

## *Tri16* Is Required for Esterification of Position C-8 during Trichothecene Mycotoxin Production by *Fusarium sporotrichioides*

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We previously characterized *Tri1*, a gene required for hydroxylation of the C-8 position during trichothecene mycotoxin biosynthesis in *Fusarium sporotrichioides* NRRL 3299. Sequence analysis of the region surrounding *Tri1* revealed a gene, named *Tri16*, which could encode an acyltransferase. Unlike the wild-type parent strain NRRL 3299, which accumulates primarily T-2 toxin along with low levels of diacetoxyscirpenol (DAS) and neosolaniol (NEO) and trace amounts of 8-propionyl-neosolaniol (P-NEO) and 8-isobutyryl-neosolaniol (B-NEO), mutants containing a disruption of *Tri16* were blocked in the production of the three C-8 esterified compounds T-2 toxin, P-NEO, and B-NEO and accumulated the C-8-hydroxylated compound NEO along with secondary levels of DAS. These data indicate that *Tri16* encodes an acyltransferase that catalyzes the formation of ester side groups at C-8 during trichothecene biosynthesis. We also report the presence of a *Tri16* ortholog in *Gibberella pulicaris* R-6380 that is likely linked to a presumably inactive ortholog for *Tri1*.

One of the major concerns regarding the presence of fungi in both agricultural settings and, more recently, indoor settings with compromised air quality is the ability of some species to produce trichothecene mycotoxins (18, 19, 33). The toxicity of these sesquiterpenoid fungal secondary metabolites to eukaryotic cells makes them important in human and animal health (19) and some cases of plant pathogenesis (5, 15).

As a group, trichothecenes are characterized by the presence of a 12,13-epoxy-trichothec-9-ene nucleus, a feature that is necessary for toxicity (13, 32). However, the addition of different combinations of side groups to this trichothecene core nucleus plays an important role in determining the level of toxicity of each trichothecene. Although there are some notable exceptions, such as the acetylation of the hydroxyl group at the C-3 position which reduces the toxicity of many trichothecenes (1), in general, the greater the number and the more elaborate the side groups, the greater the toxicity (35). One of the toxicologically most important examples involves 4,15-diacetoxyscirpenol (DAS), neosolaniol (NEO), and T-2 toxin. These three trichothecenes display increasing toxicity in mice, as demonstrated by 50% lethal doses of 23.0, 14.5, and 5.2 mg/kg, respectively (34). Significantly, they differ from one another only at the C-8 position by the presence of a hydrogen, a hydroxyl group, and an esterified hydroxyl group, isovaleroloxyl, respectively.

*Fusarium sporotrichioides* and *F. sambucinum* have been used as model organisms in studies directed toward the identification of the genes that encode the enzymes responsible for the hydroxylations and esterifications that occur during trichothecene biosynthesis. *F. sporotrichioides* NRRL 3299 makes a

closely related series of type A trichothecenes in culture, with the predominant one being T-2 toxin. Minor components include DAS, NEO, 8-propionyl-neosolaniol (P-NEO), and 8-isobutyryl-neosolaniol (B-NEO) (8). All five of these trichothecenes, except DAS, result from a hydroxylation at position C-8 and, with the exception of NEO, a subsequent esterification at C-8-OH and therefore differ from one another only at the C-8 position. Some isolates of *Gibberella pulicaris* also possess the ability to synthesize NEO, 8-acetylneosolaniol (8-Ac-NEO), and/or T-2 toxin, in addition to DAS (3, 30). Despite a well-characterized biosynthetic pathway for type A trichothecenes (12) and the fact that most of the identified trichothecene (*Tri*) genes constitute a large gene cluster (10, 17), the gene required for the esterification reaction at C-8 has not been identified.

In a previous study, we isolated several new candidate trichothecene genes on the basis of an expression pattern that is dependent upon the presence of two trichothecene regulatory genes, *Tri10* and *Tri6* (24). One of these genes was *Tri1*, which encodes a cytochrome P450 monooxygenase required for oxygenation of position C-8 in the biosynthesis of T-2 toxin (23). *Tri1* is not closely linked to the core trichothecene cluster. In the present study, we characterized *Tri16*, a gene that we identified while sequencing the DNA region adjacent to *Tri1*. The resulting data indicate that *Tri16* encodes a C-8 acyltransferase required for the production of T-2 toxin and establish the presence of a second trichothecene gene cluster. Together, *Tri16* and *Tri1* are responsible for the addition of the C-8 substituents required for the production of the most toxic trichothecenes made by *F. sporotrichioides*.

