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A Genetic and Biochemical Approach to Study Trichothecene Diversity in *Fusarium sporotrichioides* and *Fusarium graminearum*

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Brown, D. W., McCormick, S. P., Alexander, N. J., Proctor, R. H., and Desjardins, A. E. 2001. A genetic and biochemical approach to study trichothecene diversity in *Fusarium sporotrichioides* and *Fusarium graminearum. Fungal Genetics and Biology* 32, 121–133. The trichothecenes T-2 toxin and deoxynivalenol (DON) are natural fungal products that are toxic to both animals and plants. Their importance in the pathogenicity of *Fusarium* spp. on crop plants has inspired efforts to understand the genetic and biochemical mechanisms leading to trichothecene synthesis. In order to better understand T-2 toxin biosynthesis by *Fusarium sporotrichioides* and DON biosynthesis by *F. graminearum,* we compared the nucleotide sequence of the 23-kb core trichothecene gene cluster from each organism. This comparative genetic analysis allowed us to predict proteins encoded by two trichothecene genes, *TRI9* and *TRI10,* that had not previously been described from either *Fusarium* species. Differences in gene structure also were correlated with differences in the types of trichothecenes that the two species produce. Gene disruption experiments showed that *F. sporotrichioides TRI7* (*FsTRI7*) is required for acetylation of the oxygen on C-4 of T-2 toxin. Sequence analysis indicated that *F. graminearum TRI7* (*FgTRI7*) is nonfunctional. This is consistent with the fact that the *FgTRI7* product is not required for DON synthesis in *F. graminearum* because C-4 is not oxygenated.

Index Descriptors: plant pathogenic fungi; *Fusarium*; secondary metabolism; *Gibberella*; trichothecene gene cluster.

Trichothecenes belong to the sesquiterpenoid family of natural products and are potent inhibitors of eukaryotic protein synthesis (McLaughlin *et al.,* 1977; reviewed in Sharma and Kim, 1991). They are synthesized by a wide variety of fungal genera including *Fusarium, Myrothecium, Stachybotrys, Cephalosporium, Trichoderma,* and *Trichothecium,* as well as two plant species (Sharma and Kim, 1991; Jarvis, 1992). Trichothecenes have gained particular notoriety as a family of materials highly toxic to both plants (phytotoxic) and animals (mycotoxic) (Desjardins *et al.,* 1993). Research on trichothecenes has focused on understanding their biosynthesis and function in *Fusarium* species that are pathogenic to crop plants. These fungi are important pathogens of maize, wheat, and other cereal grains worldwide and are responsible for billion dollar losses yearly in these commodities due to lower yields and to lower value due to trichothecene contamination (Windels, 2000). In addition to their phytotoxicity, trichothecenes play an important role as virulence factors in ear rots of both maize and wheat caused by *Fusarium graminearum* (sexual stage *Gibberella zeae*) (Proctor *et al.,* 1995; Desjardins *et al.,* 1996; Harris *et al.,* 1999). The

Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. AF359360 and AF359361.

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mycotoxicity of trichothecenes, coupled with their frequent occurrence on a variety of agricultural commodities, has precipitated significant effort to understand their biosynthesis.

Trichothecenes comprise a very large, structurally diverse group of natural products that share a trichothecane skeleton (Jarvis, 1991). To date, more than 180 naturally occurring trichothecenes have been described in the literature. More than 60% are simple, or nonmacrocyclic, trichothecenes (Grove, 1996; Loukaci *et al.*, 2000). Structural diversity arises through the pattern of substitution of the trichothecane skeleton and, if present, the macrocyclic ring (Jarvis, 1991). Figure 1 depicts the biochemical and genetic pathway leading to the *Fusarium* nonmacrocyclic trichothecenes: T-2 toxin, deoxynivalenol (DON),[2] and nivalenol (NIV) (Desjardins *et al.*, 1993; Grove, 1996). Most of the biochemical and genetic studies that defined the pathway depicted in Fig. 1 have used biosynthesis of T-2 toxin by *F. sporotrichioides* as a model system (Desjardins *et al.*, 1993; Alexander *et al.*, 1999; Hohn *et al.*, 1999; McCormick *et al.*, 1999). The sequence of later intermediates in the biosynthesis of DON and NIV by *F. graminearum* has not yet been well defined.

Biochemical and genetic studies of *F. sporotrichioides* identified the first trichothecene biosynthetic gene cluster. To date, in strain NRRL 3299 of this species, 10 cluster genes have been described and designated (from left to right) *FsTRI8, FsTRI7, FsTRI3, FsTRI4, FsTRI6, FsTRI5, FsTRI10, FsTRI9, FsTRI11*, and *FsTRI12* (Fig. 2 and Table 1). In the *F. graminearum* trichothecene biosynthetic gene cluster, *FgTRI5* was sequenced from strain W-8 (Proctor *et al.*, 1995), and *FgTRI6, FgTRI11*, and *FgTRI12* were sequenced from strain F15 (Matsumoto *et al.*, 1999; Wuchiyama *et al.*, 2000). An 11th trichothecene biosynthetic gene, *TRI101*, is located outside of the gene clusters in both *F. sporotrichioides* and *F. graminearum* (Kimura *et al.*, 1998; McCormick *et al.*, 1999). The organization and orientation of *FgTRI5, FgTRI6, FgTRI11*, and *FgTRI12* in the *F. graminearum* gene cluster are almost identical to those of their homologues in the *F. sporotrichioides* gene cluster. In contrast, the *Myrothecium roridum* trichothecene genes *MyTRI4, MyTRI5*, and *MyTRI6* share much less nucleotide identity with either of their *Fusarium* homologues and are organized very differently within the *M. roridum* genome (Trapp *et al.*, 1998).

The biosynthesis of trichothecenes begins with the cyclization of farnesyl pyrophosphate to trichodiene and is catalyzed by TRI5 (Desjardins *et al.*, 1993). A total of 6 of the 14 putative oxygenations, isomerizations, cyclizations, and esterifications leading to T-2 toxin depicted in Fig. 1 have been assigned to genes in the trichothecene cluster. The modification of the trichodiene skeleton begins with TRI4, a putative cytochrome P450-monooxygenase, that catalyzes the oxidation of trichodiene to 2-hydroxytrichodiene (Hohn *et al.*, 1995). The gene(s) required for the hydroxylation of C-11 and C-3 of the trichodiene skeleton, producing 2,3,11-trichotriol, and the genes required for the following isomerization and cyclization, producing isotrichodermol, are unknown. The acetylation of isotrichodermol to isotrichodermin is catalyzed by TRI101 (McCormick *et al.*, 1999). The C-15 hydroxylation is catalyzed by TRI11, producing 15-decalonectrin (Alexander *et al.*, 1998), which is then acetylated by TRI3, producing calonectrin (McCormick *et al.*, 1996). The gene responsible for the oxidation of calonectrin at C-4, producing 3,15-diacetoxyspirpenol, is unknown, whereas the acetylation of the C-4 oxygen is catalyzed by TRI7 (this study). The conversion of 3,4,15-triacetoxysperpenol to 3-acetyl T-2 toxin involves an oxidation at C-7 followed by an esterification of the C-7 oxygen. TRI8 is involved in this step in an unknown manner (this study). And finally, the C-3 oxygen is deacetylated by the product of an unknown gene, producing T-2 toxin. There are four additional genes located in the cluster; *TRI6* encodes a transcriptional activator, *TRI12* encodes an efflux pump (Alexander *et al.*, 1999), and *TRI9* and *TRI10* encode proteins with poorly understood roles in trichothecene biosynthesis.

