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Bioprospecting for Trichothecene 3-*O*-Acetyltransferases in the Fungal Genus *Fusarium* Yields Functional Enzymes with Different Abilities To Modify the Mycotoxin Deoxynivalenol▽†

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The trichothecene mycotoxin deoxynivalenol (DON) is a common contaminant of small grains, such as wheat and barley, in the United States. New strategies to mitigate the threat of DON need to be developed and implemented. TRI101 and TRI201 are trichothecene 3-*O*-acetyltransferases that are able to modify DON and reduce its toxicity. Recent work has highlighted differences in the activities of TRI101 from two different species of *Fusarium* (*F. graminearum* and *F. sporotrichioides*), but little is known about the relative activities of TRI101/TRI201 enzymes produced by other species of *Fusarium*. We cloned *TRI101* or *TRI201* genes from seven different species of *Fusarium* and found genetic identity between sequences ranging from 66% to 98%. *In vitro* feeding studies using transformed yeast showed that all of the TRI101/TRI201 enzymes tested were able to acetylate DON; conversion of DON to 3-acetyl-deoxynivalenol (3ADON) ranged from 50.5% to 100.0%, depending on the *Fusarium* species from which the gene originated. A time course assay showed that the rate of acetylation varied from species to species, with the gene from *F. sporotrichioides* having the lowest rate. Steady-state kinetic assays using seven purified enzymes produced catalytic efficiencies for DON acetylation ranging from $6.8 \times 10^4\ M^{-1} \cdot s^{-1}$ to $4.7 \times 10^6\ M^{-1} \cdot s^{-1}$. Thermostability measurements for the seven orthologs ranged from 37.1°C to 43.2°C. Extended sequence analysis of portions of *TRI101*/*TRI201* from 31 species of *Fusarium* (including known trichothecene producers and nonproducers) suggested that other members of the genus may contain functional *TRI101*/*TRI201* genes, some with the potential to outperform those evaluated in the present study.

Fusarium head blight (FHB), caused by the plant pathogen *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae*), is a detrimental disease of grains such as wheat and barley (11). The fungus is a major producer of deoxynivalenol (DON), a trichothecene mycotoxin that contaminates cereal crops and compromises the health of humans and domestic animals (6). DON is a known eukaryotic protein synthesis inhibitor (20) and can cause vomiting, reproductive problems, feed refusal, and even death in mammals (26). Its acetylated derivative 3-acetyl-deoxynivalenol (3ADON) has been shown to be equivalent to or lower than DON in toxicity based on 50% lethal dose (LD_{50}) values in mice (25) and 50% inhibitory concentrations (IC_{50}s) on rabbit reticulocytes (14).

Fusarium graminearum contains a gene called *TRI101* that encodes a trichothecene 3-*O*-acetyltransferase (14) that transfers an acetyl group from acetyl coenzyme A (acetyl-CoA) to the C-3 hydroxyl moiety of the trichothecene molecule (10). This acetyltransferase reaction has been demonstrated to decrease the inhibitory effects of trichothecenes on the growth of *Saccharomyces pombe* (14), as well as that of *Chlamydomonas reinhardtii* and *Arabidopsis thaliana* (3, 7). Disruption of *TRI101* in *Fusarium sporotrichioides* (*FsTRI101*) blocked the production of T-2 toxin, a trichothecene-derived metabolite, and led to the accumulation of an intermediate, isotrichodermol, suggesting that *TRI101* is necessary for the production of trichothecenes (19). Interestingly, in trichothecene-producing *Fusarium* spp., *TRI101* is usually not located near the trichothecene biosynthetic gene cluster (15, 27, 31). *TRI101* is located between the phosphate permease gene (*PHO5*) and the UTP-ammonia ligase gene (*URA7*) in *F. graminearum* (15). In several trichothecene-nonproducing *Fusarium* species that have nonfunctioning TRI101s, C-3 acetyltransferase activity is still detected and is encoded by a gene named *TRI201* (16). *TRI201* is believed to be the result of gene duplication (31), with a different evolutionary history from that of other trichothecene biosynthetic genes.

Structural differences between *F. graminearum* TRI101 (FgTRI101) and FsTRI101 active sites demonstrated that FgTRI101 is better able to accommodate DON than is FsTRI101 (10). In addition to structural differences, there are large kinetic differences between the two enzymes. The work by Garvey et al. (10) showed that 85 times more DON substrate is required for FsTRI101 to reach the half-maximal rate than for FgTRI101. Likewise, the catalytic efficiency of FgTRI101 toward DON is considerably higher than that of FsTRI101 (10).

TRI12, another important gene involved in trichothecene

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TABLE 1. Sequences of primers used to amplify all or portions of *TRI101*/*TRI201* and *FsTRI12*[a]

Primer no. and name	Primer sequence	Gene/application
1. FsTRI101_FWD	5′-ATGGCCGCAACAAGCA-3′	Entire *TRI101*
2. FsTRI101_REV	5′-CTACCCAATATACTTTGCGTACTTTGT-3′	
3. FgTRI101_FWD	5′-ATGGCTTTCAAGATACAGCTCG-3′	Entire *TRI101*
4. FgTRI101_REV	5′-CTAACCAACGTACTGCGCATACT-3′	
5. FoTRI201_FWD	5′-ATGACTGCACTAAACGTTACAAACA-3′	Entire *TRI201*
6. FoTRI201_REV	5′-CTAGCCAATGAATTGCCCATAC-3′	
7. FfTRI201_FWD	5′-ATGACAGCACTAAACACCACAAAC-3′	Entire *TRI201*
8. FfTRI201_REV	5′-CTAGCCAATGAACTTCCCATACTTT-3′	
9. FsTRI12_FWD_BAMHI	5′-GACTGGATCCATGACTGTCGTAGTTCCAGAGG-3′	Cloning into pESC-LEU
10. FsTRI12_REV_SALI	5′-GACTGTCGACTCATTCTTTATCAGCCATCGAA-3′	
11. TRI101_Conserved_FWD	5′-MTVTTKCAGCTCAAYTTYAT-3′	Screening for *TRI101*
12. TRI101_Conserved_REV	5′-TYCRTATCCTCATCBCTCA-3′	Screening for *TRI101*
13. *Fgraminearum_URA7*_FWD	5′-CAAGCGTCATCTTTCTCAGCGC-3′	Isolation of entire *TRI101* of *F. pseudograminearum*, *F. culmorum*, and *F. crookwellense*
14. *Fgraminearum*_PHO5_REV	5′-GGATGGAACGCTTCGACCAC-3′	
15. *Fpseudograminearum*_FWD	5′-ATGGCTTTCAAGATAGAGCTCG-3′	Entire *TRI101*
16. *Fpseudograminearum*_REV	5′-CTAACCAACGTACTGCGCG-3′	
17. *Fculmorum*_FWD	5′-ATGGCTTTCAAGATACAGCTCG-3′	Entire *TRI101*
18. *Fculmorum*_REV	5′-CTAACCAACGTACTGCGCG-3′	
19. *Fcrookwellense*_FWD	5′-ATGGCTTTCAAGATACAGCTCG-3′	Entire *TRI101*
20. *Fcrookwellense*_REV	5′-CTAACCAACATACTGCGCATACTTG-3′	

[a] Underlined sequences represent restriction enzyme cut sites. Also shown are oligonucleotides for *F. graminearum URA7* and *PHO5* used to isolate the full-length *TRI101* sequence of *F. pseudograminearum*, *F. culmorum*, and *F. crookwellense*. After sequence analysis, we developed species-specific primers to amplify the coding region of each *TRI101*.

production, encodes a trichothecene efflux pump, an integral membrane protein responsible for exporting trichothecenes from hyphal cells (2). Alexander et al. (2) suggested that the expression of an efflux pump in yeast could amplify trichothecene flux into yeast cells; consequently, we predicted that we would observe increased acetylation of DON in our expression studies. Our work confirmed the interaction of TRI12 with trichothecenes when the coexpression of *TRI101*/*TRI201* and *TRI12* increased the acetylation of DON in the yeast *Saccharomyces cerevisiae*.

