

Identification of Loci and Functional Characterization of Trichothecene Biosynthesis Genes in Filamentous Fungi of the Genus *Trichoderma*^{∇†}

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Trichothecenes are mycotoxins produced by *Trichoderma*, *Fusarium*, and at least four other genera in the fungal order *Hypocreales*. *Fusarium* has a trichothecene biosynthetic gene (*TRI*) cluster that encodes transport and regulatory proteins as well as most enzymes required for the formation of the mycotoxins. However, little is known about trichothecene biosynthesis in the other genera. Here, we identify and characterize *TRI* gene orthologues (*tri*) in *Trichoderma arundinaceum* and *Trichoderma brevicompactum*. Our results indicate that both *Trichoderma* species have a *tri* cluster that consists of orthologues of seven genes present in the *Fusarium TRI* cluster. Organization of genes in the cluster is the same in the two *Trichoderma* species but differs from the organization in *Fusarium*. Sequence and functional analysis revealed that the gene (*tri5*) responsible for the first committed step in trichothecene biosynthesis is located outside the cluster in both *Trichoderma* species rather than inside the cluster as it is in *Fusarium*. Heterologous expression analysis revealed that two *T. arundinaceum* cluster genes (*tri4* and *tri11*) differ in function from their *Fusarium* orthologues. The *Ttri4*-encoded enzyme catalyzes only three of the four oxygenation reactions catalyzed by the orthologous enzyme in *Fusarium*. The *Ttri11*-encoded enzyme catalyzes a completely different reaction (trichothecene C-4 hydroxylation) than the *Fusarium* orthologue (trichothecene C-15 hydroxylation). The results of this study indicate that although some characteristics of the *tri/TRI* cluster have been conserved during evolution of *Trichoderma* and *Fusarium*, the cluster has undergone marked changes, including gene loss and/or gain, gene rearrangement, and divergence of gene function.

Trichothecenes are a group of over 200 sesquiterpenoid-derived secondary metabolites that vary by the pattern of oxygenations and esterifications of a core tricyclic structure with an epoxide function. These metabolites are produced by species in at least six genera of the fungal order *Hypocreales* (class *Sordariomycetes*): *Fusarium*, *Myrothecium*, *Spicellum*, *Stachybotrys*, *Trichoderma*, and *Trichothecium*. Trichothecenes are considered mycotoxins because they are often found as contaminants in food and animal feed and they can induce vomiting, alimentary hemorrhaging, and dermatitis in humans and livestock. These symptoms most likely result from the ability of trichothecenes to inhibit protein synthesis (40) and/or to induce apoptosis in eukaryotic cells (45). Trichothecenes also can act as immunosuppressors (51) and neurotoxins (36). Trichothecenes are phytotoxic (17). Previously (56), we observed that overproduction of trichodermin in *Trichoderma brevicompactum* reduced tomato seed germination and plant growth while increasing the size of the lesions produced when the

pathogen was artificially inoculated on tomato plants previously colonized by *Trichoderma* spp.

The biosynthesis of the *Fusarium* trichothecenes T-2 toxin (T-2), nivalenol (NIV), and deoxynivalenol (DON) has been subjected to extensive biochemical and genetic analyses (reviewed in references 4 and 29). These studies have elucidated a complex biosynthetic pathway that begins with the cyclization of farnesyl pyrophosphate (FPP) to form trichodiene, which then undergoes a series of oxygenation, isomerization, cyclization, and esterification reactions to form T-2 toxin, nivalenol, or deoxynivalenol. Most of the *Fusarium* genes directly involved in synthesis of the toxins are positioned at a locus designated the core trichothecene biosynthetic gene (*TRI*) cluster (10). In the two most thoroughly examined species, *Fusarium graminearum* and *Fusarium sporotrichioides*, the cluster can include seven genes (*TRI8*, *TRI7*, *TRI3*, *TRI4*, *TRI5*, *TRI11*, *TRI13*) encoding enzymes that catalyze 10 trichothecene biosynthetic reactions, two genes (*TRI6* and *TRI10*) encoding transcriptional regulators, one gene (*TRI12*) encoding a transport protein, and two genes (*TRI9* and *TRI14*) with uncertain functions. Some *Fusarium* spp. have two additional *TRI* loci located on chromosomes other than the one on which the core cluster is located (15, 33). One locus is a cluster that contains two genes (*TRI1* and *TRI16*), which encode biosynthetic enzymes, and the other locus contains a single gene (*TRI101*) that also encodes a biosynthetic enzyme. In some

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Fusarium species, however, *TRI1* and *TRI101* are located in the core *TRI* cluster (49). *Myrothecium roridum* has a 40-kb cluster that includes orthologs of *TRI4*, *TRI5*, and *TRI6* (57). Whether other *TRI* orthologs are present in this cluster is not known.

The genus *Trichoderma* includes species that have been evaluated extensively as biological control agents (21, 41). Strains of *Trichoderma* can produce extracellular enzymes and antibiotics but also may compete with fungal pathogens for space and nutrients through rhizosphere competence (26). Some species of *Trichoderma* can produce the trichothecenes trichodermin and harzianum A (HA). These metabolites are similar to the *Fusarium* trichothecenes T-2 toxin, NIV, and DON in that they have the core tricyclic 12,13-epoxytrichothec-9-ene (EPT) structure. They are also similar to T-2 and NIV in that they have an oxygen atom attached to carbon atom 4 (C-4). Although their structures are well characterized, biosynthesis of HA and trichodermin by *Trichoderma* has been subjected to little genetic analysis. However, orthologues of *TRI5* have been identified in a trichodermin-producing strain (IBT 40841) of *Trichoderma brevicompactum* (56) and a harzianum A-producing strain (ATCC 90237) of *Trichoderma arundinaceum* (16, 19). The identification of the *TRI5* orthologues in the two *Trichoderma* species indicates that the first committed reactions in trichothecene biosynthesis in *Fusarium* and *Trichoderma* are the same (16). However, the absence of the C-3, C-8, and C-15 oxygen atoms in trichodermin and HA indicates that there are multiple differences in trichothecene biosynthesis in the two genera. These differences probably are reflected in the presence/absence/divergence of functional orthologs of some trichothecene biosynthetic genes in the two genera. For example, in *Fusarium*, the *TRI4*-encoded cytochrome P450 monooxygenase catalyzes oxygenation of trichodiene at four carbons, C-2, C-3, C-11, and C-13 (38). In contrast, the *Myrothecium TRI4*-encoded monooxygenase catalyzes oxygenation of trichodiene at only three carbons, C-2, C-11, and C-13 (39). This difference in patterns of oxygenation is consistent with the presence of a C-3 oxygen in *Fusarium* trichothecenes and its absence in *Myrothecium* trichothecenes. The absence of a C-3 oxygen in trichodermin and HA suggests that the *Trichoderma TRI4* orthologue is more similar in function to the *Myrothecium* than the *Fusarium* orthologue. The absence of C-8 and C-15 oxygen atoms in trichodermin and HA also suggests that trichothecene-producing *Trichoderma* strains lack orthologues of the *Fusarium* trichothecene C-8 and C-15 oxygenase genes *TRI1* and *TRI11*, respectively. In contrast, the presence of the C-4 oxygen atom in trichodermin and HA suggests that *T. brevicompactum* and *T. arundinaceum* have an orthologue of the *Fusarium* trichothecene C-4 oxygenase gene *TRI13*.