The general objective of this study was to better understand trichothecene biosynthesis by *F. sporotrichioides* and *F. graminearum*. We reasoned that a careful comparison of the genetic information contained within each gene cluster could help in the characterization of the genes and their biochemical functions. Specific objectives were (1) to complete and reanalyze the sequence of the *F. sporotrichioides* cluster of 10 genes, (2) to sequence 6 additional genes to complete the *F. graminearum* cluster of 10 genes, and (3) to correlate differences in gene structure with differences in the type of trichothecenes that the two species produce.

[2] Abbreviations used: EST, expressed sequence tag; DON, deoxynivalenol; NIV, nivalenol; DAS, diacetoxyscirpenol; MAS, monoacetoxyscirpenol; TAS, triacetoxyscirpenol; B2ST, BLAST 2 Sequence Tool; YPG, yeast extract–peptone–glucose medium; GYEP, glucose–yeast extract–peptone medium; CIP, calf intestinal alkaline phosphatase; NR NCBI, nonredundant database maintained by the National Center for Biotechnology Information; GLC, gas–liquid chromatography; GC/MS, gas chromatography/mass spectrometry.

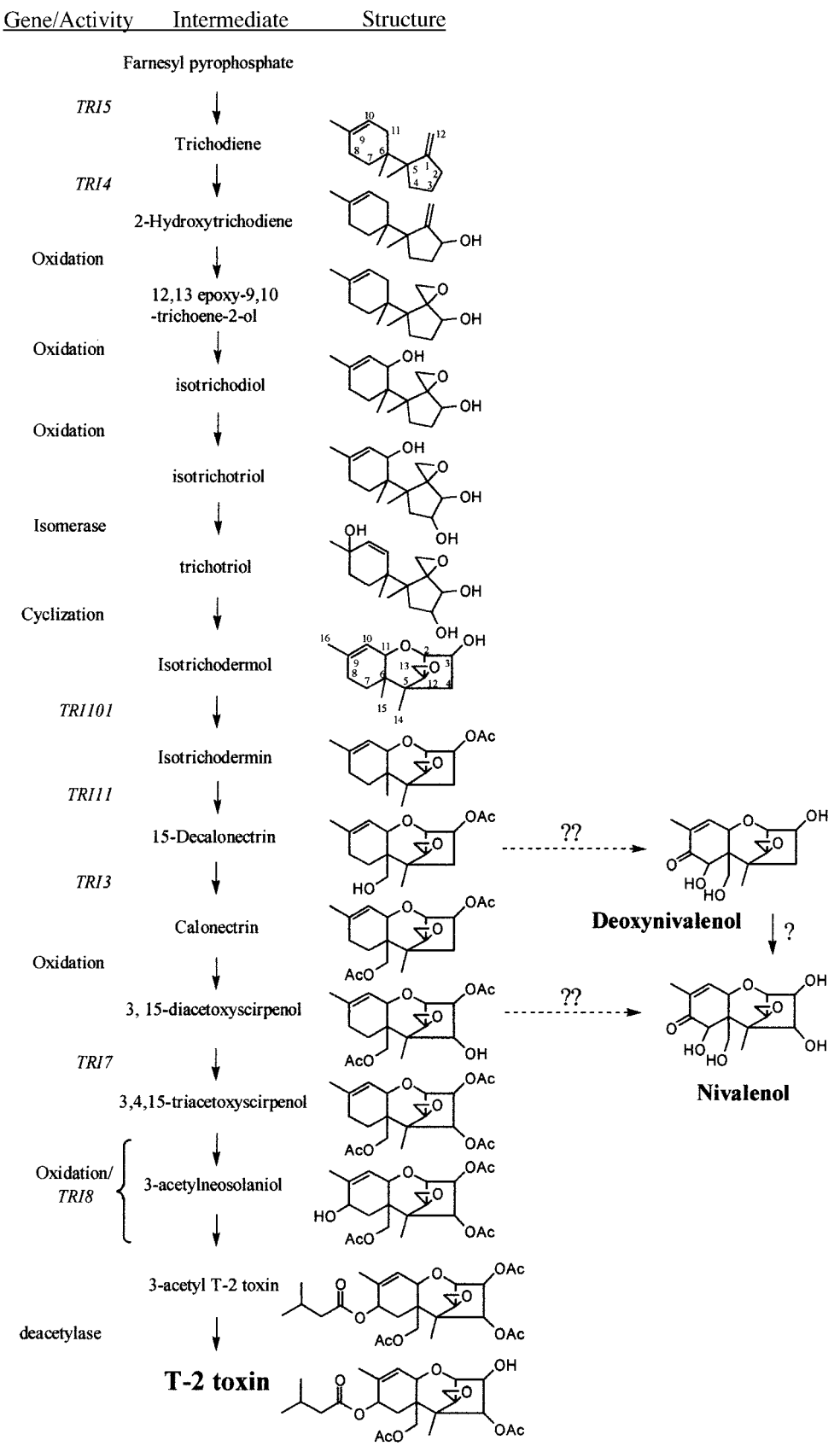

FIG. 1. Biochemical and genetic pathway for trichothecene biosynthesis in *Fusarium* species.