F. sporotrichioides TRI101 has previously been transformed into several plant systems (1). Experiments using tobacco showed that FsTRI101 reduced the phytotoxic effects of trichothecenes (22). Okubara et al. (24) obtained four transgenic wheat lines that accumulated *FsTRI101* transcripts; however, of the four lines, only one showed partial resistance against the spread of *F. graminearum* in greenhouse assays. Ohsato et al. (23) were the first to demonstrate DON acetylation in a cereal plant expressing TRI101 by determining DON resistance in rice root growth assays. Rice roots subjected to DON showed reduced growth, while roots expressing TRI101 were not inhibited and grew similarly to unexposed wild-type roots. Barley transformed with *FsTRI101* had reduced FHB in greenhouse assays, although field tests showed that transgenic plants were as susceptible to FHB as the controls (18). These noted works all illustrate the great potential of utilizing trichothecene acetyltransferase genes, such as *TRI101*, to detoxify DON. However, protection of cereal crops against DON may require the employment of a more effective and stable trichothecene acetyltransferase.

Our goal is to discover novel trichothecene acetyltransferases to facilitate the development of FHB-resistant cereal crops with reduced potential for DON contamination. As shown by Garvey et al. (10), the large disparity in the ability of TRI101 enzymes from two different species of *Fusarium* to bind DON may be explained in part by large differences in kinetic profiles. We hypothesized (i) that both producers and nonproducers of DON in the fungal genus *Fusarium* contain functional *TRI101*/*TRI201* genes and (ii) that resulting TRI101/TRI201 enzymes vary in their ability to modify DON. Bioprospecting for functional trichothecene acetyltransferases in *Fusarium* species may yield more effective and efficient enzymes that may be used to enhance disease resistance in cereal crops and reduce the threat of DON contamination. Here, we demonstrated the use of yeast feeding assays to indicate the potential functionality of trichothecene 3-*O*-acetyltransferases from different *Fusarium* species and followed this work with enzyme kinetics.

MATERIALS AND METHODS

Yeast strain. *S. cerevisiae* strain RW2802 (PDR5 *leu2 ura3-52 met5*) was used for all of the experiments (21) and was kindly provided by J. Golin, The Catholic University, Washington, DC.

Isolation of *TRI101*, *TRI201*, and *FsTRI12*. Since *TRI101* and *TRI201* do not contain introns, each *TRI101*/*TRI201* gene was amplified from the genomic DNA of the appropriate *Fusarium* species using primers specific for each species (Table 1). Entire genes were obtained from the Schmale (David Schmale, Virginia Polytechnic Institute and State University) and Leslie (John Leslie, Kansas State University) collections, which are designated with a 1 and 2, respectively, in gene and protein designations hereinafter. Complete *TRI101* gene sequences were acquired from *F. crookwellense* strain 11451 (*Fcr2TRI101*), *F. culmorum* strain 11427 (*Fcu2TRI101*), *F. pseudograminearum* strain 11435 (*Fps2TRI101*), *F. graminearum* strain Z3639 (*Fg2TRI101*), *F. graminearum* strain T1S1 (*Fg1TRI101*), *F. sporotrichioides* strain 11552 (*Fs2TRI101*), and *F. sporotrichioides* strain F4n23 (*Fs1TRI101*). Complete *TRI201* sequences were acquired from *F. fujikuroi* strain C1994 (*Ff2TRI201*), *F. fujikuroi* strain F4n17 (*Ff1TRI201*), *F. oxysporum* strain 11390 (*Fo2TRI201*), and *F. oxysporum* strain F4n26 (*Fo1TRI201*). Pairwise comparisons of nucleotide identity and divergence between full-length *TRI101*/*TRI201* orthologs were conducted using MegAlign (Lasergene version 8.1.1; DNAStar, Madison, WI). *Fusarium* genomic DNA was isolated by using a DNeasy plant minikit following the manufacturer's protocols (Qiagen, Germantown, MD). PCR amplifications were carried out in

50-μl reaction mixture volumes using Easy-A high-fidelity PCR cloning enzyme (Stratagene, La Jolla, CA) under the following conditions: one cycle at 95°C for 2 min; 30 cycles of 95°C for 40 s, 54°C for 30 s, and 72°C for 1.5 min; and finally, one cycle at 72°C for 7 min. *FsTRI12*, from *F. sporotrichioides* NRRL 3299, was amplified from the YIplac128 vector containing the *FsTRI12* gene. *FsTRI12* amplifications were carried out in 50-μl reaction mixture volumes using Easy-A high-fidelity PCR cloning enzyme (Stratagene, La Jolla, CA) as described above, with an annealing temperature of 57°C and an extension time of 2 min.

Bioprospecting for trichothecene acetyltransferases in *Fusarium* species. *TRI101* or *TRI201* sequences from 17 species of *Fusarium* were downloaded from GenBank and aligned using SeqMan Pro version 8.1 (Lasergene version 8.1.1; DNAStar, Madison, WI) (see Table S1 in the supplemental material). Degenerate primers were designed based on regions of homology among available partial *TRI101* and *TRI201* cDNA and known full-length cDNA of the *Fusarium* species listed in Table S1 in the supplemental material. Strains of *Fusarium* from the Leslie collection (see Table S2 in the supplemental material) were screened for the presence of an internal portion of *TRI101*/*TRI201* (893/890 bp) (Table 1, primers 11 and 12).

RW2802 transformations. *TRI101*/*TRI201* genes isolated from both the Leslie and Schmale collections were transformed into *Saccharomyces cerevisiae* strain RW2802 (see Table S3 in the supplemental material). *TRI101*/*TRI201* genes were cloned into Invitrogen's yeast expression vector pYES2.1 (Invitrogen, Carlsbad, CA) (Table 1). The *F. sporotrichioides TRI12* gene (*FsTRI12*) was amplified from its vector (2) and cut with restriction enzymes BamHI and SalHI obtained from New England BioLabs (NEB, Ipswich, MA) and ligated into Stratagene's pESC-LEU vector. Digestions were performed at 37°C for 1 h, and ligations were performed at 16°C for 2 h using NEB T4 DNA ligase (NEB, Ipswich, MA). Both vectors contain the GAL1 promoter and the CYC termination sequence, allowing induced expression by the addition of galactose to the liquid medium. All vector-gene combinations were first transformed into TOP10 chemically competent *Escherichia coli* cells by following Invitrogen's One Shot TOP10 chemical transformation protocol. All yeast transformations were conducted by following Invitrogen's small-scale yeast transformation protocol. Yeast transformations were performed for each *TRI101*/*TRI201* gene, and one colony was chosen from each transformation reaction mixture for the yeast assay. Double yeast transformants carrying both *TRI101*/*TRI201* and *TRI12* were made by simultaneous transformation of RW2802 using both plasmids, pTRI101YES and pTRI12ESC, and plating onto the appropriate selective medium lacking both uracil and leucine.

