The *Fusarium verticillioides FUM* Gene Cluster Encodes a Zn(II)2Cys6 Protein That Affects *FUM* Gene Expression and Fumonisin Production[▽]

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Fumonisins are mycotoxins produced by some *Fusarium* species and can contaminate maize or maize products. Ingestion of fumonisins is associated with diseases, including cancer and neural tube defects, in humans and animals. In fungi, genes involved in the synthesis of mycotoxins and other secondary metabolites are often located adjacent to each other in gene clusters. Such genes can encode structural enzymes, regulatory proteins, and/or proteins that provide self-protection. The fumonisin biosynthetic gene cluster includes 16 genes, none of which appear to play a role in regulation. In this study, we identified a previously undescribed gene (*FUM21*) located adjacent to the fumonisin polyketide synthase gene, *FUM1*. The presence of a Zn(II)2Cys6 DNA-binding domain in the predicted protein suggested that *FUM21* was involved in transcriptional regulation. *FUM21* deletion (Δ*fum21*) mutants produce little to no fumonisin in cracked maize cultures but some *FUM1* and *FUM8* transcripts in a liquid GYAM medium. Complementation of a Δ*fum21* mutant with a wild-type copy of the gene restored fumonisin production. Analysis of *FUM21* cDNAs identified four alternative splice forms (ASFs), and microarray analysis indicated the ASFs were differentially expressed. Based on these data, we present a model for how *FUM21* ASFs may regulate fumonisin biosynthesis.

The discovery of two notorious fungal toxins, fumonisins produced by *Fusarium* and aflatoxins produced by *Aspergillus*, was precipitated by disease and death that resulted from unintended consumption of contaminated maize or maize products. The surge in research that followed revealed that both families of mycotoxins share a polyketide biosynthetic origin, followed by unique and long biochemical pathways. A major long-term goal of mycotoxin research is to understand the biosynthetic mechanisms in order to develop strategies that reduce the presence of mycotoxins in crops.

The maize pathogen *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) can infect maize throughout the world and can cause disease of ears, stalks, and seedlings (29). On some occasions, this fungus can synthesize the fumonisin family of mycotoxins, which, after ingestion, have been associated with a number of animal diseases, including cancer (15), and have been epidemiologically associated with human esophageal cancer in some regions of the world (23). Of recent concern are findings that fumonisins can disrupt neural tube development in rodents and, based on human epidemiological surveys, may increase the risks of human neural tube defects (24). Fumonisins are structurally similar to the sphingolipid sphinganine and disrupt sphingolipid metabolism by inhibiting the enzyme ceramide synthase (37 and references therein). Sphingolipids play a critical role in cell membranes and a variety of cell signaling pathways. Thus, disruption of sphingolipid metabolism may account for the multiple diseases associated with fumonisins.

Over the past decade, there have been significant advances in the understanding of the genetics and biochemistry of fumonisin biosynthesis (4, 9, 10, 13, 43). In general, fumonisins consist of a 19- or 20-carbon backbone with an amine, one to four hydroxyl, two methyl and two tricarboxylic acid constituents. Eighteen of the carbons that make up the fumonisin backbone are assembled by a polyketide synthase, an enzyme class required for the biosynthesis of numerous toxins, antibiotics, and other biologically active compounds produced by fungi. The linear polyketide precursor of fumonisins undergoes up to nine oxygenation, esterification, reduction, and dehydration reactions to form mature, biologically active fumonisins (9).

All fumonisin biosynthetic (*FUM*) genes characterized to date are localized within a 42.5-kb region of the *F. verticillioides* genome (32). The clustering of genes involved in the biosynthesis of secondary metabolites in filamentous ascomycetes is common. For example, biosynthetic gene clusters have also been identified for aflatoxin/sterigmatocystin (8) and lovastatin (17) in *Aspergillus* and trichothecenes (6) and for gibberellins (41) in *Fusarium*. In most cases, transcriptional regulation is governed by a pathway-specific DNA-binding protein encoded by a gene located within the cluster (16). Despite significant effort, no gene encoding a fumonisin specific regulator was found within the *FUM* cluster nor in the 11 to 22 kb of DNA flanking the cluster (32).

Four *F. verticillioides* genes—*FCK1*, *FCC1*, *PAC1*, and *ZFR1*—that are not located in the fumonisin gene cluster can affect fumonisin biosynthesis (2, 12, 13, 36). *FCK1*, encoding a C-type cyclin-dependent kinase, and *FCC1*, encoding a cyclin-like protein, interact and are part of a signal transduction

pathway that affects both fumonisin biosynthesis and morphogenesis (36). *PAC1* encodes a transcriptional activator or repressor of pH responsive genes and acts to repress transcription of *FUM* genes under alkaline conditions (12). *ZFR1* encodes a Zn(II)2Cys6 DNA-binding protein and markedly affects fumonisin production (13). The failure of constitutively expressed *ZFR1* in a Δ*fcc1* mutant to restore fumonisin biosynthesis suggests that the FCC1 protein, Fcc1p, may activate Zfr1p (13).

In collaboration with The Institute of Genomics Research (TIGR), we previously characterized over 87,000 expressed sequence tags (ESTs) from *F. verticillioides* (5). The ESTs represent as many as 11,000 different genes that may correspond to up to 81% of the genes in the *F. verticillioides* genome. In this report, we identified a previously undescribed gene from analysis of the ESTs. The new gene is located adjacent to *FUM1*, has eight introns, and is predicted to encode a protein with two motifs found in fungal DNA transcription factors. Gene disruption and complementation analyses indicate that the gene is a positive activator of *FUM* gene transcription and plays a critical role in fumonisin biosynthesis. Microarray analysis of alternative splice form (ASF) transcripts of the gene suggests that some are differentially expressed, a result consistent with the hypothesis that ASFs play a role in fumonisin biosynthesis (5).

