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Characterization of a *Fusarium* 2-Gene Cluster Involved in Trichothecene C-8 Modification

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The *Fusarium* trichothecenes T-2 toxin and deoxynivalenol (DON) are potent inhibitors of eukaryotic protein synthesis and are a significant agricultural problem. Three coregulated loci are required for T-2 toxin synthesis by *Fusarium sporotrichioides*. The core-trichothecene gene cluster consists of 12 genes (*Tri3*−*Tri14*) while the second locus consists of a single gene (*Tri101*). The third locus was recently partially described and encodes 1−2 biosynthetic enzymes and a putative regulatory gene. Here, we describe a detailed characterization of this locus. Located adjacent to *Tri1* is *Tri16*, which is required for esterification of the C-8 hydroxyl. A putative regulatory gene, also adjacent to *Tri1*, is not required for T-2 toxin synthesis. The genomic sequence of *Fusarium graminearum* (a DON producer) contains a putative functional *Tri1* and a nonfunctional *Tri16*. The presence of the *Tri16* pseudogene is consistent with the chemical structure of DON, which has a C-8 keto group rather than the C-8 ester of T-2 toxin.

KEYWORDS: ***Fusarium*; trichothecene; T-2 toxin; deoxynivalenol; secondary metabolite; minigene cluster; P450; acyltransferase**

INTRODUCTION

Trichothecenes comprise a large family of secondary metabolites that often contaminate food and animal feed derived from maize and cereal grains (*1*). They are potent inhibitors of protein synthesis in eukaryotes and are phytotoxic (*2*), and their production by *Fusarium* is required for high levels of virulence on some plant hosts (*3*). Because of their harmful effects on both humans and animals, trichothecene-contaminated agricultural products can be substantially reduced in value (*4*). Although over 60 different trichothecenes have been isolated from *Fusarium* species and other fungi, a given strain or species typically produces only a limited number of the toxins. Over the past 16 years, genetic and metabolite feeding studies with *Fusarium sporotrichioides* have led to a generally accepted biochemical pathway leading from farnesyl pyrophosphate to T-2 toxin that includes 15 steps (*5* and references therein). Of the 10 trichothecene biosynthetic genes (*Tri*) assigned to specific biochemical steps, seven are clustered within a group of 12 open reading frames (ORFs) spanning 26 kb in the fungal genome. The eighth assigned gene, *Tri101*, is situated between two primary metabolic genes (*6*). The final biosynthetic locus consists of the trichothecene gene *Tri1* and the putative trichothecene gene *Tri16*.

All six oxygen atoms of the T-2 toxin skeleton (the ether function at position 1, the 12,13-epoxide, and the hydroxyl groups at C-3, C-4, C-8, and C-15) are derived from molecular oxygen (*7*). Four of the oxygenation reactions are known to be catalyzed by different cytochrome P450 monooxygenases (P450s). The genes for three P450s, responsible for the ether function at position 1 and the hydroxyls at C-15 and C-4, are located within the 12-gene core trichothecene gene cluster (*8*−*10*). The forth gene, *Tri1*, is located within a 2-gene cluster (*10*). The genes or enzyme(s) responsible for the remaining oxygenation steps in T-2 toxin biosynthesis have not yet been identified. However, because hydroxylation of organic substrates is a common function of P450s and because these enzymes use molecular oxygen as a source of oxygen atoms, it is possible that the remaining oxygenation steps in T-2 toxin biosynthesis for which genes have not yet been identified are catalyzed by P450s (*1*). Recently, it has been estimated that *Fusarium* genomes have approximately 40 genes encoding P450s (*11*). The challenge is to identify which, if any, of the P450 encoding genes from the remaining, uncharacterized P450s in the *F. sporotrichioides* genome are involved in T-2 toxin biosynthesis.

One way to identify new biosynthetic genes is via an expressed sequence tag (EST) approach. An EST collection from *F. sporotrichioides* was made available in 2000 by researchers at the University of Oklahoma's Advanced Center for Genome Technology (*12*, *13*; http://www.genome.ou.edu/fsporo.html). Preliminary analysis of the ESTs indicates that previously characterized trichothecene genes located in the core-gene cluster are well-represented. Thus, it is possible that some of the new, well-represented ESTs in the library with similarity

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to P450s may be responsible for catalyzing some or all of the remaining, unassigned oxygenation reactions during T-2 toxin biosynthesis.

In this paper, we demonstrate the utility of ESTs in gene discovery programs by independently identifying and characterizing *Tri1*, a putative P450 shown by Meek et al. (*10*) to be required for C-8 oxygenation of T-2 toxin. We also describe the characterization of a putative acyltransferase (*Tri16*) and putative regulatory gene (*Pdb*) that flank either side of *Tri1*. Gene deletion studies show that *Tri16* is required for esterification of the C-8 hydroxyl group leading to T-2 toxin while *Pdb* is not required for T-2 toxin synthesis.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions. *F. sporotrichioides* NRRL 3299 and *Fusarium graminearum* GZ3639 (FGSC 8630) have been described previously (*3*, *14*). *F. sporotrichioides* MB1716 (R937) was kindly provided by D. Geiser at the Fusarium Research Center, The Pennsylvania State University, and has been described previously (*14*). MB1716 was reisolated from a single conidium and was confirmed to produce 4,15-diacetoxyscirpenol (DAS) in liquid YPG medium (0.3% yeast extract, 1% peptone, and 2% glucose) prior to its use in this study. Cultures for DNA isolation and trichothecene analysis were grown in YPG medium at 28 °C with shaking at 200 rpm for 4 days. Hygromycin resistant transformants of strain NRRL 3299 were maintained on V-8 juice agar slants containing 300 μg/mL hygromycin B (Sigma, St. Louis, MO).

Nucleic Acid Manipulation. DNA and RNA analyses were conducted by using standard techniques (*15*). DNA probes were labeled with [α-^{32}P]dCTP (DuPont NEN, Boston, MA) using the Prime-A-Gene labeling system (Promega, Madison, WI). The *F. sporotrichioides* and *F. graminearum* cosmid libraries have been previously described (*5*, *16*). *F. sporotrichioides* mRNA isolation and Northern analyses were conducted essentially as previously described (*17*).

