically the most important (22). Consumption of FB_1-contaminated maize causes leukoencephalomalacia in equids, pulmonary edema in swine, and liver cancer in rats (16, 30, 40). Because of the potential health risks, guidelines for fumonisin levels in food have been established by the U.S. Food and Drug Administration and by other government agencies worldwide (11).

The initial studies on the structure of fumonisins indicated that these compounds are metabolites of a polyketide pathway (12). Proctor and coworkers confirmed this when they isolated and characterized the *FUM1* gene, which encodes a polyketide synthase (26). Strains of *F. verticillioides* with a disrupted *FUM1* gene fail to produce detectable levels of fumonisins (26). These researchers further established that *FUM1* is one of 15 *FUM* genes clustered on chromosome 1 that are involved in fumonisin biosynthesis or possible self-protection from fumonisins (27). Included in the *FUM* cluster are genes encoding proteins similar to cytochrome P450 monooxygenases (*FUM6*, *FUM12*), dehydrogenases (*FUM7*, *FUM13*), an aminotransferase (*FUM8*), a dioxygenase (*FUM9*), a fatty acyl-coenzyme A synthetase (*FUM10*), a tricarboxylate transporter (*FUM11*), a peptide synthetase (*FUM14*), longevity assurance factors (*FUM17*, *FUM18*), and an ABC transporter (*FUM19*). Mutants with the clustered genes disrupted have aided in characterization of the fumonisin biosynthetic pathway. Butchko et al. (3) provided the first biochemical evidence directly linking a *FUM* gene (*FUM13*) to a specific reaction during fumonisin biosynthesis. The Fum13 protein, which has similarity to short-chain dehydrogenases/reductases, was found to catalyze the reduction of the C-3 carbonyl of the fumonisin backbone to a hydroxyl group.

A current focus of research is the molecular mechanisms that regulate fumonisin biosynthesis. For many fungal secondary metabolites, the structural genes responsible for biosynthesis are clustered, and specific regulatory genes have been found to reside in those gene clusters (reviewed by Keller and Hohn [14]). None of the 15 genes within the *FUM* cluster appears to have a regulatory function. However, two recently described genes (*PAC1* and *FCC1*) appear to impact the regulation of fumonisin biosynthesis (10, 34). Experimental evidence showed that a *PAC1* disruption mutant of *F. verticillioides* produced more FB_1 than the wild-type strain and that only the mutant produced FB_1 under alkaline conditions (10). The results indicated that Pac1 acts as a transcriptional repressor of fumonisin structural genes. *F. verticillioides* with a disrupted *FCC1* gene fails to produce FB_1 on maize kernels (34). The deduced product of *FCC1* is similar to C-type cyclins, a class of proteins involved in the transcriptional activation or repression of genes associated with stress responses and development (7, 17, 19). Interestingly, the strain with *FCC1* disrupted produces FB_1 when cultured on synthetic media buffered at acidic pH. How acidic pH restores the ability of the *fcc1* mutant to produce FB_1 is unknown.

Here we describe the isolation of a putative regulatory gene and characterize its effect on FB_1 biosynthesis. This gene, named *ZFR1*, encodes a polypeptide with significant homology to fungal proteins that contain a DNA binding motif consisting of a Zn(II)2Cys6 binuclear cluster (36). We introduced deletion mutations in the *ZFR1* gene of *F. verticillioides* and show that the *zfr1* mutants exhibited normal growth and conidiation when cultured on maize kernels, while FB_1 biosynthesis was

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severely impaired. The results presented in this report support the hypothesis that *ZFR1* is a positive regulator of genes involved in fumonisin biosynthesis by *F. verticillioides*.

MATERIALS AND METHODS

Strains and media. *F. verticillioides* strains 7600 (M3125; Fungal Genetics Stock Center, Kansas City, Kans.) and FT536 (*fcc1*Δ mutant) were stored in 20% glycerin at −80°C. For inoculum, the strains were grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.) at 28°C. For isolation of genomic DNA, fungi were grown in stationary YEPD medium (5 g of yeast extract per liter, 10 g of peptone per liter, 20 g of glucose per liter) at 28°C. For FB$_1$ analysis and RNA isolation, fungi were grown on defined liquid (DL) medium (33) and cracked maize kernels as previously described (10).

Experiments that examined growth and fumonisin production by the wild-type, *zfr1* deletion mutant, and *ZFR1*-rescued strains were conducted with DL medium, whole cracked maize kernels, and separated germ and degermed maize kernel fractions as previously described (32, 33). Comparisons of FB$_1$ production on cracked whole maize kernels to expression profiles of *ZFR1*, *FUM1*, and *FUM8* were performed as previously described (10). All experiments were conducted at least twice with the same results.