MATERIALS AND METHODS

Strains, media, fumonisin production, and analysis. Strain M-3125 was the wild-type *F. verticillioides* strain used in the present study (19). Transformant strains were maintained on V-8 media containing 150 μg of either hygromycin (Sigma) or Geneticin (Gibco)/ml. DNA was prepared from *F. verticillioides* grown in YPG medium (0.3% yeast extract, 1% peptone, 2% glucose) at 28°C with shaking at 200 rpm for 4 days. RNA was prepared from strains grown in GYAM (0.24 M glucose, 0.05% yeast extract, 8 mM L-asparagine, 5 mM malic acid, 1.7 mM NaCl, 4.4 mM K_2HPO_4, 2 mM $MgSO_4$, and 8.8 mM $CaCl_2$; pH 3.0) medium for 2, 4, or 6 days. Fumonisin production was determined from 4-dram (2.5 g of cracked maize kernels and 1.2 ml of distilled water in a 4-dram vial and autoclaved) or flask cultures (10 g of cracked maize kernels and 4.2 ml of distilled water in a 50-ml flask and autoclaved).

Cracked maize medium was inoculated with a single mycelial plug from V-8 juice agar cultures of *F. verticillioides*. Cultures were incubated for 21 days in the dark at 28°C. Four-dram vial cultures were extracted with 12 ml, and flask cultures were extracted with 25 ml of 50% acetonitrile–50% water (vol/vol) and filtered through a 0.45-μm-pore-size Gelman filter. Culture extracts and filtrates were analyzed for fumonisins by liquid chromatography-mass spectrometry (LC-MS) as previously described (30, 31).

Nucleic acid sequence analyses. EST sequences in the *F. verticillioides* Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=f_verticill) were assembled with accession number AF155773 using Sequencher (version 4.1.4; Gene Codes Corp., Ann Arbor, MI). Accession AF155773 includes the 42.5-kb *F. verticillioides* fumonisin gene cluster, 22 kb flanking the *FUM1* end of the cluster, and 11 kb flanking the *FUM19* end of the cluster, for a total of 75 kb. The gene index is composed of 11,126 sequences derived from over 87,000 ESTs collected from 10 *F. verticillioides* cDNA libraries and 52 sequences from GenBank (5). TIGR sequence alignment software grouped the sequences into 7,198 overlapping contigs or clusters called tentative consensus sequences (TCs). None of the remaining 3,928 sequences overlapped with one another and were termed "singletons."

The TCs and singletons described in the present study were derived from ESTs from cDNA libraries FvF, FvG, and FvN (5). Library FvF and FvG were generated from mRNA prepared from GYAM cultures of strain M-3125 grown for 24 and 96 h, respectively. Library FvN was generated from mRNA from 4- and 6-day maize meal medium cultures of strain M-3125 (5). Sequence similarity searches of the nonredundant database maintained by the National Center for Biotechnology (NCBI) were performed by using the BLASTX, BLASTP, and RPS-BLAST programs (1, 44).

Gene disruption, transformation, and characterization of transformants. The deletion and genetic complementation of deletion mutants was done via the protoplast transformation method with Geneticin and hygromycin, respectively, as selectable markers (27, 33). The deletion plasmid was generated essentially as previously described (7, 10). Briefly, a 1.0-kb DNA fragment upstream of the target start codon and a similarly sized fragment downstream of the target stop codon were amplified via PCR and cloned separately into pT7Blue-3 cloning vector (Perfectly Blunt Cloning Kit; Novagen). The resulting construct contains both the upstream and the downstream regions separated by a unique AscI restriction site. The final disruption vector, pTFKOG-5, was generated by cloning a DNA fragment carrying *GenR*, which confers resistance to the antifungal agent Geneticin (39) into the AscI site between the two PCR products. Geneticin-resistant isolates recovered after transformation of strain M-3125 with vector pTF21KOG-5 were first screened by PCR for the presence of the Geneticin resistance gene with the primers rp617 (5′-CATGGCGCGCCATGCCAGTTG TTCCCAGTGATCT-3′) and rp652 (5′-TCGTGCACCAAGCAGCAGATGA-3′). Transformants were then screened by PCR for deletion of the Zn(II)2Cys6 gene coding sequence, which was expected to result from double homologous recombination between vector pTFKOG-5 and the Zn(II)2Cys6 locus. The PCR screen used two primer pairs, one for each side of the deletion construct. Each primer pair included one primer specific to *GenR* (primer rp673 5′-GCCAGG TAGGCCGAATAACTTGCAC-3′ or primer rp675 5′- GCGAAGCAGAAGA ATAGCTTAGCAG-3) and one primer specific to genomic DNA adjacent to the *F. verticillioides* DNA in the deletion vector (primer rb215 5′-AATGCTCGGT CCCAAGTTCC-3′ or primer rb217 5′-TGCCTTGAGGAGTAGACAGG-3′). PCR with the primer pairs rp673-rb215 and rp675-rb217 and DNA from transformants GmTcp172, GmTcp173, and GmTcp174 yielded amplicons of the size predicted to result from replacement of coding sequence with GenR. Loss of the coding sequence was verified by the failure of primer pairs rb215-rb219 (5′-AG GTACAGTACAAAGCAGTG-3′) and rb217-rb221 (5′-TCAATAATGTCCTA TGTGGG-3′) to amplify the amplicons produced from DNA from the wild-type progenitor strain.