### MATERIALS AND METHODS

**Strains, media, and culture conditions.** Wild-type strains *F. sporotrichioides* NRRL 3299, *G. pulicaris* (*F. sambucinum*) R-6380, and *Gibberella zeae* (*Fusarium graminearum*) GZ3639 have been described previously (6, 9). The wild-type strains were maintained on V8 juice agar (200 ml of V8 juice, 3 g of CaCO<sub>3</sub>, and

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20 g of agar per liter), while transformant strains were maintained on V8 juice agar plus 300  $\mu$ g of hygromycin B (Calbiochem, La Jolla, Calif.) per ml. Standard light and temperature conditions were used for growth of strains on V8 juice agar slants and plates (23). Conidia for all strains were stored as frozen ( $-80^{\circ}\text{C}$ ) glycerol-water (15:85) stocks. All fungal liquid cultures were incubated at  $28^{\circ}\text{C}$  and shaken at 200 rpm on an orbital shaker after being inoculated as follows with mycelia or conidia harvested from cultures grown for 7 days on V8 juice agar plates. For DNA extraction, 100 ml of YEPD-2G medium (2% glucose, 0.3% yeast extract, 1% peptone) was inoculated with mycelia (from one plate) and incubated for 24 h; for RNA extraction and toxin analysis, 100 ml (RNA) or 25 ml (toxin) of YEPD-5G medium (5% glucose, 0.1% yeast extract, 0.1% peptone) was inoculated at a density of  $10^4$  conidia/ml and incubated for 23, 27, and 36 h (RNA) or 7 days (toxin); for toxin analysis involving coinoculation of strains and whole-cell feeding experiments, cultures were inoculated at a density of  $5 \times 10^4$  conidia/ml. For whole-cell feeding studies, NEO (Sigma, St. Louis, Mo.), in acetone, was added to cultures 24 h following inoculation. The final concentration of NEO was 250  $\mu\text{M}$ , and the final concentration of acetone was 1%. Storage of clones from the cDNA library has been described previously (24). All bacterial culture media were supplemented with ampicillin at 100  $\mu\text{g}/\text{ml}$ .

**Nucleic acid isolation.** Isolation of genomic DNA and RNA has been described previously (16, 29). Plasmid DNA was isolated with the QIAprep spin miniprep kit (Qiagen, Valencia, Calif.). Restriction fragments were separated on 1.2% agarose (Boehringer Mannheim, Indianapolis, Ind.) gels and purified with the QIAquick gel extraction kit (Qiagen).

**Disruption plasmid construction and fungal transformation.** pKO16, the *Tri16* disruption plasmid, was constructed by cloning a fragment of the *Tri16* sequence obtained from a cDNA clone into the previously described fungal cloning vector pUCH3 (22). Specifically, a 963-bp fragment, including 18 bp of the pBluescript SK (Stratagene, La Jolla, Calif.) vector sequence at the 5' end, was cut from plasmid DNA of the *Tri16* cDNA clone 14J4 from our cDNA library (24) with the restriction enzymes *Bam*HI and *Sal*I (New England Biolabs, Beverly, Mass.) and ligated to *Bam*HI- and *Sal*I-cut pUCH3. The resulting plasmid, pKO16, harbors a doubly truncated fragment of the *Tri16* coding sequence adjacent to the hygromycin B resistance gene (*hygB*) in pUCH3. Protoplast formation and fungal transformation were conducted as previously described (26, 29).