Nucleic acid isolation and analysis. Bacterial plasmids were isolated with a Qiagen miniprep DNA purification system (Qiagen, Valencia, Calif.). Fungal genomic DNA was isolated by methods previously described (41). For Southern analysis, *F. verticillioides* genomic DNA was digested with EcoRI, size fractionated on a 0.7% agarose gel, and transferred to a nylon membrane (Nytran; Schleicher & Schuell, Keene, N.H.) by standard procedures (20). Total RNA was extracted by an acid-phenol extraction procedure described by de Vries et al. (8). For Northern blot analyses, 10 μg of RNA was separated by electrophoresis through a formaldehyde-denaturing gel and transferred to a nylon membrane as previously described (20). The *F. verticillioides TUB2* (β-tubulin; GenBank accession number U27303; 43) was ^{32}P labeled and hybridized as a loading control. *F. verticillioides FUM1* (GenBank accession number AF155773; 26) and *FUM8* (GenBank accession number AF155773; 30) were ^{32}P labeled and hybridized together. All hybridization probes were labeled with the Prime-It II random prime labeling kit (Amersham Biosciences, Arlington Heights, Ill.). High-stringency hybridization was performed with 7% sodium dodecyl sulfate (SDS)–0.5 M sodium phosphate (pH 7.5)–10 mM EDTA (6) and followed by two 30-min washes, the first at room temperature in a solution of 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate) and 0.5% SDS and the second at 65°C in 0.2× SSC–0.5% SDS (20). Blots were exposed to a phosphorimaging screen and scanned with a Typhoon 9200 high-performance gel blot reader (Molecular Dynamics, Inc., Amersham Biosciences). Images were resized and adjusted for contrast with Adobe Photoshop software (Adobe Systems Incorporated, San Jose, Calif.).

Isolation of *ZFR1*. A 45-kb cosmid clone, designated pZFRcos1, was identified by screening a genomic library of *F. verticillioides*. Standard protocols were used to plate and screen the library (20) with a ^{32}P-labeled insert from expressed sequence tag (EST) clone wt_1_O18 (GenBank accession number CF452892).

Nucleotide sequence analysis. The nucleic acid sequence of *ZFR1* was obtained from plasmids pZFR-pst (6.1-kb) and pZFR-bam (6.3-kb). Both plasmids are subclones from cosmid pZFRcos1 (45-kb) and contain the full-length *ZFR1* sequence. DNA sequencing reactions were performed by the Plant-Microbe Genomics Facility, Ohio State University, Columbus.

To obtain cDNA clones of *ZFR1*, total RNA was extracted from strain 7600 grown on cracked maize kernels for 7 days. Ten micrograms of total RNA was used in the first-strand cDNA synthesis reaction mixture with Superscript II RNase H$^-$ reverse transcriptase (Invitrogen, Carlsbad, Calif.) in a 20-μl reaction volume incubated at 42°C for 2 h. Regions of the *ZFR1* cDNAs were amplified with the following PCR primer sets: zinc1 (5′-ATGCTCGTTGACCG-3′) and zinc2 (5′-GCTAAGCTCAGTAG-3′), based on the 5′ region (1.2-kb product), and zinc3 (5′-GTGAAGAACCAAAG-3′) and zinc4 (5′-ACGAGAACAGCTTTAG-3′), based on the 3′ region (1.4-kb product). The reaction conditions were the same as those described for the isolation of *ZFR1*. The PCR products were cloned into the pGEM T-easy vector and sequenced by the Purdue Genomics Core Facility, West Lafayette, Ind. All DNA sequences were analyzed and predicted amino acid sequences were deduced with MacDNASIS software (Hitachi Software Engineering America, Ltd., San Bruno, Calif.). Homology searches were conducted with the BLAST algorithm (1). The PSORTII program was used to analyze the deduced Zfr1 peptide sequence for predictions of subcellular localization (23). Multiple alignments were conducted with the ClustalW software (http://www.ch.embnet.org/software/ClustalW.html) (35).

Disruption of *ZFR1*. The *ZFR1* deletion vector (pZFR-Δ1) was constructed by insertion of a 1.4-kb HpaI fragment that contained a hygromycin resistance gene cassette from pCB1003 (Fungal Genetics Stock Center), into the EcoRV sites (405 bp apart in pZFR1-TA) that are located in the *ZFR1* coding sequence. pZFR-Δ1 was amplified by PCR with primers zfrV5 (5′-ACTGGCAGTCTCTTCAG-3′) and zfrV3 (5′-CGCATGCGATTGTG-3′). A 2.4-kb product that consisted of the 1.4-kb hygromycin marker surrounded by ∼500 bp of *ZFR1* flanking sequence was gel purified and used for fungal transformation.

A second *ZFR1* deletion plasmid (pZFR-Δ2) was constructed from plasmid pHYG-TA, which consists of the 1.4-kb hygromycin resistance gene cassette from pCB1003 cloned into the pGEM T-easy cloning vector (Promega). Regions (500 bp) located at both ends of *ZFR1* were cloned immediately up- and downstream of the hygromycin gene cassette contained within pHYG-TA. A 2.4-kb amplicon that contained the hygromycin gene cassette flanked by 500 bp of *ZFR1* was amplified from pZFR-Δ2 by PCR with primers zfrD1 (5′-GATATACCTGCCTG-3′) and zfrD2 (5′-TCATCTCATGCAGCG-3′) and used for fungal transformation.