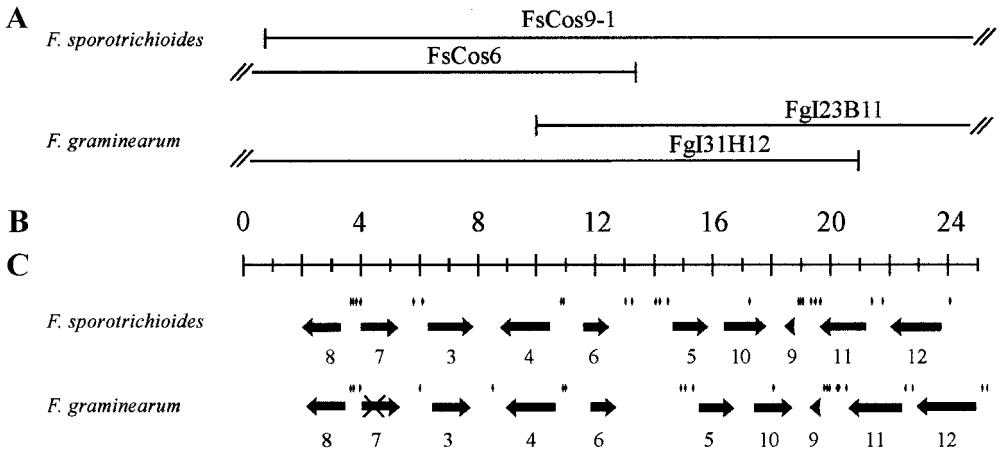

FIG. 2. Genomic organization of the *Fusarium* trichothecene biosynthetic gene cluster. (A) Overlapping cosmid clones representing the genomic region comprising the trichothecene biosynthetic gene cluster. (B) Relative scale in kilobases. (C) Organization of the predicted ORFs for *F. sporotrichioides* and *F. graminearum*. The arrowheads indicate the direction of transcription. Numbers underneath each large arrow refer to the specific genes, e.g., 5 indicates *TRI5*. Genes from different *Fusarium* species with the same number are homologues. The symbol ◆ indicates TRI6-binding sites YNAGGCC, GGCCTNR, or YNAGGCCTNR. The X over *F. graminearum TRI7* indicates that this gene does not encode a functional protein.

MATERIALS AND METHODS

Strains, media, and culture conditions. *F. sporotrichioides* NRRL 3299 and *F. graminearum* GZ3639 have been described previously (Beremand, 1987; Proctor *et al.*, 1995). Cultures were grown in YPG medium (0.3% yeast extract, 1% peptone, 2% glucose) at 28°C with shaking at 200 rpm for 4 days prior to DNA isolation. Strains 7-4-7 (Δ*FsTRI7*) and 8-5-6 (Δ*FsTRI8*) are hygromycin-resistant transformants of strain NRRL 3299 and were maintained on V-8 juice agar slants containing 150 μg/ml hygromycin B (Sigma, St. Louis, MO). Strain MB1716 is a T-2 toxin-deficient strain created by UV-mutagenesis from NRRL 3299 (Beremand, 1987). Strain 1716cos9-1-3 was derived from MB1716 by transformation with *F. sporotrichioides* Cos9-1 and was engineered to produce high levels of 4,15-diacetoxyscirpenol (4,15-DAS) (Hohn *et al.*, 1993). *Saccharomyces cerevisiae* strain JG 436 (TRI101/TRI12) has been described previously (McCormick *et al.*, 1999).

TABLE 1
Genes of the Trichothecene Cluster and Their Function

Gene	Putative activity	Reference
TRI8	Esterase/oxygenase	TS[a]
TRI7	Acetylesterase	TS
TRI3	Acetylesterase	McCormick *et al.* (1996)
TRI4	Mono-oxygenase	Hohn *et al.* (1995)
TRI6	Regulatory protein	Proctor *et al.* (1995)
TRI5	Trichiodiene synthase	Hohn and Beremand (1989)
TRI10	Unknown	—
TRI9	Unknown	—
TRI11	Mono-oxygenase	Alexander *et al.* (1999)
TRI12	Efflux pump	Alexander *et al.* (1998)

[a] TS, this study.

Plasmid construction and F. sporotrichioides transformation. The *FsTRI7* disruption plasmid was created as follows: A 657-bp DNA fragment of *FsTRI7* coding sequence (base 4266 to base 4923) was generated by PCR and cloned into pCRII (Invitrogen, La Jolla, CA) creating pTOX7-2. The entire fragment was then released by digestion with *Sac*I and *Xho*I and cloned into the *Sac*I/*Sal*I sites of the fungal transformation vector pUCH1 (Hohn *et al.*, 1993) to yield plasmid pTOX7-3. The *FsTRI8* disruption plasmid was created as follows: a 569-bp DNA fragment of *FsTRI8* coding sequence (base 2642 to base 3211) was created by digestion with *Eco*47III/*Sac*I and cloned into the *Sac*I/*Sma*I sites of pUCH1 to yield plasmid pTri8-1. Transformation of *F. sporotrichioides* and the selection and isolation of transformants were performed essentially as previously described (McCormick *et al.*, 1996).

F. graminearum cosmid library construction. A genomic library was constructed using *F. graminearum* strain GZ3639 and the pSuperCos1 cosmid vector (Strata-

gene, La Jolla, CA) following the protocol supplied by the manufacturer. For preparation of the high-molecular-weight genomic DNA, mycelia were lyophilized, ground to a fine powder in liquid nitrogen, and solubilized with isolation buffer (10 mM NaCl, 20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 100 μg/ml proteinase K, 0.5% SDS) with gentle rocking. The supernatant from a short, low-speed centrifugation was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform:isoamyl alcohol (24:1). The DNA was further purified by a CsCl density gradient. The DNA was partially digested with *Mbo*I to generate fragments >40 kb and then dephosphorylated with calf intestinal alkaline phosphatase (CIP). The pSuperCos1 cosmid vector was prepared first by digestion with *Xba*I, followed by CIP treatment and then by digestion with *Bam*HI. The *Mbo*I fragments were then ligated with the prepared pSuperCos1 and packaged into phage particles in accordance with the manufacturers instructions (Stratagene). The library was titered using bacterial host strain XL1-Blue. Approximately 5000 clones were picked to 96-well plates. All enzymes were purchased from New England BioLabs (Beverly, MA).

***F. graminearum* trichothecene cosmid identification.** Heterologous probes derived from two *F. sporotrichioides* trichothecene genes (hereafter referred to as *FsTRI#*) were used to identify *F. graminearum* cosmids that contain trichothecene genes (hereafter referred to as *FgTRI#*). DNA was generated by PCR using primers flanking either *FsTRI6* (primer tri6F, CTCTTTGATCGTGTTGCGTCTC, and tri6R, CGCCAAACTCGTCATCATTTACG) or *FsTRI12* (primer tri12F, GCAGACAAAGCCACGAGTATC, and tri12R; GTGTCGACCTTGAATCTCAGCC) and *F. sporotrichioides* cosmid Cos9-1 as a template. DNA probes were labeled with [α-^{32}P]dCTP (DuPont NEN, Boston MA) using the Prime-A-Gene labeling system (Promega, Madison, WI). *F. graminearum* cosmids FgI23B11 and FgI5C2 were chosen for further analysis.

***F. sporotrichioides* cDNA library construction and screening.** The *F. sporotrichioides* cDNA library was created using the Librarian cDNA Construction System (Invitrogen) in the pYES2 vector essentially as recommended by the manufacturer (Trapp, 1997; Alexander *et al.,* 1998). The library was stored as a pool of clones and was screened for clones corresponding to transcripts from genes within the trichothecene gene cluster by hybridization with DNA derived from *F. sporotrichioides* cosmid Cos9-1 using standard techniques (Sambrook *et al.,* 1989; Hohn *et al.,* 1993).