Vector pTHF21 was constructed to complement the fumonisin nonproduction phenotype of *F. verticillioides* transformant GmTcp172. pTHF21 contained 738 bp of the *F. graminearum* translation elongation factor gene promoter (*TEFp*) fused to the *F. verticillioides* Zn(II)2Cys6 gene coding region. pTHF21 also included the *HygB* gene, which confers resistance to hygromycin B. The *TEFp* DNA fragment was generated by PCR from *F. graminearum* genomic DNA with the primers FgTEFF (5′-GCTATAGAACAGCTGTACTACTCC-3′) and FgTEFR (5′-GTGGCGCGCCGACGGTTGTGGATGGTTTG-3′) and cloned into pT7Blue-3 with the Perfectly Blunt Cloning Kit (Novagen). The *TEFp* fragment was then released by digestion with BamHI and HindIII and cloned into pUCH2-8 creating pTH1. pUCH2-8 contains *HygB* (42). The Zn(II)2Cys6 gene coding region and 3′ flanking DNA was amplified by PCR from *F. verticillioides* genomic DNA with the primers FvBF1EF (5′-GTGGCGCGCCTTGTT TTATATCTTATTCTACTTG-3′) and FvBF1ER (5′-GTGGCGCGCCCTCTC GTATATAACTACAGTTG-3′) cloned into pT7Blue-3 with the Perfectly Blunt Cloning Kit, creating pFUM21. The Zn(II)2Cys6 gene fragment was released from pFUM21 by digestion with AscI and cloned into the AscI site of pTH1, generating pTHF21.

Hygromycin- or Geneticin-resistant putative transformants were isolated from a single conidium prior to DNA and fumonisin analysis. Putative hygromycin transformant were examined by PCR with hygromycin-specific primers 741 (5′-GGATGCCTCCGCTCGAAGTA-3′) and 742 (5′-CGTTGCAAGACCTGCCT GAA-3′). Putative Geneticin transformants were examined by PCR with Geneticin-specific primers described above.

RT-PCR analysis of wild-type and deletion mutants. The wild type and the three deletion mutants (see above) were cultured in liquid fumonisin production medium, GYAM, for 2, 4, or 6 days. Mycelium was harvested by vacuum filtration, rinsed with sterile water, flash frozen in liquid nitrogen, and stored at −80°C. Samples were lyophilized prior to RNA extraction with the RNeasy minikit (QIAGEN). RNA was quantified spectrophotometrically, and an aliquot was treated with Turbo Free DNase (Ambion) prior to reverse transcriptase PCR (RT-PCR). RT-PCR was conducted using the StrataScript One-Tube System (Stratagene) with the following modifications. The total reaction volume was reduced to 20 μl: 2 μl of buffer, 1 μl of each primer, 1 μl of RNA template, 1 μl of DNA polymerase, 1 μl of RT, and 14 μl of water. Template RNA amount in each reaction was approximately 100 ng. The primers rp32 (5′-ACAAGTGTC CTTGGGGTCCAGG-3′) and rp33 (5′-GATGCTCTTGGAAGTGGCCTAC G-3′) were used to detect *FUM1* transcripts, the primers rp311 (5′-CGTGTGC ATCTGGCTCCTTTG-3′) and rp573 (5′-CGCGGATCCACCCAGGAAATCA CTACGA-3′) were used to detect *FUM8* transcripts, and the primers rp992
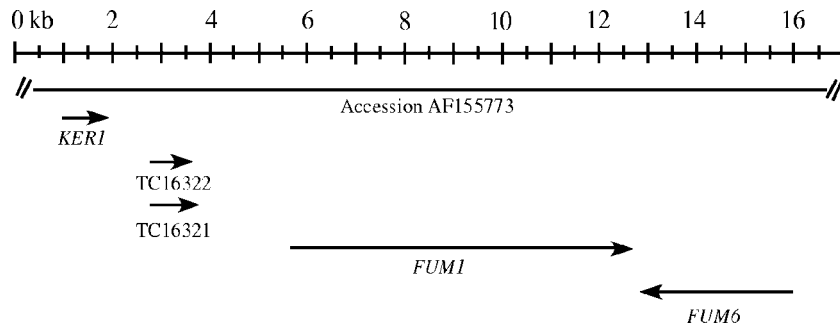
FIG. 1. Diagram of sequence alignment of TC16321 and TC16322 from *F. verticillioides* Gene Index Release 4.0 to a portion of the fumonisin cluster sequence and relative position of flanking gene *KER1*. *KER1* encodes a putative carbonyl reductase and is not likely involved in fumonisin biosynthesis since it is not expressed in a manner similar to cluster genes. *FUM1* and *FUM6* both encode proteins that are required for fumonisin biosynthesis.

(5′-ATGGGTAAGGARGACAAGAC-3′) and rp993 (5′-GGARGTACCAGT SATCATGTT-3′) were used to detect *TEF* transcripts.

Microarray analysis of Zn(II)2Cys6 gene expression. The development and initial characterization of a *F. verticillioides* NimbleGen Systems, Inc. (Madison, WI) microarray chip containing 16,250 probe sets has been described (5a). Briefly, wild-type *F. verticillioides* strain M-3125 was cultured in liquid fumonisin production medium, GYAM, for 12, 24, 48, 72, 96, or 120 h. Mycelium was harvested from duplicate cultures at each time point, and RNA was extracted as described above. Total RNA samples were tested for purity and integrity by NimbleGen using an RNA LabChip on an Agilent 2100 Bioanalyzer before labeling. Microarray hybridization, data acquisition, and normalization were conducted by NimbleGen. Normalized data from probe sets were compared from the multiple arrays using the Acuity 4.0 microarray analysis software package (Molecular Devices Corp.). Each probe set includes up to 12 24-mer perfectly matching oligonucleotide probes.