Protoplasts of strain 7600 were obtained and transformed as described by Proctor et al. (26). Transformants were selected on regeneration medium containing hygromycin B (Roche Molecular Biochemicals, Indianapolis, Ind.) at 60 μg/ml. Transformants were screened by PCR with primers that distinguished homologous crossover events. Primers zincD5 (5′-GGACTCTGTACTTGTTCG-3′) and h3P (5′-CGATAGTGGAAACCGACG-3′) produced a 500-bp DNA product from the homologous crossover at the 5′ end of the insertion DNA, and primers zincD3 (5′-GACCTGTGAGAGGTAG-3′) and h5P (5′-GATCAGAAACTTCTCGACAG-3′) produced a 1,020-bp product from the 3′ end. Conditions for PCR amplification were the same as those described for the isolation of *ZFR1*, with the exception of the annealing temperature (54°C).

Two deletion mutants (ZN27ss and *zfr1*Δ), one generated from each type of deletion vector construct, were complemented with pZFRsub-G418. This plasmid was constructed by insertion of a 4.3-kb DNA fragment that spans *ZFR1* from 500 bp upstream of the ATG to 1.8 kb downstream of the translational stop codon into vector pUC-G418, which harbors a Geneticin resistance gene (21). The *ZFR1* fragment used to construct pZFRsub-G418 was obtained by PCR from pZFRcos1 in a reaction mixture containing a high-fidelity polymerase (*Pwo*; Roche Molecular Biochemicals) with primers zincA (5′-CGAATCTGCGTAACG-3′) and zincB (5′-ATCTTGAGAGACACG-3′). Conditions for amplification were as follows: 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, and 2.5 min at 72°C. Geneticin-resistant transformants were selected on regeneration medium containing Geneticin (Sigma Chemical Co., St. Louis, Mo.) at 75 μg/ml. Analysis of the transformants with primer sets zincA-zincB and zincD3-zincD5 (described above) confirmed that the transformants contained a full-length *ZFR1* gene (data not shown).

Overexpression of *ZFR1*. The entire coding sequence of *ZFR1* was amplified by PCR with primers ZFR-5p (5′-GAGTTACCATGGTTATGCTCGTTG-3′) and ZFR-3p (5′-TTCCTCCATGGTTTGCAGGTG-3′p) and a high-fidelity polymerase (*Pwo* polymerase; Roche Molecular Biochemicals); the resulting DNA fragment was cloned in frame immediately downstream of the *GPDA* promoter contained within pGPD-G418, a modified pNOM102 vector (28) that contains a 2.2-kb XbaI fragment from plasmid pSM334 that harbors a Geneticin resistance gene cassette (10). The resulting plasmid, pGPD-ZFR1, was used to transform both the *zfr1* and *fcc1* deletion mutants. Single conidia of Geneticin-resistant transformants were isolated, and integration of the pGPD-ZFR1 vector was confirmed by Southern blot analysis. Overexpression of *ZFR1* was verified by Northern blot analysis.

Fumonisin analysis. Fumonisins were extracted from cultures, and concentrations of FB$_1$ were determined by high-pressure liquid chromatography (HPLC) as previously described (33). Briefly, fumonisins in samples were extracted overnight with acetonitrile-water (1:1, vol/vol). The extracts were passed through C$_{18}$ solid-phase extraction columns (J & W Scientific, Folsom, Calif.). The fumonisins were eluted with 2 ml of acetonitrile-water (7:3, vol/vol), derivatized with *o*-phthaldehyde (Sigma Chemical Co.), and analyzed with an HPLC apparatus (Shimadzu Scientific Instruments, Inc., Kyoto, Japan) equipped with an analytical C$_{18}$ column (150 by 4.6 mm) and a variable-wavelength spectrofluorometric detector (excitation wavelength, 335 nm; emission wavelength, 440 nm). FB$_1$ was quantified by comparison with an FB$_1$ standard (Sigma Chemical Co.).

Nucleotide sequence accession numbers. The nucleic acid sequence and predicted amino acid sequence of *ZFR1* were submitted to the GenBank database and assigned accession number AY493199.

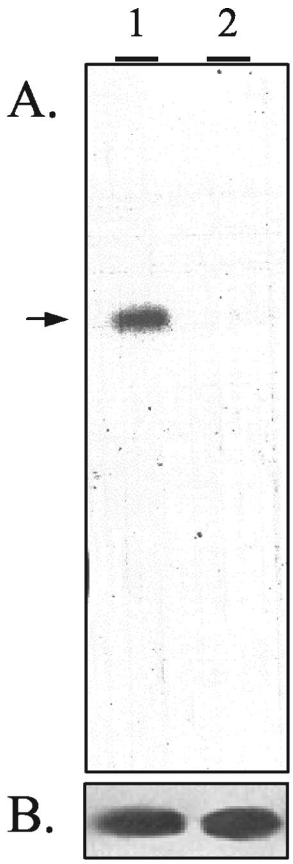

FIG. 1. Northern blot analysis of wild type and *fcc1* mutant cells cultured on cracked maize kernels. Total RNAs (10 μg) isolated from the wild-type strain (lane 1) and the *fcc1*Δ mutant strain (lane 2) cultured for 7 days were separated by electrophoresis in a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled *ZFR1*-specific probe (A) and a ^{32}P-labeled β-tubulin-specific probe (B).