Sequencing analysis. All sequence data were obtained using the ABI Prism BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA). Reactions were analyzed using a PE Biosystems 377 automated DNA sequencer. Sequencing templates were generated using the GPS-1 kit from New England BioLabs, Inc., following the manufacturer's instructions. This kit created sequencing templates by placing a transposon containing universal-priming sites into target DNA at random locations *in vitro.* 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 (Altschul *et al.,* 1997; Zhang *et al.,* 1998). Nucleotide or amino acid sequence comparisons were carried out using the DNAMAN software (Lynnon BioSoft, Vaudreuil, Quebec, Canada). Nucleotide sequence comparison was carried out using the BLAST 2 Sequence Tool (B2ST) (Tatusova and Madden, 1999).

Trichothecene toxin assays. Transformants and other strains of *F. sporotrichioides* were screened for trichothecene production on GYEP medium (5% glucose, 0.1% yeast extract, 0.1% peptone) as previously described (McCormick *et al.,* 1996). Liquid shake cultures were harvested after 7 days of incubation at 28°C on a gyratory shaker at 200 rpm, extracted with ethyl acetate, and analyzed by gas–liquid chromatography (GLC) and gas chromatography/mass spectrometry (GC/MS). Trichothecene concentrations were determined using appropriate standard curves.

Extraction and isolation of trichothecenes. Trichothecenes produced by the *F. sporotrichioides* mutant strains were isolated from liquid shake cultures grown as described above. Cultures were extracted twice with ethyl acetate and the combined extracts were concentrated under reduced pressure. The extract was separated on a silica gel column eluted with 5% methanol in dichloromethane (v:v). Column fractions were monitored for the presence of trichothecenes by GLC and TLC. 4-Deacetyl T-2 toxin (HT-2 toxin)-containing fractions from strain Δ*FsTRI7* were combined and further purified by silica gel chromatography with 5% methanol in ether (v:v). 4,15-DAS-containing fractions from strain Δ*FsTRI8* were combined and recrystallized from ether.

Trichothecene standards. 4,15-DAS was isolated from strain 1716cos9-1-3, and 15-monoacetoxyscirpenol (15-MAS) was prepared by treating 4,15-DAS with 0.1 N NaOH; 3,4,15-triacetoxyscirpenol (3,4,15-TAS) was pre-

pared from 4,15-DAS treated with acetic anhydride in pyridine, and 3,15-DAS was prepared by feeding 15-MAS to liquid cultures of *S. cerevisiae* strain JG 436. HT-2 toxin was isolated as described above from strain Δ*FsTRI7*. T-2 tetraol was prepared by hydrolysis of T-2 toxin with 0.1 N NaOH. All standards were greater than 95% pure as determined by GLC.

Whole-cell feeding experiments. Liquid cultures of *F. sporotrichioides* strains Δ*FsTRI7*, Δ*FsTRI8*, and MB1716 were inoculated with conidia washed from V-8 juice agar plates, at a starting density of 5×10^4 conidia/ml in 20 ml GYEP medium in a 50-ml Erlenmeyer flask, and incubated at 28°C as described above. After 24 h, a 25 mM stock solution of the trichothecene in acetone was added to the culture to a final concentration of 250 μM and 1% acetone (v:v). Control cultures had acetone added to a final concentration of 1% acetone (v:v). Cultures were incubated for up to 6 additional days, extracted with ethyl acetate, and analyzed by GLC.

RESULTS AND DISCUSSION

Reanalysis of the F. sporotrichioides Gene Cluster

The *F. sporotrichioides* NRRL 3299 trichothecene gene cluster is represented by five GenBank nucleotide entries: U22463, U22462, U22150, M27246, and AF011355. We resequenced this region and found that the aggregate GenBank sequence agreed well with our new sequence data. Over the total of 22,037 bases, we found evidence to make 33 edits. One edit, the deletion of a base at 5228 (base 3403 in Accession No. U22463), had a dramatic effect on the predicted FsTRI7 protein described in Accession No. U22463. The resulting shift in reading frame altered the sequence of the last 19 amino acids of the original predicted protein and added 43 amino acids to the carboxy-terminal end of the new predicted protein. Additional differences noted between the original and the new sequences include a 27-bp gap in Accession No. U22150 located 748 bp upstream from the end of *FsTRI6* and a 16-bp duplication in Accession Nos. U22462 and U22150 that is not present in the new sequence. A total of eight predicted proteins are described in the previous trichothecene cluster entries: FsTRI8, FsTRI7, FsTRI3, FsTRI4, FsTRI6, FsTRI5, FsTRI11, and FsTRI12. Two additional open reading frames (ORFs), designated *FsTRI10* and *FsTRI9*, are located between *FsTRI5* and *FsTRI11*. Both *FsTRI10* and *FsTRI9* have been referred to very briefly in the literature and little information on their structure and function is available (Beremand and Hohn, 1995; Hohn *et al.*, 1999).

In a preliminary report from our research group, former members described *FsTRI10* as 555- and 911-bp ORFs that overlap by 41 bp located proximal to *FsTRI5* (Beremand and Hohn, 1995). DNA fragments corresponding to these ORFs hybridize to the same size transcript (i.e., 1400 bp) and display a transcription profile that parallels other pathway genes, suggesting that the ORFs are portions of a single coding sequence (Beremand and Hohn, 1995). In 1999, nucleotide sequences including *FsTRI10* and *FsTRI9* were first made publicly available via an update to Accession No. M27246 that was originally submitted in 1995. In addition, predicted proteins for these genes were depicted as bars in figures describing the genetic organization of the trichothecene gene cluster (McCormick *et al.*, 1998; Hohn *et al.*, 1999).

In the present study of *FsTRI10*, we identified perfect intron border sequences flanking a frameshift and a conserved internal consensus sequence or lariat site (Gurr *et al.*, 1987), indicating further that the two ORFs represent a single coding sequence that is interrupted by an intron (this study). The predicted FsTRI10 protein is 420 aa and does not share similarity with any proteins in GenBank. A comparison of the sequence located between *FsTRI5* and *FsTRI11* to an expressed sequence tag (EST) library derived from a *F. sporotrichioides TRI10* overproducing strain derived from NRRL 3299 (Roe *et al.*, 2000) by BLASTN identified two cDNA clones, represented by sequences j3h01fs.rl and d3g05fs.fl, that overlap the predicted *FsTRI10*. Clone d3g05fs.fl appeared to be an antisense transcript and includes a portion of the predicted *FsTRI10* intron. It is possible that the RNA corresponding to this EST is involved in antisense gene regulation, a strategy observed in *Aspergillus flavus* during aflatoxin biosynthesis (Woloshuk *et al.*, 1994).

In the present study, we found evidence for *FsTRI9* through a routine screen of a *F. sporotrichioides* cDNA library with DNA from cosmid Cos9-1 (Trapp, 1997). The sequence of the putative *FsTRI9* transcript is identical to the genomic sequence upstream from *FsTRI10* between bases 17,993 and 18,560, and the direction of transcription of *FsTRI9* is opposite that of *FsTRI10*, as previously reported (Hohn *et al.*, 1999). A comparison of the sequence located between *FsTRI5* and *FsTRI11* to the *F. sporotrichioides* EST library (Roe *et al.*, 2000) by BLASTN identified two clones almost identical to the *FsTRI9* cDNA found in this study.

