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Disruption of *TRI101*, the Gene Encoding Trichothecene 3-*O*-Acetyltransferase, from *Fusarium sporotrichioides*

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We screened a *Fusarium sporotrichioides* NRRL 3299 cDNA expression library in a toxin-sensitive *Saccharomyces cerevisiae* strain lacking a functional *PDR5* gene. Fourteen yeast transformants were identified as resistant to the trichothecene 4,15-diacetoxyscirpenol, and each carried a cDNA encoding the trichothecene 3-*O*-acetyltransferase that is the *F. sporotrichioides* homolog of the *Fusarium graminearum TRI101* gene. Mutants of *F. sporotrichioides* NRRL 3299 produced by disruption of *TRI101* were altered in their abilities to synthesize T-2 toxin and accumulated isotrichodermol and small amounts of 3,15-didecalonectrin and 3-decalonectrin, trichothecenes that are not observed in cultures of the parent strain. Our results indicate that TRI101 converts isotrichodermol to isotrichodermin and is required for the biosynthesis of T-2 toxin.

Trichothecenes are sesquiterpene epoxide mycotoxins produced by species of *Fusarium*, *Trichothecium*, and *Myrothecium* that act as potent inhibitors of eukaryotic protein synthesis (21). The broad spectrum of trichothecene toxicity probably is due to their ability to interact with highly conserved elements of the protein-synthetic apparatus. While trichothecene vertebrate toxicity has been the focus of numerous studies due to the frequent occurrence of trichothecenes in agricultural products, the observed phytotoxicity of trichothecenes has stimulated research into the role these toxins may play in plant diseases caused by some *Fusarium* species (4, 6, 11, 24).

The biosynthesis of the trichothecene T-2 toxin in *Fusarium sporotrichioides* has been studied with the aid of mutant strains blocked at specific steps in the trichothecene pathway (5, 12, 18–20). Many of the trichothecene pathway genes in *F. sporotrichioides* (12) are localized in a gene cluster of at least nine genes including genes for P450 oxygenases (1, 9), an acetyltransferase (19), a transcription factor required for pathway gene expression (25), and a toxin efflux pump (2).

Trichothecenes are antibiotics, and their biosynthesis likely requires special adaptations by the producing organisms for self-protection. Antibiotic-producing microorganisms use various mechanisms for self-protection including alteration of target proteins, pumps to reduce the intracellular concentration of the antibiotic, and metabolism to reduce toxicity (3). Trichothecene 3-*O*-acetyltransferase (TRI101) catalyzes the conversion of toxic *Fusarium* trichothecenes to less-toxic products and has, therefore, been proposed as a metabolic self-protection mechanism in *Fusarium graminearum* (13). *TRI101* is not tightly linked to other trichothecene biosynthetic genes in either *F. graminearum* or *F. sporotrichioides* (13–15). The enzyme encoded by *TRI101* can modify a number of trichothecenes and, when expressed in *Schizosaccharomyces pombe*, confers resistance to trichothecenes (13). It was suggested that TRI101 acetylation, rather than modification or replacement of target ribosomes, is the primary defense mechanism against 3-hydroxylated trichothecenes in *Fusarium* and that a mutation resulting in a loss of *TRI101* would be lethal (13).

Our objectives in this study were to determine (i) if inactivation of *TRI101* was lethal and (ii) if *TRI101* functions in both *Fusarium* self-protection against trichothecenes and trichothecene biosynthesis. We found that disruption of *TRI101* was not lethal and that *TRI101* deletion mutants accumulated isotrichodermol, a 3-hydroxytrichothecene. These mutants could both germinate and grow in the presence of isotrichodermol and other 3-hydroxytrichothecenes. These results show that although expression of *TRI101* in *Saccharomyces cerevisiae* and *S. pombe* increased their resistance to trichothecenes, *TRI101* is not an essential self-defense mechanism for *F. sporotrichioides*. Our results also show that *TRI101* is an essential trichothecene-biosynthetic gene and are consistent with the hypothesis that much of the T-2 toxin pathway is via 3-acetylated intermediates.

MATERIALS AND METHODS

Strains. *F. sporotrichioides* NRRL 3299 was obtained from the USDA Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research, Peoria, Ill., and maintained on V-8 juice agar slants (29). *F. sporotrichioides* FsTri101-3D contains disrupted sequences for *TRI101* as described below. *S. cerevisiae* RW2802 (PDR5 *leu2 ura3-52 met5*) and JG436 (pdr5::Tn*5* *leu2 ura3-35 met5*) were obtained from J. Golin, The Catholic University of America (22).