The objective of the current study was to identify trichothecene biosynthetic genes in *T. arundinaceum* and *T. brevicompactum* and to compare the functions of these genes with those of previously identified orthologs in *Fusarium*. The results of our analyses revealed that both *Trichoderma* species have a trichothecene biosynthetic gene cluster with orthologues of seven genes in the *Fusarium TRI* cluster but that the *TRI5* orthologue is not within this core cluster. Functional analyses demonstrate a key difference in trichothecene biosynthesis among different genera, namely, that the same structural modification of trichothecenes (i.e., C-4 oxygenation) is cata-

TABLE 1. Strains of filamentous fungi used in this study

Species	Strain no. ^a	Alternate strain no.	Trichothecene production ^b	Reference
<i>T. arundinaceum</i>	IBT 40837	<i>Ta37</i>	Harzianum A	44
	ATCC 90237		Harzianum A	14, 16
<i>T. brevicompactum</i>	IBT 40841	<i>Tb41</i>	Trichodermin	44
<i>T. harzianum</i>	CECT 2413		None	22
<i>M. roridum</i>	ATCC 52485		Roridin E	31
<i>F. sporotrichioides</i>	ATCC 24630	NRRL 3510	T-2 toxin	58
<i>F. verticillioides</i>	FRC M-3125	NRRL 20956	None	34

^a IBT, IBT Culture Collection of Fungi, Denmark; ATCC, American Type Culture Collection, United States; CECT, Spanish Type Culture Collection, Spain; NRRL, Agricultural Research Service (ARS) Culture Collection (NRRL), United States; FRC, *Fusarium* Research Center, United States.

^b The metabolites listed are the predominant trichothecenes produced by each strain.

lyzed by nonorthologous enzymes in *Trichoderma* and *Fusarium*.

MATERIALS AND METHODS

Strains, media, and culture conditions. Strains of filamentous fungi used in this work are listed in Table 1.

For HA analysis and RNA isolation, a two-step culture procedure was employed. The media for this procedure were malt broth medium (CM) (5 g/liter malt extract, 5 g/liter yeast extract, 5 g/liter glucose) and modified potato dextrose broth (PDB) (24 g/liter PDB supplemented with 0.1 g/liter ZnSO₄ · 7H₂O and 0.05 g/liter CuSO₄ · 5H₂O) (Difco Becton Dickinson, Sparks, MD) (44). CM medium was inoculated with *T. arundinaceum* conidia at 1 × 10⁷ conidia per ml and then incubated at 28°C in the dark with shaking (250 rpm). After 24 h, 10 ml of the resulting CM culture was transferred to 50 ml of modified PDB medium and incubated at 28°C in the dark with shaking (250 rpm) for 24 to 96 h. Finally, the mycelium was recovered by filtration through a 30-μm-pore-diameter nylon filter (Sefar AG, Heiden, Switzerland), and the supernatant was used for HA purification and quantification. The mycelia were then washed with 0.9% NaCl and dried on absorbent filter paper (Albet-Hahnemuehle, Barcelona, Spain) prior to RNA isolation.

Genetic nomenclature. Trichothecene biosynthetic genes have been characterized most extensively in *Fusarium* species. In these species, the gene names employ three uppercase letters and a number (e.g., *TRI4*, *TRI5*), following conventions of genetic nomenclature developed for *Fusarium* (61). For consistency with conventions of *Trichoderma* genetic nomenclature, we designate trichothecene biosynthetic genes in *Ta37* and *T. brevicompactum* with three lowercase letters and a number (*tri4*, *tri5*). When it is appropriate to distinguish between orthologues from these two *Trichoderma* species, the gene names are preceded by *Ta* (e.g., *TaTri4*, *TaTri5*) for *T. arundinaceum* and *Tb* (e.g., *TbTri4*, *TbTri5*) for *T. brevicompactum*. Protein designations follow a format consistent with the nomenclature for yeast, *Aspergillus*, and multiple plant pathogenic fungi (e.g., *TaTri4*, *TaTri5*) (13).

Nucleic acid manipulation. A genomic library of *T. arundinaceum* strain 37 was constructed in the bacteriophage vector lambda DASH II (Stratagene, La Jolla, CA) by following the manufacturer's instructions.

To optimize the conditions for Southern hybridization, genomic DNAs were hybridized in buffers containing 25, 30, or 40% formamide. Twenty-five-percent formamide was optimal for detecting the *TaTri4* gene signal from a λ genomic library, and a buffer with 40% formamide was optimal for chromosome walking. Filters with bound genomic or phage DNA were prehybridized and hybridized at 42°C for 2 and 12 h, respectively. The filters were then washed twice with 2× saline sodium citrate buffer (SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) and 0.1% sodium dodecyl sulfate (SDS) for 5 min each at room temperature and twice with 0.5× SSC and 0.1% SDS for 15 min each at 65°C.

Probes used in Southern analysis were labeled by using the digoxigenin (DIG) DNA labeling kit (Roche, Mannheim, Germany) by following the manufacturer's instructions. Labeling, hybridization, and immunological detection were carried out with a nonradioactive labeling and immunological detection kit with CDP-Star as the chemiluminescent substrate (Roche, Mannheim, Germany), as previously described (12).

For the isolation of genomic DNA, 1 × 10⁷ spores/ml of the *Ta37* strain were inoculated in 100 ml of CM and incubated for 24 to 48 h at 28°C and 250 rpm. The mycelia were recovered by filtration through a 30-μm-pore-diameter nylon

filter and washed with 0.9% NaCl, and the excess liquid was removed with absorbent filter paper. Finally, the mycelia were lyophilized, and the dried mycelia were used for genomic DNA isolation as previously described (11, 53). To isolate total RNA, the *Ta37* strain was grown using the two-step procedure in CM followed by PDB as described above. Mycelia were recovered from PDB medium by filtration, washed with 0.9% NaCl, and dried on absorbent filter paper. RNA was extracted with the phenol-SDS method (6) and treated with DNase and RNase protector (Fermentas, Vilnius, Lithuania). One microgram of total RNA was used for cDNA synthesis in the reverse transcription (RT) system (Promega, Madison, WI). For the amplification, cDNAs were synthesized as described above and subcloned in the TOPO cloning system (Invitrogen, Carlsbad, CA) by following the manufacturer's instructions. To distinguish gene amplification products from genomic DNA and cDNA, we used primers (see Table S1a in the supplemental material) that span the putative introns in the genomic DNA and should yield amplification products from the mRNA that are smaller than the corresponding regions amplified from genomic DNA. The sizes of the amplified *Tatri* cDNA fragments were as follows: *Tatri5*, 390 bp; *Tatri14*, 494 bp; *Tatri12*, 861 bp; *Tatri10*, 452 bp; *Tatri3*, 798 bp; *Tatri4*, 449 bp with oligonucleotides T44int3/T44int5 or 668 bp with oligonucleotides T437CT/T43E4bNt; and *Tatri11*, 1,426 bp.

For sequence analysis, CLC Protein Workbench 5.4 (CLC bio) and Jalview 2.6.1 (59) programs were used to compare multiple amino acid sequences (Clustal method) and for pairwise protein alignments between two sequences (Lipman-Pearson method). The Lasergene (DNASTAR, Inc., Madison, WI) PROTEAN program was used to analyze structural features of the proteins (Hoop-Woods algorithm) (25). For the assembly of individual sequence fragments into a contig, the Lasergene program SEQMAN was employed, while EDITSEQ was used for basic sequence editing. Comparative analyses involving protein databases were performed with the BLASTX (5) algorithm. Evolutionary distances between the *tri/TRI* genes of the different fungi were determined by using a pairwise distance analysis as implemented in MEGA 4.