TABLE 2
Comparison of Nucleotide and Predicted Protein Sequences for *F. sporotrichioides* and *F. graminearum* Trichothecene Biosynthetic Genes[a]

Trichothecene gene	Fs aa	Fg aa	Protein % ID	NT % ID
TRI8	447	445	76	82
TRI7	441	NA	NA	75
TRI3	519	439	76	84
TRI4	520	520	87	84
TRI6	217	218	86	85
TRI5	374	375	91	84
TRI10	420	420	88	85
TRI9	43	43	97	92
TRI11	492	488	90	86
TRI12	598	623	78	80
Average			85.4	84.6

[a] Fs, *F. sporotrichioides*; Fg, *F. graminearum*; aa, amino acid; Fs or Fg aa, number of amino acids of the predicted protein; ID, identity; NT, nucleotide; Protein % ID is the percentage amino acid identity shared between homologous proteins; NT % ID is the percentage nucleotide identity shared between homologous genes; Avg, average; and NA, not applicable.

Analysis of the F. graminearum Gene Cluster

In a screen of a *F. graminearum* GZ3639 genomic library, we identified a series of overlapping cosmid clones containing DNA sequences that share significant identity to *F. sporotrichioides* trichothecene genes (Fig. 2). BLASTX analysis of the cosmid sequence with the NR NCBI database identified seven putative trichothecene genes, *FgTRI8*, *FgTRI3*, *FgTRI4*, *FgTRI6*, *FgTRI5*, *FgTRI11*, and *FgTRI12*, with similarity to the corresponding *F. sporotrichioides* trichothecene genes in the five GenBank entries (Table 2). The *FgTRI5* allele was previously described from a different strain of *F. graminearum* (W-8) (Accession No. U22464) (Proctor *et al.*, 1995). Only three bases differ between the *FgTRI5* alleles of strains W-8 and GZ3639: two within the coding sequence that have no effect on the predicted protein sequence and one within the noncoding sequence. Identification of the three remaining genes in the trichothecene biosynthetic core cluster was facilitated by the application of comparative nucleotide sequence analysis as described below.

Comparative Cluster Analysis

Comparative genetics is a powerful tool to help elucidate biological processes. A comparison of genetic material that is responsible for a similar function (e.g., trichothecene biosynthesis), but has been obtained from different organisms, can identify both nucleotide differences and similarities that define biologically important genetic elements. For example, a comparison of *F. graminearum* sequence located between *FgTRI5* and *FgTRI11* to the homologous region from *F. sporotrichioides* provided support for the proposed *FsTRI10* intron and allowed the concomitant prediction of *FgTRI10*. In *F. graminearum*, the *FgTRI10* sequence region has two contiguous ORFs similar in length to the two *FsTRI10* ORFs. A putative *FgTRI10* intron was identified between these ORFs based on the presence of intron border sequences and a conserved internal motif. The processing of this intron would eliminate the stop codon and create a single ORF in the resulting transcript. Additional evidence for the *TRI10* introns was evident when we compared the putative *TRI10* genomic sequence between *Fusarium* species. The identity shared between the putative introns was 14% lower than the identity shared between flanking, coding sequence. This value is similar to the degree of identity shared between noncoding sequence, both intron and intragenic, located elsewhere in the cluster (Table 3).

Comparative nucleotide sequence analysis may also help define conserved intron features enabling more accurate prediction of fungal coding sequences. The genes examined in this study contain a total of 37 introns, all of which have the highly conserved intron border sequence

TABLE 3
Comparison of the Intergenic/Noncoding Sequences within the *F. sporotrichioides* and *F. graminearum* Trichothecene Biosynthetic Gene Clusters[a]

Location	Fs IVS	Fg IVS	IVS % ID
Before *TRI8*	2000	2000	42
IVS8/7	697	681	68
IVS7/3	766	893	53
IVS3/4	765	953	56
IVS4/6	1158	1168	68
IVS6/5	2303	2887	50
IVS5/10	616	667	67
IVS10/9	460	425	68
IVS9/11	1089	1167	56
IVS11/12	645	557	71
After *TRI12*	770	779	60
Average			60

[a] Fs, *F. sporotrichioides*; Fg, *F. graminearum*; IVS, intervening sequence in basepairs; IVS % ID is the percentage identity shared between the intervening sequences; IVS8/7 is the intervening sequence between *TRI8* and *TRI7*; Before *TRI8* is the sequence 5′ to *TRI8*; and After *TRI12* is the sequence 3′ to *TRI12*.

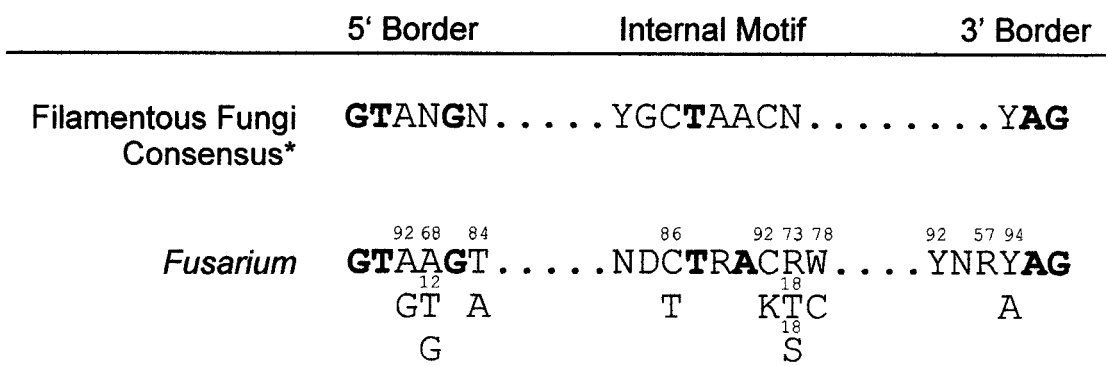

FIG. 3. Comparison of 5′ and 3′ borders and internal motif for introns identified in *F. graminearum* and *F. sporotrichioides* trichothecene biosynthetic genes. The boldface letters indicate invariant nucleotides. Numbers above the nucleotides refer to the percentage occurrence of that nucleotide in 37 introns examined. The invariant A of the internal conserved motif in *Fusarium* species is located, on average, 14 bp from the 3′ border (range = 11 to 24 bp). The average intron length was 60 bp and the range was 49 to 90 bp. *Adapted from Gurr *et al.*, 1987.

and internal motif typical of filamentous fungal spliceosomal introns (Gurr *et al.*, 1987). In *A. nidulans* and *Neurospora crassa*, only the fourth position T of the internal motif is invariant. In yeast, the sixth-position A is thought to be critical for the formation of the branch site of the lariat splicing intermediate (Gallwitz *et al.*, 1987). We have found that in the *Fusarium* species trichothecene biosynthetic gene introns, the fourth-position T and the sixth-position A are both invariant (Fig. 3). This trend may be characteristic of *F. sporotrichioides/graminearum* genes in general. A search of GenBank found 29 introns in likely primary and secondary metabolic genes from these species that also contain conserved internal motifs with the A and T invariant nucleotides (Accession Nos. AB014492, AF107883, AF107876, AB011417, and U85575).