Media and culture conditions. All *Fusarium* cultures were grown initially on V-8 juice agar plates under an alternating cycle of 12 h of light at 25°C and 12 h of dark at 22°C. Conidia were washed from the plates and grown in YPG medium (0.3% yeast extract, 1% peptone, 2% glucose) for DNA isolation or in GYEP medium (5% glucose, 0.1% yeast extract, 0.1% peptone) for toxin production (33). For yeast transformations, cells were grown on YPD (1% yeast extract, 2% peptone, 2% glucose) plates for 1 to 3 days; otherwise, cultures were maintained on glucose minimal media with appropriate supplements (1 g of leucine, 0.2 g of uracil, and 0.2 g of methionine/liter). For feeding studies, yeasts were grown in Ygal (1% yeast extract, 2% peptone, 2% galactose) to induce plasmid expression.

Physical analyses. Gas chromatography (GC) measurements were made by flame ionization detection with a Hewlett-Packard 5890 gas chromatograph fitted with a 30-m fused-silica capillary column (DB1; 0.25 μm; J&W Scientific Co., Palo Alto, Calif.). For routine screening of the trichothecene toxin phenotype, the column was held at 120°C at injection, then heated to 210°C at 15°C/min and held for 1 min, and then heated to 260°C at 5°C/min and held for 8 min. Low-resolution mass spectra were obtained by GC-mass spectrometry (MS) with a Hewlett-Packard 5891 mass-selective detector fitted with a DB-5 MS column (15-m by 0.25-mm film thickness).

DNA and RNA manipulations. The cDNA expression library was constructed with mRNA from an *F. sporotrichioides* NRRL 3299 culture grown for 23 h in GYEP. cDNA was cloned into the yeast expression vector pYES2 (Invitrogen, Carlsbad, Calif.). Plasmid preparation methods were as previously described (2).

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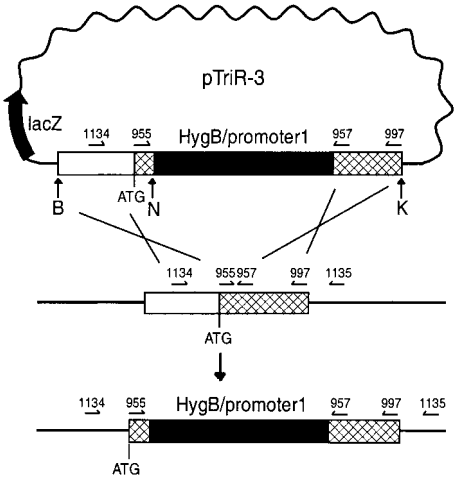

FIG. 1. Disruption vector for gene disruption of *TRI101*. B, *Bam*HI site; K, *Kpn*I site; N, *Nde*I site; ATG, start codon of *TRI101*. Numbers reference primers used in PCR analysis. p*TriR-3* is the chimeric plasmid. The middle diagram indicates the wild-type DNA with the crossover events; the bottom diagram indicates a gene replacement to form the gene disruption.

A cosmid library of *F. sporotrichioides* NRRL 3299 genomic DNA was made in the SuperCos1 vector (Stratagene, La Jolla, Calif.) in accordance with the manufacturer's instructions and was screened with a ^{32}P-labeled probe corresponding to the *TRI101* coding region. DNA labeling was performed with the Prime-a-Gene kit (Stratagene). Sequencing was performed with the DYEdeoxy sequencing kit (Applied Biosystems, Foster City, Calif.). Southern hybridizations were performed as described by Sambrook et al. (26).

Gene disruption and transformations. To disrupt *TRI101* we subcloned a PCR (*Pfu* polymerase; Stratagene) fragment of about 5 kb from cosmid 5-1 that contains *TRI101* into plasmid pBluescript II SK(+) (Stratagene) digested with *Bam*HI/*Ecl* 136 to form p*TriR*-2. A chimeric hygromycin B phosphotransferase gene (*hyg*) containing promoter 1 from *Cochliobolus heterostrophus* (32) was cloned into the unique *Nco*I site located 90 bp downstream of the ATG start site in *TRI101*. The resulting plasmid, p*TriR*-3 (Fig. 1), was used to transform *F. sporotrichioides* as previously described (25). The PCR analysis employed primers 955 (5′ GCGCTGCAGATCAAAATGGCCGAACAAGC 3′), 957 (5′ GTT TCCTTCGCTGATGCC 3′), 997 (5′ GGCGGTACCACAGAAAAGAGTAAA AGG 3′), 1134 (5′ GTCGATCGATACGCACGC 3′), and 1135 (5′ CTGGTC GTTGTATGTAGCC 3′). The underlined sequence in 955 indicates an added *Pst*I site, and that in 997 indicates an added *Kpn*I site. These primers have complementary sequences that are located both inside and outside the *TRI101* sequences present in plasmid p*TriR*-3 (Fig. 1). In the Southern analysis, genomic DNA of transformants was digested with *Bam*HI/*Xba*I and hybridized to a ^{32}P-labeled probe consisting of the *TRI101* coding regions.