Sequence Analysis. All sequence data were obtained by using the ABI Prism BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA). Reactions were analyzed with a PE Biosystems 377 automated DNA sequencer. Cosmid sequencing templates were generated using the GPS-1 kit from New England BioLabs, Inc. (Beverly, MA) following the manufacturer's instructions. Sequence information was assembled and edited using the Sequencher program from Gene Codes Corp. (Ann Arbor, MI). Sequence similarity searches of the nonredundant (NR) database maintained by the National Center for Biotechnology Information (NCBI) were performed using the BLAST program (*18*, *19*). Nucleotide and/or amino acid (aa) sequences were analyzed by the GAP, PILEUP, and PRETTY programs of the Genetics Computer Group (GCG) analysis software package version 10.2-UNIX (*20*). Primers were synthesized on a PerSeptive Biosystems Expedite Nucleic Acid Synthesis System (PE Biosystems) and processed according to the manufacturer's recommendations. Sequence data from this article have been deposited with the GenBank Data Library under accession nos. AY217783 and AY226098.

Deletion Plasmids and Transformation. Gene deletion plasmids pΔTri1, pΔP450B, pΔPdb, and pΔTri16 were constructed to independently delete the coding regions of *Tri1*, *P450B*, *Pdb*, and *Tri16*, respectively. The general strategy used to construct each deletion vector was described previously (*9*). In brief, a 2 kb DNA fragment upstream of the predicted start codon of the target gene and a similarly sized fragment downstream of the predicted stop codon of the target gene were amplified via polymerase chain reaction (PCR) and cloned separately into PCR cloning vector pT7 Blue-3 with the Perfectly Blunt Cloning Kit (Novagen, WI). One *Asc*I site was engineered into each PCR product at the end proximal to the start or stop codon. Unique restriction sites also were engineered into the opposite end of each PCR product. This design enabled one of the products to be cloned into a vector adjacent to the second product such that the orientation of their sequences was the same as in the *Fusarium* genome. Construction of each deletion vector was completed by cloning the 2.5 kb Hyg/P1 fragment from pHygAsc into the *Asc*I (*9*) between the two PCR products. The completed deletion vector consisted of Hyg/P1 sandwiched between two 2 kb *Fusarium*-derived DNA fragments from up- and downstream of the target coding region. Sequence analysis of the *Fusarium*-derived portion of each disruption vector did not identify any nucleotide differences from the corresponding genomic sequence. Transformation of *F. sporotrichioides* and selection and isolation of transformants were performed essentially as described previously (*21*). Deletion mutants were identified by Southern analysis of hygromycin resistant isolates recovered following transformation. In deletion mutants, the target coding region was replaced by Hyg/P1. This resulted from the double homologous recombination event between sequence on both sides of the target coding region and the homologous sequences on both sides of Hyg/P1 in the deletion vector.

The *Tri1* complemention vector, pTri1F, carried a 2.4 kb DNA fragment that included the entire *Tri1* coding region plus 424 nt of the 5′ flanking region. The 5′ flanking region included four putative Tri6 binding sites (*22*). The 2.4 kb *Tri1* fragment was amplified via PCR from cosmid clone Fs1.4 with primers FsAF2 (5′-GCATAGCAACTTAGTTAAGGAG-3′) and FsAR2 (5′-CTACTGATAAGATATTCACCCC-3′) and cloned into pT7 Blue-3 with the Perfectly Blunt Cloning Kit (Novagen). The entire fragment was then cloned into the *BamH*I/*Sal*I sites of the fungal transformation vector pUCH1 (*16*) to yield vector pTri1F. The sequence of the fungal-derived portion of pTri1F was confirmed to be identical to the corresponding genomic sequence in cosmid Fs1.4.

Extraction and Analysis of Trichothecenes. Filtrates from YPG cultures of deletion mutants and 4−6 nondeleted hygromycin resistant strains derived from the same transformation were analyzed for trichothecene production by high-performance liquid chromatography−mass spectroscopy (HPLC-MS). Culture filtrates were diluted 10-fold with 1:1 acetonitrile/water, and 10 μL was analyzed by injection into a 150 mm × 3.0 mm Intersil ODS-3 5 μm HPLC column. The flow rate was 0.3 mL/min. After a 1 min flow at 65:35 with water:methanol, the solvent was changed in a linear gradient from 65:35 water:methanol to 20:80 water:methanol over 10 min and held at the final composition for a further 9 min. The solvent was then returned to the 65:35 composition over the next minute. The eluant was coupled into the MS in positive ion electrospray (ESI) mode. The MS detector was scanned from *m*/*z* 190−1100, and data were collected for 25 min after injection. Trichothecenes were identified by comparison of retention time and positive ESI spectra with authentic standards.

RESULTS

Identification of Candidate P450s. The *F. sporotrichioides* EST library and collection of contigs include sequences for six putative P450s genes in addition to the three previously described P450 genes (*Tri4*, *Tri11*, and *Tri13*) involved in trichothecene biosynthesis. Four of the putative P450s were represented by a single EST each in the library, while two others, originally designated *P450A* and *P450B*, were represented by 15 and 32 ESTs, respectively. By comparison, *Tri4*, *Tri11*, and *Tri13* were represented by 123, 9, and 18 ESTs, respectively (**Table 1**). We selected *P450A* and *P450B* for further analysis because they were represented by multiple ESTs in a manner similar to the previously identified P450 genes required for trichothecene biosynthesis. Meek et al. (*10*) recently showed that the gene corresponding to *P450A* is in fact *Tri1*. Therefore, *P450A* is referred to hereafter as *Tri1*.