Comparative nucleotide sequence analysis also played a key role in the characterization of *TRI9.* As noted above, we identified the *F. sporotrichioides FsTRI9* on a 578-bp cDNA. However, we could not identify the likely protein encoded by the transcript due to the presence of two initiation codons near the 5′ end that resulted in possible proteins of 43 and 68 aa. A B2ST (Tatusova and Madden, 1999) comparison of this region to the comparable *F. graminearum* region identified a 146-bp sequence with 91% identity. The fact that the *F. sporotrichioides* ORF encoding the 43-aa protein lies entirely within this conserved sequence suggests that this is the protein product of the *F. sporotrichioides* transcript. The similarity between the *F. sporotrichioides* transcript and the *F. graminearum* sequence dropped to 50% over the next 100 bp, averaging out to 64% over the rest of the sequence. The predicted TRI9 proteins from both *F. sporotrichioides* and *F. graminearum* do not share significant similarity with any protein in GenBank. Functional characterization of *TRI9* is in progress in our laboratory and characterization of *TRI10* is being carried out by Beremand and co-workers (Beremand and Hohn, 1995).

Intergenic Sequence Analysis

One focus of this study was the analysis of intergenic regions of sequence within the *Fusarium* trichothecene gene cluster. Our objective was to compare the same intergenic regions of homologous genes in *F. sporotrichioides* and *F. graminearum* in an effort to identity new protein coding or regulatory regions. Eleven noncoding regions of *F. sporotrichioides* and *F. graminearum*, including nine intergenic regions, the region 3′ to *TRI8*, and the region 5′ to *TRI12*, were compared. DNAMAN was used to determine the degree of identity and B2ST was used to identify local regions of conservation. The degree of identity shared between intergenic regions, as calculated by DNAMAN, ranged from 50 to 68% with an average of 60% (Table 3). In contrast, the identity shared between coding regions ranged from 80 to 92% with an average of 85% (Table 2). Although the average intergenic identity was low, B2ST analysis identified local regions of sequence ranging in size from 66 to 300 bp that shared a high level of identity more typical of coding regions. However, none of these regions of sequence contained ORFs capable of encoding proteins greater than 20 amino acids.

One function for the conserved intergenic sequence regions may be transcriptional regulation. Recently, Hohn *et al.* (1999) identified a nucleotide motif (YNAGGCC) that is necessary but not sufficient for transcriptional activation of trichothecene genes by FsTRI6, a Cys_2His_2

zinc-finger DNA-binding protein. Twenty-three possible TRI6-binding motifs are dispersed within seven of the eight intergenic regions analyzed upstream from predicted start codons in *F. sporotrichioides.* Nineteen possible TRI6-binding motifs are dispersed within the same regions in *F. graminearum* (Fig. 2). Sixteen of these motifs align by B2ST. Experiments are in progress to determine the transcriptional importance of the TRI6-binding motifs that are not well conserved between different *Fusarium* species. Nine of the 10 trichothecene genes described have putative TRI6-binding motifs located within their promoter regions. The *TRI10* promoter region does not have this motif and therefore appears to be the only gene in both *Fusarium* species that is not directly regulated by TRI6.

There are a number of possible TRI6-binding motifs that are located outside of likely gene promoter regions. For example, there is a TRI6-binding motif located within the predicted coding sequence for both *FgTRI10* and *FsTRI10.* Hohn *et al.* (1999) found that the putative TRI6-binding motif in *FsTRI10* did not bind FsTRI6 *in vitro* and, as a result, concluded that additional elements were required for protein binding.

Trichothecene Genes from Other F. graminearum Strains

The sequences of three trichothecene cluster genes, *FgTRI6, FgTRI11,* and *FgTRI12,* from *F. graminearum* strain F15 are also present in GenBank (Matsumoto *et al.,* 1999; Wuchiyama *et al.,* 2000). Strains GZ3639 and F15 belong to different *Fusarium* lineages defined, in part, by sequence differences between *FgTRI101* alleles (O'Donnell *et al.,* 2000). To date, *TRI101* is the only trichothecene biosynthetic gene that has not been located within the core gene cluster. At the nucleotide level, the *TRI101* alleles from strains GZ3639 and F15 share more than 99% identity. In contrast, the coding sequences of the *FgTRI6, FgTRI11,* and *FgTRI12* alleles share 90 to 95% identity. These differences are scattered throughout the sequence and are composed almost entirely of basepair substitutions. It will be interesting to see whether a phylogenetic comparison of genes located within the cluster supports the proposed lineages of *F. graminearum* strains (O'Donnell *et al.,* 2000).

Functional Analysis of F. sporotrichioides FsTRI7 and FsTRI8

The roles that *FsTRI7* and *FsTRI8* play in trichothecene biosynthesis have not previously been established (Keller and Hohn, 1997; McCormick *et al.,* 1998; Hohn *et al.,* 1999). The requirement of *FsTRI7* and *FsTRI8* for trichothecene biosynthesis has been inferred based on the following observations: (1) the close proximity of these genes to other trichothecene genes, (2) the presence of putative FsTRI6-binding sites within the *FsTRI8*/*FsTRI7* intragenic region, and (3) a pattern of transcript expression during trichothecene biosynthesis that is identical to other pathways (McCormick *et al.,* 1998; Hohn *et al.,* 1999). The predicted proteins for both of these genes were compared to the NR NCBI database. FsTRI7 shared no similarity to previously described proteins; thus we were not able to propose a function for this gene based on sequence alone. FsTRI8 shared similarity to a family of lipases from *Candida albicans.* The similarity was both significant (score = 174, $E = 2 \times 10^{-42}$; Accession No. AAF69522) and sufficiently unique to suggest a possible role for FsTRI8 in trichothecene biosynthesis: the addition of isovalerate to the C-8 hydroxyl group to generate 3-acetyl T-2 toxin (Fig. 1).

Gene disruption and trichothecene feeding experiments provided conclusive evidence for the role of *FsTRI7* in trichothecene biosynthesis in *F. sporotrichioides.* The *TRI7* disruption mutant, strain Δ*FsTRI7,* did not produce T-2 toxin, but accumulated HT-2 toxin (Fig. 4) by an alternate pathway that presumably included C-8 oxidation of 3,15-DAS to produce 3,15-diacetyl-T-2 tetraol, C-8 esterification to 3-acetyl HT-2 toxin, and C-3 deacetylation to HT-2 toxin. The presence of an acetyl group on the C-4 oxygen in T-2 toxin and the lack of an acetyl group on the C-4 oxygen of HT-2 toxin suggest that the product of *FsTRI7* is responsible for this specific acetylation (Fig. 1). This conclusion was confirmed by feeding studies, which showed that a C-4 acetyl group was required for strain Δ*FsTRI7* to convert trichothecenes to T-2 toxin (Table 4).