RESULTS

Isolation and characterization of *ZFR1*. A cDNA subtraction library was previously constructed from RNA isolated from an *fcc1*Δ mutant and a wild-type strain of *F. verticillioides* cultured on cracked maize kernels (34). One of the 655 ESTs sequenced from the library enriched with transcripts from the wild-type strain exhibited high similarity to genes encoding known transcription factors harboring a Gal4-like zinc finger DNA-binding motif (18). This EST (wt_1_O18) was used as a probe to verify differential expression via Northern blot analysis of total RNA extracted from maize kernels colonized by a wild-type or *fcc1*Δ mutant strain (Fig. 1). The same probe was used to screen a cosmid genomic library of *F. verticillioides*, and a single 45-kb cosmid designated pZFRcos1 was isolated. Sequence analysis of a subclone of pZFR1cos1, designated pZFRsub1, revealed the entire coding region of *ZFR1*. Comparison of the genomic sequence of *ZFR1* to sequences from three overlapping cDNA clones revealed a single uninterrupted open reading frame that is predicted to encode a peptide of 705 amino acids. The deduced protein product of *ZFR1* contains (at amino acid residues 157 to 187) a conserved zinc finger DNA binding domain similar to that of known transcription factors such as Gal4 of *Saccharomyces cerevisiae* (18) and AflR of *Aspergillus flavus* (42). Zfr1 also contains the peptide sequence RRKDPSCDACRERKVKC beginning at amino acid 152, which fits the pattern of a bipartite nuclear localization signal (23). Zfr1 exhibits the highest overall similarity (22%) to Acu-15 of *Neurospora crassa* (2) and exhibits 8% similarity to Gal4 and 10% similarity to AflR.

Expression of *ZFR1*. Northern analysis was conducted with total RNA of colonized germ and degermed tissues that were inoculated with strain 7600 and incubated for 7 days. When probed with *ZFR1*, a ~2.4-kb band of hybridization was observed in the lane loaded with RNA isolated from the colonized degermed tissue (Fig. 2). No hybridization signal was detected for RNA isolated from colonized germ tissue (Fig. 2). FB_1 production was considerably higher in the degermed kernel fraction (280 ± 20 μg of FB_1/g of kernel tissue) than in the germ fraction (40 ± 2 μg of FB_1/g of tissue) (Fig. 2C). Thus, *ZFR1* transcript levels correlated with increased levels of fumonisin biosynthesis.

Disruption of *ZFR1*. *ZFR1* deletion mutants of strain 7600 were produced by homologous recombination. Of the 28 hygromycin-resistant transformants obtained, 1 contained a disrupted *ZFR1* gene. The disrupted strain was designated ZN27ss. Southern blots probed with *ZFR1* indicated a 1.0-kb larger band of hybridization in ZN27ss than observed for the wild type (Fig. 3). The increase in size corresponds to the replacement of 405 bp within *ZFR1* (nucleotides +1590 to +1995 relative to the translational start codon, ATG) with the 1.4-kb hygromycin gene cassette. PCR products generated from the ends of the hygromycin cassette indicated that the disruption occurred by a homologous double-crossover event (data not shown).

To rule out the possibility that ZN27ss may produce a functional (or partially functional) Zfr1 protein despite the gene disruption event described above, we constructed an additional vector (pZFR-Δ2) to delete nearly all of the coding sequences of *ZFR1* (−50 to +1933 relative to ATG). Of the 41 hygromycin-resistant colonies resulting from the transformation of strain 7600 with pZFR-Δ2, two contained a deleted *ZFR1* gene. The *zfr1*Δ mutants were not distinguishable from strain ZN27ss and exhibited the same fumonisin phenotype as strain ZN27ss on all of the substrates tested. Southern blot analysis of genomic DNAs isolated from both types of deletion mutations confirmed a homologous double-recombination event at the *ZFR1* locus (Fig. 3).

Impact of *ZFR1* on fumonisin biosynthesis. On whole cracked maize kernels, the wild-type strain produced 300 μg of FB_1/g of maize after 7 days and 450 μg after 14 days (Fig. 4A). In contrast, a ZFR1 deletion strain, ZN27ss, produced 25 μg of FB_1/g of maize after 7 days and 35 μg after 14 days of growth (Fig. 4A). FB_1 biosynthesis was restored in ZN27ss and the *zfr1*Δ mutant when they were transformed with a plasmid (pZFR-G418) containing a full-length *ZFR1* gene and a Geneticin resistance gene cassette. Southern analysis confirmed the presence of the *ZFR1* gene in the ZN27ss and ZFRΔ2 genomes (Fig. 3). The *ZFR1*-rescued strains were designated ZN27-R and ZFRΔ2-R. ZN27-R produced the same amount of FB_1 as the wild type over the duration of the time course (data not shown).