RESULTS

Characterization of ESTs located adjacent to *FUM1*. *F. verticillioides* Gene Index Release 4.0 included two overlapping TCs that aligned to genomic DNA sequence next to *FUM1* (Fig. 1). TC16321 was 880 bp and, compared to the genomic sequence, contained four gaps that ranged in size from 56 to 61 bp. The length of the gaps and the presence of typical fungal intron splice site sequences in three of four of the genomic regions corresponding to the gaps were consistent with the gaps being spliced introns. TC16322 was 774 bp and differed from TC16321 in that the first putative intron was 9 bp longer
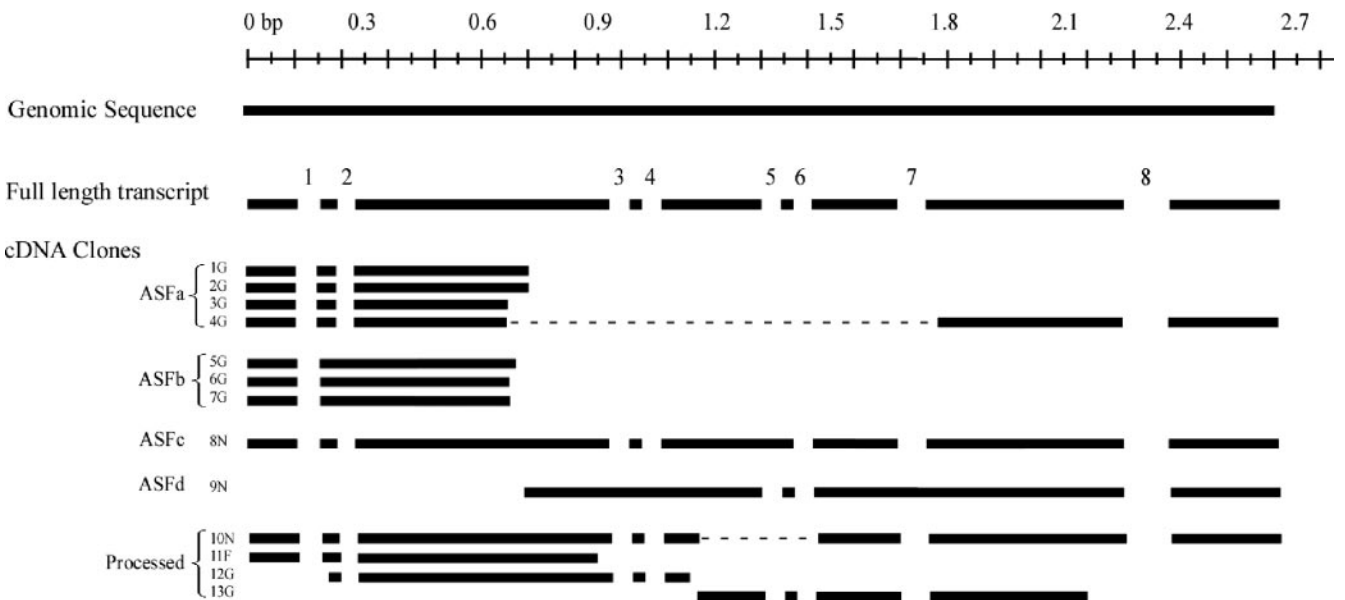
FIG. 2. Diagram of multiple sequence alignment of Zn(II)2Cys6 gene EST/cDNA sequences. The first bar under the base pair scale represents genomic sequence, and the second bar represents the full-length predicted Zn(II)2Cys6 transcript, where each numbered gap represents an intron. Translation of this full-length transcript would yield a 672-amino-acid protein. The next series of bars represent sequences of cDNAs from EST libraries FvF (11F), FvG (1G to 7G, 12G, and 13G) and FvN (8N to 10N). ASFa through ASFd represent ASFs of the Zn(II)2Cys6 transcript. ASFa transcripts, represented by four sequences, had nine fewer nucleotides in the first intron than other ASFs. ASFb transcripts, represented by three sequences, retained the second intron. ASFc, represented by one sequence, retained the fifth intron, and ASFd, represented by one sequence, retained the third, fourth, and seventh introns. Processed transcripts, represented by four sequences, had all of possible introns fully excised, based on available sequence data. Within a cDNA or ASF, dashed lines between two bars represent a portion not sequenced. The three ASFb ESTs were part of TC30496, while all remaining ASF ESTs were from TC30495.

FIG. 3. Partial sequence alignment of a representative of ASFa and ASFb and genomic sequence, showing the positions of the first and second introns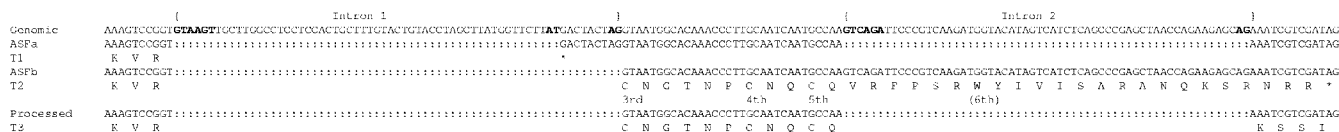 and the corresponding predicted amino acid sequence. The intron splice site sequences are indicated in boldface in the genomic sequence. T1, T2, and T3 refer to the putative translated protein. The number below each cysteine refers to the position of that cysteine in the Zn(II)2Cys6 motif. Excision of the shorter first intron in ASFa introduces a stop codon indicated by the asterisk. The failure to excise the second intron in ASFb results in a frameshift and termination of translation indicated by the asterisk. The putative 72-amino-acid protein would contain a tryptophan in place of the sixth cysteine. The excision of both introns in the "processed" transcript would allow for the translation of a complete Zn(II)2Cys6 motif.

at the 3′ border and the second intron was not excised. The exons flanking the third and fourth introns observed in TC16321 were not part of TC16322. BLASTX analysis of TC16321 and TC16322 indicated that they shared low but significant similarity to putative Zn(II)2Cys6 transcriptional factors (score ≈ 50 bits, E ≈ 2 × 10^{-5}) of the GAL4 family. In contrast, BLASTX analysis of the corresponding genomic sequence did not reveal any significant matches. Although the removal of the intron sequence shed light on possible gene function, the predicted protein lacked a complete six-cysteine motif (40). For example, translation of TC16321 would generate proteins lacking the sixth cysteine, while the translation of TC16322 transcripts would terminate just prior to the third cysteine.