***Tatri* gene expression in yeast.** cDNAs of *Tatri4*, *Tatri5*, and *Tatri11* were amplified by RT-PCR (Superscript III one-step RT-PCR with Platinum *Taq* polymerase; Invitrogen, Carlsbad, CA) using primer pairs 2096/2097, 2114/2115, and 2105/2106, respectively (see Table S1b in the supplemental material). Each amplicon was gel purified with the UltraClean DNA purification kit (MoBio, Carlsbad, CA) and then subjected to an incubation at 72°C for 10 min with *Taq* polymerase to introduce 3' A overhangs at each end of the amplicon to facilitate cloning into the yeast expression vector pYES2.1/V5-His-TOPO (*URA3+*) (Invitrogen, Carlsbad, CA). Correct orientation of cDNA inserts in the vector was confirmed by PCR. This procedure yielded an expression plasmid for each of the three genes: pYETri4 for *Tatri4*, pYETri5 for *Tatri5*, and pYETri11 for *Tatri11*. Each plasmid was transformed separately into *Saccharomyces cerevisiae* strain INVSc1 (*MATa his3D1 leu2 trp1-289 ura3-52*) by following the high-efficiency yeast transformation protocol described by Gietz et al (20). Transformation reaction mixtures were plated onto MD (minimal medium plus dextrose) (8) amended with leucine (1 mg/ml), histidine (0.2 mg/ml), and tryptophan (0.2 mg/ml) (MD+LHT) for selection of transformants carrying pYES2.1 plasmids.

***Tatri4* expression in *Fusarium verticillioides*.** The *Ta37 tri4* gene was amplified by PCR using *Pfu* polymerase (Fermentas, Vilnius, Lithuania) with primers Tri437-5 and Tri437-3b (see Table S1b in the supplementary material), using the plasmid pF42E4 as the template, which included the entire 1,791-bp *Tatri4* coding region. The *Tatri4* amplicon was treated with T4 polynucleotide kinase and ligated into plasmid pAN52.1 (50) that had been digested with BamHI and then treated with Klenow fragment and calf intestine alkaline phosphatase (CIAP; Fermentas, Vilnius, Lithuania). The resulting plasmid, pSPT4-1, contained a 4,914-bp *Tatri4* expression cassette that consisted of the *Tatri4* coding region fused to the *Aspergillus nidulans gpdA* gene promoter region and *trpC* gene terminator region. The expression cassette was then isolated by digestion of pSPT4-1 with BglIII and EheI and treated with the Klenow fragment followed by gel purification. The isolated expression cassette was ligated to pAN7-1 (50) that had been digested with HindIII and treated with Klenow and CIAP. Ten micrograms of the resulting plasmid, pSPT4-4, was then transformed into *F. verticillioides* via the protoplast method with selection of transformants on regeneration medium containing 300 µg/ml hygromycin B as previously described (48).

Bioassay with *Kluyveromyces marxianus* GK1005. Fifty milliliters of malt agar medium (MA) (20 g/liter glucose, 20 g/liter malt extract, and 1 g/liter mycopeptone) was inoculated with one single colony of *K. marxianus* GK1005, a yeast strain sensitive to trichothecenes (18, 55), and incubated overnight at 30°C and 250 rpm. Cell density was measured as an optical density at 560 nm (an OD₅₆₀ of 1 corresponds to 1.1 × 10⁹ cells). Bioassay plates were made by adding 2.2 × 10⁸ cells to 100 ml of 1% MA. Sixty microliters of the high-performance liquid chromatography (HPLC) fractions taken each minute was added into holes with

a 5.5-mm diameter and 3.5-mm depth that were formed in the bioassay plates. Plates were maintained at 4°C for 5 h to allow the solution to diffuse and then incubated for 14 h at 30°C to visualize the growth inhibition zone.

Purification and analysis of HA. Two-day-old PDB cultures of the *Ta37* strain (1.5 liters) were filtered through a 30-µm-pore-diameter nylon filter, the resulting supernatant was mixed with one volume of acetone, and the solution was evaporated to the original filtrate volume, or less, with a rotary evaporator at room temperature and 40 rpm. The concentrated solution was extracted three times with equal volumes of ethyl acetate. The extracts were pooled, mixed vigorously with anhydrous Na₂SO₄, and then allowed to stand for 1 h before filtration through filter paper. The filtrate was evaporated to dryness with a rotary evaporator at room temperature, and the residue was resuspended in acetonitrile to a tenth of the original filtrate volume and stored at -30°C until analysis.

A Waters 600 HPLC with a 996 photodiode detector set at 306 nm with an ODS-H80 preparative column (150 mm, 10 mm internal diameter, 4 µm particle size, 8 nm pore diameter; YMC Waters), was used to purify the HA. The method used in this work was based on the one described by Lee et al. (32), with some modifications.

The mobile phase consisted of 60:40 water plus 0.1% trifluoroacetic acid-acetonitrile. Two hundred microliters of sample was applied and eluted with a 3-ml/min flux of mobile phase for 40 min, reaching 100% of acetonitrile in 7 min, held for 10 min, and then returned to the initial conditions (total time of the program, 62 min). Under these conditions, HA was eluted at 34.8 min. HA-containing fractions were combined and completely evaporated on a rotary evaporator at 4°C. Finally, the solid obtained was resuspended in acetonitrile and stored at -30°C. HA was identified by nuclear magnetic resonance (NMR) (27) and quantified by using a standard curve.

For routine analysis, *Ta37* cultures were extracted with equal volumes of ethyl acetate, and the upper phase was recovered, evaporated to dryness in a rotary evaporator at room temperature, and resuspended in acetonitrile to a tenth of the initial volume. Twenty microliters of the concentrated extract was applied to a YMC Waters analytical column of 150 by 4.6 mm and eluted with a 1-ml/min flux of mobile phase (as described above) for 30 min, reaching 100% of acetonitrile in 10 min, held for 5 min, and then returned to the initial conditions (total time of the program, 50 min). Using these conditions, HA was eluted at 22.9 min.

Chemical analysis of HA intermediates. Gas chromatography low-resolution mass spectrometry (GCMS) measurements were made with a Hewlett Packard 6890 gas chromatograph fitted with an HP-5MS column (30-mm by 0.25-mm film thickness) and a 5973 mass detector. The carrier gas was helium with a 20:1 split ratio and a 20-ml/min split flow. Two temperature programs were used: (i) the column was held at 120°C at injection, 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 3 min (total run time of 20 min) or (ii) the column was held at 120°C at injection and heated to 260°C at 25°C/min and held for 4.4 min (total run time of 10 min). The first program was used to detect isotrichodiol, and the second program was used to detect trichodiene and trichodermol. HA intermediates were identified by comparison of retention times and mass spectral fragmentation patterns with standards.

Precursor feeding experiments. For yeast experiments, cells were grown in MD+LHT medium at 28°C and 200 rpm for 48 h and then transferred to YGal medium (YD plus 2% galactose) supplemented with trichodiene (Tri4 assays) or trichothecene (Tri11 assays) dissolved in acetone to a final concentration of 250 µM. No substrate was added for Tri5 assays. Untransformed yeast cultures were used as negative controls. A 3-ml aliquot was removed from each culture after 3 to 4 days and mixed with 3 ml acetone to precipitate the yeast cells. The supernatant was then extracted with hexane-ethyl acetate (85:15), concentrated under nitrogen, and analyzed by GCMS.

For *F. verticillioides* experiments, spores were inoculated into 5-GYEP (50 g/liter glucose, 1 g/liter yeast extract, 1 g/liter peptone) medium, and the cultures were incubated at 28°C and 200 rpm. After 24 h, trichodiene dissolved in acetone was added to the cultures to a final concentration of 250 µM. After 4 days, cultures were extracted with ethyl acetate, and the extracts were analyzed by GCMS for trichothecene content. As a negative control, the transformant FvTaTri4#19-2 was grown in 5-GYEP medium without trichodiene.