Yeast transformations were performed by the methods of Gietz et al. (7, 27) for high-efficiency transformation or quick and easy transformation. Transformants were screened by PCR analysis with *TRI101* primers to ensure the presence of *TRI101* in the transformant.

Germination and growth on toxin-containing media. To determine what effect the loss of *TRI101* had on the growth of *F. sporotrichioides* in the presence of toxin, YPG agar plates containing 4,15-diacetoxyscirpenol (4,15-DAS) or T-2 toxin at 0, 100, 500, or 1,000 μg/ml were inoculated with a dilution containing approximately 10 spores of *F. sporotrichioides* NRRL 3299 or mutant strain FsTri101-3D. Plates were incubated at 28°C.

Trichothecene toxin assays. Liquid cultures of *F. sporotrichioides* were harvested after 7 days, extracted with ethyl acetate, and analyzed by gas-liquid chromatography (GLC) as described previously (19). Trichothecene concentrations were determined from the appropriate standard curves. Compound identifications were confirmed by GC-MS. For deoxynivalenol assays, trimethylsilyl ether derivatives were prepared with Tri-Sil TBT (Pierce, Rockford, Ill.).

Extraction and isolation of trichothecenes. We isolated trichothecenes produced by the mutant strain from liquid shake cultures of *F. sporotrichioides* FsTri101-3D grown for 7 days at 28°C and 200 rpm on YPD (1 liter in 2-liter Erlenmeyer flasks). Cultures were extracted twice with 600 ml of ethyl acetate, and the combined extracts were concentrated under reduced pressure. The syrup was separated on a silica gel column eluted with dichloromethane-methanol (95:5). Twelve 40-ml fractions were collected, and separation was monitored by thin-layer chromatography and GLC. Fractions 4 to 6 contained isotrichodermol. Fractions 8 to 10 contained 3,15-didecalonectrin.

Trichothecene standards. 4,15-DAS was isolated from *F. sporotrichioides* 1716cos9-1 (12); 15-monoacetoxyscirpenol was prepared by treating 4,15-DAS with 0.1 N NaOH; 3,4,15-triacetoxyscirpenol (3,4,15-TAS) was prepared from 4,15-DAS treated with acetic anhydride in pyridine. Isotrichodermin, 8-hydroxyisotrichodermin, and 8-hydroxyisotrichodermol were isolated from *F. sporotrichioides* mutant Allb (18); isotrichodermol was prepared by hydrolysis of isotrichodermin with 0.1 N NaOH. Deoxynivalenol and 15-acetyldeoxynivalenol were isolated from *Gibberella zeae* 3639 (24) grown on cornmeal. T-2 toxin was isolated from *F. sporotrichioides* NRRL 3299. 15-Decalonectrin and 3,15-didecalonectrin were isolated from *F. sporotrichioides* mutant strain O2 (19). All standards were greater than 95% pure as determined by GLC.

Whole-cell feeding experiments. Liquid cultures of *F. sporotrichioides* FsTri101-3D were inoculated with conidia washed from the V-8 plates, at a starting density of 5×10^4 conidia/ml in 10 ml of GYEP medium in a 50-ml Erlenmeyer flask, and incubated on a gyratory shaker (200 rpm) at 28°C. 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 (1% acetone). Six substrates were tested: 4,15-DAS, 3,4,15-TAS, isotrichodermin, 15-decalonectrin, 3,15-didecalonectrin, and 8-hydroxyisotrichodermin. Control cultures had acetone added to a final concentration of 1% acetone. Cultures were incubated on a rotary shaker (200 rpm) at 28°C for up to six additional days and then were extracted with ethyl acetate and analyzed by GLC.

Liquid yeast cultures were inoculated with a loop of yeast cells from a minimal medium plate. For feeding studies, yeast was grown on supplemented minimal media for 2 days and centrifuged ($1,600 \times g$, 5 min), and the pellet was resuspended in Ygal to induce plasmid expression. Cultures were normalized for optical density. After 2 h, the cultures were amended with a solution of the trichothecene (1 mg/10 ml of medium) in acetone (final concentration of 1% acetone). Six substrates were tested: 4,15-DAS, isotrichodermol, 15-monoacetoxyscirpenol, T-2 toxin, deoxynivalenol, and 15-decalonectrin. Cultures were incubated on a rotary shaker (200 rpm) at 28°C for up to five additional days and then were extracted with ethyl acetate and analyzed by GLC.