F. verticillioides Gene Index Release 7.0 included sequences from additional cDNA libraries and significantly extended the length of TC16321 generating TC30495. The new TC was more than 2,240 nucleotides in length and included all of the TC16321 ESTs plus five FvN ESTs and two additional FvG ESTs. TC16322 did not include any additional ESTs in release 7 of the Gene Index but was renamed TC30496. TC30495 had five gaps compared to genomic DNA. Four of these gaps corresponded to the introns described above. BLASTX analysis of TC30495 revealed significant similarity to a second domain referred to in the NCBI Conserved Domain Database (CDD) as a fungal specific transcription factor domain (pfam04082) (26).

Alignment and analysis of the 16 ESTs that comprise TC30495 and TC30496 (Fig. 2) provided insight into the pu-

tative Zn(II)2Cys6 gene structure and allowed us to predict a full-length protein. TC30495 consisted of 13 ESTs derived from 10 different cDNAs, and TC30496 consisted of three ESTs derived from three different cDNAs. Analysis of all sequences from the 13 cDNAs indicated that at least nine had at least one intron that was not excised or that had an alternative 3′ splice site. EST sequences in which an intron was not excised or had an alternative 3′ splice site relative to the predicted full-length transcript are referred to hereafter as ASFs. Four different Zn(II)2Cys6 gene ASFs were observed. The first ASF, ASFa, has an alternative 3′ splice site in the first intron. This 3′ splice site is a noncanonical "AT" rather than the "AG" in all other Zn(II)2Cys6 gene introns (Fig. 3) and typical of fungal introns in general. The second ASF, ASFb, retained the second intron. All three TC30496 ETSs were of this type. Translation of ASFa (Fig. 3, translation 1 [T1]) or ASFb (Fig. 3, T2) would yield truncated proteins with incomplete Zn(II)2Cys6 DNA-binding domains. The location of introns 1 and 2, relative to a predicted Zn(II)2Cys6 domain, is indicated in Fig. 4B. In the third ASF, ASFc, the fifth intron was retained, while the other seven introns were excised. The fifth intron is located in sequence predicted to encode a second domain associated with fungal transcription factors (see below) and is indicated in Fig. 4B. In the fourth ASF, ASFd, the third, fourth, and seventh introns were retained, while the fifth, sixth, and eighth introns were excised.

The 13 cDNAs corresponding to the Zn(II)2Cys6 gene were present in three EST libraries. FvF included one (0.013%), FvG included nine (0.098%), and FvN included three (0.015%).
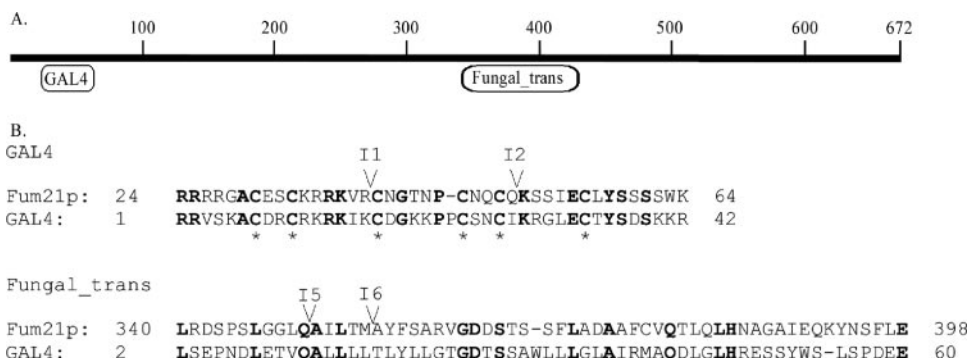
FIG. 4. Reverse position specific BLAST of the predicted Zn(II)2Cys6 protein (Fum21p) against the NCBI CDD. (A) The bar represents the Fum21p in 100s of amino acids. GAL4 refers to GAL4-like Zn(II)2Cys6 binuclear cluster DNA-binding domain (smart00066) and Fungal_trans refers to a fungal specific transcription factor domain (pfam04082). (B) Alignment of portions of the putative Fum21p with the GAL4 (E = 3 × 10^{-7}) and Fungal_trans domains (E = 2 × 10^{-4}). I1, I2, I5, and I6 indicates positions of introns 1, 2, 5, and 6, respectively. The asterisks mark the position of the six, highly conserved cysteines that form the Zn(II)2Cys6 DNA-binding motif.

TABLE 1. Fumonisin analysis of cracked maize cultures

Strain	Genotype[a]	Fumonisin[b]
M-3125	Wild-type	3.5
GmTcp172	Δfum21	≥0.2
GmTcp173	Δfum21	≥0.2
GmTcp174	Δfum21	≥0.2
GmTcp192	Δfum21	≥0.2
GmTcp193	Δfum21	≥0.2
CFUM21.72.2	FUM21 CS	2.45
CFUM21.72.6	FUM21 CS	6.17
GmTcp190	Geneticin R	5.44
GmTcp191	Geneticin R	5.50

[a] Δfum21 indicates a disrupted FUM21, and FUM21 CS indicates FUM21 complemented strains derived from strain ΔFUM21.172. Geneticin R indicates a transformant in which the FUM21 deletion vector integrated ectopically, leaving a wild-type copy of FUM21 intact.