Sequence Analysis of *P450B*. The *F. sporotrichioides* cosmid library was screened to obtain a genomic copy of the *P450B* gene. Sequences obtained from the EST database corresponding to *P450B* (contigs 1026 and 581) were used to design primer pair FsBF (5′-CAGCGCCCATCTCATAATGTG-3′) and FsBR (5′-GCTGAGAATGGCCAGTACGG-3′). PCR with *F. sporotrichioides* genomic DNA template and the *P450B* specific primers generated an amplicon similar to the predicted size of 1400 nts. The amplicon was gel-purified, labeled with ^{32}P, and used as a hybridization probe to screen the cosmid library, as

Table 1. EST Representation in the ACGT EST Library

gene (locus)	no. of clones	gene (locus)	no. of clones
Main Cluster			
Tri7	9	Tri9	2
Tri3	7	Tri11	9
Tri4	123	Tri12	12
Tri6	0	Tri13	18
Tri5	76	Tri14	32
Tri10	2		
P450B			
FsP450B	32		
Minicluster			
Pdb	0	Tri16	6
Tri1	15	Orf1	6
Tri101			
Tri101	51		

previously described (*5*). We isolated and partially sequenced cosmid clones Fs2.3 and Fs2.4 that included *P450B* (accession AY226098).

A BLASTN comparison of the *P450B* genomic sequence obtained from cosmids Fs2.3 and Fs2.4 to the *F. sporotrichioides* EST database indicated that contigs 1026 and 581 overlap by a single nucleotide. Alignment of the genomic sequence to the reconstituted *P450B* cDNA sequence identified two typical fungal introns, each with a length and border and internal sequence motifs typical of fungal introns (*5*) and no base pair differences. BLASTX analysis to the NR-NCBI database indicated that *P450B* shares significant similarity with a number of putative P450s including a probable benzoate 4-hydroxylase from *Neurospora crassa* (score = 138 bits, $E = 2e^{-31}$; accession AL670007) and the trichothecene C-15 hydroxylase (*Tri11*) from *Gibberella zeae* (score = 134 bits, $E = 2e^{-30}$; accession AY102588). There were no Tri6 binding sites within 1000 nts upstream of the *P450B* predicted start codon.

Sequence Analysis of *Tri1*/*Tri16* Locus. We also screened the *F. sporotrichioides* cosmid library to obtain a genomic sequence for *Tri1*. Sequences obtained from the EST database corresponding to *Tri1* (contigs 956 and 993) were used to design primer pair FsAF (5′-CAAGGCATCGACTGAATCGGC-3′) and FsAR (5′-TGTCGTACACCAGCTCAGTGG-3′). PCR with *F. sporotrichioides* genomic DNA template and the *P450A* specific primers generated an amplicon similar to the predicted size of 1100 nts. The *Tri1* amplicon was gel-purified, labeled with ^{32}P, and used as a hybridization probe to screen a *F. sporotrichioides* cosmid library (*5*). We isolated and partially sequenced the cosmid clones Fs1.4 and Fs3A that included *Tri1* (accession AY217783). The original cDNA sequence corresponding to *Tri1* submitted to GenBank (accession AY032743) by Beremand and co-workers contained three nt differences at nucleotides 598 G→T, 602 A→G, and 658 G→T. As noted by Meek et al. (*10*), a comparison between the cDNA and the genomic sequences identified four introns.

The genomic region flanking *Tri1* included three ORFs, which potentially could also be involved in trichothecene biosynthesis. BLASTX analysis of the 18.1 kb sequence from the *Tri1* cosmids with the NR-NCBI database identified two of the three ORFs. As expected, BLASTX analysis of *Tri1* genomic sequence was consistent with its assignment as a P450. In addition to *Tri1* exhibiting a high level of identity to accession AY032743 (score = 391 bits; E = 0.0), it also exhibited high levels of similarity to putative P450s from *N. crassa* (score 193 bits; $E = 1e^{-109}$; accession T49413) and *Gibberella fujikuroi* (score = 190 bits; $E = 1e^{-77}$; accession Y15277). As noted by

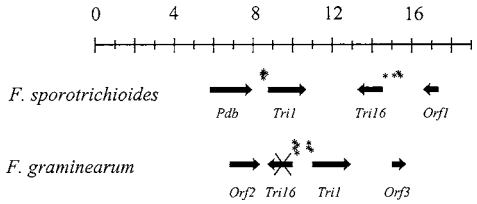

Figure 1. Comparison of the genomic organization of the *F. sporotrichioides* and *F. graminearum* trichothecene mini-gene clusters. Relative scale in kilobases is shown. Organization of the predicted ORFs. Gene names are indicated underneath each large arrow. Arrowheads refer to direction of gene transcription. The X over *F. graminearum Tri16* indicates that this gene in nonfunctional. * refers to Tri6 protein binding sites (TNAGGCC) (*22*). The functions of Orf1, Orf2, and Orf3 are unknown.

Meek et al. (*10*), an ORF located upstream from *Tri1* shared significant similarity with a putative GAL4-like transcriptional activator from *Colletotrichum lindemuthianum* (score = 162 bits; $E = 1e^{-38}$; accession AF190427) and is referred to as *Pdb* for putative DNA binding protein coding gene hereafter. Also as noted by Meek et al. (*10*), the second ORF, located upstream from *FsTri1*, shared significant similarity to *Tri101* (trichothecene 3-O-acetyltransferase) from *F. cerealis* (score = 83.6 bits; $E = 7\ e^{-15}$; accession AAG43718) and *F. sporotrichioides* (score = 82.4 bits; $E = 2\ e^{-14}$; accession AAD19745). On the basis of BLAST analysis and the observation that transcripts to this ORF appeared to be regulated in a manner similar to trichothecene biosynthetic genes, Meek et al. (*10*) concluded that this gene is likely involved in trichothecene biosynthesis and designated it *Tri16*.