In contrast to *FsTRI7,* gene disruption and precursor feeding experiments have provided conflicting evidence for the specific role of *FsTRI8* in trichothecene biosynthesis in *F. sporotrichioides.* The *TRI8* disruption mutant, strain Δ*FsTRI8,* did not produce T-2 toxin, but accumulated 4,15-DAS (Fig. 4), presumably by 3-deacetylation of 3,4,15-DAS. The absence in this strain of any C-8 oxygenated trichothecenes suggests that *FsTRI8* is involved in this specific oxygenation. On the other hand, sequence similarity to lipases, the lack of sequence similarity to any known oxygenases, and the inability of Δ*FsTRI8* to add the isovalerate moiety and convert T-2 tetraol to T-2 toxin (Table 4) suggest that *FsTRI8* is responsible for esterification of the C-8 oxygen.

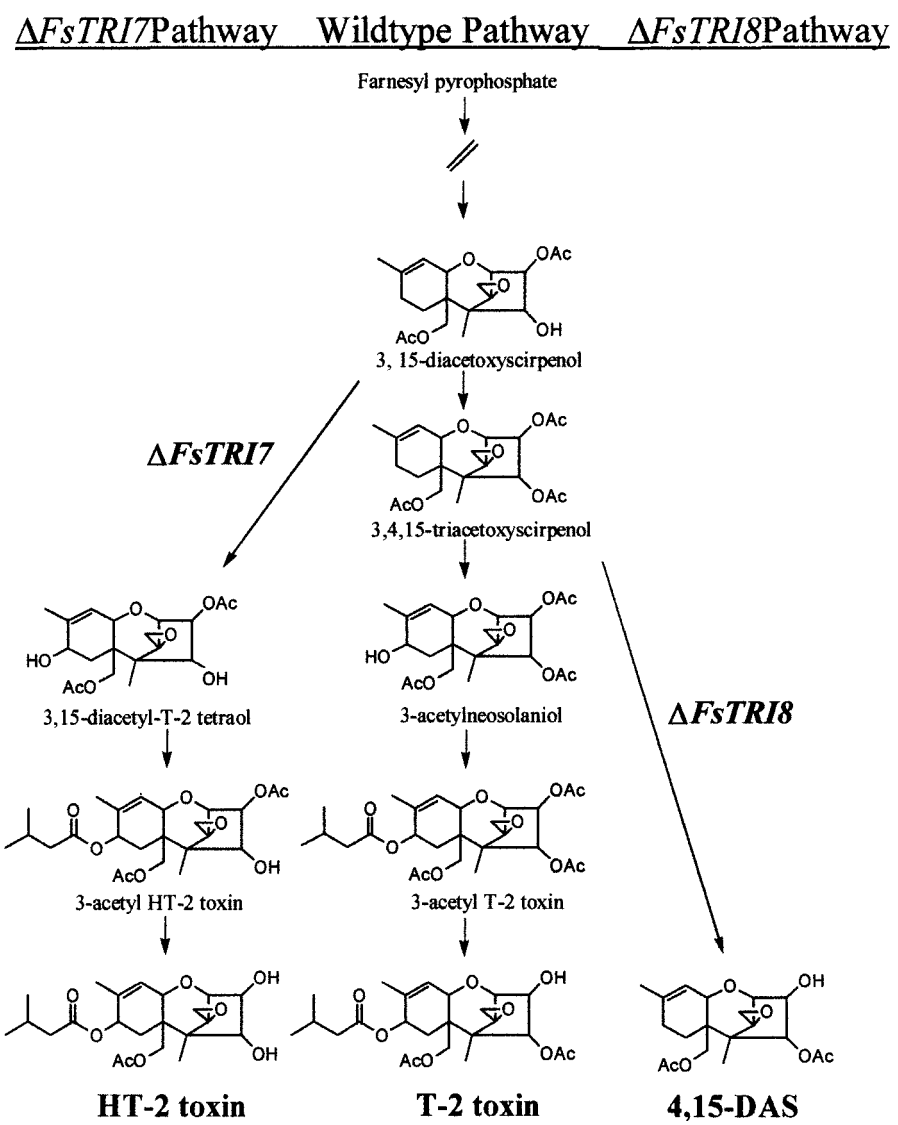

FIG. 4. Proposed biochemical pathway leading to the trichothecenes that accumulate in *F. sporotrichioides* strain Δ*FsTRI7* (HT-2 toxin) and strain Δ*FsTRI8* (4,15-DAS).

In previous studies, the functions of trichothecene esterase genes *FsTRI3* and *FsTRI101* were confirmed by their expression in a *S. cerevisiae* system. Trichothecene precursor feeding studies demonstrated that heterologously expressed *FsTRI3* was able to acetylate the C-15 hydroxyl group and *FsTRI101* was able to acetylate the C-3 hydroxyl group (McCormick *et al.*, 1999). In contrast, efforts to determine the function of *FsTRI8* by heterologous expression were not successful. *S. cerevisiae* strains transformed with *FsTRI8* were not able to oxygenate or esterify added trichothecenes (data not shown).

Strain Δ*FsTRI8* is phenotypically similar to a previously described *F. sporotrichioides* T-2 toxin-deficient mutant, designated MB1716, in that neither produces T-2 toxin and both accumulate 4,15-DAS (Beremand, 1987; Hohn *et al.*, 1993). However, feeding studies with a number of trichothecenes have differentiated the biochemical phenotypes of these two mutants. Strain MB1716 is unable to oxygenate C-8, but is able to add an isovalerate group to the C-8 oxygen of T-2 tetrol for conversion to T-2 toxin (Table 4). To date, the mutation in strain MB1716 has not been complemented by any of the cosmids that contain

TABLE 4
Conversion of Trichothecene Substrates to T-2 Toxin by *F. sporotrichioides* Strains Δ*FsTRI7*, Δ*FsTRI8*, and MB1716

Substrate	Δ*FsTRI7*	Δ*FsTRI8*	MB1716
4,15-DAS	Yes[a]	No	No
3,4,15-TAS	Yes	No	No
15-MAS	No	No	No
Scirpenetriol	No	No	No
3,15-DAS	No	No	No
HT-2	No	Yes	Yes
T-2 tetraol	No	No	Yes

[a] Yes indicates that T-2 toxin was detected in culture extracts following the addition of the specified intermediate. No indicates that T-2 toxin was not detected.

the trichothecene core cluster, and the location of this gene within the genome is unknown.