[b] Fumonisin = total FB_1, FB_2, and FB_3 in μg/ml of culture extract extracted after 21 days of growth. Values of ≥0.2 were below detection limits.

No Zn(II)2Cys6 gene transcripts were found in FvM, a library that was generated from mRNA from 48- and 72-h GYAM cultures, time points between the 24-h(FvF) and the 96-h (FvG) libraries. The single FvF EST, two of the nine FvG ESTs, and one of three FvN ESTs were derived from transcripts that could encode a putative full-length Zn(II)2Cys6 protein that contained all six cysteine residues. These four ESTs are referred to as "processed" in Fig. 2.

The sequence of the ESTs differed from the FUM cluster sequence (accession number AF155773) 379 nucleotides from the predicted Zn(II)2Cys6 gene start codon. All EST sequences lacked a thymidine at this position that was present in accession number AF155773. Sequence analysis of a PCR product generated from F. verticillioides M-3125 genomic DNA spanning this position matched the EST sequences, and the accession was updated accordingly.

Analysis of the predicted Zn(II)2Cys6 protein. Excision of all eight introns from the putative Zn(II)2Cys6 gene transcript would result in a 2,016-nucleotide open reading frame (ORF) that could encode a 672-amino-acid protein (Fig. 2). Reverse position specific BLAST of the putative Zn(II)2Cys6 gene protein against the CDD at NCBI generated alignments with significant scores to domains included in smart00066 (E = 3 × 10^{-7}) and pfam04082.7 (E = 2 × 10^{-4}) (Fig. 4A). smart00066.11 is the GAL4-like Zn(II)2Cys6 binuclear cluster DNA-binding domain in the SMART (for Simple Modular Architecture Research Tool) database, and pfam04082.7 is a domain C-terminal to the Zn(II)2Cys6 domain in some fungal transcription factors (25, 26). The Pfam 20.0 database is a collection of 8,296 multiple sequence alignments and hidden Markov models covering many common protein families (11). The failure to detect the Zn(II)2Cys6 protein based on translation of genomic sequence was most likely due to the presence of sequences corresponding to introns 1, 2, 5, and 6. Introns 1 and 2 are located within the sequence encoding the Zn(II)2Cys6 domain, whereas introns 5 and 6 are located within the sequence encoding the fungal specific transcription factor domain (Fig. 4B). The presence of any of these four introns in transcripts introduces stop codons and/or frameshifts in the Zn(II)2Cys6 gene ORF that lead to dramatic changes in the amino acid sequence of the predicted protein. Thus, BLASTX analyses

with genomic sequence would not reveal significant identity to either conserved domain described above.

Functional analysis of Zn(II)2Cys6 gene. Transformation of wild-type strain M-3125 with the deletion plasmid pTFKOG-5 yielded 35 transformants, three of which, designated GmTcp172, GmTcp173, and GmTcp174, were shown via PCR to lack the Zn(II)2Cys6 gene coding sequence. A second transformation with pTFKOG-5 yielded additional transformants, two of which, designated GmTcp192 and GmTcp193, were shown via PCR to lack the Zn(II)2Cys6 gene coding sequence. Two transformants in which pTFKOG-5 integrated ectopically, leaving the Zn(II)2Cys6 coding sequence intact, were designated GmTcp190 and GmTcp191 and were arbitrarily chosen for further analysis. All seven transformants exhibited wild-type growth and conidiation on a variety of growth media. LC-MS analysis of 21-day cracked maize culture extracts indicated that the deletion mutants did not produce detectable levels of fumonisins (Table 1). The two strains in which the deletion vector integrated ectopically produced fumonisin at levels similar to the wild-type progenitor strain. Based on the proximity of the Zn(II)2Cys6 gene to the FUM cluster and its involvement in fumonisin biosynthesis, the Zn(II)2Cys6 gene is hereafter referred to as FUM21.

Deletion mutant GmTcp172 was transformed with vector pTHF21 to determine whether introduction of a wild-type copy of FUM21 could restore fumonisin production in the mutant. Two hygromycin-resistant isolates recovered after transformation of GmTcp172 with pTHF21, designated CFUM21.72.2 and CFUM21.72.6, were arbitrarily selected for further analysis. PCR analysis with primers specific to the hygromycin coding sequence (741 and 742) indicated that both transformants contained the HygB gene. LC-MS analysis of 21-day cracked maize cultures of the transformants revealed that fumonisins were present at levels similar to those detected in cultures of the wild-type progenitor strain (Table 1).

Transcriptional analysis of Δfum21 strains. Total RNA from three Δfum21 mutants (GmTcp172, GmTcp173, and GmTcp174) and wild-type strain M-3125 was subjected to RT-PCR for the detection of FUM1, FUM8, and the transcription elongation factor gene (TEF) (Fig. 5). The expected TEF amplicon served as a positive control and was observed as an intense band in all samples that included RT for each time point. The amplicon for FUM1 and FUM8 transcripts were detected in the wild-type strain M-3125 as expected. Low levels
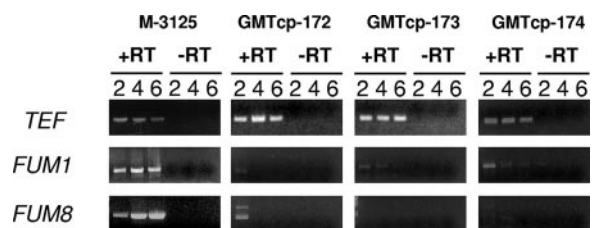
FIG. 5. RT-PCR analysis of genes in fum21 mutants. Total RNA from three Δfum21 mutants (strains GMTcp-172, GMTcp-173, and GMTcp-174) and wild-type M-3125 was used as a template for RT-PCR. The 2, 4, and 6 refer to the time point in days. Control reactions without RT are indicated as "−RT"; reactions that included RT are indicated as "+RT." The primer pairs used for amplification of each gene are described in Materials and Methods.