We identified the final ORF in the region flanking *Tri1* by BLASTN analysis of the 18.1 kb of cosmid clone sequence with the *F. sporotrichioides* EST library. There were six ESTs to an ORF (referred hereafter as *Orf1*) upstream from *Tri16* that were contained in a single contig (contig 961) that perfectly matched the genomic sequence. The longest possible predicted Orf1 protein consisted of 239 amino acids and did not share any significant identity to any protein in the NR-NCBI database by BLASTP analysis. BLASTN analysis also identified six *Tri16* ESTs that were present in two contigs (contigs 799 and 873) and matched perfectly with genomic sequence but failed to overlap by 167 nts (**Table 1**). No *Pdb* ESTs were found.

The promoter regions to *Pdb*, *Tri1*, *Tri16*, and *Orf1* were examined for the presence of Tri6 binding sites (TNAGGCC) (*22*). Tri6 is a DNA binding regulatory protein encoded by a gene (*Tri6*) located within the core-trichothecene gene cluster and is required for trichothecene biosynthesis (*12*). All seven of the genes encoding structural enzymes located in the core-trichothecene cluster have Tri6 binding sites within their promoter regions (*5*). The promoter region of *Tri1* and *Tri16* contain four Tri6 binding sites each and are located within 500 nts of the predicted start codon (**Figure 1A**). The promoter region of *Pdb* and *Orf1* does not contain any Tri6 binding sites within 1000 nts of their predicted start codons.

Northern Analysis. RNA isolated from *F. sporotrichioides* grown under trichothecene-inducing conditions was probed with DNA corresponding to *Tri1*, *Tri16*, *Pdb*, *Orf1*, and *P450B* coding regions. Transcripts to *Tri1*, *Tri16*, and *P450B* shared a similar pattern of expression to that of *Tri5*, previously shown to be required for T-2 toxin biosynthesis. Transcripts to *Pdb* were not detected and transcripts to *Orf1* displayed a different pattern to that of *Tri5* (data not shown and *23*).

Identification of Deletion Mutants. Wild-type strain NRRL 3299 was transformed independently with deletion vectors pΔTri1, pΔP450B, pΔPdb, and pΔTri16 in order to delete the

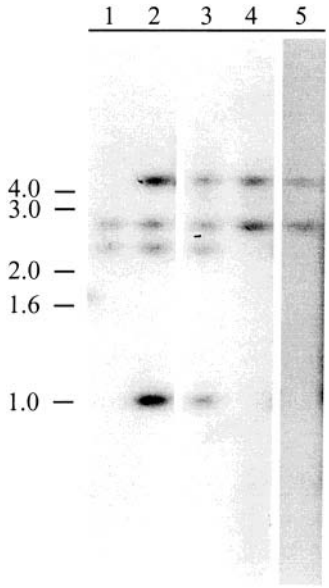

Figure 2. Southern analysis of *Fusarium* wild type and pΔP450A transformants. Genomic DNA was cut with *Hind*III and probed with a 2 kb DNA fragment downstream of the *P450A* start codon. Lane 1, GZ3639; lane 2, ΔFsTri1.5; lane 3, ΔFsTri1.6; lane 4, ΔFsTri1.1; and lane 5, ΔFsTri1.9. Molecular size in kilobases is indicated.

coding region of *Tri1*, *P450B*, *Pdb*, and *Tri16*, respectively. Between 13 and 17 hygromycin resistant isolates were recovered from each transformation. Mutants were identified by Southern analysis of genomic DNA digested with an appropriate enzyme and probed with DNA corresponding to the up- or downstream arm of the deletion vector. Double homologous integration events, whereby the target gene had been deleted from the genome and replaced with the selectable marker Hyg/P1, were detected by an increase in the DNA band size relative to the wild type. For example, the integration of pΔTri1 by double homologous recombination and the resulting replacement of the *Tri1* coding region with the Hyg/P1 results in the loss of two *Hind*III restriction sites present in the *Tri1* coding region. Southern analysis of *Hind*III digested wild-type genomic DNA probed with a 2 kb DNA fragment downstream of the *Tri1* start codon (D probe) hybridized to the expected 2.7 and 2.2 kb DNA fragments (**Figure 2**). Loss of the two *Hind*III sites by replacement of the *Tri1* coding region with Hyg/P1 would result in hybridization of the D probe with a 4.1 kb fragment rather than the wild-type 2.2 kb fragment. Two of the 16 putative pΔTri1 transformants (12%) had the hybridization pattern (e.g., 2.7 and 4.1 kb bands) predicted for replacement of the *Tri1* coding region with Hyg/P1 (**Figure 2**). Using the same approach, two pΔP450B transformants (15%), two pΔPdb transformants (12%), and four pΔTri16 transformants (31%) that had the respective coding regions replaced by Hyg/P1 were identified.

Trichothecene Production by Deletion Mutants. Culture filtrates from the wild-type progenitor strain NRRL 3299 contained primarily T-2 toxin and much smaller amounts of DAS, neosolaniol (8-hydroxy, 4,15-diacetoxyscirpenol) (Neo), and acetylated and propyl esters of Neo (**Figure 3A**) (*24*). Culture filtrates from two *F. sporotrichioides P450B* deletion mutants (ΔFsP450B.5 and ΔFsP450B.7) and the two *Pdb* deletion mutants (ΔFsPdb1.3 and ΔFsPdb3.6) were essentially identical to wild-type both in the quantity and in the profiles of trichothecenes produced. Culture filtrates from the two *Tri1* deletion mutants (ΔFsTri1.1 and ΔFsTri1.9) contained DAS but no detectable T-2 toxin, Neo, or Neo esters (**Figure 3B**). DAS presumably is formed by C-3 deacetylation of 3,4,15-triacetoxyscirpenol (TAS), a reaction catalyzed by the Tri8 protein (*25*). Culture filtrates from the four *Tri16* deletion mutants (ΔFsTri16.45, ΔFsTri16.49, ΔFsTri16.52, and ΔFsTri16.53) contained Neo but no detectable T-2 toxin (**Figure 4B**). Neo presumably is formed by C-3 deacetylation of 3-acetylneosolaniol, a reaction that should also be catalyzed by the Tri8 protein (*25*). Hygromycin B resistant *F. sporotrichioides* mutant strains derived from transformation with either pΔTri1 or pΔTri16 where the target gene remained intact synthesized T-2 toxin at levels similar to wild type.