**PCR, Northern, and Southern analyses.** PCR analyses of *Tri16* transformants were conducted with primers A-90 (5'-GCCCTTCTAGATGCCTGAG-3'), A-91 (5'-GTTGGTTGCCTCCGCTCTGG-3'), and S-12 (5'-CAGGTGGGCCTTGACATGTG-3'). PCR conditions were 30 cycles of 30 s at  $95^{\circ}\text{C}$ , 60 s at  $51^{\circ}\text{C}$ , and 90 s at  $72^{\circ}\text{C}$  and 1 cycle of 10 min at  $72^{\circ}\text{C}$ . All reactions were performed with *Taq* DNA polymerase (Promega, Madison, Wis.) in accordance with the manufacturer's instructions. For Northern analyses, RNA electrophoresis was conducted with 1% agarose gels containing 1.1% formaldehyde and each lane was loaded with 5  $\mu\text{g}$  of total RNA (27). For Southern analyses of *Tri16* transformants, electrophoresis was performed in accordance with standard protocols (27) and 5  $\mu\text{g}$  of DNA from each transformant strain (1  $\mu\text{g}$  from NRRL 3299) was used in restriction digests. Genomic DNA from all strains was digested with *Bgl*II (New England Biolabs), which does not cut the pKO16 vector. For Southern analysis of the *Tri16* and *Tri1* genes in *F. sporotrichioides* and *G. pulicaris*, 5  $\mu\text{g}$  of DNA was used for each restriction digest with *Apa*I, *Sma*I, or *Eco*RI (New England Biolabs, Boehringer Mannheim). These enzymes were chosen because sequence information indicated that *Tri16* and *Tri1* in *F. sporotrichioides* are on the same *Eco*RI and *Sma*I fragments but on different *Apa*I fragments. The *Tri16* and *Tri1* probes for all Southern and Northern analyses were the cDNA insert of library clone 14J4 excised with *Bam*HI and *Sal*I and the cDNA insert of library clone 1J3 cut with *Apa*I and *Sma*I, respectively. Individual radioactively labeled DNA probes were prepared using [ $\alpha$ - $^{32}\text{P}$ ]dCTP (Amersham, Piscataway, N.J.) with the Nick Translation System (GIBCO BRL, Rockville, Md.). All Northern and Southern blots were made on Hybond-N+ nylon membranes (Amersham) and hybridized, washed, and stripped under high stringency as specified by the Gene Images nonisotopic nucleic acid detection kit (United States Biochemical, Cleveland, Ohio). Autoradiographic images were produced on BIOMAX MR or X-Omat LS imaging film (Kodak, Rochester, N.Y.).

**DNA sequencing and sequence homology searches.** Inserts isolated from plasmid DNA preparations of cDNA clones were sequenced by using either the ABI Prism Dye Terminator cycle sequencing core kit or the BigDye Terminator cycle sequencing core kit (Perkin-Elmer, Boston, Mass.). All reactions were run on a 373 or 377 DNA sequencer (Applied Biosystems, Foster City, Calif.) by the Gene Technologies Laboratory at Texas A&M University. Nucleotide sequences and their putative corresponding amino acid sequences were compared to the National Center for Biotechnology Information nonredundant database (GenBank+

RefSeq Nucleotides+EMBL+DDBJ+PDB sequences) by using the blastn and blastp algorithms (4).

**Toxin analyses.** Ethyl acetate extractions of whole-culture samples were performed as previously described (29). Trichothecenes in the ethyl acetate extracts were detected and identified by gas chromatography-mass spectrometry with a Hewlett-Packard 5890 gas chromatograph directly interfaced to a Hewlett-Packard 5970B mass spectrometer via a heated capillary interface. The gas chromatograph was configured with a splitless injection port and a DB-5ms fused-silica capillary column coated with a 0.25- $\mu\text{m}$  film of stationary phase (30 m by 0.25 mm [internal diameter]; J&W Scientific, Folsom, Calif.). Sample volumes of 2  $\mu\text{l}$  were introduced to a heated injection port. The injection and transfer line temperatures were  $300^{\circ}\text{C}$ . The oven temperature program consisted of an initial temperature of  $175^{\circ}\text{C}$  that was held for 2.0 min, ramped to  $275^{\circ}\text{C}$  at  $23^{\circ}\text{C}/\text{min}$ , held at  $275^{\circ}\text{C}$  for 2 min, ramped to  $290^{\circ}\text{C}$  at  $30^{\circ}\text{C}/\text{min}$ , and held at  $290^{\circ}\text{C}$  for 16 min. Helium was the carrier gas at a constant flow rate of 0.75 ml/min. A mass-selective detector was used in selective ion monitoring mode with an ionization energy of 70 eV and an ion source temperature of  $180^{\circ}\text{C}$ . T-2 toxin and NEO were purchased from Sigma and used without further purification. T-2 toxin was below the detection limit ( $<1$  ppm) in the NEO standard.

**Nucleotide sequence accession number.** The GenBank accession number for the *Tri16* nucleotide and predicted protein sequences is AY187275.