Cell-free system. Cell extracts of *F. sporotrichioides* NRRL 3299 and FsTri101-3D were made from liquid GYEP cultures incubated for 42 h on a gyratory shaker (200 rpm) at 28°C in the dark. Cultures were vacuum filtered, washed with nitrogen, and extracted with 3.5 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol. The extract was centrifuged at $3,000 \times g$ for 10 min at 4°C, and the supernatant was decanted and centrifuged at $3,000 \times g$ for an additional 5 min. Assays were run at 30°C and were initiated by the addition of 100 μl of the cell extract to a reaction mixture containing 250 μl of potassium phosphate buffer (pH 7.5), 10 μl of the trichothecene substrate in acetone (1 mg/50 μl of acetone), 100 μl of 20 mM $MgSO_4$, and 50 μl of acetyl coenzyme A (Sigma, St. Louis, Mo.) in water (25 mg/200 μl). Controls contained an additional 100 μl of potassium phosphate buffer in place of the cell extract. Immediately after addition of the cell extract and at 1, 2, and 4 h following addition, 100-μl aliquots of the reaction mixture were transferred to a glass vial containing 60 μl of ethanol. The samples were dried under a stream of nitrogen, redissolved in 50 μl of ethyl acetate or methanol, and analyzed by GLC. Six substrates were tested: isotrichodermol, 4,15-DAS, 15-monoacetoxyscirpenol, deoxynivalenol, 15-acetyldeoxynivalenol, and T-2 toxin.

Isolation of *TRI101*. Trichothecene-sensitive *S. cerevisiae* JG436 was used as a host for the expression of an *F. sporotrichioides* cDNA library. The increased trichothecene sensitivity of yeast strain JG436 results from the absence of a functional pleiotropic drug resistance gene, *PDR5* (22). cDNA library construction employed the yeast expression vector pYES2 and mRNA harvested under growth conditions supporting maximum levels of trichothecene pathway gene expression. Following the initial transformation of JG436, cells were plated under plasmid selection conditions and the transformants were pooled. Dilutions of the transformant pools were then selected for resistance to trichothecenes by plating them onto media containing 4,15-DAS. Of the 45 resistant colonies isolated, 16 were analyzed further. Plasmids rescued from these transformants could retransform JG436 to the trichothecene resistance phenotype. Of the 16 analyzed clones, 14 contained the same cDNA.

Nucleotide sequence accession number. The nucleotide sequence of *TRI101* has been submitted to GenBank under accession no. AF127176.

RESULTS

TRI101 expression in yeast. Analysis of trichothecenes from liquid cultures of yeast transformed with *TRI101* amended with 4,15-DAS indicated that about 50% of the 4,15-DAS was converted to 3,4,15-TAS in 3 days. Based on the proposed pathway for biosynthesis of T-2 toxin (5), we think that isotrichodermol is the likely substrate for TRI101 in the T-2 toxin pathway. Isotrichodermol is the first intermediate in *Fusarium* pathways to possess the trichothecene core structure and also has a C-3 hydroxyl group.

Whole-yeast cell-feeding experiments using isotrichodermol as a precursor indicated that nontransformed yeast converted only a very small amount to the acetylated product isotri-

chodermin. Yeast transformed with *TRI101* showed complete conversion to isotrichodermin within 44 h. Conversion of other 3-*O*-hydroxyl trichothecenes to their 3-*O*-acetyl analogs was slower. Conversion rates for all trichothecene substrates were improved in yeast expressing both *TRI101* and the trichothecene efflux pump *TRI12*. *TRI12* has been reported to increase conversion rates when paired with *TRI3* in yeast (2).

Characterization of *TRI101*. Analysis of the cDNA and corresponding genomic sequences for *TRI101* indicated that the coding region consists of 1,380 bp with no introns and that *TRI101* encodes a protein of 459 amino acids. A putative TRI6 binding site with the sequence TNAGGCCT (10, 25) is located 315 bp upstream of the start codon. Cosmid clones containing *TRI101* do not appear to overlap cosmids carrying the other described trichothecene pathway genes, in agreement with published reports indicating *TRI101* is not closely linked to the pathway gene cluster (13, 14).