Yeast media. Yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose [D-glucose]) was used to grow untransformed *S. cerevisiae* strain RW2802. Transformed *S. cerevisiae* lines were grown on synthetic drop-out medium containing yeast nitrogen base without amino acids (0.67%), 2% glucose, and supplemented with amino acids. The drop-out medium without leucine contained all standard amino acids (76 mg/liter final) except leucine (Sigma, St. Louis, MO). The drop-out medium without uracil contained all standard amino acids (76 mg/liter final) plus leucine (380 mg/liter final) and lacked uracil (Sigma, St. Louis, MO). The drop-out medium without uracil and leucine contained all standard amino acids (76 mg/liter final) except leucine and uracil (Sigma, St. Louis, MO). The induction medium was similar to the synthetic drop-out medium except that glucose was supplemented with filter-sterilized galactose (final concentration of 2%) and raffinose (final concentration, 1%). Glucose and raffinose were filter sterilized using a Millex GP 0.22-μm filter unit (SLGP033RS; Millipore, Billerica, MA), and galactose was filter sterilized using a 0.45-μm filter unit.

Acetylation assays with TRI101/TRI201 constructs. Liquid drop-out medium, containing the appropriate selective amino acids, was inoculated with transformed yeast cells from a plate and grown at 28°C overnight until an optical density at 600 nm (OD_{600}) greater than 3.0 was reached. Optical density was measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). A volume of culture was spun down and resuspended in induction medium at an OD_{600} of 3.0. Cultures were transferred to a culture tube containing an amount of dried DON that, upon resuspension, equaled 10 ppm. Cultures were placed on a rotary shaker at 200 rpm and 28°C for 30 h or 1 day, depending on the experiment. After the selected time had passed, 500 μl of yeast culture was removed and added to 3.5 ml of acetonitrile. Cultures were placed on a shaker at 200 rpm for 1 h at room temperature. The yeast-acetonitrile mixture was then passed through an alumina:C_{18} column for cleanup. A 2-ml aliquot of eluant was transferred to a glass test tube and evaporated to dryness using a nitrogen evaporator set at 55°C. One hundred microliters of the derivatization agent *N*-trimethylsilylimidazole (TMSI) was then added to the dried-down samples. After 30 min, 500 μl of isooctane was added to each tube, followed by 500 μl of water to quench the reaction. Samples were exposed to 10 s of vortexing, and 150 μl of the isooctane supernatant was removed and transferred to chromatography vials for gas chromatography-mass spectrometry (GC-MS) analysis. Conversion of DON to 3ADON was determined by calculating a percent based on the amount of 3ADON produced in relation to total toxin (DON plus 3ADON).

GC-MS analysis. GC-MS analysis was conducted using an Agilent 6890/5975 system operating in selected ion monitoring (SIM) mode. DON, 3ADON, and mirex eluted off the column (HP-5MS, 30.0 m, 250 μm, 0.25 μm) at 6.23 6.70, and 6.89 min, respectively. Mirex was used as an internal control at 0.5 ppm. SIM mode detected DON and 3ADON target ions at a mass/charge ratio of 512 and 392, respectively, with reference ions at 422 and 497 for DON and a reference ion at 467 for 3ADON. SIM mode detected mirex target ions at a mass/charge ratio of 272 with reference ions at 276 and 237. The initial column temperature was held at 150°C for 1 min, increased to 280°C at a rate of 30°C/min, and held constant for 5 min. The injection temperature was set at 300°C, and the flow rate of the column was 1 ml/min. DON and 3ADON were quantified in the samples using a quadratic regression model using pure DON and 3ADON standards (Biopure, Austria) at concentrations of 0.5 ppm, 1.0 ppm, 5 ppm, 10 ppm, and 30 ppm.

Cloning and expression of TRI101/TRI201 enzymes in *E. coli*. *TRI101*/*TRI201* genes were amplified by PCR from pYES2.1 plasmids bearing the appropriate insert by using *Pfu* Herculase(r) II fusion DNA polymerase (Stratagene). Amplified products were digested with NheI and BlpI restriction enzymes (New England BioLabs) and ligated into a modified pET31b (Novagen, Darmstadt, Germany) vector containing an N-terminal His_6 tag followed by a TEV protease cleavage site (29). The TRI101/TRI201 orthologs were overexpressed in *E. coli* strain BL21 Codon Plus (DE3). Starter cultures from a single colony were grown overnight in Luria broth (LB) supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. Fifteen milliliters of the starter culture was used to inoculate 1 liter of LB medium including ampicillin and chloramphenicol. Cultures were grown to mid-log phase ($OD_{600} \sim 0.8$) at 37°C, cooled to 16°C, and induced with 1 mM β-D-thiogalactopyranoside (IPTG; Sigma Chemical Co.). Cells were harvested after 20 h by centrifugation at 3,000 × *g*, washed with a buffer containing 10 mM HEPES and 100 mM NaCl at pH 7.6, and flash-frozen in liquid nitrogen. Cells were stored at −80°C.

Purification of TRI101/TRI201 proteins. All purification steps were carried out on ice or at 4°C. Seven grams of transformed *E. coli* cells were resuspended in 50 ml lysis buffer [20 mM HEPES, 50 mM NaCl, 20 mM imidazole, 0.2 mM tris(2-carboxyethyl)phosphine (TCEP), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 25 mg lysozyme at pH 7.6]. Cells were lysed with 5 pulses of 20 to 40 s using a Misonix XL2015 sonicator. The NaCl concentration was brought to 300 mM by the addition of 4 M NaCl, and the lysate was clarified by centrifugation at 40,000 rpm in a Beckman 45 Ti rotor for 30 min. The supernatant was loaded onto a 4-ml column of nickel-nitrilotriacetic acid-agarose (Ni-NTA) equilibrated with buffer A (50 mM potassium phosphate, 300 mM NaCl, 20 mM imidazole, 0.2 mM TCEP at pH 7.8). After washing with 80 ml buffer A and 40 ml buffer A containing 40 mM imidazole, the protein was eluted in a linear gradient of 40 to 300 mM imidazole in buffer A. Fractions containing the TRI101/TRI201 protein were identified by SDS-PAGE and Coomassie blue staining. Tobacco etch virus (TEV) protease, purified by following the protocol of Blommel and Fox (5), was added at approximately a 1:40 molar ratio to remove the His_6 tag, and the fractions were dialyzed overnight against buffer B (10 mM HEPES, 100 mM NaCl, 0.2 mM TCEP). The NaCl and imidazole concentrations were brought to 300 mM and 20 mM, respectively, and the solution was passed over a 2.5-ml Ni-NTA column equilibrated with buffer A. The flowthrough was collected; the column removed the TEV protease and undigested TRI101/TRI201 enzyme. The resultant protein was concentrated using a stirred-cell pressure concentrator (Amicon) fit with a 30,000 molecular-weight-cutoff membrane (Millipore) and dialyzed against storage buffer (10 mM HEPES, 100 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol [DTT] at pH 7.6). Protein was drop-frozen in liquid nitrogen and stored at −80°C.