Characterization of *Tri1* in *Tox1* Mutant. Beremand et al. (1987) identified a locus in *F. sporotrichioides* that conferred the ability to hydroxylate trichothecenes at the C-8 position. The *Tox1* locus was identified in a single UV-induced mutant (designated MB1716) that produced DAS but not T-2 toxin or Neo in liquid culture. Recently, Meek et al. (*10*) reported that the *Tox1* locus and *Tri1* are the same gene. We confirmed this result by complementing the *Tox1* mutant with a wild-type copy of *Tri1*. Seven of the eight hygromycin resistant isolates recovered from the transformation with p450AF produced T-2 toxin similar to the wild-type strain.

We also determined the nucleotide sequence of the *Tri1* allele in the *Tox1* mutant, strain MB1716. To do this, PCR primers FsPAF2 (5′-CGCACTTTCTATGGCTCAGC-3′) and FsPAR2 (5′-CCTGAGTGAGTAGGAACTG-3′) were used along with genomic DNA of strain MB1716 to amplify a 2.6 kb DNA fragment, which was then used as a sequencing template. The nucleotide sequence of the *Tri1* allele from strain MB1716 was identical to the wild-type allele except for a single A→G transition that changed the specificity of codon 378 from lysine in the wild type to glutamic acid in strain MB1716.

Search for *F. graminearum Tri1* Homologue. *F. graminearum* produces the trichothecenes deoxynivalenol (DON) and nivalenol (NIV). Both of these toxins are considered B-type trichothecenes because they have a carbonyl function at the C-8 position. This is in contrast to A-type trichothecenes (e.g., T-2 toxin, Neo, and DAS) that do not have a carbonyl function at C-8. A possible mechanism for the formation of the C-8 carbonyl is hydroxylation followed by dehydrogenation. Thus, *F. graminearum* may have a *Tri1* homologue (*FgTri1*). However, several attempts to identify a homologue were unsuccessful. ^{32}P-labeled hybridization probes consisting of the *F. sporotrichioides Tri1* coding region failed to hybridize to *F. graminearum* genomic DNA in Southern blot analyses or to a *F. graminearum* cosmid library during three different screens of the library. In contrast, probes consisting of the *F. sporotrichioides Pdb* coding region were used successfully to isolate four cosmid clones with the *F. graminearum Pdb* (*FgPdb*) homologue. Nucleotide sequence analysis of a 13.6 kb portion of cosmid FgG3 revealed the presence of the *FgPdb*. GCG GAP analysis of *FgPdb* and *FsPdb* and homologous sequence upstream from the predicted *Pdb* start codons indicated that they share 81 and 83% nucleotide identity, respectively. GCG GAP comparison of downstream sequence showed no similarity.

Recently, a draft sequence of the *F. graminearum* NRRL 31084 (strain PH-1) genome was published by the Whitehead Institute/MIT Center for Genome Research (http://www-genome.wi.mit.edu). Strains PH-1 and Gz3639 were both isolated in North America and are members of the phylogenetically defined lineage 7 (*26*). A search of the database with *FsTri1* identified a portion of sequence on contig 1.4 with significant similarity (score = 290 bits; E value = e^{-124}). Contig 1.4 is part of scaffold 1, which is approximately 8.82 million bp (mbp). Primers

