Disruption of TRI101, the Gene Encoding Trichothecene 3-0-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-diacetoxysecrpenol, and each carried a cDNA encoding the trichothecene 3-0-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 isotrichodermin and small amounts of 3,15-didecalonectrin and 3-decaloectrin, trichothecenes that are not observed in cultures of the parent strain. Our results indicate that TRI101 converts isotrichodermin 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 3-0-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 isotrichodermin, a 3-hydroxytrichothecene. These mutants could both germinate and grow in the presence of isotrichodermin 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 F5Tri101-3D contains disrupted sequences for TRI101 as described below. S. cerevisiae RW2802 (PDR5 leu2 ana3-52 met5) and JG436 (pdr5::Tn5 leu2 ana3-35 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. Cornda 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 (DBI; 0.25 mm; 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 5971 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 1299 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)
1716cos9-1 (12); 15-monoacetoxyscirpenol was prepared by treating 4,15-DAS (95:5). Twelve 40-ml fractions were collected, and separation was monitored by gas chromatography and the combined extracts were concentrated under reduced pressure. The syrup from FsTri101-3D grown for 7 days at 28°C and 200 rpm on YPD (1 liter in 2-liter 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-deacelodermol, 3,15-didecalcemycin, and 8-hydroxyisotrichodermin. Culture 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 5 additional days and then were extracted with ethyl acetate and analyzed 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 concentration of 5 × 10^7 conidia/ml in 10 mM GYE 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 0.1 N NaOH; Deoxynivalenol and 15-acetyldeoxynivalenol were isolated after 7 days, extracted with ethyl acetate, and analyzed by gas-liquid chromatography with 0.1 N NaOH. Deoxynivalenol and 15-acetyldeoxynivalenol were isolated from Gibberella zeae 3639 (24 grown on concom. 2-T toxin was isolated from F. sporotrichioides NRRL 3299. 15-Decalonecin and 3,15-didecalcemycin were isolated from F. sporotrichioides NRRL 3299 and FsTri101-3D were made from liquid GYEI 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 × g for 10 min at 4°C, and the supernatant was decanted and centrifuged at 3,000 × 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 0.1 M potassium phosphate buffer (pH 7.5), 10 µl of the trichothecene extract in acetone (1 mg/50 µl of acetone), 100 µl of 20 mM MgSO4, 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: isotrichodermin, 4,15-DAS, 15-monoacetoxyscirpenol, deoxynivalenol, 15-acetyldeoxynivalenol, and 2-T 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 conditions supporting maximum levels of trichothecene pathway gene expression. The initial transformants of JG436 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 isolated 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 isotrichodermin is the likely substrate for TRI101 in the T-2 toxin pathway. Isotrichodermin 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 isotrichodermin 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 isochodermin 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 pTriR-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 pTriR-3 and chromosomal TRI101 sequences, since the hyg gene is 2.5 kb (Fig. 1). If pTriR-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 pTriR-3. The PCR results were confirmed via Southern hybridization with BamHI/XbaI-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 two hydroxyl groups and was tentatively identified as didecalonectrin. 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.

The accumulation of isochodermin 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 (isochodermin, 3,15-didecalonectrin, and 4,15-DAS) were not converted into T-2 toxin by cultures of F. sporotrichioides FsTri101-3D, but isochodermin 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 TR3 (13), a trichothecene 15-O-acetyltransferase encoded by a gene that resides within the pathway gene cluster (19), both TR3 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 (TNAGG CCT) (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 hydroxy 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 monoxygenase (1) and the TRI5-encoded C-15 acetyltransferase (19) to accept isotrichoderin 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 isotrichodermol is the primary biosynthetic reaction catalyzed by TRI101.

The disruptant strain FsTri101-3D can convert isotrichoderin 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 TR3 and TR11 accumulate 15-decalonectrin and isotrichodermol, 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 isotrichodermol, 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 ro-ridium 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