Nucleotide sequence accession numbers. Sequences have been deposited in the GenBank database. Accession numbers of the *T. arundinaceum tri* genes are as follows: FR715494 for *Tatri5*, FN394491 for *Tatri14*, FN394492 for *Tatri12*, FN394493 for *Tatri11*, FN394494 for *Tatri10*, FN394495 for *Tatri3*, FN394496 for *Tatri4*, and FN394497 for *Tatri6*. Accession numbers of the *T. brevicompactum tri* genes sequenced in this work are as follows: FR775447 for *Tbtri14*, FR839627 for *Tbtri12*, FR839626 for *Tbtri11*, FR775446 for *Tbtri10*, FR775444 for *Tbtri3*, FR822202 for *Tbtri4*, and FR775445 for *Tbtri6*.

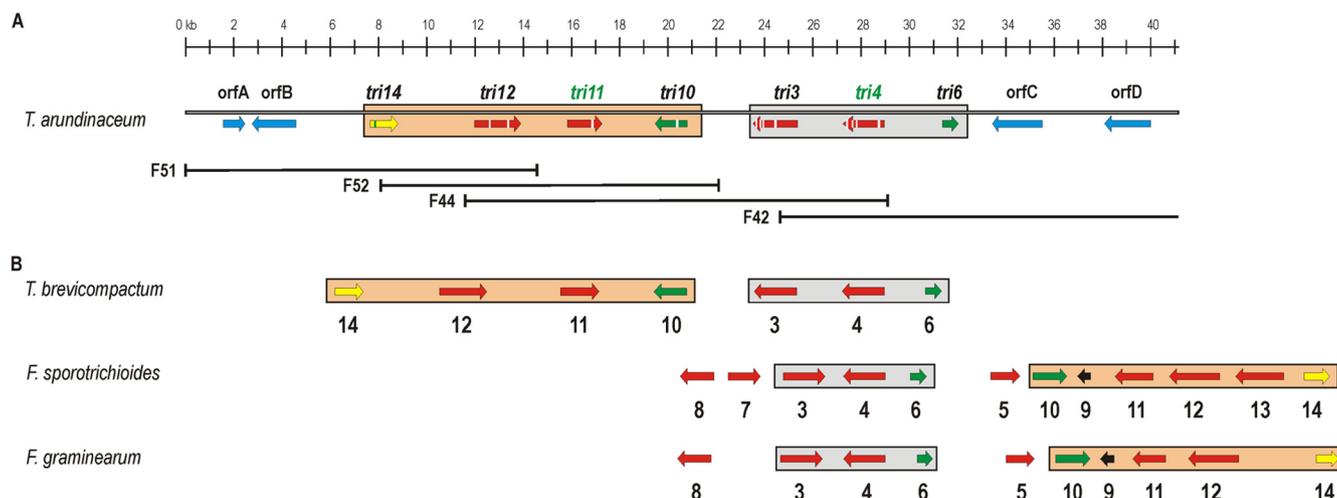


FIG. 1. (A) Genomic organization of the *Trichoderma arundinaceum* IBT 40837 HA core cluster of *tri* genes. Overlapping phages representing the genomic region comprising the trichothecene biosynthetic gene cluster are also represented. The arrows indicate predicted positions and directions of transcription of individual genes/open reading frames (ORFs). Small rectangles inside each gene indicate the introns of each ORF. The names above each arrow correspond with their *Fusarium* orthologue designation. The red arrows refer to those structural genes involved in the HA biosynthesis (*tri11*, *tri3*, *tri4*) or in the export of HA outside the cell (*tri12*), the yellow arrow (*tri14*) has an unknown function, and the blue arrows indicate non-*tri* genes. The genes *tri11* and *tri4*, whose names are illustrated in green, were those whose role in the biosynthesis have been studied in the present work by expression in yeast and, in the case of *tri4*, also in *F. verticillioides*. Note that the *tri5* gene has not been illustrated, even when it has been also expressed in yeast, since it is located in another region of the *Ta37* genome outside the core cluster of *tri* genes. (B) Genomic organization of the trichothecene gene cluster of *T. arundinaceum* in comparison with *T. brevicompactum* and *Fusarium* species (10). The arrows indicate predicted positions and directions of transcription of individual genes/ORFs. Numbers indicate gene designations based on their *Fusarium* orthologues (e.g., 6 refers to *tri6* or *TRI6*). Colored designations illustrate their role in trichothecene biosynthesis, as indicated above. Colored rectangles represent those genes that are grouped in a similar fashion in the different clusters.

RESULTS

Identification of HA in *T. arundinaceum* culture broth.

HPLC analysis of culture supernatants of the *T. arundinaceum* *Ta37* strain resolved three main peaks at 21.1, 22.9, and 39.0 min. The antifungal activity of HA had been previously reported (14), and our bioassay confirmed this. *K. marxianus* was sensitive only to the fractions collected around the 22.9-min peak. We confirmed that the bioactive fraction contained HA with ^1H NMR and ^{13}C NMR spectroscopy.

Identification of trichothecene biosynthetic loci in *T. arundinaceum* and *T. brevicompactum*. Initially, an internal *Tatri5* fragment from genomic DNA of the *Ta37* strain was amplified with the previously described *tri5* PCR primers Tri5F and Tri5R (see Table S1c in the supplemental material). The resulting amplicon was sequenced to confirm its similarity to previously described *TRI5* orthologues from other trichothecene-producing fungi (e.g., *Fusarium* and *Myrothecium*) and then used as a hybridization probe to screen the *Ta37* lambda library. This screen yielded two overlapping clones containing *Tatri5*, F72 and F73, which included a 1,226-bp coding region that was 50 to 66% identical to previously characterized *TRI5* orthologues from *F. graminearum* (47), *F. sporotrichioides* (23), *M. roridum* (57), *Stachybotrys echinata* (30), and *Trichothecium roseum* (30).

Since some of the trichothecene biosynthetic genes are clustered in *Fusarium* and in *Myrothecium*, we examined the regions flanking *Tatri5* in clones F72 and F73. We were unable to identify any other known *TRI* orthologues in the 18 kb of DNA of these two clones.

To identify other *TRI* orthologues in *T. arundinaceum*, we used PCR-amplified fragments of *F. sporotrichioides* *TRI4* (*FsTRI4*) and *M. roridum* *TRI4* (*MrTRI4*) as hybridization probes to screen the lambda library prepared from the *Ta37* strain. We selected *TRI4* as a hybridization probe because *TRI4* is essential for trichothecene production in both *Fusarium* and *Myrothecium*. The library screen identified three clones (F41, F42, and F44) that hybridized to the *FsTRI4* and *MrTRI4* fragments. Based on partial sequence analysis, these clones contain orthologs of the *Fusarium* *TRI4*, *TRI6*, and *TRI12* genes. An F44 subclone that included the *TRI12* orthologue was used as a hybridization probe to rescreen the *Ta37* lambda library to obtain two additional clones (F51 and F52) that overlapped clone F44.

Sequence analysis of 41 kb of DNA spanned by lambda clones F42, F44, F51, and F52 identified seven orthologs of *Myrothecium* and/or *Fusarium* *TRI* genes in the order *tri14*, *tri12*, *tri11*, *tri10*, *tri3*, *tri4*, and *tri6* (Fig. 1). There also were two genes, designated *orfA* and *orfB*, in the region flanking *tri14* and two more genes, *orfC* and *orfD*, flanking *tri6* (Fig. 1). These *tri6*- and *tri14*-flanking genes have no detectable DNA sequence similarity to known *TRI* genes, but their deduced amino acid sequences exhibit significant levels of similarity (BLAST) to metallo- β -lactamase superfamily proteins (*orfA*), α -*N*-acetylglucosaminidases (*orfB*), proteins of unknown function (*orfC*), and oligopeptide transporter proteins (*orfD*). Based on these results, we hypothesize that the region of genomic DNA that spans 24,291 bp from *tri14* to *tri6* is the core *tri* cluster in *T. arundinaceum*.