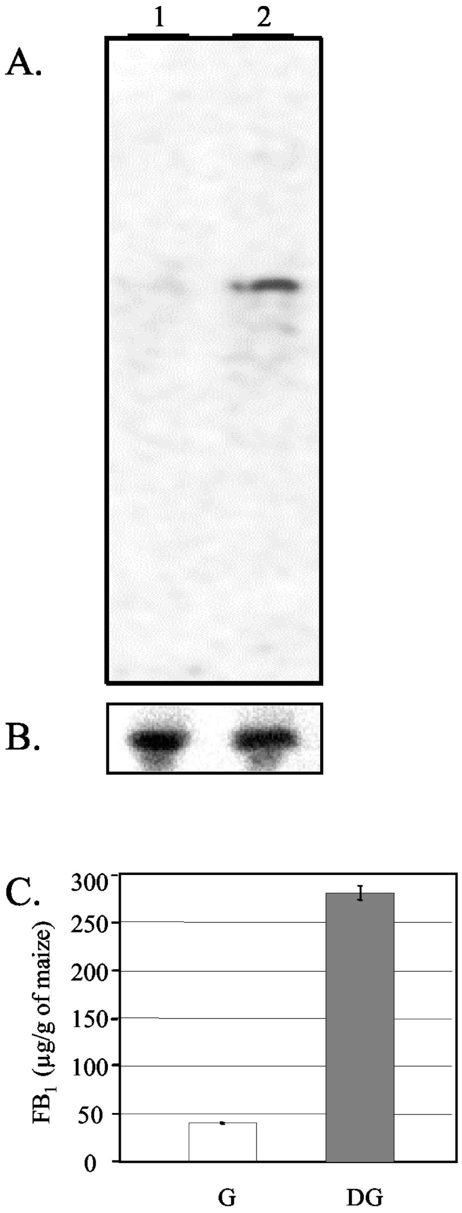

FIG. 2. Northern blot analysis and FB_1 production of the wild type cultured on separated maize kernel germ and degermed tissues. Total RNA (10 μg) isolated from the wild-type strain was cultured for 7 days on maize kernel germ (lane 1) and degermed (lane 2) fractions. The blot was probed with *ZFR1* (A) and *TUB2* (B) as a loading control. (C) Quantification of FB_1 as determined by HPLC extracted from germ (G) and degermed (DG) kernel fractions. Averages of three repetitions are shown with error bars showing standard errors.

FB_1 production was also examined on separated maize-kernel germ and degermed tissue fractions. On degermed kernels, the wild-type strain produced high levels of FB_1 (250 ± 25 μg/g of tissue), while on the germ fraction, lower levels of FB_1 were produced (26 ± 2 μg/g of tissue) (Fig. 4B). In contrast, the *zfr1* mutant exhibited reduced levels of FB_1 production (25 μg/g of tissue) on the degermed kernels compared to either the wild type or the rescued strain, but it produced nearly the same amounts of FB_1 on the germ tissue (Fig. 4B). Furthermore, the *zfr1* mutant failed to produce detectable levels of FB_1 when grown on DL medium while the wild-type and ZN27-R strains each produced in excess of 120 μg of FB_1/ml of medium (Table 1). The *zfr1* mutant produced mycelial mass equivalent to that of the control strains (Table 1). All of the experiments described above that included strains ZN27ss and ZN27-R were repeated to include strains ZFRΔ2 and ZFRΔ2-R, and the same results were obtained (data not shown).

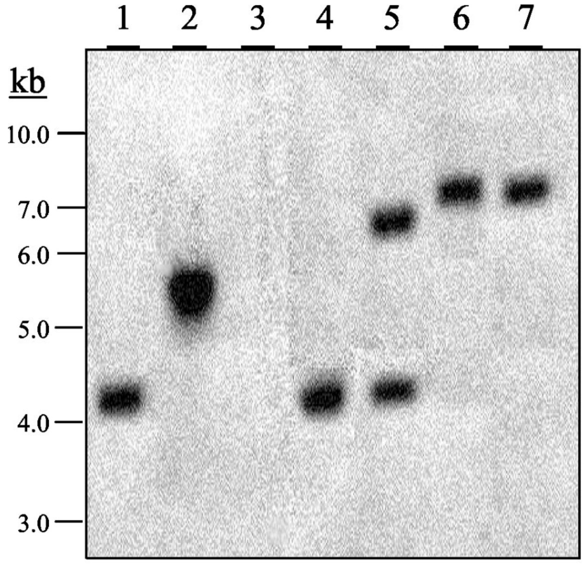

FIG. 3. Analysis of the *ZFR1* deletion and rescued strains. Shown is a Southern analysis of genomic DNAs (2 μg) from the wild-type (strain M-3125, lane 1), zn27ss (*zfr1* deletion strain 1, lane 2), *zfr1*Δ (*zfr1* deletion strain 2, lane 3), *fcc1*Δ (*fcc1* deletion strain, lane 4), *fcc1*Δ *GPD*::*ZFR1* (*fcc1*Δ mutant transformed with *GPD*::*ZFR1*, lane 5), *zfr1*Δ *GPD*::*ZFR1* (*zfr1* deletion strain 2 transformed with *GPD*::*ZFR1*, lane 6), and *zfr1*Δ *ZFR1* (*zfr1*Δ strain transformed with *ZFR1*, lane 7) strains digested with EcoRI, separated by electrophoresis in a 0.7% agarose gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled DNA fragment of *ZFR1*. Molecular size standards are indicated on the left.