Disruption of *TRI101*. Disruption of *TRI101* was accomplished via transformation of wild-type strain *F. sporotrichioides* NRRL 3299 with plasmid p*TriR*-3, which included the entire *TRI101* coding region into which the chimeric *hyg* gene had been inserted. Four of sixteen transformants, e.g., FsTri101-3D, were identified as *TRI101* disruptants by PCR analysis with primers 955 or 1134 paired with 957, 997, or 1135 (Fig. 1). The single fragment amplified from FsTri101-3D genomic DNA with each primer combination was 2.5 kb larger than the single product amplified from genomic DNA of the wild-type progenitor strain. The increased size of FsTri101-3D PCR products is consistent with disruption of *TRI101* via two homologous recombination events between p*TriR*-3 and chromosomal *TRI101* sequences, since the *hyg* gene is 2.5 kb (Fig. 1). If p*TriR*-3 had integrated ectopically or via a single homologous recombination event, PCR with primer pairs 955 and 957 would have yielded two amplification products: a 2.0-kb fragment corresponding to the native *TRI101* and a 2.7-kb fragment corresponding to the *hyg*-interrupted *TRI101* in p*TriR*-3. The PCR results were confirmed via Southern hybridization with *Bam*HI/*Xba*I-digested genomic DNA hybridized to a probe consisting of the *TRI101* coding region (data not shown). In the *TRI101*-disrupted strains the hybridizing band was 2.5 kb larger than the hybridizing band from the wild-type progenitor strain.

Spores of transformant FsTri101-3D were able to germinate and grow on media amended with 4,15-DAS or T-2 toxin. Its radial growth was only slightly reduced at the higher concentrations of 4,15-DAS or T-2 toxin (500 to 1,000 μg/ml) in comparison to growth on media without toxins, although the transformant had more aerial growth in the presence of higher concentrations of toxin.

F. sporotrichioides NRRL 3299 produces a number of closely related trichothecenes in liquid cultures of GYEP medium. Culture filtrates typically contain T-2 toxin, neosolaniol, propylneosolaniol, butylneosolaniol, and 4,15-DAS as well as the modified trichothecene apotrichodiol. In these mixtures, T-2 toxin usually constitutes 60 to 80% of the total trichothecenes (100 to 250 μg/ml) produced by 7-day-old shake cultures. Culture filtrates from transformant FsTri101-3D had no detectable levels of the trichothecenes normally produced by the parent strain, although apotrichodiol was still detected. However, three novel peaks were present in the GLC traces of FsTri101-3D liquid culture extracts (Fig. 2).

The electron impact (EI) mass spectrum of the first component exhibited a molecular ion at *m*/*z* 250 consistent with a trichothecene with one hydroxyl group and was tentatively identified as isotrichodermol. The EI mass spectrum of the second component exhibited a molecular ion at *m*/*z* 266 consistent with a trichothecene with two hydroxyl groups and was tentatively identified as didecalonectrin. The EI mass spectrum of the third component had fragments at *m*/*z* 248 and 220 characteristic of 3-deacetylcalonectrin. Compound identifications were confirmed by comparison with a mass spectrum library and published nuclear magnetic resonance and mass spectral data and by cochromatography with standard compounds.

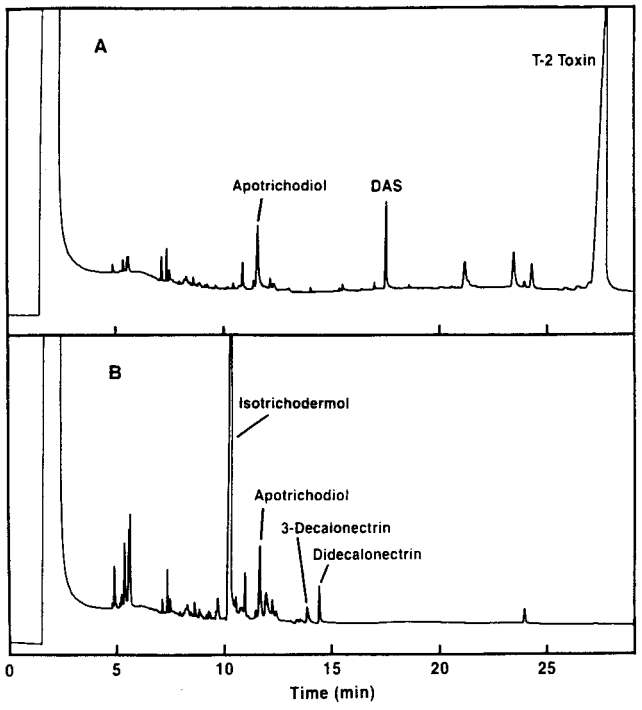

FIG. 2. Chromatograms of *F. sporotrichioides* culture extracts analyzed by GLC. Cultures were grown on GYEP medium for 7 days. (A) NRRL 3299 (*TRI101*$^+$) chromatogram. The locations of T-2 toxin, DAS, and apotrichodiol are indicated. (B) Strain FsTri101-3D (mutant *TRI101*). The locations of isotrichodermol, didecalonectrin, 3-decalonectrin, and apotrichodiol are indicated.