FIG. 6. Microarray analysis of *FUM21* expression. (A) The upper bars represent the predicted full-length *FUM21* transcript, where each numbered gap corresponds to an intron. Each small bar below the full-length transcript represents a single 24-mer oligonucleotide probe. For the exon probes, *FUM21* expression was measured by using three different probe sets of between 10 and 12 oligonucleotides each. Exon probes of each probe set were scattered over the length of the *FUM21* exon sequence as indicated. For the intron probes, the probe sets for the second, third, fourth, and seventh introns are indicated by the stack of small bars under introns 2, 3, 4, and 7, respectively. (B) Ratio of expression at 96 versus 12 h in liquid GYAM medium for *FUM21*; *FUM21* ASFs retaining introns 2, 3, 4, or 7; *FUM1*; and *FUM8*. Relative expression change is indicated on the vertical axis in the logarithmic base 2 ratio. A positive value indicates an increase in expression over time, while a negative value indicates a decrease in expression over time. (C) Fumonisin levels in μg of FB$_1$ per ml of GYAM medium (●) on the left vertical axis and ratio change in expression in logarithmic base 2 scale over time of *FUM1* (◆) and *FUM8* (■) on the right vertical axis.

of transcripts to both genes were detected in at least one time point for all three Δ*fum21* strains. The exclusion of RT in a duplicate set of reactions served as a negative control. In each case, no PCR products were observed, indicating that the RNA was free of contaminating genomic DNA (Fig. 5).

Microarray analysis of *FUM21* transcription. Expression of *FUM21* transcripts, including those that retained introns 2, 3, 4, or 7, were examined by microarray analysis (Fig. 6). The arrays included three different probe sets, of between 10 and 12 probes each (Fig. 6A, exon probes), for *FUM21* exon sequences. Each set of probes were essentially scattered over the entire length of the *FUM21* ORF. The data generated with these sets were similar and indicated an increase in expression of *FUM21* between 12 and 48 h, but no change between 48 and 120 h. Transcripts of *FUM* structural genes (e.g., *FUM1* and *FUM8*) increase most significantly between 48 and 72 h (Fig. 6C). The log$_2$ value of the ratio of expression at 12 and 96 h was determined for the *FUM21* exon and intron sequences and plotted (Fig. 6B). A positive value in this plot indicates an increase in expression of a given sequence from 12 to 96 h, while a negative value indicates a decrease in expression over the same period. Expression of transcripts retaining the second intron decreased from 12 to 96 h a little more than twofold, while transcripts retaining the seventh intron increased from 12 to 96 h a little more than twofold. Transcript retaining the

third and fourth intron did not change over the same time period.

DISCUSSION

Transcriptional regulation of secondary metabolite gene clusters is positively and/or negatively affected by both narrow- and broad-domain transcription factors and global regulators of transcription (16). Narrow-domain factors are typically encoded by genes located within a metabolite biosynthetic gene cluster and positively affect transcription of all cluster genes. Broad-domain factors and global regulators are encoded by genes located elsewhere in the genome, influence multiple physiological processes, and generally respond to environmental cues such as pH, temperature, and nutrition (16). To date, four *F. verticillioides* genes have been found to affect fumonisin biosynthesis. Two, *ZFR1* and *PAC1*, encode broad-domain transcription factors, and two, *FCC1* and *FCK1*, encode global regulators of gene expression (2, 12, 13, 36). Efforts to exploit these factors to control mycotoxin production have generally been frustrated by a limited understanding of the additional roles they play in growth and development. Prior to this report, efforts to identify a narrow-domain fumonisin transcription factor have not been successful. Although a few candidate regulatory genes were found within the 11- to 22-kb regions

flanking each end of the cluster, transcriptional analysis suggested that none were likely involved in fumonisin biosynthesis (32). Strains in which one of the genes (*ZNF1*) was disrupted exhibited wild-type fumonisin production (R. H. Proctor and R. D. Plattner, unpublished observations).

The development of the *F. verticillioides* Gene Index by TIGR, with its collection of over 87,000 ESTs derived from conditions that do and do not support fumonisin production, provided a tool to search for a narrow-domain transcription factor for fumonisin biosynthesis (5). ESTs corresponding to *FUM* genes were found primarily in three cDNA libraries. Two of these libraries were constructed from RNA harvested after growth in GYAM, a liquid medium that supports fumonisin production, for 48/72 h (FvM) or 96 h (FvG). The third library (FvN) was constructed after growth on maize meal, which also supports fumonisin production.

A search for ESTs that correspond to previously characterized *FUM* cluster genes led to the identification of ESTs that mapped to a genome location located 663 bp upstream from *FUM1*, the fumonisin polyketide synthase gene, and that had not previously been associated with a gene. BLASTX analysis of initial EST sequence data suggested that the new gene may encode a zinc binuclear cluster type fungal transcription factor but with an incomplete Zn(II)2Cys6 DNA-binding motif. Analysis of additional ESTs resolved the motif and allowed the prediction of a full-length, potentially functional protein. Gene deletion and complementation studies indicated that the protein was required for fumonisin production. RT-PCR comparison of deletion mutants and the wild type indicated that the gene is required for almost all *FUM* gene transcription. Based on these facts, we have designated the Zn(II)2Cys6 gene *FUM21*, thereby extending the fumonisin gene cluster approximately 3.5 kb to a total of 46 kb.