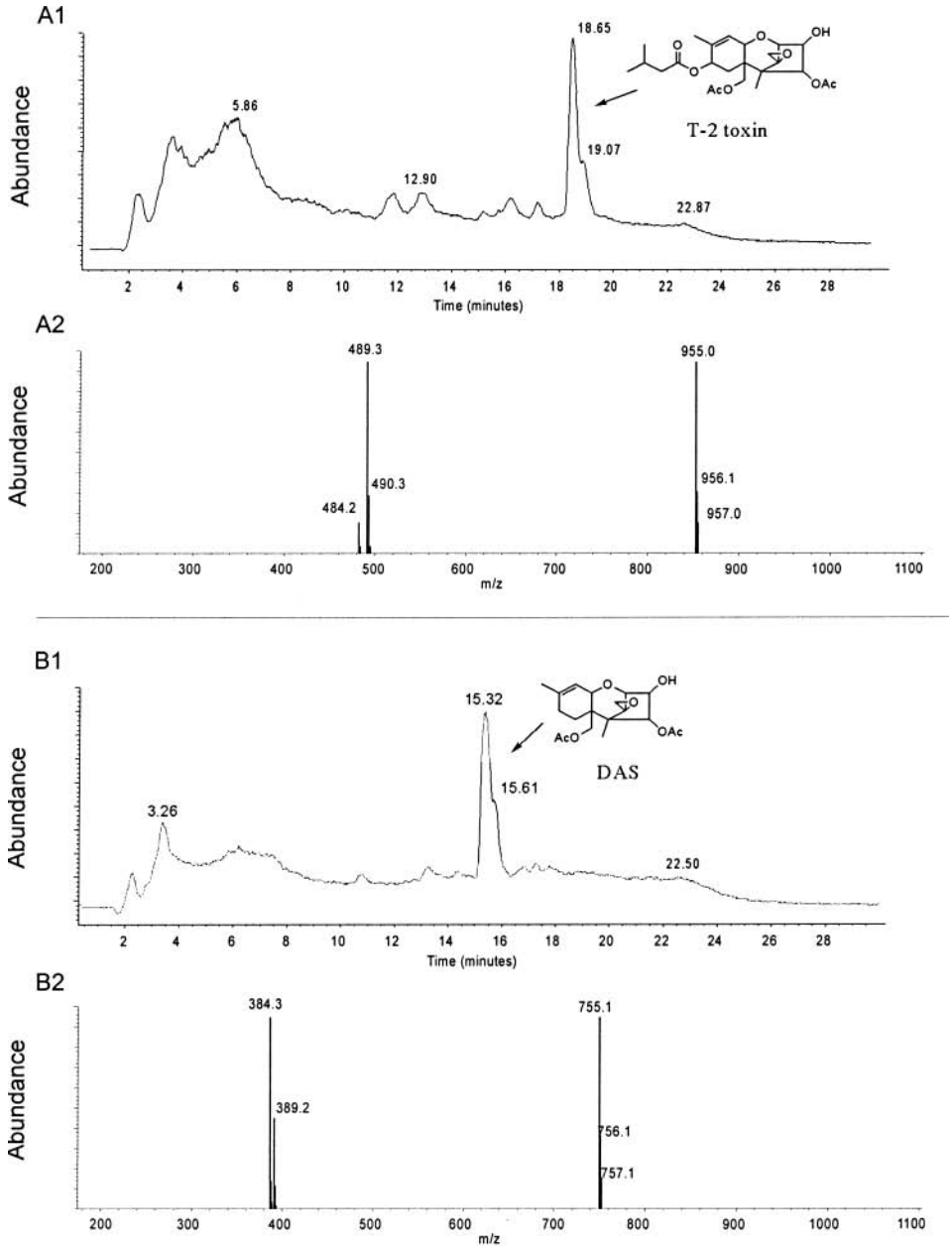

Figure 3. Reconstructed ion chromatograms and mass spectra of extracts from 3 day old cultures of (**A**) NRRL 3299 and (**B**) ΔFsTRI1.1. A2 corresponds to the mass spectra of the peak at 18.65 min in A1. The amount of 489.3 mass units represents the molecular weight of T-2 toxin (M) plus sodium. The amount of 955.0 mass units represents 2 M plus sodium. B2 corresponds to the mass spectra of the peak at 15.32 min in B1. The amount of 384.3 mass units represents the molecular weight of DAS plus sodium. The amount of 755.1 mass units represents 2 M plus sodium.

(FgT1ExpF, 5′-CTGGATCCACCATTTGTAGTTTCAAGG-3′; and FgT1ExpR, 5′-GTGGATCCTGAGTTCGATATTCCCATG-3′) designed to amplify the *FgTri1* promoter, coding, and terminator sequences from Gz3639 generated the expected sized amplicon. The two *FgTri1* alleles differ by 21 nucleotides of which 15 are located within the four predicted introns. The remaining seven nucleotide differences do not affect the sequence of the predicted protein. A search of the PH-1 genome sequence with Gz3639 Pdb predicted protein by TBLASTN located the nucleotide sequence to scaffold 3 (approximately 5.33 mbp) and confirmed our initial results indicating that the genomic location of the putative *Fusarium Tri1* homologues are different.

The putative *FgTri1* shares 69% nucleotide identity and 64% amino acid identity with *F. sporotrichioides Tri1* by GCG GAP analysis The extent of nucleotide similarity is consistent with the failure to identify this gene by heterologous probing. Analysis of sequence flanking *FgTri1* identified a region that shared limited similarity with *FsTri16* (score = 61 bits; E value = $1e^{-14}$). It was clear from analysis of the predicted protein fragments from this region that *FgTri16* was nonfunctional as there were numerous stop codons and frame shifts. An examination of the promoter regions to both *FgTri1* and nonfunctional *FgTri16* identified three and four Tri6 binding sequence motifs (YNAGGCC), respectively (**Figure 1B**).

DISCUSSION

Studies of trichothecene biosynthesis over the past 15 years have focused on understanding the genetic and biochemical pathway leading to trichothecene formation in order to better understand their role in plant disease and the factors that regulate