TRI101/TRI201 enzymatic assay. Enzymatic assays were performed as described in reference 10. Briefly, the acetyltransferase reaction was monitored by following the production of CoA in a 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) coupled continuous assay (8). Reactions were carried out at 25°C in a cuvette containing 100 mM potassium phosphate at pH 8.0, 1.5 mM acetyl-CoA (Sigma), 0.6 mM DTNB (Sigma), 200 μg/ml bovine serum albumin (BSA), 34 ng/ml recombinant TRI101/TRI201 enzyme, and various concentrations of DON in 4.5% dimethyl sulfoxide (DMSO). The change in absorbance at 412 nm was followed, and data were fit by nonlinear regression to the Michaelis-Menton equation.

Circular dichroism. Thermal melting curves of the purified, recombinant TRI101/TRI201 proteins were generated using circular dichroism spectroscopy. The TRI101/TRI201 proteins were diluted to 0.2 mg/ml in a degassed buffer

containing 100 mM potassium phosphate at pH 8, and spectra were recorded from 190 to 260 nm in a model 202SF circular dichroism spectrophotometer (Aviv Biomedical). The temperature was increased in 2.5-degree increments from 10°C to 60°C. The melting point (T_m) was determined from the change in molar ellipticity at 222 nm, which resulted in a sigmoidal curve that was fit to a modified version of the van't Hoff equation (28).

TRI101/TRI201 Western blot analysis. Protein extraction for each yeast transformant was conducted based on the method of Kushnirov (17). Yeast was grown in 5 ml of liquid drop-out medium containing the appropriate amino acids for selection. The liquid medium was inoculated with transformed yeast cells from a plate and grown at 28°C overnight in a shaking incubator until an optical density (OD_{600}) greater than 2.5 was reached. A volume of liquid culture necessary to achieve an OD_{600} of 2.5 in a 5-ml volume was removed and spun down in a 1.5-ml centrifuge tube. The pellet was resuspended in 100 μl of distilled H_2O, and then 100 μl of 0.2 M NaOH was added to the reaction mixture and held at room temperature for 5 min. Following room temperature incubation, the yeast cells were centrifuged and resuspended in 100 μl of SDS sample buffer (17) and boiled for 3 min. Ten microliters of supernatant was loaded onto a 12% acrylamide–SDS-PAGE gel and run at 150 V for 1 h. Precision plus protein dual color (Bio-Rad, Hercules, CA) standard was used to determine protein size. Following separation, protein transfer to a nitrocellulose membrane (Bio-Rad) was conducted in a transfer chamber at 55 mA for 1 h at room temperature. The transfer buffer was composed of 25 mM Tris, 190 mM glycine, 2% SDS, and 20% liquid chromatography-mass spectrometry-grade methanol. The membrane was then blocked in 7% nonfat dry milk in TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The membrane was incubated with rabbit anti-FsTri101 primary antibody for 1 h in 7% milk TBST (1:5,000). After incubation with the primary antibody, the membrane was washed with 7% milk TBST three times for 15 min each time. The membrane was incubated with secondary antibody (alkaline phosphatase-conjugated anti-rabbit) for 1 h in 7% milk TBST solution (1:10,000). The membrane was washed in TBST three times for 15 min each time and then washed in TBS (no Tween 20) once for 15 min. The membrane was incubated with Lumi-Phos WB substrate (Fisher Scientific, Pittsburgh, PA) at a volume of 0.125 ml for every square centimeter of membrane for 3 min. X-ray film was exposed to the membrane for 1 min and developed. Protein levels were quantified using the freeware software program ImageJ version 1.43u. The band intensities for known concentrations of FsTR101 and FfTRI201 purified from *E. coli* were scored in ImageJ and used to generate standard curves to calculate protein levels for the unknown TRI101 and TRI201 proteins from seven *Fusarium* spp. A correlation analysis was conducted using PROC CORR in SAS (version 9.2; SAS Institute, Cary, NC) to determine the relationships between (i) predicted protein concentrations based on standard curves generated using FsTRI101 or FfTRI201 and (ii) percent conversion of DON to 3ADON at 8 or 24 h and predicted protein concentrations based on standard curves generated using FsTRI101 or FfTRI201.

RESULTS

Presence of *TRI101*/*TRI201* in at least 31 species of *Fusarium*. An internal region of *TRI101*/*TRI201* was amplified from 33 *Fusarium* strains from the Leslie collection (see Table S2 in the supplemental material). Together with the sequence from an additional four strains from the Schmale collection and 17 sequences from GenBank (see Table S1 in the supplemental material), a phylogenetic tree was constructed in MegAlign using ClustalW (Lasergene version 8.1.1; DNAStar, Madison, WI) (Fig. 1), based on a total of 688 bp of sequence internal to the 3′ end of *TRI101*/*TRI201*. The tree shows two large clusters that consist mostly of trichothecene producers (top cluster) and nonproducers (bottom cluster) (Fig. 1). The large top cluster (*F. graminearum*, *F. culmorum*, etc.) contains portions of characterized and uncharacterized *TRI101* genes, and the large bottom cluster (*F. oxysporum*, *F. fujikuroi*, etc.) contains portions of characterized and uncharacterized *TRI201* genes.

TRI12 enhances the conversion of DON to 3ADON. In the absence of FsTRI12, our transformed yeasts expressing FgTRI101 alone were extremely limited in their ability to acetylate DON, converting only 4.7% ± 0.5% (mean ± standard deviation) of DON to 3ADON in 24 h. Yeast transformed with *FsTRI12* alone converted 7.4% ± 0.7% of DON to 3ADON in 24 h, which was greater than both the untransformed control and *Fg1TRI101*-transformed yeast, most likely due to the existence of the endogenous 3-*O*-acetyltranferase encoded by *S. cerevisiae AYT1* (4). In transformed yeast carrying both *Fg1TRI101* and *FsTRI12*, the conversion of DON to 3ADON was 66% in 24 h.

Comparisons of sequence identity. Eleven full-length *TRI101*/*TRI201* sequences from seven species of *Fusarium* from the Leslie and Schmale collections were compared for sequence identity (Table 2). The seven selected species were placed into two groups: trichothecene producers (*F. graminearum*, *F. sporotrichioides*, *F. crookwellense*, *F. culmorum*, and *F. pseudograminearum*) and trichothecene nonproducers (*F. fujikuroi* and *F. oxysporum*) as described by Desjardins (6). Pairwise comparisons of genetic identity between full-length *TRI101*/*TRI201* DNA sequences from the seven *Fusarium* species ranged from 66% to 98% (Table 2). The trichothecene nonproducers had sequence similarity to each other but lower levels of identity to trichothecene producers (Table 2).