TABLE 2. Amino acid comparison of *TRI* orthologues from *Trichoderma* to other fungi

<i>T. arundinaceum</i> <i>TRI</i> orthologue	No. of amino acids	Intron length(s) (bp)	% identity to ^a :		
			<i>T. brevicompactum</i>	<i>M. roridum</i>	<i>F. sporotrichioides</i>
<i>tri3</i>	520	57, 55, 56, 54	92	ND	48
<i>tri4</i>	517	70, 58, 56, 53	98	67	73
<i>tri5</i>	388	59	99	56	57
<i>tri6</i>	218	None	99	51	46
<i>tri10</i>	422	70	98	ND	58
<i>tri11</i>	499	80	96	ND	38
<i>tri12</i>	602	55, 68	92	ND	55
<i>tri14</i>	363	59	99	ND	64

^a ND, not done.

Using the sequences of the *Tatri* orthologues, we designed PCR primers (see Table S1d in the supplemental material) to amplify and sequence the corresponding orthologues in *Tb41*. The resulting sequence contig spanned 25,498 bp and included orthologues of the seven *TRI* genes in the same order and orientation as those in the *T. arundinaceum* core cluster. The deduced amino acid sequences of *TaTri* and *TbTri* orthologues exhibited $\geq 92\%$ identity (Table 2). The most notable difference between the *T. arundinaceum* and *T. brevicompactum* clusters is the size of the *tri11-tri12* intergenic region, which is 1,793 bp in *T. arundinaceum* and 3,000 bp in *T. brevicompactum*. Thus, the core *tri* cluster in these species contains seven orthologs of trichothecene biosynthetic genes in *Fusarium* but does not include *tri5* (Fig. 1).

The numbers and organization of genes in the cluster are the same in both *Trichoderma* species examined but markedly different from the organization in *Fusarium*. The *Trichoderma* cluster has orthologues of only seven of the 10 to 14 genes that occur in the *Fusarium* cluster. Although the arrangement of genes differs from that in the *Fusarium* cluster, the *Trichoderma tri* cluster can be divided into two regions (or blocks) that exhibit conservation of gene content and order relative to blocks within the *TRI* clusters of *F. graminearum* and *F. sporotrichioides* (Fig. 1). A region orthologous to the *tri3-tri4-tri6* block in *Trichoderma* also exists in the *F. graminearum* and *F. sporotrichioides* cluster (i.e., *TRI3-TRI4-TRI6*). Likewise, a region orthologous to the *tri14-tri12-tri11-tri10* block in *Trichoderma* also exists in the *F. graminearum* and *F. sporotrichioides* cluster but includes two additional genes, *TRI9* and *TRI13*, which are not in the *Trichoderma* cluster. Within the two blocks, gene orientation is also largely the same in *Trichoderma* and *Fusarium*; only *tri3/TRI3* and *tri14/TRI14* differ in orientation with respect to other genes in the blocks.

Functional characterization of *Tatri* genes. We examined the functions of *Tatri4*, *Tatri5*, and *Tatri11* by expressing each gene independently in yeast. For *Tatri5* expression, transformed yeast cells were analyzed without the addition of a trichothecene biosynthetic intermediate, because the substrate for the *Fusarium* *Tri5* enzyme is farnesyl pyrophosphate, which is an intermediate of primary metabolism in yeast. Extraction and GCMS analysis of pYETri5-transformed yeast cultures following galactose induction revealed the presence of trichodiene (Fig. 2). This is the same product of the reaction catalyzed by the *Fusarium* *Tri5* enzyme and the first intermediate in the trichothecene biosynthetic pathway. The presence

of trichodiene was observed in four independent yeast transformants carrying pYETri5. These results indicate that the *TaTri5* enzyme catalyzes cyclization of farnesyl pyrophosphate to form trichodiene.

For *Tatri4* expression, cultures of pYETri4-transformed yeast were induced with galactose and then fed trichodiene, the substrate of the *Tri4* enzymes in *Fusarium* and *Myrothecium*. Extraction and GCMS analysis of the resulting cultures revealed the presence of the trichothecene biosynthetic intermediate isotrichodiol (Fig. 3). This result was confirmed in eight yeast transformants. The function of *TaTri4* was also assessed by introducing a *Tatri4* expression cassette, on plas-

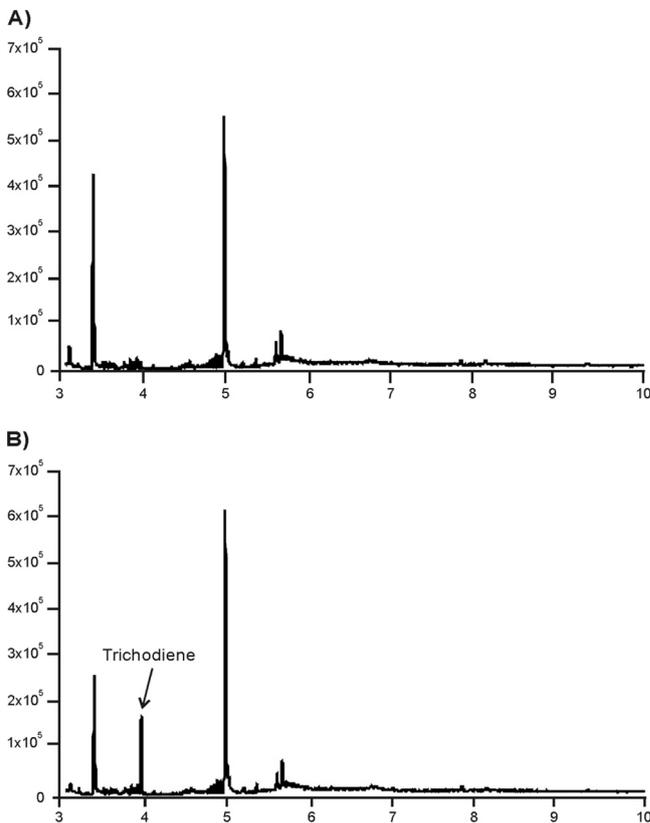


FIG. 2. Reconstructed ion chromatograms of extracts from 4-day-old *Saccharomyces cerevisiae* YGal liquid cultures. (A) INVSc; (B) INVSc expressing *Tatri5* (transformant INVtri5#4). The arrow indicates trichodiene (TD) at 4.0 min.