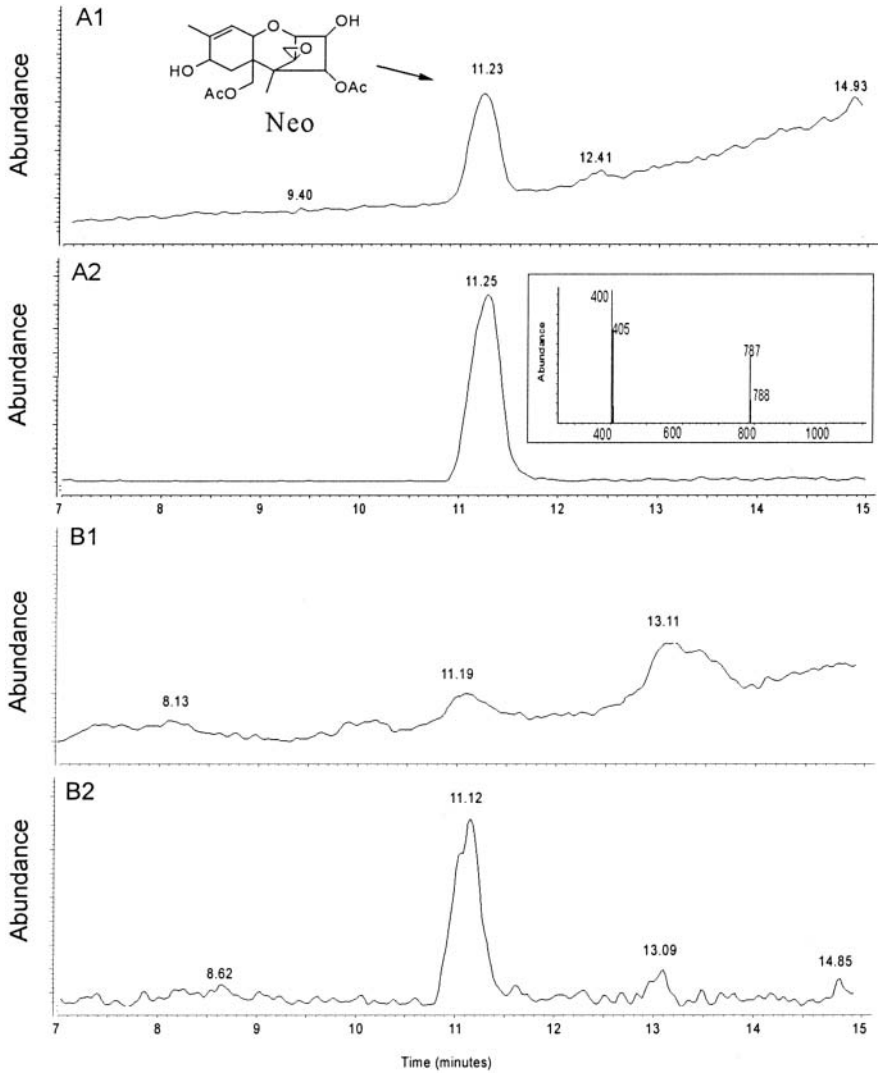

Figure 4. Partial reconstructed ion chromatograms of a standard solution of neosolaniol (**A**) and of extracts from a 3 day old culture of ΔFsTri16.49 (**B**). A1 and B1 correspond to the total ion current (TIC) while A2 and B2 correspond to the selected ion of 405 mass units. The insert in A2 corresponds to the mass spectra of the peak at 11.25 min in A2. The amount of 405 mass units represents the molecular weight of neosolaniol (M) plus sodium plus H_2O. The amount of 787 mass units represents 2 M plus sodium plus H_2O. The mass spectra of the peak at 11.12 min in B2 contained the same ions from neosolaniol as the standard at the same relative abundance.

their synthesis. Three genetic loci involved in trichothecene biosynthesis have been identified, *Tri101*, the 12-gene core cluster, and a 2-gene minicluster. An examination of published *Fusarium* genome sequence suggests that these loci are separated by millions of base pairs; *Tri101* is located on scaffold 4 (5.0 mbp), the core cluster is located on scaffold 2 (5.38 mbp), and the minicluster is located on scaffold 1 (8.82 mbp). To our knowledge, this is the first fungal secondary metabolite whose biosynthesis requires structural genes from three distant loci. This observation has potential implications for our understanding of the evolution of fungal secondary metabolite gene clusters. Genomic location of aflatoxin biosynthetic genes has been show to be critical for transcription regulation in *Aspergillus* (*27*). Perhaps this level of regulation is not required for fungal metabolites that are important virulence factors.

During the course of this study, Meek et al. (*10*) describe the use of a differential screen of cDNA derived from *F. sporotrichioides* T-2 toxin overproducing and underproducing mutants to identify a new trichothecene gene called *Tri1*. Gene disruption studies show that *Tri1* is required for C-8 oxygenation. The authors also describe the partial characterization of flanking genes through BLAST and Northern analysis. On one side of *Tri1* is a gene encoding a putative acyltransferase that appears to be transcriptionally regulated similarly to *Tri1* and on the other side of *Tri1* is a gene encoding a putative GAL4 type DNA binding protein that appears to be regulated differently (*10*). Functional studies of either gene were not described.

Here, we described the identification of *Tri1* using a different approach from that of Meek et al. (*10*). In 2000, a collection of 7495 ESTs from a trichothecene-overproducing transgenic strain of *F. sporotrichioides* became available (*12*, *13*; http://www-.genome.ou.edu/fsporo.html). It was clear from an examination of this collection that transcripts for previously characterized trichothecene genes were well-represented (**Table 1**). It seemed reasonable that transcripts for new trichothecene genes may also be represented to a similar degree in this collection. We focused on putative P450s because this family of enzymes could be involved in any of the unassigned oxygenation pathway steps at the time this study was initiated. Analysis of the *F. sporotrichioides* EST collection identified at least nine different P450s; four were represented by single ESTs while five were represented by multiple ESTs. Of these five, three corresponded

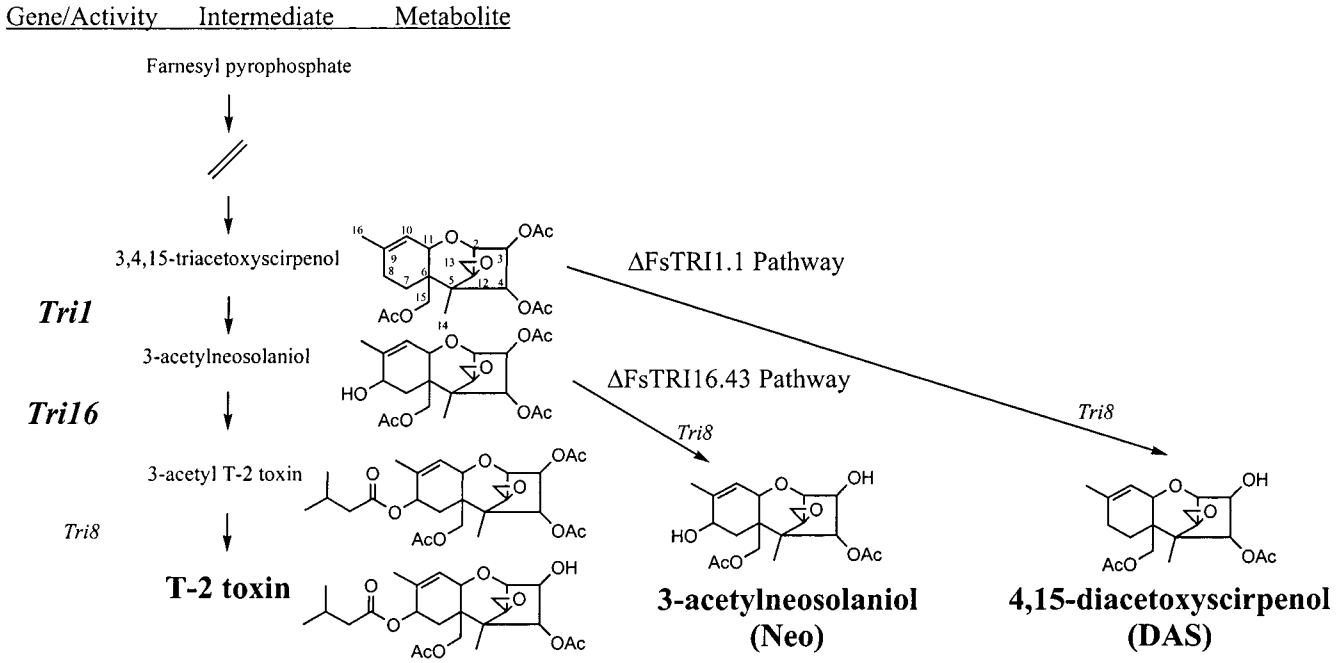

Figure 5. Proposed biochemical pathways leading to T-2 toxin in wild-type *F. sporotrichioides* and to Neo and DAS, the major trichothecenes that accumulate in *F. sporotrichioides* mutant strains ΔFsTri16.49 and ΔFsTri1.1, respectively.