The accumulation of isotrichodermol in cultures of *F. sporotrichioides* FsTri101-3D implies that the C-3 acetyl group is necessary for the next step in the pathway to occur. This modification also may be important for substrate recognition by other pathway enzymes, and the biosynthetic pathway may require subsequent acetylation and deacetylation events involving the C-3 hydroxyl group. Cultures of FsTri101-3D were amended with a number of trichothecenes that are efficiently converted to T-2 toxin by mutant strains blocked at earlier steps in the trichothecene pathway (20). Trichothecenes lacking a C-3 acetyl group (isotrichodermol, 3,15-didecalonectrin, and 4,15-DAS) were not converted into T-2 toxin by cultures of *F. sporotrichioides* FsTri101-3D, but isotrichodermin and 15-decalonectrin, both of which have an acetyl group at the C-3 position, were efficiently converted into T-2 toxin by this mutant strain. A third C-3 acetylated trichothecene, 3,4,15-TAS, was not converted to T-2 toxin.

DISCUSSION

The sequence of *TRI101* from *F. sporotrichioides* NRRL 3299 has 94% similarity to the sequence from the previously reported *TRI101* (14), resulting in a 93% protein similarity. No sequence similarities between TRI101 and the several fungal *O*-acetyltransferases that have significant similarity to each

other were detected (8, 16, 17). Although TRI101 also appears unrelated to TRI3 (13), a trichothecene 15-*O*-acetyltransferase encoded by a gene that resides within the pathway gene cluster (19), both TRI3 and TRI101 share distinct motifs with a group of plant acyltransferases and their genes may be part of an extended acyltransferase gene family. TRI101 is similar in size to these plant acyltransferases and is conserved in both sequence and position for two motifs thought to be involved in catalysis (30).

The regulation of *TRI101* in the presence of exogenously added trichothecenes is rapid and unlike that of other trichothecene biosynthetic genes (13). However, there is a putative TRI6 binding site (TNAGGCCT) (10) located in the *TRI101* promoter region, which suggests that *TRI101* may be regulated by the pathway transcription factor TRI6.

Disruption of the *TRI101* gene in *F. sporotrichioides* confirms that it encodes an enzyme essential for T-2 toxin biosynthesis. A role for *TRI101* as a self-protection mechanism in *Fusarium* was proposed (13) based on the fact that acetylation, like other modifications of the C-3 hydroxyl group of trichothecenes, significantly reduces their toxicity (13, 28). Although we found that yeasts transformed with *TRI101* have increased tolerance of 4,15-DAS, the loss of *TRI101* in *F. sporotrichioides* does not result in a significant inhibition of growth on trichothecene-containing media. Therefore, *F. sporotrichioides* most likely has additional self-protection mechanisms such as the toxin efflux pump encoded by *TRI12* (2).

The possibility that multiple 3-*O*-acetyltransferases are present in *Fusarium* species has been proposed (14). No residual trichothecene 3-*O*-acetyltransferase activity was detected in mycelial extracts of the *TRI101* disruptant strain, arguing against the possibility that *TRI101* activity was still expressed in this mutant or that other trichothecene 3-*O*-acetyltransferases were expressed in *F. sporotrichioides*.

Mutants lacking a functional *TRI101* produced an altered profile of trichothecenes. Specifically, no T-2 toxin was synthesized and isotrichodermol accumulated as the major trichothecene component. Isotrichodermol accumulation suggests that 3-O-acetylation is required for the subsequent C-15 oxygenation step to occur. Formation of small amounts of 3,15-didecalonectrin and 3-decalonectrin may be due to the presence of relatively high concentrations of isotrichodermol and the ability of the *TRI11*-encoded C-15 monooxygenase (1) and the *TRI3*-encoded C-15 acetyltransferase (19) to accept isotrichodermol and didecalonectrin as substrates under these conditions. We think that TRI101 acts as an integral biosynthetic enzyme and that while C-3 acetylation of other trichothecenes may occur, conversion of isotrichodermol to isotrichodermin is the primary biosynthetic reaction catalyzed by TRI101.

The disruptant strain FsTri101-3D can convert isotrichodermin and 15-decalonectrin, both of which have a C-3 acetyl, to T-2 toxin, suggesting that biosynthetic steps after that performed by the *TRI101* product are not affected by the disruption reported. Our results are consistent with the hypothesis that most of the *F. sporotrichioides* trichothecene biosynthetic pathway is via intermediates with a C-3 acetyl protecting group. Mutants with disrupted *TRI3* and *TRI11* accumulate 15-decalonectrin and isotrichodermin, respectively, indicating that trichothecenes with an acetyl group at C-3 accumulate as predicted. 3,4,15-TAS was not converted to T-2 toxin by the disrupted strain FsTri101-3D, which suggests that there may be deacetylation and reacetylation required at C-3 in the later steps of biosynthesis.