Overexpression of *ZFR1*. The entire coding sequence of *ZFR1* was placed under the transcriptional control of the *GPDA* gene of *A. nidulans* (28) and introduced into the *zfr1*Δ and *fcc1*Δ mutant strains by transformation. Two transformants of each mutant were identified, and single-conidium isolates were obtained. Northern blot analysis confirmed that *ZFR1* transcript was overproduced (Fig. 5). In the *zfr1*Δ transformant with constitutive expression of *ZFR1*, FB_1 production was restored (Fig. 6), as well as transcription of *FUM1* and *FUM8* (Fig. 5). In contrast, constitutive expression of *ZFR1* in the *fcc1*Δ mutant failed to restore FB_1 biosynthesis (Fig. 6) or transcription of the *FUM* genes (Fig. 5).

DISCUSSION

The members of the Zn(II)2Cys6 binuclear cluster family of proteins are unique regulators of a wide range of processes in fungi, including primary and secondary metabolism (36). Among those described as positive regulators of secondary metabolism are AflR of *A. flavus*, Crg1 of *Cercospora nicotianae*, Pig1 of *Magnaporthe grisea*, and Cmr1 of *Colletotrichum*

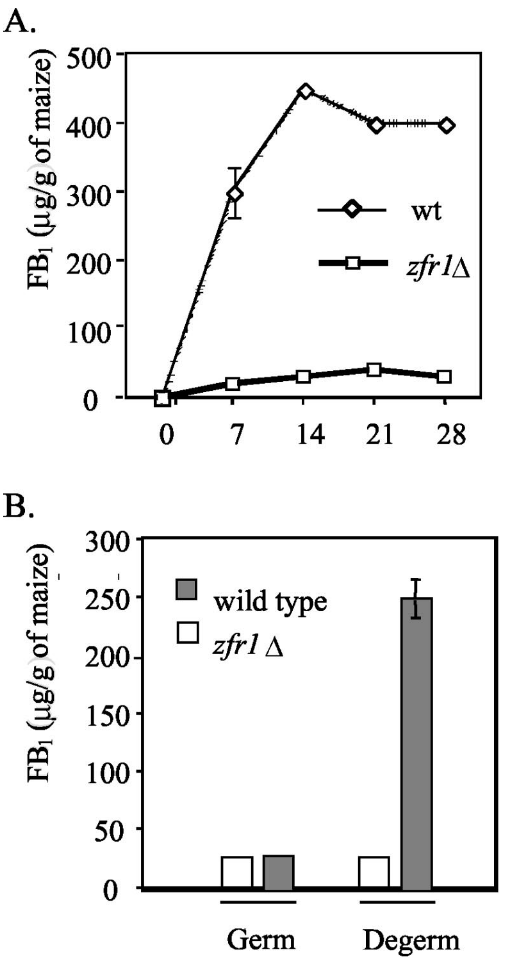

FIG. 4. FB$_1$ production by the wild type (wt) and the *zfr1* deletion mutant on whole cracked maize kernels and separated maize kernel germ and degermed tissues. (A) Wild-type and *zfr1*Δ mutant (*zfr1* deletion) strains cultured on whole cracked maize kernels for 28 days and analyzed for FB$_1$ every 7 days. (B) Wild-type and *zfr1*Δ mutant strains cultured for 7 days on separated germ and degermed maize kernel tissue fractions and analyzed for FB$_1$ production. All values are averages of three repetitions, and error bars indicate standard errors greater than 5% of the mean value.