## RESULTS

**Sequence of *Tri16*.** The largest putative open reading frame with a characteristic translational start site for *Tri16* is 1,479 bp, which would encode a protein of 493 amino acids. Comparison of the genomic DNA sequence with cDNA sequence data generated by the *F. sporotrichioides* cDNA Sequencing Project (contig 799 and contig 873) and within our laboratory (clone 14J4) indicates the absence of any introns. *Tri16*'s direction of transcription is the same as that of *Tri1*. The predicted protein sequence of TRI16 has the greatest identity (23 to 24%) with TRI101 (trichothecene 3-*O*-acetyltransferase) from various *Fusarium* species and with AYT1 from *Saccharomyces cerevisiae* (22%), which was designated an acetyltransferase on the basis of its ability to acetylate the C-3 position of isotrichodermol (2). Like those of TRI101 and AYT1, the TRI16 protein sequence contains a motif (HXXXDG) that is conserved among members of the BAHD superfamily of plant acyltransferase genes (28). However, the HXXXDG motif in TRI16 more closely resembles that of ATF1 (alcohol-*O*-acetyltransferase) from *S. cerevisiae* and TRI3 (trichothecene 15-*O*-acetyltransferase) from *F. sporotrichioides* and *G. zeae* because it does not contain the two-amino-acid insertion (HXXXDXXG) that is found in TRI101 and AYT1. A second motif in the carboxy terminus of BAHD acyltransferases, DFGXGKP, may be present in TRI16, but it would represent a unique case since there is an insertion of 11 amino acids (DFGXXXXXXXXXXXXXXXXGKP) rather than two amino acids (DFXGXGKP) as found in TRI101 and AYT1. TRI3 and ATF1 do not possess this C-terminal motif (2).

**Disruption of *Tri16*.** *Tri16* mutants were generated by transforming *F. sporotrichioides* NRRL 3299 with pKO16. This vector contains a doubly truncated fragment of the *Tri16* coding region. Homologous integration of a single copy of this construct results in two nonfunctional copies of the *Tri16* gene, one truncated at the amino terminus (by 88 amino acids) and the other truncated at the carboxy terminus (by 91 amino acids) (Fig. 1A). Transformation of the wild-type strain yielded 28 hygromycin-resistant transformants. Southern analysis revealed that at least 11 of these transformants no longer contained a wild-type *Tri16* gene. Two of these 11, Tx #7 and Tx #20, resulted from the homologous integration of a single copy