Accepting *TRI101* as part of an intracellular protection mechanism implies the existence of an extracellular esterase to activate trichothecenes. In cultures of mutants that accumulate trichothecenes with a C-3 acetyl group, e.g., 15-decalonectrin or isotrichodermin, these acetylated compounds are slowly deacetylated. Four esterases that may remove acetyl groups from various positions of the trichothecene skeleton are known (23). Kimura et al. (14) reported that there was no conversion to an acetylated product when T-2 toxin was added to *F. sporotrichioides* cultures, although crude extracts of recombinant *TRI101* from *Escherichia coli* and from *F. graminearum* cultures both could convert T-2 toxin to 3-acetyl T-2 toxin. They interpreted this observation as evidence that *TRI101* was not strongly induced by T-2 toxin. An alternate hypothesis is that competing esterases mask *TRI101* expression by removing the C-3 acetyl group in the final steps in T-2 toxin biosynthesis. This hypothesis is supported by our results with several trichothecenes containing a C-3 acetyl group. When these compounds were added to cultures of the disruptant strain FsTri101-3D, deacetylation did not appear to be blocked and deacetylated products were recovered.

The presence of a 3-hydroxyl group differentiates *Fusarium* trichothecenes from most other fungal trichothecenes. Even though the early enzymes and regulatory mechanisms for the biosynthesis of macrocyclic trichothecenes in *Myrothecium roridum* are related to their counterparts in *Fusarium* trichothecene pathways (31), macrocyclic trichothecenes do not contain a 3-hydroxyl group. Therefore, identification of isotrichodermol as the primary *TRI101* substrate makes this enzyme essential for biosynthesis of the most toxic *Fusarium* trichothecenes. TRI101 therefore appears to be a critical and unique enzyme in the *Fusarium* trichothecene biosynthetic pathway.

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REFERENCES

1. **Alexander, N. J., T. M. Hohn, and S. P. McCormick.** 1998. The *TRI11* gene of *Fusarium sporotrichioides* encodes a cytochrome P-450 monooxygenase required for C-15 hydroxylation in trichothecene biosynthesis. Appl. Environ. Microbiol. **64:**221–225.
2. **Alexander, N. J., S. P. McCormick, and T. M. Hohn.** 1999. *TRI12*, a trichothecene efflux pump from *Fusarium sporotrichioides*: gene isolation and expression in yeast. Mol. Gen. Genet. **261:**977–984.
3. **Cundliffe, E.** 1989. How antibiotic-producing organisms avoid suicide. Annu. Rev. Microbiol. **43:**207–233.
4. **Desjardins, A. E., and T. M. Hohn.** 1997. Mycotoxins in plant pathogenesis. Mol. Plant-Microbe Interact. **10:**147–152.
5. **Desjardins, A. E., T. M. Hohn, and S. P. McCormick.** 1993. Trichothecene biosynthesis in *Fusarium* species: chemistry, genetics, and significance. Microbiol. Rev. **57:**595–604.
6. **Desjardins, A. E., R. H. Proctor, G. Bai, S. P. McCormick, G. Shaner, G. Buechley, and T. M. Hohn.** 1996. Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. Mol. Plant-Microbe Interact. **9:**775–781.
7. **Gietz, R. D., A. St. Jean, R. A. Woods, and R. H. Schiestl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:**1425.
8. **Goyon, C., G. Faugeron, and J.-L. Rossignol.** 1988. Molecular cloning and characteristics of the *met2* gene from *Ascobolus immersus*. Gene **63:**297–308.
9. **Hohn, T. M., A. E. Desjardins, and S. P. McCormick.** 1995. The *Tri4* of *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase involved in trichothecene biosynthesis. Mol. Gen. Genet. **248:**95–102.
10. **Hohn, T. M., R. Krishna, and R. H. Proctor.** 1999. Characterization of a transcriptional activator controlling trichothecene toxin biosynthesis. Fungal Genet. Biol. **26:**224–235.
11. **Hohn, T. M., S. P. McCormick, N. J. Alexander, A. E. Desjardins, and R. H. Proctor.** 1998. Function and biosynthesis of trichothecenes produced by Fusarium species, p. 17–24. *In* K. Kohmoto and O. C. Yoder (ed.), Molecular genetics of host-specific toxins in plant disease. Kluwer Academic Publishers, Dordrecht, The Netherlands.
12. **Hohn, T. M., S. P. McCormick, and A. E. Desjardins.** 1993. Evidence for a gene cluster involving trichothecene pathway biosynthetic genes in *Fusarium sporotrichioides*. Curr. Genet. **24:**291–295.