Time course yeast feeding assays. A time course assay was conducted using seven *TRI101*/*TRI201* genes from the Schmale and Leslie collections (see Table S3 in the supplemental material) expressed in yeast to illustrate rate differences of DON acetylation across a 30-h period (Table 3). Trichothecene extractions were conducted over six different time points: 0 h, 4 h, 8 h, 16 h, 24 h, and 30 h. At a concentration of 10 ppm, DON was acetylated at various rates by each protein encoded by a *TRI101*/*TRI201* gene. Ff1TRI201 reached 50% and 90% DON acetylation at 10.3 h and 15.1 h, respectively. During the first 8 h of our assay, Ff1TRI201 had the highest level of acetylation, converting an average of 30.8% ± 2.2% of DON to 3ADON at a rate of 3.6% per hour. At 16 h, Fcu2TRI101 and Ff1TRI201 had relatively the same conversion percentage, at 97.4% ± 0.7% and 97.5% ± 1.1%, respectively; however, Fcu2TRI101 had the highest conversion rate from 8 to 16 h, converting 9.3% DON per hour. Within 24 h, Ff1TRI201, Fcr2TRI101, and Fcu2TRI101 reached complete conversion of DON to 3ADON. Fo1TRI201 was the fourth best converter of DON to 3ADON, reaching complete conversion in 30 h. During the times tested, Fs1TRI101 converted the smallest amount of DON overall, converting only 50.5%. Unexpectedly, Fg1TRI101 and Fps2TRI101 showed only intermediate abilities at acetylating DON, converting 92.6% and 81.5% in 30 h, respectively. Pairwise comparisons using the Tukey-Kramer honestly significant difference test between TRI101/TRI201 of each species showed that Ff1TRI201, Fcu2TRI101, Fcr2TRI101, and Fo1TRI201 were not significantly different at the 30-h time point ($P = 0.05$).

Steady-state kinetic analysis. Steady-state kinetic analyses were conducted in order to compare *in vitro* enzymatic differences among the seven TRI101/TRI201 proteins (Table 4). A chemically coupled assay was used to follow the rate of CoA production generated by acetyl transfer to the DON substrate. TRI101 from *F. pseudograminearum*, *F. graminearum*, *F. crookwellense*, and *F. culmorum* (all are trichothecene producers) had similar catalytic efficiencies for DON acetylation. Fps2TRI101 had the largest turnover number, 195 s^{-1}. TRI201 from *F. fujikuroi* (a trichothecene nonproducer) had an inter-

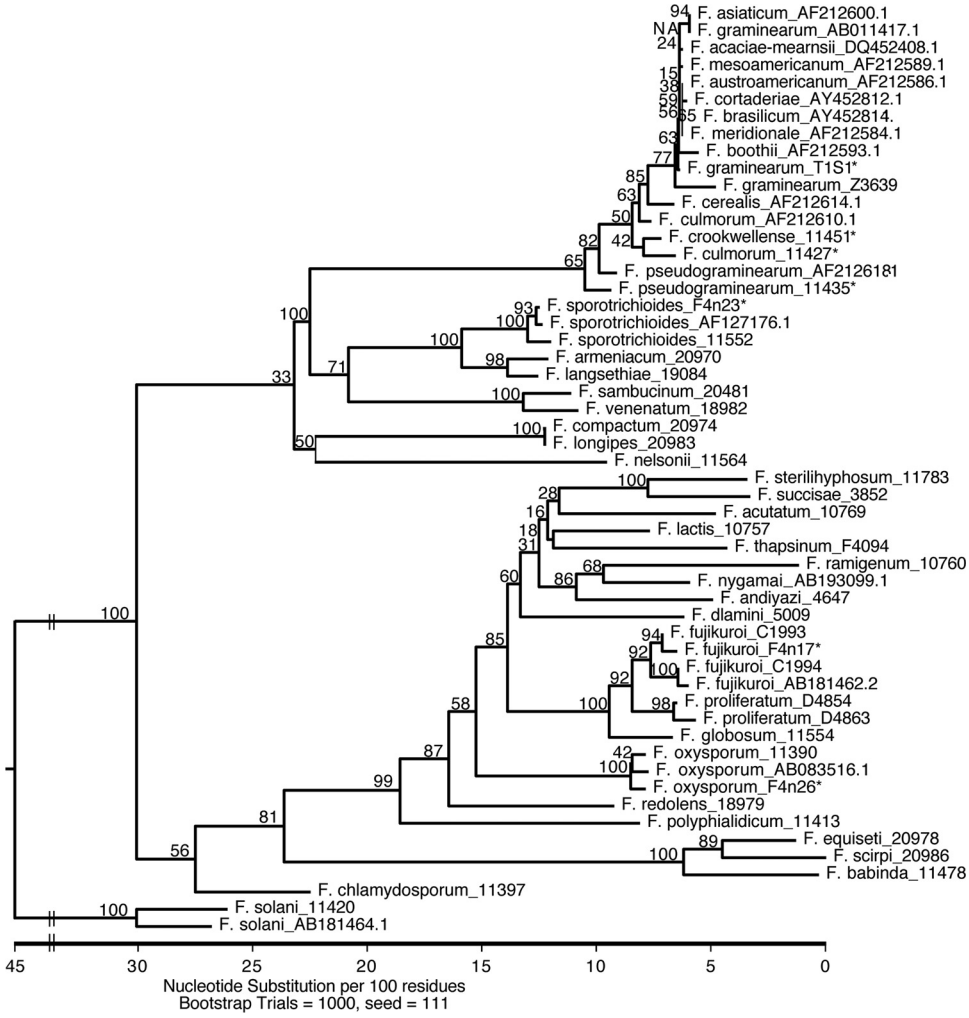

FIG. 1. Phylogenetic tree based on a total of 688 bp of sequence internal to the 3′ end of *TRI101*/*TRI201*. Sequences included in the tree are from a total of 54 strains of *Fusarium*: 33 strains from the Leslie collection (see Table S2 in the supplemental material), four strains from the Schmale collection, and 17 strains from GenBank (see Table S1 in the supplemental material). Strain numbers or GenBank accession numbers follow the underscores. *, species whose full-length *TRI101*/*TRI201* gene products were analyzed in this study for their ability to detoxify DON. Numbers at branch points represent bootstrap values.

mediate catalytic efficiency of $4.8 \times 10^5\ M^{-1} \cdot s^{-1}$, with a lower turnover number and higher K_m. TRI201 from *F. oxysporum* (a trichothecene nonproducer) had both the lowest k_{cat}/K_m value ($6.8 \times 10^4\ M^{-1} \cdot s^{-1}$) and the lowest turnover number ($34\ s^{-1}$) for DON acetylation of the seven orthologs tested. TRI101 from *F. sporotrichioides* (a T-2 toxin trichothecene producer) had a $K_{m\ of}$ 550 ± 70, suggesting a low affinity for DON.