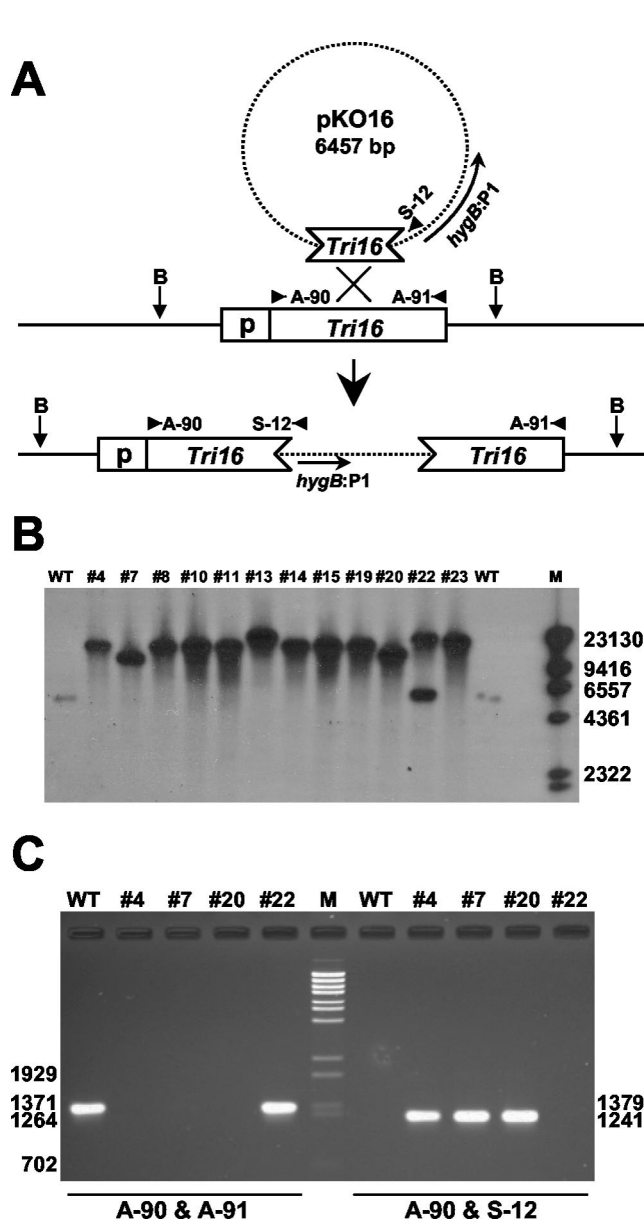


FIG. 1. Disruption of *Tri16* in *F. sporotrichioides* NRRL 3299. (A) Expected result for insertion of the *Tri16* gene disruption construct pKO16 into the host genome via a single homologous integration event. (B) Southern blot analysis of selected transformant strains. Wild-type (WT) and transformant genomic DNAs were cut with *Bgl*II, and blots were probed with a doubly truncated fragment of the *Tri16* gene. M, lambda DNA cut with *Hind*III. All transformants except Tx #22 lack the wild-type *Tri16* *Bgl*II fragment. Instead, transformants Tx #7 and Tx #20 contain a single fragment consistent with the expected combined size (approximately 13 kb) of the wild-type *Tri16* *Bgl*II fragment and one homologously integrated copy of pKO16. The remaining transformants contain a larger fragment indicative of the presence of two (expected size, approximately 20 kb) or more tandem, homologously integrated copies of pKO16. Band sizes (bases) of the marker are shown at the right. (C) PCR analysis of wild-type and selected transformant strains. PCRs were performed with primers A-90 and A-91 and primers A-90 and S-12 (positions are shown in panel A). PCR products were resolved on an agarose gel and stained with ethidium bromide. M, lambda DNA cut with *Bst*EII. Band sizes (bases) of the marker are shown at the left, and band sizes of the PCR products are shown at the right.

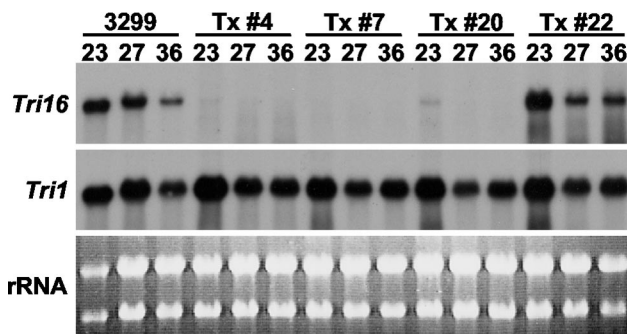


FIG. 2. Northern analysis of *Tri16* and *Tri1* transcript accumulation by *F. sporotrichioides* strains grown in liquid culture. Total RNA was isolated for each strain from mycelia collected from cultures grown for 23, 27, and 36 h in YEPD-5G medium. Strains are (left to right) the wild type (NRRL 3299),  $\Delta$ *Tri16* transformants (Tx #4, Tx #7, and Tx #20), and a transformant (Tx #22) resulting from ectopic integration of pKO16. rRNA bands from the agarose gel stained with ethidium bromide are shown.

of pKO16; the remainder resulted from the homologous integration of multiple tandem copies, e.g., Tx #4 (Fig. 1B). In contrast, transformant Tx #22 contained a wild-type-sized *Tri16* *Bgl*II fragment, as well as a second, larger *Tri16*-hybridizing *Bgl*II fragment indicative of the ectopic integration of the transforming vector. These integration events were confirmed in a PCR with primer A-90 and vector-specific primer S-12 (Fig. 1A) that would yield a 1,241-bp product only if homologous integration occurred. Transformants Tx #4, Tx #7, and Tx #20 all produced this product (Fig. 1C), while neither the wild-type strain nor transformant Tx #22 did. We also conducted a PCR with primers A-90 and A-91 (Fig. 1A) and found that the wild-type strain and Tx #22 yielded the expected 1,379-bp PCR product for the wild-type *Tri16* gene sequence but that Tx #4, Tx #7, and Tx #20 did not (Fig. 1C). Thus, Tx #4, Tx #7, and Tx #20 are transformants resulting from homologous integration of pKO16 while the transformed DNA in Tx #22 appears to have been inserted into the genome ectopically.

**Northern analyses.** When RNA blots were hybridized with a probe for the *Tri16* gene (Fig. 2), *Tri16* transcripts accumulated at all three of the time points examined (23, 27, and 36 h) for the wild-type strain (NRRL 3299) and ectopic strain (Tx #22) while the three *Tri16* disruption transformants (Tx #4, Tx #7, and Tx #20) accumulated little, if any, of this transcript. *Tri1* transcript accumulation was similar in all of the strains and at all three time points. These results confirm the loss of *Tri16* gene expression in the  $\Delta$ *Tri16* transformants.

**Toxin analyses.** To determine the effect of *Tri16* gene disruption on T-2 toxin production, trichothecene profiles were examined for the wild-type and four transformant strains (Fig. 3A). The wild-type strain, NRRL 3299, produced T-2 toxin, DAS, NEO, P-NEO, and B-NEO as expected. While the ectopic transformant, Tx #22, retained a wild-type toxin profile, all three *Tri16* disruption transformants, Tx #4, Tx #7, and Tx #20, produced no detectable T-2 toxin. Instead, they accumulated primarily NEO and DAS. In addition, no detectable amounts of P-NEO or B-NEO were produced by these three transformants. Minor peaks with retention times similar to