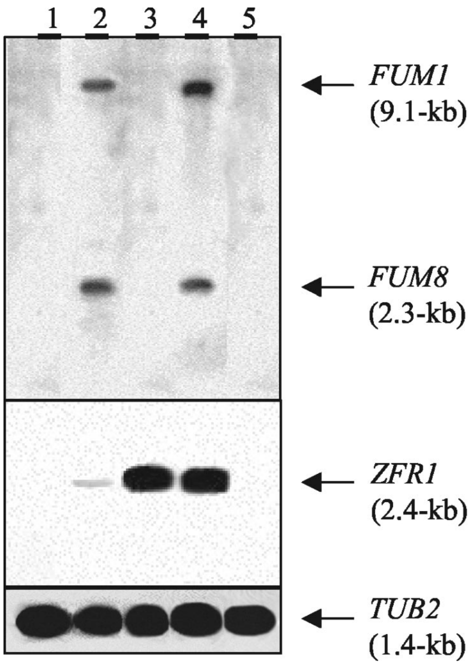

FIG. 5. Northern blot analysis of wild-type and *ZFR1* overexpression strains. Total RNAs (10 µg) isolated from the *zfr1*Δ mutant (*zfr1* deletion strain, lane 1), the wild type (lane 2), the *fcc1*Δ/*GPD*::*ZFR1* mutant (*fcc1*Δ mutant strain transformed with a *ZFR1* overexpression construct [*GPD*::*ZFR1*], lane 3), the *zfr1*Δ *GPD*::*ZFR1* mutant (*zfr1* deletion strain transformed with *GPD*::*ZFR1*, lane 4), and the *fcc1*Δ mutant (*fcc1* deletion strain) extracted from cultures grown for 7 days on whole cracked maize kernels were separated by electrophoresis in a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled *FUM1*- and *FUM8*-specific probes (top), a ^{32}P-labeled *ZFR1*-specific probe (middle), and a ^{32}P-labeled β-tubulin-specific probe (bottom).

lagenarium. The gene that encodes AflR is the pathway-specific regulatory gene of aflatoxin biosynthesis (9). Crg1 appears to be involved in the activation of genes associated with production of and resistance to cercosporin (5). Pig1 and Cmr1 are involved in controlling gene expression leading to melanin biosynthesis (40). In the study presented here, a cDNA with sequence similarity to the Zn(II)2Cys6 binuclear cluster family of proteins was identified among >700 sequences obtained from a subtraction library enriched for *F. verticillioides* transcripts expressed during fumonisin biosynthesis on maize kernels. The EST was used to isolate a genomic cosmid clone that contained the entire gene designated *ZFR1*. *F. verticillioides* contains a single copy of *ZFR1*, which possesses a single uninterrupted open reading frame of 2,115 nucleotides and is predicted to encode a peptide of 705 amino acids. Sequence analysis of Zfr1 reveals a DNA binding motif at the N terminus of the deduced peptide characteristic of the Zn(II)2Cys6 binuclear cluster family.

TABLE 1. Comparison of FB$_1$ production by wild-type and *zfr1*Δ, and *zfr1*Δ-*ZFR1* mutant strains on DL medium[a]

Strain	Mycelial mass[b]	FB$_1$[c]
Wild type	1.36 ± 0.20	120 ± 10
*zfr1*Δ	1.38 ± 0.18	ND[d]
*zfr1*Δ-*ZFR1*	1.30 ± 0.22	110 ± 20

[a] FB$_1$ was extracted from cultures grown for 14 days on DL medium (pH 5.6).
[b] Dry weight (grams) of fungal tissue after vacuum filtration is shown. Each value represents the mean of three replicates ± the standard error.
[c] Micrograms of FB$_1$ per milliliter. Each value represents the mean of three replicates ± the standard error.
[d] ND, none detected.

Zfr1 exhibits the highest amino acid identity to the acetate regulatory proteins of *A. nidulans* and *N. crassa* (FacB and Acu-15, respectively) (2, 37). *FACB* and *ACU-15* mutants fail to utilize acetate and therefore exhibit impaired growth when cultured on plates containing acetate as the sole carbon source (2, 37). Deletion of *ZFR1* by homologous double-crossover transformation resulted in mutant strains that exhibited normal growth and conidiation on cracked maize kernels. The mutants grew at the same rate as the wild type on medium containing acetate as the sole carbon source (data not shown), indicating that *ZFR1* is not orthologous to *FACB* or *ACU-15*.

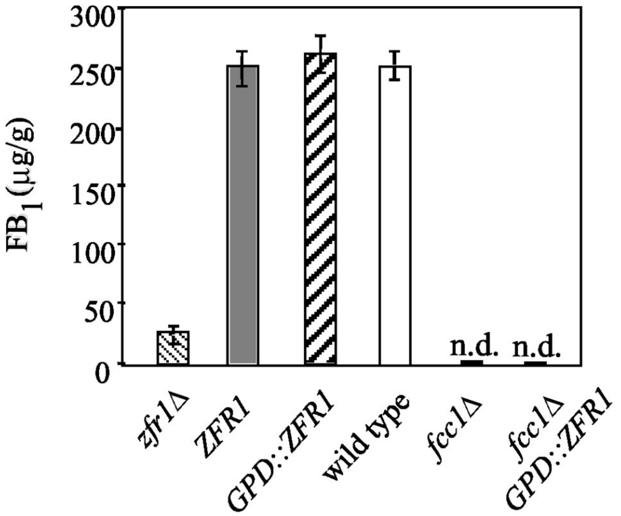

FIG. 6. Effect of *ZFR1* overexpression on FB$_1$ production. The wild-type, *zfr1*Δ mutant (*zfr1* deletion mutant), *zfr1*Δ-R mutant (*zfr1*Δ mutant transformed with *ZFR1*), *zfr1*Δ *GPD*::*ZFR1* mutant (*zfr1*Δ mutant strain transformed with a *GPD*::*ZFR1* overexpression construct), *fcc1*Δ mutant, and *fcc1*Δ *GPD*::*ZFR1* mutant (*fcc1*Δ mutant transformed with a *GPD*::*ZFR1* overexpression construct) strains were cultured for 7 days on whole cracked maize kernels and analyzed for FB$_1$ production. All values are averages of three repetitions, and error bars indicate standard errors. n.d. = none detected.