Thermostability measurements. Thermal melting curves were generated by circular dichroism spectroscopy to assess differences in thermostability among the TRI101/TRI201 proteins (Table 4). FsTRI101 had the highest T_m, 43.3°C, while Ff1TRI201 had the lowest T_m, 37.1°C. Fps2TRI101, which had the highest catalytic efficiency for DON acetylation, had a T_m of 37.8°C, indicating that the differences in T_m did not correlate with measured kinetic parameters.

TRI101/TRI201 Western blot analysis. A Western blot assay was performed to detect and quantify the TRI101/TRI201 protein produced by each yeast transformant at 24 h (Fig. 2) and at 12 h (data not shown) to resolve whether acetylation rates may have been influenced by the amount of protein produced. The predicted protein concentrations based on a standard curve using FsTRI101 were the following: 16.5 ng/μl of Fps2TRI101, 21.2 ng/μl of Fcr2TRI101, 26.9 ng/μl of Fg1TRI101, 27.4 ng/μl of Fo1TRI201, 34.4 ng/μl of Fcu2TRI101, 45.1 ng/μl of Fs1TRI101, and 65.3 ng/μl of Ff1TRI201. Predicted protein concentrations based on a standard curve using Ff1TRI201 were the following: 18.8 ng/μl of Fps2TRI101, 24.1 ng/μl of Fcr2TRI101, 30.6 ng/μl of Fg1TRI101, 31.2 ng/μl of Fo1TRI201, 39.2 ng/μl of Fcu2TRI101, 51.3 ng/μl of Fs1TRI101, and 74.3 ng/μl of Ff2TRI201. The relative band intensities did not vary between the 12- and 24-h time points (data not shown). Yeast strain RW2802 produced Ff1TRI201 at the highest levels, and this enzyme also had the highest rate of acetylation (Fig. 2 and Table 3). The Fps2TRI101 enzyme was produced in the smallest amount and had the second lowest rate of DON

TABLE 2. Pairwise comparisons between full-length *TRI101* and *TRI201* DNA sequences evaluated in the present study[a]

Gene and strain	% Identity or divergence of:										
	Fg1*TRI101* (+)	Fg2*TRI101* (+)	Fcr2*TRI101* (+)	Fcu2*TRI101* (+)	Fps2*TRI101* (+)	Fs1*TRI101* (+)	Fs2*TRI101* (+)	Ff1*TRI201* (−)	Ff2*TRI201* (−)	Fo1*TRI201* (−)	Fo2*TRI201* (−)
Fg1*TRI101* (+)		99.9	97.7	97.0	95.9	80.2	80.3	65.6	65.8	67.6	67.3
Fg2*TRI101* (+)	0.1		97.6	96.9	95.8	80.1	80.3	65.5	65.7	67.5	67.2
Fcr2*TRI101* (+)	2.3	2.4		97.7	96.5	80.4	80.6	65.5	65.6	67.5	67.3
Fcu2*TRI101* (+)	3.1	3.2	2.3		96.7	80.7	81.7	65.9	67.0	67.6	67.3
Fps2*TRI101* (+)	4.3	4.3	3.6	3.4		81.7	81.7	67.0	67.0	68.1	67.8
Fs1*TRI101* (+)	23.3	23.4	23.0	22.6	21.2		99.6	69.6	69.7	69.7	69.3
Fs2*TRI101* (+)	23.1	23.2	22.8	22.6	21.2	0.4		69.5	69.6	69.6	69.4
Ff1*TRI201* (−)	46.8	47.0	47.2	46.3	44.2	39.8	39.9		99.3	86.2	86.0
Ff2*TRI201* (−)	46.5	46.7	46.9	45.9	44.1	39.6	39.8	0.7		86.1	85.8
Fo1*TRI201* (−)	46.8	43.1	43.2	42.9	42.1	39.8	39.7	15.5	15.6		98.8
Fo2*TRI201* (−)	43.5	43.6	43.5	43.3	42.5	40.1	40.0	15.6	15.9	1.2	

[a] Percent identity is above the diagonal, and percent divergence is below the diagonal. Strain abbreviations: 1, strain from Schmale collection; 2, strain from Leslie collection; Fg, *F. graminearum*; Fcr, *F. crookwellense*; Fcu, *F. culmorum*; Fps, *F. pseudograminearum*; Fs, *F. sporotrichioides*; Ff, *F. fujikuroi*; Fo, *F. oxysporum*; +, trichothecene producer; −, trichothecene nonproducer.

acetylation in our yeast assay (Fig. 2 and Table 3). A Western blot analysis of four dilutions (1:1, 1:2, 1:4, and 1:16) of Fg1TRI101 and Ff1TRI201 did not reveal variations in the binding affinity of the rabbit anti-FsTri101 primary antibody (data not shown). There was perfect correlation between predicted protein concentrations based on the standard curves generated using Fs1TRI101 and Ff1TRI201 ($r^2 = 1.0$, $P < 0.0001$). The percent conversion of DON to 3ADON at 8 h and predicted protein concentrations based on the standard curves generated using Fs1TRI101 and Ff1TRI201 were weakly correlated ($r^2 = 0.63$, $P = 0.13$ for both Fs and Ff). The percent conversion of DON to 3ADON at 24 h and predicted protein concentrations based on the standard curves generated using Fs1TRI101 and Ff1TRI201 were not correlated ($r^2 = 0.02$, $P = 0.96$ for both Fs and Ff).

DISCUSSION

In principle, there are two ways in which a trichothecene 3-*O*-acetyltransferase might be improved to facilitate detoxification of trichothecenes *in planta*. One way would be to genetically engineer the enzyme to enhance its kinetic properties and bioavailability. Given the structural and functional knowledge for TRI101, this is a reasonable approach, but current methods for predicting functional changes induced by site-directed mutagenesis are still not entirely reliable. Furthermore, given the time and effort needed for each new version of the enzyme, it is not possible to create all combinations that might yield a superior enzyme. Bioprospecting offers an alternative approach to isolating better enzymes that have evolved through natural selection. In our study, *TRI101*/*TRI201* sequence differences were observed within and among different species of *Fusarium*, illustrating the potential for utilizing natural variation to find an improved enzyme (Table 2). Years of selection and a wide range of environmental conditions are expected to yield a number of TRI101/TRI201 isoforms, some of which might be expected to be better than either FgTRI101 or FsTRI101. The main challenge with this approach is to select the most likely candidates for detailed study. Here, we have used the known structures and kinetic characteristics of FgTRI101 and FsTRI101 to identify alternative promising isoforms with the future goal of determining the parameters that control the specificity and stability of the enzyme.