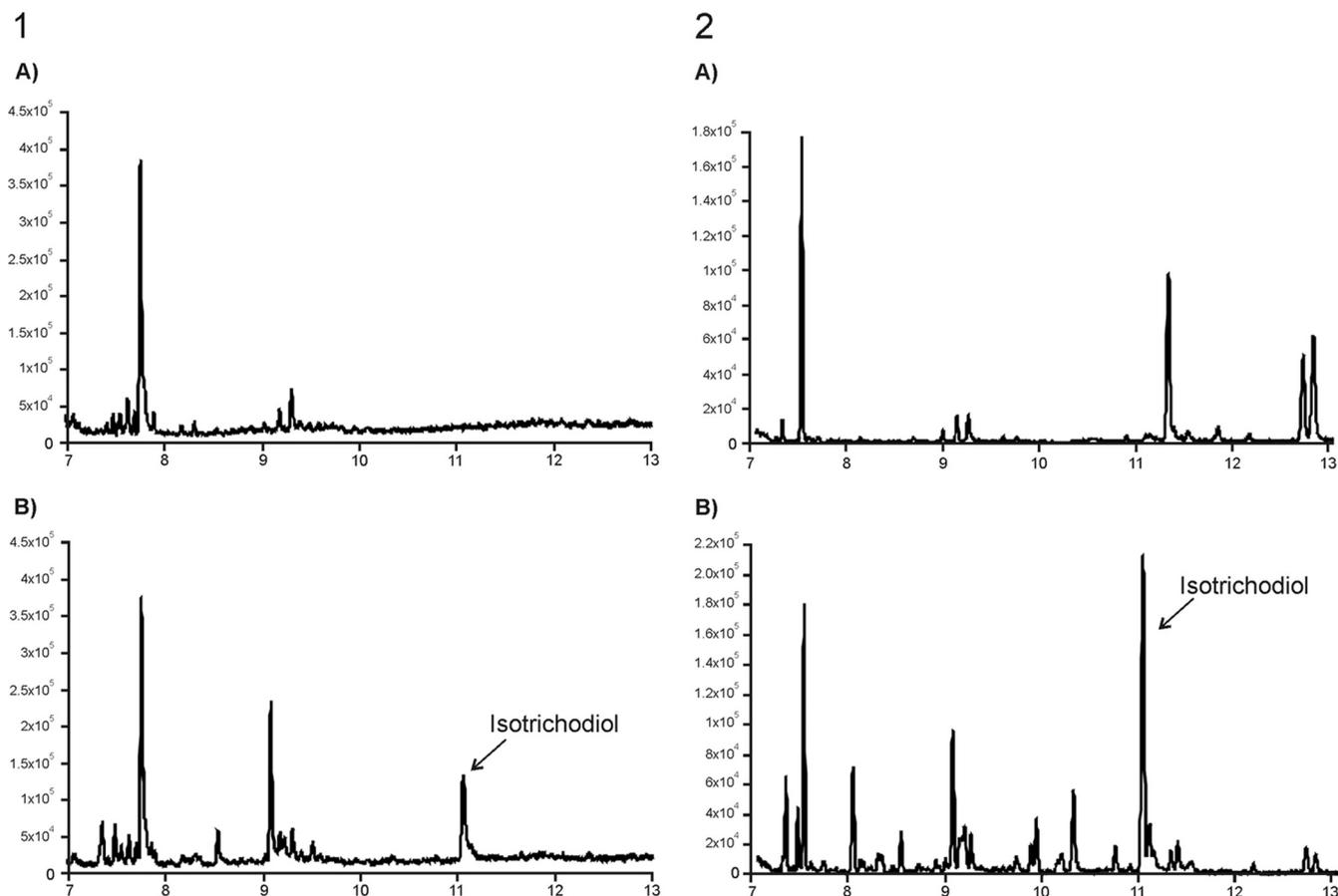


FIG. 3. (1) Reconstructed ion chromatograms of extracts from 3-day-old *Saccharomyces cerevisiae* YGal liquid cultures after feeding trichodiene: INVSc yeast (A) and INVtri4#3 (B). (2) Reconstructed ion chromatograms of extracts from 4-day-old 5-GYEP liquid cultures of *F. verticillioides* expressing *Tatri4* (transformant FvTaTri4#19-2): control culture with no trichodiene (A) and after feeding with trichodiene (B). The arrows indicate the position of isotrichodiol at 11.1 min.

mid pSPT4-4, into the trichothecene-nonproducing fungus *F. verticillioides*. The addition of trichodiene to cultures of eight *F. verticillioides* transformants carrying the expression cassette also resulted in the formation of isotrichodiol (Fig. 3).

For *Tatri11* expression, cultures of pYETri11-transformed yeast cells were induced with galactose and then fed 12,13-epoxytrichothec-9-ene (EPT). Extraction and GCMS analysis of the cultures of five independent transformants revealed the presence of 4-hydroxytrichothecene (trichodermol) (Fig. 4). These results indicate that the *TaTri11* enzyme catalyzes hydroxylation of C-4 of the trichothecene skeleton. This activity differs from that of the *Fusarium* Tri11 enzyme, which catalyzes hydroxylation of C-15 of the trichothecene skeleton.

In silico characterization of *TaTri* proteins. Analysis of the deduced protein sequence of *TaTri5* showed two conserved metal binding motifs that coordinate Mg²⁺ ion-bridged binding of the diphosphate moiety of FPP, which is similar to other proteins belonging to class 1 of *cis-trans* terpene cyclases that catalyze the cyclization of FPP to trichodiene. The first motif is a DDXX consensus sequence, DDSR, in *TaTri5*. The first D coordinates with the second conserved region, (N/D)XXX(S/T)XXXE, which corresponds to amino acids (aa) 234 to 242 (NDLFSFYKE) of *TaTri5*.

Both *TaTri4* and *TaTri11* were identified as cytochrome

P450 monooxygenases and contained all the conserved structural features described for this family of proteins. The highest structural conservation occurs in the core of the protein around the heme and reflects a common mechanism of electron and proton transfer and oxygen activation. This region comprises (i) the heme-binding loop, containing the most characteristic P450 consensus sequences (FXXGXRXCXG), FSQGSRQCIG (positions 445 to 454) in *TaTri4* and FNVGPRNCVG (positions 435 to 444) in *TaTri11*, with the absolutely conserved Cys that serves as the fifth ligand to the heme iron, (ii) the absolutely conserved motifs (EXXR), EGLR (positions 369 to 372) in *TaTri4* and EAFR (positions 352 to 355) in *TaTri11*, probably required to stabilize the core structure, and (iii) the central part containing the consensus sequences of the P450 signature [(A/G)GX(D/E)T(T/S)], AGTETT (positions 311 to 316) in *TaTri4* and AGSETT (positions 294 to 299) in *TaTri11*, which corresponds to the protein transfer groove (1, 60). In spite of the high conservation of the consensus motifs for P450 monooxygenases between *TaTri11* and *TaTri4*, they showed a low amino acid sequence similarity of 19% to each other.

TaTri3 most likely belongs to the chloramphenicol acetyltransferase family (CAT) of acetyltransferases, as it contains the consensus sequence HXXXDG (HLFWDG) (at positions

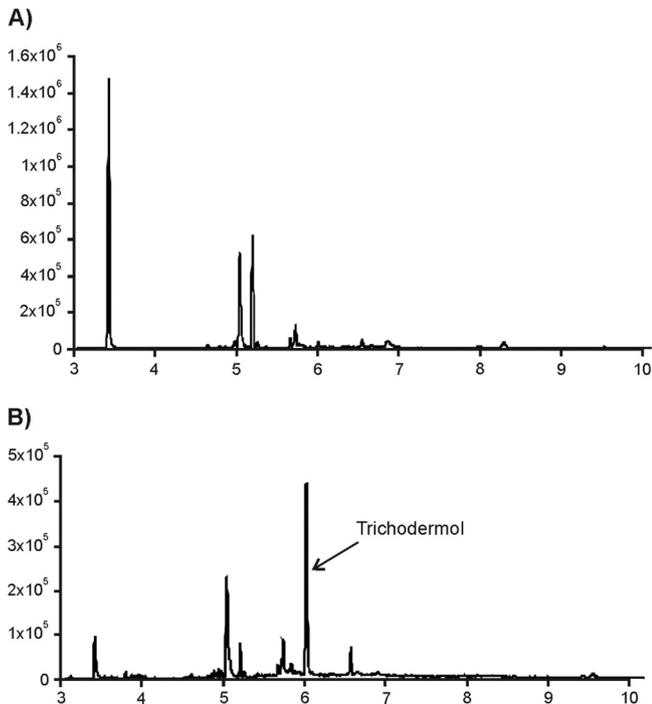


FIG. 4. Reconstructed ion chromatogram of extracts from 3-day-old *Saccharomyces cerevisiae* YGal liquid cultures after feeding 12,13-epoxytrichothec-9-ene (EPT): INVSc (A) and INVSc expressing *Tatri11* (transformant INVTri11#3) (B). The arrow indicates the position of trichodermol at 6.0 min.