Correlating Gene Structure with Trichothecene Diversity

The pattern of oxygenation of the trichothecane skeleton can be used to differentiate *Fusarium* species and/or populations (Grove, 1996). For example, incorporation of oxygen at the C-7 position differentiates the synthesis of DON and NIV by *F. graminearum* from the synthesis of T-2 toxin by *F. sporotrichioides.* Incorporation of oxygen at the C-4 position differentiates the synthesis of T-2 toxin by *F. sporotrichioides* and of NIV by some strains of *F. graminearum* from the synthesis of DON by other strains of *F. graminearum* (Fig. 1) (Desjardins *et al.,* 1993). These differences in trichothecene oxygenation could be due to the absence of genes, transcriptional regulation, and/or posttranscriptional modification. For example, genes for enzymes that modify the C-7 or C-4 positions may be missing or regulated differently among strains or populations. Precedence for the first possibility may be found in *Aspergillus* species. The inability of *A. nidulans* to synthesize aflatoxin appears to be due, in part, to the lack of a gene encoding a monooxygenase that in *A. parasiticus* converts sterigmatocystin to aflatoxin (Brown *et al.,* 1996; Yu *et al.,* 1998).

T-2 toxin synthesis requires oxygenation at C-4 by an enzyme encoded by an unknown gene, followed by acetylation that requires *FsTRI7.* As noted above, DON lacks the C-4 oxygenation. If *F. graminearum* evolved from an ancestral *Fusarium* that produced C-4 oxygenated trichothecenes, the current lack of C-4 oxygenation and acetylation might be due to inactivation of the genes responsible for C-4 hydroxylation and C-4 acetylation, e.g., *FgTRI7.* Our analysis of the *F. graminearum* trichothecene cluster from the DON-producing strain GZ3639 indicates that the *FgTRI7* homologue is not functional. The initial BLAST analysis of GZ3639 DNA 1 to 3 kb upstream from *FgTRI8* suggested the presence of a *FgTRI7* homologue: score = 121; $E = 4 \times 10^{-76}$. The BLASTX alignment with FsTRI7 suggested that translation of a functional FgTRI7 protein was highly unlikely for the following reasons: (1) the lack of a translation initiation codon, (2) the presence of six frameshifts due to either the insertion (once) or the deletion (five times) of one or more nucleotides, (3) the presence of a stop codon within a portion of predicted amino acid sequence that otherwise shares significant similarity with FsTRI7, and finally, (4) the likely incorrect processing of the *FgTRI7* intron. Although the predicted intron shares similarity with the *FsTRI7* intron, it lacks the internal sequence motif and contains an insert of five repeats of the sequence CACAATATTAG. This 11-base sequence is not present elsewhere in the cluster, and its significance is unknown.

If *FsTRI8* is involved in esterification of the C-8 oxygen, we would expect this gene to be unnecessary in *F. graminearum,* which does not produce C-8 esterified trichothecenes. In DON and NIV, the two major trichothecenes produced by this species, the C-8 oxygen is present in an oxidized form as a ketone group. Our analysis of the *F. graminearum* trichothecene gene cluster indicates that the *FgTRI8* homologue in the DON-producing strain GZ3639 should be able to produce a functional protein. The predicted FsTRI8 and FgTRI8 proteins are similar in length and share 76% amino acid identity. As with FsTRI8, FgTRI8 shares similarity over its entire length to a lipase (Accession No. AAF69522) from *C. albicans* (score = 214, $E = 2 \times 10^{-54}$). However, if *F. graminearum* evolved from an ancestral species that produced C-8 hydroxylated trichothecenes, then oxidation of the C-8 hydroxyl group to a ketone may be a recently acquired trait. Thus, evolutionary time may not have been sufficient for extensive mutation and inactivation of a C-8 esterase gene.

Conclusions

Genetic and/or biochemical approaches have identified or confirmed the roles that six clustered genes play in the biosynthesis of T-2 toxin by *F. sporotrichioides* and in the biosynthesis of DON by *F. graminearum. TRI5* encodes trichodiene synthase, *TRI4* and *TRI11* encode cytochrome P450 monooxygenases; *TRI6* encodes a transcriptional activator; *TRI12* encodes a transporter; and *TRI3* encodes an acetylase. Gene disruption and precursor feeding experi-

ments have established that *FsTRI8* is required for some aspect of the oxygenation and/or esterification of C-8 in T-2 toxin biosynthesis, but the specific role of FsTRI8 is not known. Disruption of *FgTRI8* in a DON-producing strain of *F. graminearum* should help clarify the role that this gene plays in trichothecene biosynthesis. In addition, the roles of two other genes in the core cluster, *TRI9* and *TRI10,* have not yet been established.

Finally, *FsTRI7* encodes a C-4 acetylase that is required for T-2 biosynthesis in *F. sporotrichioides.* In contrast, in the DON-producing *F. graminearum* strain GZ3639, the *TRI7* homologue included a base deletion(s) and insertion(s) that would preclude a functional protein being translated from this gene. Perhaps these mutations have been retained because the *FgTRI7* protein product is not required for biosynthesis of DON, which is not oxygenated at C-4. Recently, we identified a Nepalese population of *F. graminearum,* some strains of which produce only DON while other strains also produce high levels of NIV and related trichothecenes that are oxygenated and esterified at C-4 (Desjardins *et al.,* 2000). We are currently investigating whether these NIV-producing strains have retained a functional *FgTRI7* or have acquired novel genes for C-4 oxidation and esterification. Furthermore, if gene inactivation is a general strategy leading to trichothecene diversity, then we should be able to make a number of genetic predictions based on comparison of structural differences among trichothecenes produced by various *Fusarium* species and related fungi.

Comparative nucleotide sequence analysis and biochemical approaches should further our understanding, not only of trichothecene biosynthetic pathways, but also of the origin and evolution of trichothecenes in *Fusarium.* Progress toward a complete understanding of trichothecene evolution will require the identification of additional trichothecene biosynthetic genes because the functions identified to date are insufficient to account for all known structures in the trichothecene family. Efforts to identify additional trichothecene biosynthetic genes adjacent to the core cluster and elsewhere in the genome are in progress. Current data support the hypothesis that trichothecene genes in *F. sporotrichioides* and *F. graminearum* are homologues in the strict sense; i.e., they are related by divergence from a common ancestor.

Although trichothecene production by economically important *Fusarium* species has received the most attention, trichothecenes also are produced by a wide range of plant parasitic Ascomycetes and form subclasses with ascomycetous affinities, such as Coelomycetes and Hyphomycetes. Trichothecenes play an important role as virulence factors in plant diseases caused by *F. sporotrichioides* and *F. graminearum.* If they play a similar role in other plant parasitic Ascomycetes, trichothecenes may have evolved at a very early stage in the association of Ascomycetes with land plants. If trichothecene genes are strongly selected and highly conserved, they may prove useful for phylogenetic studies of the evolution of plant parasitism in Ascomycetes.

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