13. **Kimura, M., I. Kaneko, M. Komiyama, A. Takatsuki, H. Koshino, K. Yoneyama, and I. Yamaguchi.** 1998. Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of *Tri101*. J. Biol. Chem. **273:**1654–1661.
14. **Kimura, M., G. Matsumoto, Y. Shingu, K. Yoneyama, and I. Yamaguchi.** 1998. The mystery of the trichothecene 3-O-acetyltransferase gene. Analysis of the region around *Tri101* and characterization of its homologue from *Fusarium sporotrichioides*. FEBS Lett. **435:**163–168.
15. **Kimura, M., Y. Shingu, K. Yoneyama, and I. Yamaguchi.** 1998. Features of *Tri101*, the trichothecene 3-O-acetyltransferase gene, related to the self-defense mechanism in *Fusarium graminearum*. Biosci. Biotechnol. Biochem. **62:**1033–1036.
16. **Langin, T., G. Faugeron, C. Goyon, A. Nicolas, and J.-L. Rossignol.** 1986. The *MET2* gene of *Saccharomyces cerevisiae*: molecular cloning and nucleotide sequence. Gene **49:**283–293.
17. **Mathison, L., C. Soliday, T. Stephan, T. Aldrich, and J. Rambosek.** 1993. Cloning, characterization, and use in strain improvement of the *Cephalosporium acremonium* gene *cefG* encoding acetyl transferase. Curr. Genet. **23:**33–41.
18. **McCormick, S. P., and T. M. Hohn.** 1997. Accumulation of trichothecenes in liquid cultures of a *Fusarium sporotrichioides* mutant lacking a functional trichothecene C-15 hydroxylase. Appl. Environ. Microbiol. **63:**1685–1688.
19. **McCormick, S. P., T. M. Hohn, and A. E. Desjardins.** 1996. Isolation and characterization of *Tri3*, a gene encoding 15-O-acetyltransferase from *Fusarium sporotrichioides*. Appl. Environ. Microbiol. **62:**353–359.
20. **McCormick, S. P., S. L. Taylor, R. D. Plattner, and M. N. Beremand.** 1990. Bioconversion of possible T-2 toxin precursors by a mutant strain of *Fusarium sporotrichioides* NRRL 3299. Appl. Environ. Microbiol. **56:**702–706.
21. **McLaughlin, C. S., M. H. Vaughn, J. M. Campbell, C. M. Wei, and M. E. Stafford.** 1977. Inhibition of protein synthesis by trichothecenes, p. 263–273. *In* J. V. Rodricks, C. W. Hesseltine, and M. A. Mehlman (ed.), Mycotoxins in human and animal health. Pathotoxin Publishers, Park Forest, Ill.
22. **Meyers, S., W. Schaur, E. Balzi, M. Wagner, A. Goffeau, and J. Golin.** 1992. Interaction of the pleiotropic drug resistance genes *PDR1* and *PDR5*. Curr. Genet. **21:**431–436.
23. **Park, J. J., and F. S. Chu.** 1996. Immunochemical studies of an esterase from *Fusarium sporotrichioides*. Food Agric. Immunol. **8:**41–49.
24. **Proctor, R. H., T. M. Hohn, and S. P. McCormick.** 1995. Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. Mol. Plant-Microbe Interact. **8:**593–601.
25. **Proctor, R. H., T. M. Hohn, S. P. McCormick, and A. E. Desjardins.** 1995. *Tri6* encodes an unusual zinc finger protein involved in regulation of trichothecene biosynthesis in *Fusarium sporotrichioides*. Appl. Environ. Microbiol. **61:**1923–1930.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Shiestl, R. H., and R. D. Geitz.** 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. **16:**339–346.
28. **Shima, J., S. Takase, U. Takahashi, Y. Iwai, H. Fujimoto, M. Yamazaki, and K. Ochi.** 1997. Novel detoxification of the trichothecene mycotoxin deoxynivalenol by a soil bacterium isolated by enrichment culture. Appl. Environ. Microbiol. **63:**3825–3830.
29. **Stevens, R. R.** 1974. Mycology guidebook, p. 703. University of Washington Press, Seattle.
30. **St. Pierre, B., P. Laflamme, A.-M. Alarco, and V. DeLuca.** 1998. The terminal O-acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for coenzyme A-dependent acyl transfer. Plant J. **14:**703–713.
31. **Trapp, S. C., T. M. Hohn, S. P. McCormick, and B. B. Jarvis.** 1998. Characterization of the gene cluster for biosynthesis of macrocyclic trichothecenes in *Myrothecium roridum*. Mol. Gen. Genet. **257:**421–432.
32. **Turgeon, B. G., R. C. Garber, and O. C. Yoder.** 1987. Development of a fungal transformation system based on selection of sequences with promoter activity. Mol. Cell. Biol. **7:**3297–3305.
33. **Ueno, Y., M. Sawano, and K. Ishii.** 1975. Production of trichothecene mycotoxins by *Fusarium* species in shake culture. Appl. Microbiol. **30:**4–9.