188 to 193) (43) but not the 3' consensus DFGWGKP (see reference 3 for acetyltransferase comparisons).

The analysis of the 422-aa *TaTri10* protein with BLASTP, CDART, and CDD (Conserved Domain Database) algorithms detected no putative transmembrane conserved domains. However, other algorithms, such as DAS (Dense Alignment Surface), TMPred (TransMembrane Prediction), SMART (Simple Modular Architecture Research Tool), and TMHMM (transmembrane helices), did detect putative transmembrane sites, with aa 196 to 218 receiving the highest score. The last program (TMHMM) gave a lower probability score to the sequence from *Tb41* than to *Ta37*, most likely due to the two amino acid differences at aa 209 and 210 in the two strains. MotifFinder found no conserved domains in either Tri10 protein. Compared to the *FsTRI10* sequence, the sequence from the *TaTri10* protein has a 58% identity (Table 2).

The analysis of the *TaTri6* protein revealed the presence of three regions in the carboxy-terminal end with similarity to zinc finger domains found in a wide range of gene regulatory proteins. These domains are important for DNA binding. Motif ZF1 has a high similarity to the consensus sequence (Ψ/Y)XCX_{2,4}CX₃FX₅ΨX₂HX_{3,5}H (Ψ denotes a hydrophobic amino residue) (7) of zinc finger binding proteins and correlates well with the ZF1 sequence from *M. roridum* and *F. sporotrichioides* (Fig. 5A). The sequences of ZF2 and ZF3 of *TaTri6*, while retaining the CCFHH amino acids of the consensus sequence, have additional amino acid inserts and correlate highly with those regions of Tri6 from *M. roridum* and *F. sporotrichioides* (Fig. 5A). When the carboxy-terminal region of the *TaTri6* protein was compared with the same region of the Tri6 from

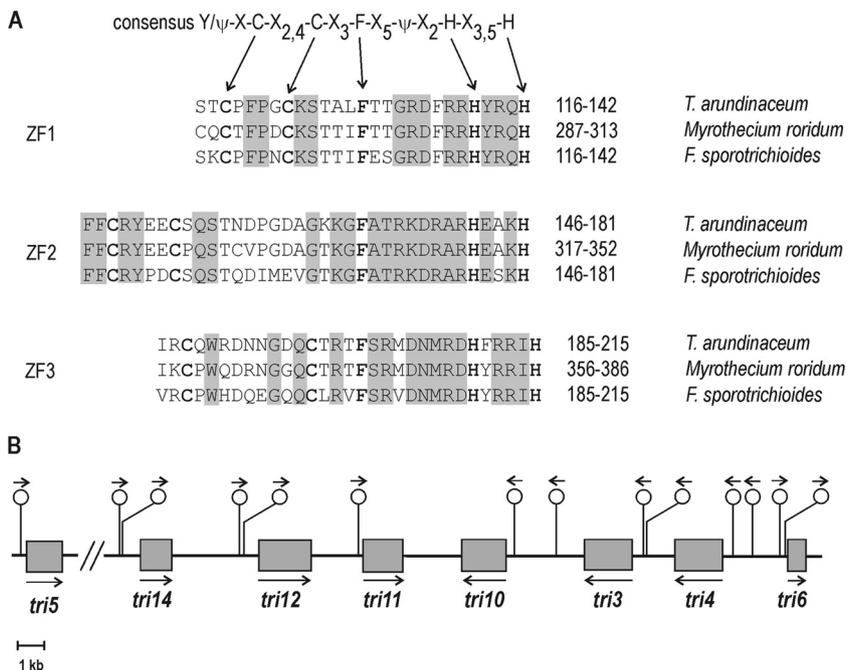


FIG. 5. (A) Sequence alignment between the ZF1, ZF2, and ZF3 domains, located at the carboxy-terminal end of the predicted Tri6 protein sequences from *Ta37*, *M. roridum*, and *F. sporotrichioides*. The Cys and His residues within the proposed zinc fingers, as well as other residues conserved with respect to the consensus sequence proposed for these zinc finger domains, are shown in bold (7). Those residues conserved within each zinc finger domain between the four analyzed Tri6 sequences are shaded. (B) Localization of the putative binding sequences for the transcription factor Tri6 localized in the promoter region of the *Tatri* genes.

M. roridum and *F. sporotrichioides*, the identities were 84% and 73%, respectively. However, when the entire *TaTri6* amino acid sequence was compared to *M. roridum* and *F. sporotrichioides*, the amino acid identities were only 51% and 47%, respectively.

In *Fusarium*, the *Tri6* protein induces expression of *TRI* cluster genes, in part by binding to the DNA sequence YNAGGCC that is present in the promoter regions of the genes (24). This same sequence motif was observed in the promoter regions of all the *Tatri* genes (Fig. 5B). Thus, *Trichoderma Tri6* likely binds to the promoter regions of *Trichoderma tri* cluster genes and thereby regulates their expression as described for *Fusarium* (24).

The analysis of the *TaTri12* sequence identified the protein as having a relatively high predicted amino acid sequence similarity with *Fusarium Tri12* (Table 2), which is known to serve as a trichothecene efflux pump (2). The high sequence similarity with proteins in the MFS (major facilitator superfamily) indicates that *TaTri12* should facilitate transport, presumably of HA, across the cytoplasmic or other intracellular membranes. The PROTEAN program (25) predicts that *TaTri12* contains 14 transmembrane alpha helix domains (TM) connected by hydrophilic groups. This structure is consistent with the presence of 272 hydrophobic amino acids, 45% of the total 602 amino acids of the protein, a percentage similar to that of the *Tri12* protein from *F. sporotrichioides* (44%) and higher than that detected in any of the other *Tri* proteins.

The analysis of the deduced *TaTri14* protein sequence found no conserved domains compared against the CDD. Thus, *TaTri14* function remains unknown.

DISCUSSION

Within the fungal order *Hypocreales*, trichothecene biosynthesis and the *TRI* cluster have been examined extensively in selected species of *Fusarium*, and there is evidence for a *TRI* cluster in *Myrothecium*. In the current study, we describe trichothecene biosynthesis and a *tri* cluster in a third trichothecene-producing genus, *Trichoderma*. The presence of a *tri/TRI* cluster in species of *Trichoderma*, *Myrothecium*, and *Fusarium* could result if an ancient hypocrealean fungus, a common ancestor of all three genera, produced trichothecenes and had a *tri/TRI* cluster. Although the *Trichoderma* cluster (seven genes) differs markedly in gene content and organization from the larger *Fusarium* cluster (10 to 14 genes), the *Trichoderma* cluster includes genes encoding a range of functions similar to those encoded by the *Fusarium* cluster. These functions include biosynthetic enzymes (*Tri3*, *Tri4*, *Tri11*), regulatory proteins (*Tri6* and *Tri10*), and a transport protein (*Tri12*).

The difference in gene content of the clusters indicates that genes have been gained and/or lost during evolution of *Trichoderma* and *Fusarium*. Perhaps the most notable difference is the absence of a *tri5/TRI5* orthologue in the *Trichoderma* cluster. Whether the *Trichoderma tri5* and *tri* clusters are physically linked on the same chromosome or located on different chromosomes has not been determined. Results from the current study and those from previous studies (23) indicate that the *Trichoderma* and *Fusarium tri/TRI5* orthologues encode trichodiene synthase, the enzyme that catalyzes the first committed step in trichothecene biosynthesis. Thus,

tri5/TRI5 is essential for trichothecene production in both fungi. The location of the *tri5/TRI5* orthologue outside the *tri* cluster in *Trichoderma* but inside the cluster in *Fusarium* and *Myrothecium* (57; R. H. Proctor, unpublished data) suggests that the ancestral *tri/TRI* cluster included a *tri5/TRI5* gene but that the gene moved out of the cluster to another genomic location during evolution of *Trichoderma*. One hypothesis for the function of secondary metabolite biosynthetic gene clusters is that they facilitate vertical and horizontal inheritance of all genes directly involved in the biosynthesis of a secondary metabolite (28). A second hypothesis for the function of secondary metabolite biosynthetic gene clusters is that they facilitate coordinated expression of all genes directly involved in biosynthesis of a metabolite (28). The location of *tri5* outside the *Trichoderma tri* cluster runs counter to both hypotheses in that *tri5* would not necessarily be inherited with the rest of the cluster and its expression is correlated with other *tri* genes and with trichothecene production by *Trichoderma* (S. Gutiérrez, unpublished data).