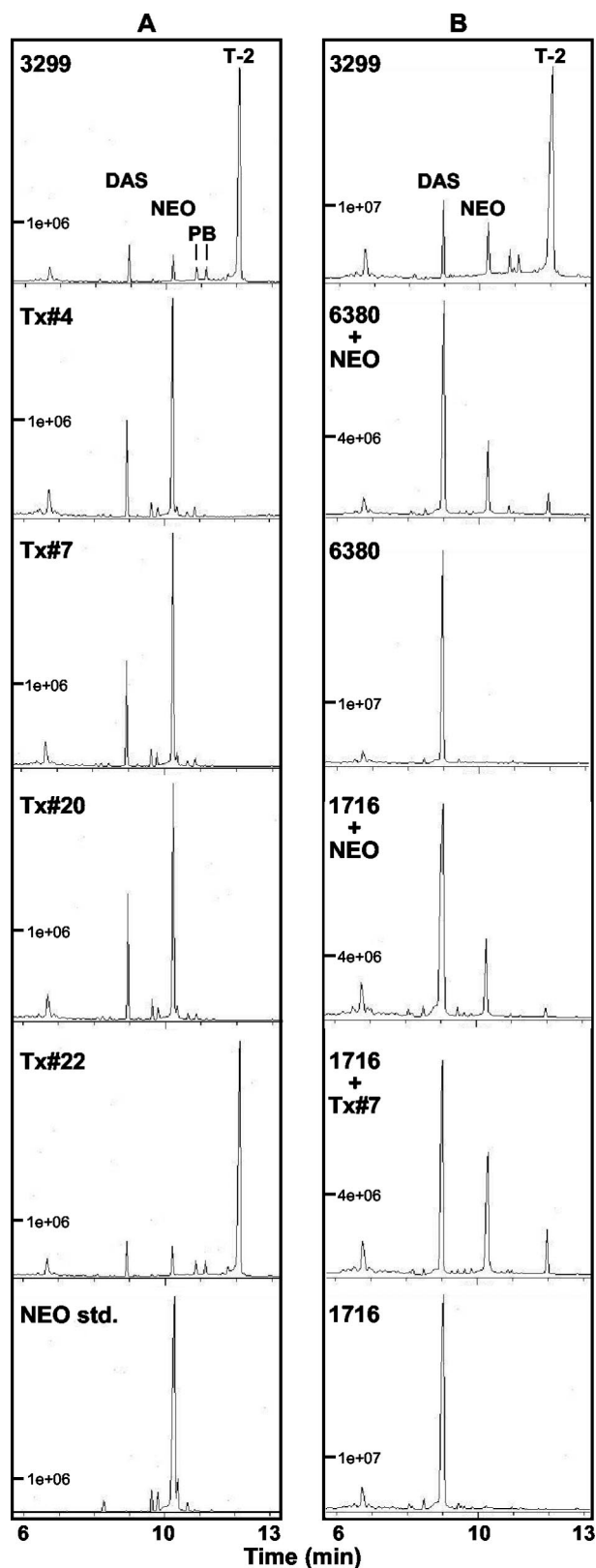


FIG. 3. Chromatograms of ethyl acetate extracts of 7-day *Fusarium* cultures. (A) Analysis of the *Tri16* transformants. P, P-NEO; B, B-NEO. (B) Precursor feeding and coinoculation experiments. std., standard.

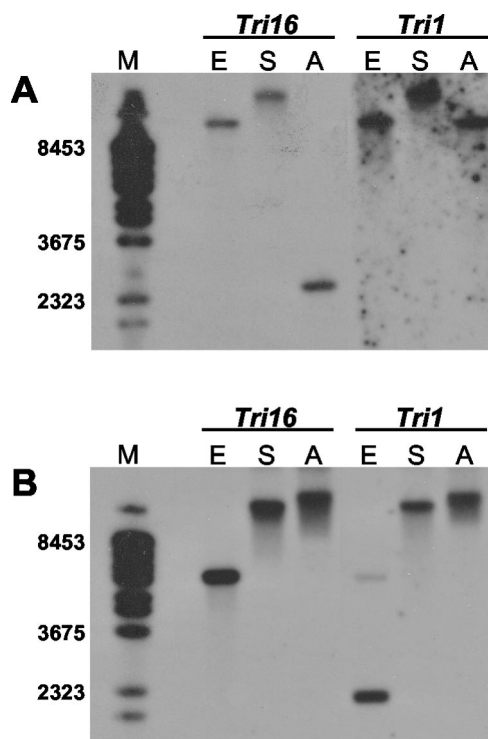


FIG. 4. Southern blots of *F. sporotrichioides* NRRL 3299 (A) and *G. pulicaris* (*F. sambucinum*) R-6380 (B) probed with the *Tri16* and *Tri1* genes. E, *EcoRI*; S, *SmaI*; A, *ApaI*; M, lambda DNA digested with *BstEII*. Sizes (bases) of selected marker bands are shown at the left.

those of P-NEO and B-NEO were present; however, analysis of the mass spectra of the compounds in these peaks failed to identify a base peak of  $m/z$  382 that is common to both P-NEO and B-NEO. Thus, the disruption of *Tri16* blocks C-8 esterification during trichothecene biosynthesis.