The detection of a low level of *FUM1* transcript in all three Δ*fum21* mutants and *FUM8* transcript in one mutant after 2 days of growth in GYAM medium but none after 4 or 6 days of growth suggests that, at least during early stages of growth, there are other factors that can activate low-level *FUM* expression (Fig. 5). The detection of low levels of transcripts for some genes involved in fungal secondary metabolite biosynthesis in narrow-domain transcription factor mutants is not without precedence. Low-level expression of a trichothecene biosynthetic gene was detected in mutants in which *TRI6*, a narrow-domain transcriptional activator, was deleted (34). An *F. sporotrichioides tri6* mutant was completely blocked in production of the trichothecene T-2 toxin and *TRI4* expression but exhibited a low level of *TRI5* expression and production of the trichothecene biosynthetic intermediate trichodiene, the synthesis of which is catalyzed by the *TRI5* protein.

During our characterization of *FUM21*, we noted a significant number of ASF transcripts in the EST libraries. Previously, we found that seven of the 16 *FUM* cluster genes have one to three ASF types (5). The percentage of ASFs for cluster genes (47%) is significantly higher than the 3.1% predicted for the *F. verticillioides* transcriptome (unpublished observation). Of the 13 *FUM21* transcripts examined, 9 consisted of four different ASF types. As with other *FUM* gene ASFs in the EST libraries, *FUM21* ASFs accumulate late in culture (5). The single *FUM21* transcript from the 24-h cDNA library (FvF) appeared to be fully processed, although the available se-

quence only included the first three exons. In contrast, seven of the nine ESTs from the 96-h cDNA library (FvG) were ASFs. Microarray analysis of *FUM21* transcripts indicated that different *FUM21* ASFs were differentially expressed. Transcripts that retained the seventh intron accumulated later in culture, whereas transcripts that retained the second intron decreased over time. Transcripts that retained the third or forth introns did not change over time (Fig. 6).

ASFs have been previously described for a few genes in other filamentous fungi (e.g., *Aspergillus niger* [3] and *Neurospora crassa* [35]), and many more examples can be found in large-scale fungal EST collections (The Gene Index Databases) maintained by the Dana-Farber Cancer Institute, Boston, MA (http://combio.dfci.harvard.edu/tgi/). One practical consequence of the prevalence of ASFs in fungi has been to complicate gene annotation efforts of genomic sequence (14). Analysis of genomic and cDNA data of the basidiomycetous yeast *Cryptococcus neoformans* has found evidence for 277 ASFs which represent 4.2% of the transcriptome (21). The authors of that study suggest that the diverse genomic distribution of the ASFs support a role in regulation. Recently, work characterizing nonsense-mediated mRNA decay (NMD) in *Aspergillus nidulans* (28) highlighted the potential link of ASFs and NMD to gene regulation (20). In this case, alternative splicing may affect gene expression by shunting mRNAs toward degradation (reviewed in reference 18).

Fumonisins were first detected in GYAM culture filtrates at 72 h and increased in concentration through 120 h (Fig. 6C). Changes in *FUM* gene expression paralleled this production pattern. Transcripts of most fumonisin biosynthetic genes (e.g., *FUM1* and *FUM8*) were low through 48 h but increased dramatically at 72 h and remained high at 96 and 120 h (Fig. 6C). *FUM21* ASFs that retain the seventh intron exhibited a similar increase. The seventh intron includes stop codons in all reading frames, thus potentially targeting transcripts that retain it for degradation via NMD. The overall affect of the decreasing pool of *FUM21* transcripts could be to limit fumonisin synthesis. Alternatively, ASFs that retain the seventh intron may enable the production of a truncated protein (Fum21pi7) with altered function relative to that of the full-length *FUM21* protein (Fum21p). The predicted Fum21pi7 retains the complete Zn(II)2Cys6 and fungal transcription factor motifs but lacks the 227 carboxy-terminal amino acids present in the full-length Fum21p. The absence of these amino acids in Fum21pi7 could cause it to function differently than Fum21p and/or interfere with Fum21p by competing for DNA-binding sites. A comparison of Fum21p to GAL4, the archetypical Zn(II)2Cys6 transcriptional activator from *Saccharomyces cerevisiae*, suggests how Fum21pi7 may function differently from Fum21p (22, 38). The carboxy-terminal portion of GAL4 contains a domain required for activating transcription. The corresponding region in Fum21p could also be required for activation of transcription, and its absence in Fum21pi7 could limit this activity.

Initial efforts to examine the effects of Fum21p variants generated from mutant versions of *FUM21* designed to mimic different ASFs have thus far been inconclusive. For each version, the border sequence of one intron was altered by site-directed mutagenesis to prevent its excision and thereby introduce one to two stop codons. All of the *F. verticillioides* strains containing the mutant forms of *FUM21* exhibit wild-type fu-

monisin production in cracked corn cultures (unpublished results).

An alternative or additional function of ASFs is suggested by the decrease over time of ASFs that retain the second intron. These ASFs may serve as a reservoir of transcripts that can undergo more complete processing at a later time. The dramatic increase in structural *FUM* gene transcripts between 48 h and 72 h may be linked, in part, to this available reservoir. The predicted 73-amino-acid protein (Fum21pi2) translated from such ASFs lacks a complete Zn(II)2Cys6 motif and therefore is probably nonfunctional.

Our results demonstrate that *FUM21*, a predicted a Zn(II)-2Cys6 DNA-binding transcription factor, positively regulates *FUM* gene expression and is required for fumonisin synthesis. The *FUM21* EST collection and microarray study has provided intriguing insights into a potential role for ASFs in fumonisin biosynthesis. The role of some of these ASFs and their relative importance to fungal secondary metabolism will be the focus of further study by our laboratory. The information reported here may lead to strategies that reduce fumonisin production in the field and thereby limit toxin contamination of maize. Thus, a more complete understanding of the role ASFs play in fumonisin biosynthesis may lead to novel methods to solve the fumonisin mycotoxin problem.

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