Our study confirmed that TRI101/TRI201 enzymes from fusaria classified as producers and nonproducers of trichothecenes were able to acetylate DON; all seven enzymes tested,

TABLE 3. Percentage of DON converted to 3ADON for each time point for each combination of *TRI101* or *TRI201* with *TRI12* and respective time to reach 50% and 90% DON conversion[a]

Gene combination	% DON converted to 3ADON (mean ± SD) at indicated time point (h)						Time (h) to reach indicated % conversion	
	0	4	8	16	24	30	50	90
Ff1TRI201/*FsTRI12*	1.7 ± 0.2	7.2 ± 0.8	30.8 ± 2.2	97.5 ± 1.1	100.0 ± 0.0	99.9 ± 0.2	10.3	15.1
Fcu2TRI101/*FsTRI12*	1.6 ± 0.1	3.7 ± 0.4	22.7 ± 0.7	97.4 ± 0.7	99.9 ± 0.2	99.8 ± 0.3	10.9	15.2
Fcr2TRI101/*FsTRI12*	2.3 ± 0.3	3.8 ± 0.8	19.7 ± 1.7	88.2 ± 3.6	99.0 ± 0.6	99.7 ± 0.4	11.5	17.3
Fo1TRI201/*FsTRI12*	2.7 ± 0.9	2.8 ± 0.6	9.3 ± 1.1	70.7 ± 4.4	97.0 ± 0.8	99.2 ± 0.5	13.3	21.9
Fg1TRI101/*FsTRI12*	1.5 ± 0.3	2.2 ± 0.1	9.1 ± 0.6	70.2 ± 2.7	89.8 ± 3.3	92.6 ± 3.1	13.4	24.4
Fps2TRI101/*FsTRI12*	1.9 ± 0.3	2.1 ± 0.3	3.7 ± 0.8	34.2 ± 3.1	69.1 ± 1.2	81.5 ± 0.4	19.6	ND
Fs1TRI101/*FsTRI12*	1.5 ± 0.2	2.9 ± 0.5	6.5 ± 0.6	21.0 ± 1.0	39.0 ± 1.4	50.5 ± 1.0	29.7	ND
RW2802, untransformed	1.7 ± 0.2	2.4 ± 0.6	1.9 ± 0.4	1.1 ± 0.2	1.7 ± 0.6	2.4 ± 0.7	ND	ND

[a] The study was conducted for 30 h, and cultures were exposed to 10 ppm DON at the time of induction. Results are based on four replications. TRI101 from *F. graminearum* (Fg), *F. sporotrichioides* (Fs), *F. crookwellense* (Fcr), *F. culmorum* (Fcu), and *F. pseudograminearum* (Fps) and TRI201 from *F. fujikuroi* (Ff) and *F. oxysporum* (Fo) were analyzed in this study; 1 and 2 indicate strains from the Schmale or Leslie collection, respectively. ND, not determined.

TABLE 4. Kinetic constant K_m and k_{cat} values and T_m values for TRI101/TRI201 enzymes[a]

TRI101/TRI201 variant	K_m (μM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	T_m (°C)
Fps2TRI101	41 ± 2	195 ± 5	4.7×10^6	37.8
Fg1TRI101	24 ± 2	106 ± 3	4.4×10^6	39.2
Fcr2TRI101	37 ± 2	153 ± 4	4.2×10^6	38.8
Fcu2TRI101	36 ± 2	140 ± 4	3.9×10^6	41.9
Ff1TRI201	170 ± 15	84 ± 3	4.8×10^5	37.1
Fs1TRI101	550 ± 70	73 ± 4	1.3×10^5	43.2
Fo1TRI201	510 ± 90	34 ± 3	6.8×10^4	38.2

[a] Results are listed in order of decreasing k_{cat}/K_m ratio. Species abbreviations: Fs, *F. sporotrichioides*; Fg, *F. graminearum*; Fo, *F. oxysporum*; Ff, *F. fujikuroi*; Fcr, *F. crookwellense*; Fcu, *F. culmorum*; Fps, *F. pseudograminearum*; 1, obtained from the Schmale collection; 2, obtained from the Leslie collection.

two of which were isolated from trichothecene nonproducers, converted DON to 3ADON. Since TRI101 from *F. graminearum* has been shown to have a high relative affinity for DON (10), we hypothesized (i) that TRI101 from *F. sporotrichioides* (a producer of T-2 toxin) would have an intermediate ability to acetylate DON and (ii) that TRI201 from *F. oxysporum* and *F. fujikuroi* (nonproducers of DON) would have lesser abilities (if any) to acetylate DON. We found that FsTRI101 acetylated DON more slowly than FgTRI101; however, we were surprised to find that the TRI201s from the two trichothecene-nonproducing species were highly capable of acetylating DON. This led to the comparison of DNA sequences among the tested strains. Pairwise comparisons of TRI101/TRI201 DNA sequences between trichothecene nonproducers (*F. fujikuroi* and *F. oxysporum*) and producers (*F. graminearum*) showed identities of about 67%. In contrast, sequence identities between trichothecene producers (e.g., *F. graminearum* and *F. sporotrichioides*) were 80% and higher.

C-3 acetyltransferase activity can be detected in trichothecene-nonproducing species and is encoded by *TRI201* (16). The trichothecene-nonproducing strains examined in this study have fully functional TRI201s, suggesting that certain nonproducing strains have maintained trichothecene acetyltransferase function. The fact that TRI201 from the trichothecene nonproducer *F. fujikuroi* was demonstrated to have higher acetylation efficiency than TRI101 from the trichothecene producers was unexpected. The demonstration of TRI201 functional activity in our assays begs the question as to why nonproducers have a nonfunctional TRI101 and yet an active TRI201. *TRI201* is believed to be the result of gene duplication (31). Based on our DNA sequence analysis, *TRI201* genes from the nonproducers and *FgTRI101* genes from the trichothecene producers are the most divergent. There may be selective pressures (9, 30) yet to be studied that act to maintain a functional *TRI201* in trichothecene nonproducers. Perhaps TRI201 functions as an antibiotic resistance gene (31) or plays an ecological role in fungi that coexist with trichothecene producers. It is also interesting to note that when *TRI101* is inactivated in trichothecene producers by gene disruption, there is no C-3 acetyltransferase activity (19), suggesting that there is no active *TRI201* gene in trichothecene producers.

TRI101/TRI201 amino acid sequence identity in all seven species examined is considerably higher in the cofactor and substrate binding regions than in other regions, with all seven species maintaining the conserved HXXXMDXG and DFGXG structural motifs that are important for catalytic activity (10). There is also divergence in the sequences from the nonproducers and producers occurring in the flexible surface loop that is between Pro^{215} and Pro^{226} in FgTRI101 (see Fig. S1 in the supplemental material). The influence of these residues on enzyme activity is uncertain, but the trichothecene nonproducers FoTRI201 and FfTRI201 contain four proline residues in this region. Interestingly, these enzymes show the lowest catalytic efficiency of the orthologs tested.