When cultured on DL medium otherwise conducive to FB$_1$ production by the wild type, the *ZFR1* mutants failed to produce detectable levels of FB$_1$ although they produced equivalent mycelial mass. Despite data from extensive growth experiments on cracked maize kernels and DL medium that suggest that *ZFR1* is not involved in fungal growth or development, *zfr1* mutants were observed to produce very few conidial chains when cultured on defined agar plates such as Czapek's solution agar (10) and carnation leaf agar (34) compared to the wild-type and *ZFR1*-rescued strains (data not shown). Therefore, we cannot rule out the possibility that *ZFR1* can conditionally influence conidiation. Interestingly, FB$_1$ production by the *ZFR1* mutants was less than 10% of the amount produced by the wild type when cultured on cracked maize kernels, and transcripts of *FUM1* and *FUM8* were not detectable in the mutants by Northern blot analysis. Fumonisin production was restored in the mutant strains when transformed with a functional copy of *ZFR1*. The observed effects of *ZFR1* on fumonisin biosynthesis are analogous to that of *AFLR* in *A. flavus* and *A. parasiticus* (4, 42). Strains containing a mutated *AFLR* gene produce little aflatoxin, and transcription of the aflatoxin pathway genes is not detectable by Northern blot analysis.

Previous studies have shown that the greatest amount of FB$_1$ is produced in the tissues of the degermed kernel (32). The concentration of FB$_1$ produced by wild-type *F. verticillioides* cultured on the degermed kernel fraction, which is composed of the seed coat, aleurone layer, and endosperm, was nearly 10 times greater than that produced on colonized germ tissue, even though both kernel fractions supported equivalent growth of the fungus (32). In this study, expression of *ZFR1* was greatest in the degermed kernel fraction. Furthermore, *zfr1* mutants failed to produce higher levels of FB$_1$ on degermed maize kernel fractions compared to the germ. These results indicate that elevated FB$_1$ production on the degermed tissue fraction requires a functional *ZFR1* gene.

A final question addressed by this study was if overexpression of *ZFR1* can restore FB$_1$ biosynthesis in an *fcc1* mutant. *ZFR1* was identified in a cDNA library that was constructed after subtractive hybridization with cDNAs from an *fcc1* disruption mutant that fails to produce FB$_1$ when cultured on cracked maize kernels (34). Northern blot analysis also indicated that no *ZFR1* transcript was detectable in the *fcc1* mutant. When grown on maize, two transformants of the *fcc1* mutant that overexpressed *ZFR1* failed to produce FB$_1$ or detectable transcripts of *FUM1* and *FUM8*. An explanation for the failure of constitutive *ZFR1* expression to restore FB$_1$ biosynthesis in the *fcc1* mutant may be that *FCC1* is required for transcription of the *FUM* genes, as well as *ZFR1*. Transcription of the *FUM* genes may also require activated Zfr1. Such a mechanism exists in yeast, where the C-type cyclin Srb11 (Ume3) is required for activation of the transcription factor Gal4, also a Zn(II)2Cys6 binuclear cluster protein (13). If a similar posttranslational activation mechanism exists in *F. verticillioides*, constitutive production of *ZFR1* would not necessarily lead to production of the active protein without the corresponding Fcc1 (cyclin)–cyclin-dependent kinase interaction. In addition, constitutive expression of *ZFR1* in the wild-type strain does not result in constitutive production of FB$_1$ in culture (data not shown), supporting the hypothesis that additional factors are required for Zfr1 activity. Further experiments are required to confirm that Zfr1 is a direct regulator of fumonisin pathway gene expression and also to prove that Zfr1 interacts with Fcc1 and/or a cyclin-dependent kinase.

On the basis of the evidence presented in this report, we conclude that *ZFR1* is a positive regulator of fumonisin biosynthesis. Because *ZFR1* is predicted to encode a protein that contains a DNA binding motif similar to that found in other fungal transcription factors such as Gal4 and AflR, it remains to be determined if Zfr1 binds to specific sequences in the promoter regions of the *FUM* genes. Other questions concerning specificity are raised because *ZFR1* does not reside within the *FUM* gene cluster, as there are several examples in fungi of close linkage of pathway-specific transcription factors to their respective structural genes. Furthermore, a DNA sequence with high similarity to that of *ZFR1* is present within the genome of *F. graminearum*, a non-fumonisin-producing species that does not harbor *FUM* gene sequences, which reinforces the plausibility that Zfr1 may not be a specific regulator of fumonisin pathway genes. Future experiments will address these questions of specificity, as well as determine the involvement of Fcc1 in regulating Zfr1 activity.

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