to genes previously shown to be required for T-2 toxin biosynthesis (*Tri4*, *Tri11*, and *Tri13*) (**Table 1**). We decided to focus on the two remaining P450s and determine if either was involved in T-2 toxin biosynthesis.

Gene deletion studies indicated that only one of the two P450s, referred to as *Tri1*, was required for T-2 toxin biosynthesis, confirming the analysis described by Meek et al. (*10*). We also independently confirmed by complementation of the *Tox1* mutant MB1716 that *Tri1* and *Tox1* are the same gene. In addition, sequence analysis of the *Tri1* allele in the *Tox1* mutant identified a possible mechanism for the inability of MB1716 to produce wild-type levels of T-2 toxin; a single A→G transition was found that alters amino acid 378 from a lysine to a glutamic acid. Amino acid 378 is adjacent to an absolutely conserved motif in P450s that is thought to play a role in stabilizing the heme group (*28*, *29*). A glutamic acid at position 378 would likely disrupt proper folding of the Tri1 protein (J. Peterson, personal communication).

Sequence analysis of the genomic region flanking *Tri1* identified three additional ORFs. *Tri16*, located immediately downstream of *Tri1*, was suspected to be involved in trichothecene biosynthesis for three reasons: (i) it had four Tri6 binding sites in its promoter region, (ii) it was predicted to encode an acyltransferase, which could be involved in the addition of the isovalerate group to the C-8 oxygen, and (iii) it was represented in the EST library to a degree similar to some other trichothecene genes (**Table 1**). Transcriptional studies described here as well as by Meek et al. (*10*) support the third line of evidence. Chemical analysis of four independent transformants whereby the entire Tri16 coding region was deleted shows that this gene is required for esterification of isovalerate to the C-8 hydroxyl during T-2 toxin biosynthesis (**Figure 5**).

In contrast to *Tri16*, the putative regulatory gene *Pdb*, located immediately upstream of *Tri1*, did not have any Tri6 binding sites within its promoter and was not represented in the EST library. Nevertheless, it was possible that this gene was involved in trichothecene biosynthesis. The lack of *Pdb* transcripts in the EST library could be due to a low expression of this gene and the lack of Tri6 binding sites could reflect a different regulatory mechanism than previously described for trichothecene genes. Gene deletion demonstrated that *Pdb* is not required for trichothecene production. Interestingly, Pdb shares the greatest similarity with a protein from *Colletotrichum lindemuthianum* that is involved in the switch between biotrophic and necrotrophic phases of the infection process (*30*). Whether Pdb is involved in pathogenic growth has yet to be determined. The third ORF (*Orf1*), located adjacent to *Tri16*, does not share a significant similarity with any protein in the database. Although *Orf1* is represented in the EST library to the same degree as *Tri16*, it did not have any Tri6 binding sites and we decided not to pursue its characterization by gene deletion studies.

The principal trichothecenes produced by *F. graminearum*, DON and NIV, have a carbonyl function at the C-8 position. It is therefore reasonable that this species would require a *Tri1* homologue for C-8 oxygenation. Despite repeated efforts, we were unable to detect a *F. graminearum Tri1* homologue (*FgTRI1*) by standard Southern blotting and cosmid library screening methods. We were able to identify a *F. graminearum Pdb* homologue that shares 81% nucleotide identity to the *FsPdb*. Sequence comparison of the region flanking *Pdb* between the two *Fusarium* species indicated that the genomic regions containing these genes are very different. The recent publication of the genome sequence of *F. graminearum* NRRL 31084 (strain PH-1) allowed us to identify by sequence comparison a potential *FgTri1* homologue. Analysis of flanking sequence identified a nonfunctional version of *Tri16*. The presence of three and four Tri6 binding sites within the promoter region of *FgTri1* and *FgTri16*, respectively, provides additional circumstantial evidence that *FgTri1* is involved in DON biosynthesis and that *FgTri16* was once involved in trichothecene biosynthesis by a *F. graminearum* ancestor.

Recently, we proposed that the core trichothecene biosynthetic gene cluster in *F. sporotrichioides* represents an ancestral version of the cluster in *F. graminearum* (*5*). We (*5*, *9*) and Lee et al. (*31*) have shown that trichothecene structural diversity

at C-4 in *F. graminearum* evolved through inactivation of genes located in the core cluster that are involved in the oxygenation (*Tri13*) and subsequent acetylation (*Tri7*) at C-4. In a similar manner, we propose that the trichothecene minicluster in *F. sporotrichioides* represents an ancestral version of the minicluster present in *F. graminearum*. In this case, structural diversity at C-8 evolved through inactivation of the gene (*FgTri16*) involved in acylation of the C-8 oxygen.

The characterization of *Tri1* and *Tri16* also provides an example of a second evolutionary strategy leading to secondary metabolite structural diversity: the acquisition of new modifying genes located elsewhere in the genome. According to this hypothesis, the progenitors of *Tri1* and *Tri16* would have been present in the genome until natural mutations and unknown selective pressures led to the acquisition of trichothecene modifying functions. However, we cannot discount the possibility that the minicluster was once part of the core cluster and attained its present genomic location through chromosomal rearrangement. The breakup of the cluster may reflect unknown changes in selective pressures involved in developing and maintaining the original gene organization.

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