The difficulty lies in explaining why the products of *TRI201* genes isolated from the trichothecene nonproducers *F. oxysporum* and *F. fujikuroi* converted DON to 3ADON at levels similar to the levels of conversion of the products of *TRI101* genes from the trichothecene producers, such as *F. culmorum*, by the end of our 30-h assay but at different rates. Western blot analysis showed that TRI101/TRI201 enzyme levels might play a role in affecting the levels of DON conversion to 3ADON. The percent conversion of DON to 3ADON in our yeast assay at 8 h was weakly correlated with predicted protein concentrations for all of the TRI101/TRI201 enzymes tested. Thus, small variations (less than 4-fold differences in concentrations) in enzyme levels may explain some of the observed variation in acetylation activity at early time points (<12 h). However, at 24 h there was no correlation between the percent conversion of DON to 3ADON and the predicted protein concentrations based on standard curves, perhaps because most of the reactions were close to completion. Our kinetic measurements indicated lowered levels of catalytic efficiency for TRI201 enzymes and for TRI101 from *F. sporotrichioides*. This might explain in part the low rate of conversion of DON to 3ADON for TRI101 from *F. sporotrichioides*, but it does not resolve the ability of TRI201 from *F. fujikuroi* to have one of the highest conversion rates of DON to 3ADON in 24 h. Together, our data suggest that both enzyme levels and kinetics may contribute to conversion rates of DON to 3ADON, at least during the time course experiments conducted as part of this work.

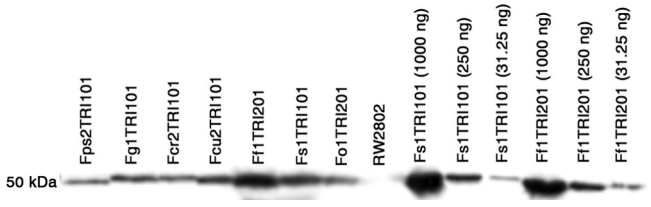

FIG. 2. Western blot of TRI101/TRI201 enzymes from *F. pseudograminearum* (Fps2TRI101), *F. graminearum* (Fg1TRI101), *F. crookwellense* (Fcr2TRI101), *F. culmorum* (Fcu2TRI201), *F. fujikuroi* (Ff1TRI201), *F. sporotrichioides* (Fs1TRI101), and *F. oxysporum* (Fo1TRI201) (1 and 2 indicate that the strain is from the Schmale or Leslie collection, respectively). Protein extracts from yeast cultures were collected following 24 h of expression. Untransformed yeast strain RW2802 represents the negative control. Ten microliters of each protein extract was loaded onto a 12% acrylamide–SDS-PAGE gel and run at 150 V for 1 h. Precision plus protein dual color standard was used to determine protein size. Ten microliters of TRI101 from *F. sporotrichioides* and TRI201 from *F. fujikuroi*, both purified from *E. coli*, were loaded as positive controls at known amounts of 1,000 ng, 250 ng, and 31.25 ng. The Western blot was probed with rabbit anti-FsTRI101 primary antibody and detected with alkaline phosphatase-conjugated anti-rabbit antibody.

The exploitation of TRI101/TRI201 activity may be helpful in the production of fuel ethanol coproducts with reduced levels of DON. At present, corn is the predominant grain used in the United States for the fermentation of starch to fuel ethanol by yeast. The dried residue remaining is a nutrient-rich feed for domestic animals, called dried distillers grains with solubles (DDGS) (13). If the demand for fuel ethanol increases, wheat and barley may be used as a fermentation source (12). Any DON-contaminated grains may also be toxic to yeast used for the bioconversion process to ethanol. However, *S. cerevisiae* is known to carry the gene *AYT1*, which can acetylate the C-3 position of trichothecenes (4). The addition of appropriate *TRI101*/*TRI201* genes to fermentation yeast might increase the acetylation activity and allow greater ethanol conversion in contaminated grains. The engineering of either yeast or grains to contain more efficient TRI101s/TRI201s may also lower the amount of residual DON in the DDGS.

This work has important implications for the use of TRI101/TRI201 to combat FHB. For example, a transgenic plant expressing *FsTRI101*, which has a lower rate of activity against DON, might not be able to reduce DON levels faster than the invading fungus is releasing the mycotoxin, thus causing greater disease. Inefficient enzymes would likely, in time, convert most DON to 3ADON; however, given that the C-3 acetyl in plants may be unstable, a high rate of acetylation would be preferred *in planta* (32). Putting into plants an engineered *TRI101*/*TRI201* that is kinetically geared toward DON acetylation so that rapid modification occurs as the fungus invades plant tissue might be successful in limiting FHB. An unknown in all this, however, is the effects of plant or *Fusarium* esterases that may remove the C-3 acetyl group as soon as a TRI101/TRI201 acetylates the trichothecene.

Our work confirmed the value of FsTRI12 to enhance the conversion of DON. If the influx of mycotoxin into yeast cells is through passive diffusion, then the expression of an efflux pump could raise trichothecene flux into yeast cells, thereby increasing the acetylation of DON (2). In our feeding studies, in the absence of *FsTRI12*, transformed yeasts with *FgTRI101* alone demonstrated little to no ability to acetylate DON. In yeast transformed with *FsTRI12* alone, the acetylation activity was greater than that in both the untransformed control and FgTRI101-transformed yeast. This is likely due to the FsTRI12 enzyme working together with the native 3-*O*-acetyltransferase AYT1 that is present in yeast (4). The added benefit of a trichothecene efflux pump should also be factored into the design of engineering wheat and barley to resist FHB in the future.

Most phylogenetic studies, including this one, compare gene sequences of a specific gene in order to deduce a tree that suggests the evolutionary relationships among species. Rarely do they examine the sequence for continuity of the reading frame. When Kimura et al. (16) examined the sequence between *PHO5* and *URA7* in *F. oxysporum*, they found a pseudo-*TRI101* whose coding region was interrupted by 2 stop codons. Although the sequence of the pseudo-*TRI101* was similar to that of *FgTRI101*, the FoTRI101 was nonfunctional. In the same study, the authors found an open reading frame (ORF) for *TRI101* from *Fusarium moniliforme* (later assigned to *F. fujikuroi*), yet no transcriptional product was found, suggesting that no enzyme activity was present. This shows the importance of examining the sequence for an entire ORF for a gene, as well as performing functional studies, because the isolation of a full-length ORF does not indicate that the translated product will be functional. The results from our phylogenetic analysis are consistent with those of Proctor et al. (27). Our tree identified two large clusters that separate many known trichothecene producers (top cluster) from nonproducers (bottom cluster). Interestingly, these large clusters also appear to contain either *TRI101* (top cluster) or *TRI201* (bottom cluster). Future functional work with TRI101 and TRI201 from fusaria that have not yet been characterized for trichothecene production and acetyltransferase activity may help to clarify the relationships among members within each of these clusters.

Our work demonstrates the tremendous potential for bioprospecting for additional trichothecene acetyltransferase genes in members of *Fusarium* and other fungal genera in the near future. There are other fungi which carry trichothecene acetyltransferase sequences, but it is unknown whether these genes are active. Perhaps a fungus which has to compete in the environment with *Fusarium* has a method to combat trichothecene toxicity. Also, nature may be able to provide clues to better engineer more stable and efficient trichothecene acetyltransferase enzymes in the future or even deliver an improved TRI101/TRI201 to directly combat FHB through the implementation of transgenic plants. However, obtaining full-length sequences of *TRI101*/*TRI201* genes from new and/or understudied *Fusarium* species may be limited by challenges associated with the location and orientation of these genes in the genomes of these species (27). Isolating and screening additional orthologs of *TRI101*/*TRI201* will undoubtedly play an important role in finding more stable and efficient enzymes to modify DON.

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