**Detection of *Tri16* in *G. pulicaris*.** We previously identified a putative, presumably inactive, *Tri1* ortholog in *G. pulicaris* (*F. sambucinum*) R-6380 (23). In this study, we detected an ortholog of *Tri16* in the same strain (Fig. 4B). The Southern hybridization pattern for *Tri1* and *Tri16* in *F. sporotrichioides* NRRL 3299 (Fig. 4A) is consistent with the restriction sites predicted from sequence data that placed *Tri1* and *Tri16* on the same *EcoRI* and *SmaI* fragments but on different *ApaI* fragments. In *G. pulicaris* R-6380 (Fig. 4B), the *Tri16* ortholog lies on restriction fragments (for all three enzymes) of the same size as *Tri1*. Thus, as in *F. sporotrichioides*, *Tri16* and *Tri1* appear to be physically linked in *G. pulicaris*.

**C-8 acylation in *G. pulicaris*.** To test whether the *Tri16* ortholog in *G. pulicaris* R-6380 could be functional, we assessed the ability of this strain to convert NEO to T-2 toxin by growing it with exogenously added NEO for 7 days in YEPD-5G liquid medium. We found that R-6380 could convert NEO to T-2 toxin at a very low rate (Fig. 3B), as could MB1716, an *F. sporotrichioides* NRRL 3299-derived *tri1* mutant that retains C-8 acylation activity (7). Both of these results are consistent with the previously observed poor conversion of NEO to T-2 toxin by the *F. sporotrichioides* NRRL 3299-derived  $\Delta$ *Tri5* transformant 8-3-9 (25). These data indicate that R-6380, like MB1716, can esterify C-8 although it cannot hydroxylate this

carbon. Since MB1716 converted the exogenously supplied NEO to T-2 toxin inefficiently, this strain was coinoculated with a *Tri16* mutant (Tx #7) into YEPD-5G medium to test for production of T-2 toxin under these conditions. This mixed culture was capable of producing T-2 toxin, but it still accumulated primarily DAS and NEO (Fig. 3B).

## DISCUSSION

We previously reported the discovery of a putative acyltransferase gene 2.5 kb downstream of *Tri1* that has a *Tri10*- and *Tri6*-dependent expression pattern similar to that of other *Tri* genes (23). In the present study, we demonstrated that this gene is necessary for esterification of the C-8 position during T-2 toxin biosynthesis and have thus designated it *Tri16*. Disruption of the *Tri16* coding region in *F. sporotrichioides* prevents the production of compounds esterified at C-8 and results in accumulation of the less toxic metabolite NEO. The predicted TRI16 protein sequence possesses motifs that are found in a group of plant acyltransferase enzymes and thus appears to be a C-8 acyltransferase.

The most obvious phenotype of the *Tri16* disruption mutants is that they accumulate NEO and do not produce T-2 toxin. This result is consistent with the inability to add the isovaleryl moiety to the C-8 hydroxyl group. A previous study (8) showed that a leucine auxotroph of NRRL 3299 produced less T-2 toxin and more P-NEO and B-NEO than the wild-type parent when leucine was limited during growth in YEPD-5G medium. A wild-type toxin profile was restored by the addition of sufficient levels of leucine to the medium. Since leucine is a precursor to the C-8 isovaleryl moiety (8), the overproduction of P-NEO and B-NEO was attributed to increased competitive esterification of propionyl and isobutyryl groups at C-8 due to the decreased availability of isovalerate. If the addition of the propionyl and butyryl moieties were catalyzed by an enzyme other than TRI16, then P-NEO and B-NEO levels should increase in the  $\Delta$ *Tri16* mutants. They did not. Instead, they declined such that P-NEO and B-NEO were no longer detectable. The failure of  $\Delta$ *Tri16* mutants to accumulate P-NEO and B-NEO indicates that TRI16 also is responsible for the esterification reactions that result in the production of P-NEO and B-NEO.

In the proposed metabolic grid for trichothecene biosynthesis (23), the placement of *Tri16* would fall between *Tri1* and *Tri8*, which encodes a C-3 esterase (20). 3-Acetylneosolaniol is thought to be the preferred substrate for TRI16. However, the production of 4-deoxy-T-2 toxin and HT-2 toxin by *Tri13* (C-4 hydroxylase) deletion mutants and *Tri7* (C-4 acetylase) deletion mutants (10, 11), respectively, suggests that 8-hydroxycalonectrin and 3,15-diacetyl-T-2 tetraol also may serve as substrates for TRI16. Therefore, in addition to being able to catalyze the addition of multiple acyl (isovaleryl, propionyl, and butyryl) groups to C-8-OH, TRI16 appears to accommodate multiple trichothecene substrates.

*Tri16* is the fourth cloned acyltransferase gene for trichothecene biosynthesis, but among the protein products of those genes, TRI16 is significantly similar only to TRI101. Aside from one conserved motif in TRI3, TRI3 and TRI7 are not significantly similar to TRI16 or TRI101 and have previously

been reported not to be similar to known fungal acetyltransferases (10, 21, 22).

*Tri1* and *Tri16* are adjacent to each other but not to the core trichothecene gene cluster (20). On the basis of the toxicological differences between DAS and T-2 toxin, this organizational pattern has potential implications for the evolution of the production of more toxic trichothecenes and for the evolution of gene clusters for fungal secondary metabolism. *Tri1* and *Tri16* encode enzymes for the two consecutive steps that extend the trichothecene pathway beyond DAS to the most toxic type A trichothecene, T-2 toxin. A similar pattern exists for another fungal secondary metabolite. The last three steps in the cephalosporin biosynthetic pathway in *Acremonium chrysogenum* are catalyzed by two adjacent genes that are not located in the cluster of genes required for the first portion of the pathway (14). Adjacent genes also encode enzymes that catalyze consecutive biosynthetic steps in fungal secondary metabolic pathways for the gibberellins (31) and the aflatoxins (36), but they are not organized in a separate second gene cluster. Certainly, the comparative analysis of *Tri1* and *Tri16* relative to the core trichothecene gene cluster in other *Fusarium* species will be most interesting.

As determined by Southern hybridization, *G. pulicaris* R-6380 contains a putative *Tri16* ortholog that is physically linked to a potential ortholog of *Tri1*. This DAS-accumulating strain, like the *tri1* DAS-accumulating mutant of *F. sporotrichioides*, can convert NEO to T-2 toxin at a low rate and therefore can add an isovalerate moiety to C-8-OH. Although some naturally occurring isolates of *G. pulicaris* produce T-2 toxin (3, 30), to our knowledge, this is the first report that R-6380 can produce T-2 toxin under laboratory conditions. The ability of R-6380 to produce T-2 toxin from NEO suggests that it lacks only the ability to hydroxylate the C-8 position of trichothecenes. Disruption of *Tri16* in R-6380 will be necessary to prove that the TRI16 homolog catalyzes the C-8 esterification. Some strains of *G. pulicaris* produce 8-Ac-NEO as their primary trichothecene metabolite (7). Whether TRI16 or another enzyme in *Fusarium* can add the acetyl side group to C-8 also remains to be determined. In addition, both genetic and molecular studies can be used to reveal the location of *Tri16* relative to the other *Tri* genes in *G. pulicaris*.

We previously reported the failure to detect a *Tri1* ortholog in *G. zeae* (*F. graminearum*) GZ3639, a type B trichothecene producer, by Southern analysis (23). Similarly, we were not able to detect an ortholog of *Tri16* in GZ3639. However, examination of the initial release of the draft sequence of the *F. graminearum* PH-1 genome (*Fusarium graminearum* Sequencing Project, Whitehead Institute/MIT Center for Genome Research [http://www-genome.wi.mit.edu]) revealed possible orthologs for both of these genes. As in *F. sporotrichioides* NRRL 3299, the putative orthologs are adjacent to each other and are not associated with the core trichothecene gene cluster, but their directions of transcription diverge rather than converge. While the *Tri16*-like sequence appears to be inundated with stop codons and therefore nonfunctional, the *Tri1* ortholog could be functional. Analysis of the function of the *Tri1* ortholog in *F. graminearum* would be critical to answering the question of whether this species uses this gene or a different one to hydroxylate the C-8 position in the biosynthesis of type B trichothecenes.

The characterization of *Tri16* completes the assignment of genes for the last eight steps in the trichothecene biosynthetic pathway in *F. sporotrichioides* NRRL 3299 and completes the identification of genes for the esterifications required at C-5, C-4, C-15, and C-8 (10, 21, 22; this study) in the synthesis of T-2 toxin. In contrast to TRI101, the activity of TRI16, like that of TRI7, increases trichothecene toxicity.

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