Multiple sesquiterpene-derived metabolite biosynthetic gene clusters have been characterized in filamentous fungi. These include the abscisic acid and botrydial clusters in *Botrytis cinerea* (46, 52), the Ao cluster in *Aspergillus oryzae* (42), and the gibberellin cluster in *Fusarium fujikuroi* (35). Of these clusters, only the *B. cinerea* abscisic acid cluster lacks a sesquiterpene cyclase gene that encodes the enzyme that catalyzes conversion of farnesyl pyrophosphate to the first committed intermediate in synthesis of the metabolite.

In this study, we demonstrated that the *Tatri11*-encoded cytochrome P450 monooxygenase catalyzes trichothecene C-4 hydroxylation. This finding was unexpected, because C-4 hydroxylation is catalyzed by a different monooxygenase, encoded by the *TRI13* gene, in *Fusarium*. Thus, although *Trichoderma* and *Fusarium* employ the same enzymatic mechanism (i.e., cytochrome P450 monooxygenase) for trichothecene C-4 hydroxylation, the two genera do not employ orthologous enzymes to catalyze the reaction. This pattern indicates that the enzymes responsible for trichothecene C-4 hydroxylation may have evolved independently in *Trichoderma* and *Fusarium*. As far as we are aware, this is the first report of nonorthologous enzymes catalyzing the same structural modification during synthesis of structurally related secondary metabolites in different fungal genera. Although the structural modifications catalyzed by *TaTri11* and *FsTri13* are the same, the substrates and products of the reactions are not. The substrate for *TaTri11* is the trichothecene skeleton, EPT, whereas the substrate for *FsTri13* is calonectrin, i.e., a trichothecene that has been oxygenated and acetylated at C-3 and C-15 (9).

The role of *TaTri11* in trichothecene C-4 hydroxylation differs from the role of the *Fusarium Tri11* enzyme in trichothecene C-15 hydroxylation. Despite this difference in function, we used the *tri11* designation for the *Trichoderma* orthologue, because in BLASTX analyses, *Tatri11* exhibited significant levels of identity (score = 382, E value of 1e-88) to *Fusarium TRI11* orthologues but did not exhibit significant levels of identity to *Fusarium TRI13* orthologues. However, of the eight *Tatri* genes identified in this study, *Tatri11* exhibits the lowest level of sequence identity to the corresponding *Fusarium* orthologues; the deduced *Tatri11* sequence is 38% identical to the *FsTRI11* sequence, whereas other *Tatri* genes exhibit 46 to

73% identity to their corresponding *F. sporotrichioides* orthologues (Table 2). In addition, the number and pattern of introns in *Tatri11* and *FsTRI11* are markedly different from those of other *Trichoderma* and *Fusarium tri/TRI* orthologues; *Tatri11* has one intron near the 3' end of the coding region, whereas *FsTRI11* has four introns that are dispersed across the coding region. The deduced *TaTri11* sequence also exhibits higher levels of sequence identity to proteins from more distantly related fungi than it does to *Fusarium Tri11* orthologues. For example, the deduced amino acid sequence for *TaTri11* exhibits 58% and 42% identity to protein ATEG_08832 from *Aspergillus terreus* (class *Eurotiomycetes*) and SS1G_13923 from *Sclerotinia sclerotiorum* (class *Leotiomyces*), respectively. These data suggest that although *Tatri11* and *FsTRI11* share an ancestor, the most recent common ancestor was likely not a trichothecene biosynthetic gene and could have occurred in a fungus that was distantly related to the *Hypocreales*. One possible scenario is that the ancestral trichothecene cluster lacked a *tri11/TRI11* homologue and that acquisition of the homologues by the ancestral *Trichoderma* and *Fusarium* clusters occurred independently and from different sources. Another possible scenario is that the ancestral trichothecene cluster included a *tri11/TRI11* homologue but that the gene was lost from either the ancestral *Trichoderma* cluster or the ancestral *Fusarium* cluster, and subsequently the cluster that lost *tri11/TRI11* acquired a more distantly related homologue. Despite the likely distant evolutionary origins of *Tatri11* and *FsTRI11*, the position of these homologues relative to other *tri/TRI* genes is conserved in the core clusters in *Trichoderma* and *Fusarium*. Whether this position within the cluster is fortuitous or has a functional significance remains to be determined.

In this study, functional analysis also indicated that the *T. arundinaceum tri4*-encoded enzyme (*TaTri4*) catalyzes oxygenation of trichodiene at C-2, C-11, and C-13. These are the same three oxygenations catalyzed by the *M. roridum Tri4* enzyme (*MrTri4*) (39). In contrast, *F. graminearum Tri4* (*FgTri4*) catalyzes trichodiene oxygenation at C-3 as well as C-2, C-11, and C-13 (38). The inability of *TaTri4* and *MrTri4* to catalyze C-3 oxygenation is consistent with the absence of a C-3 oxygen in trichothecenes produced by *Trichoderma* and *Myrothecium* (39, 44). Although *TaTri4* and *MrTri4* have the same activity, the identity (67%) of their predicted amino acid sequences is lower than the identity (73%) of *TaTri4* and *FsTri4* (Table 2). Thus, identity of *Tri4* orthologues is not correlated with differences in their functions among the three genera examined. This finding raises the question of how differences in *Tri4* function arise among trichothecene-producing genera. Did the ancestral *Tri4* catalyze four oxygenation reactions and then lose the ability to catalyze the C-3 oxygenation during evolution of *Trichoderma* and *Myrothecium*, or did the ancestral *Tri4* catalyze only three reactions and then gain the ability to catalyze the C-3 oxygenation during the evolution of *Fusarium*? Functional and phylogenetic comparisons of orthologues from additional trichothecene-producing genera (e.g., *Stachybotrys* and *Trichothecium*) with those of *Trichoderma*, *Myrothecium*, and *Fusarium* should provide further insight into the evolution of *Tri4*.

Although we did not characterize the function of the *TaTri3*, its similarity to the corresponding orthologues (*Tri3*) in *Fusarium* suggests a function. The numbers and positions of in-

trons are the same in *Tatri3* and *FsTRI3*, and their deduced amino acid sequences exhibit a higher level of identity (48%) than those of *TaTri11* and *FsTri11*. Together, these data indicate that *Tatri3* and *FsTRI3* are more closely related orthologues than *Tatri11* and *FsTRI11* and more likely to be descended directly from the same ancestral trichothecene biosynthetic gene. In *Fusarium*, the *TRI3*-encoded enzyme (*Tri3*) is an acetyltransferase; it catalyzes acetylation of the oxygen at C-15 of the trichothecene skeleton (37). However, *Trichoderma* trichothecenes (e.g., HA and trichodermin) lack an oxygen at C-15 and, therefore, are not acetylated at this position. The functional analyses of *TaTri4* and *TaTri11* demonstrate that *Tri* orthologues can have different functions in *Trichoderma* and *Fusarium*. Thus, *Trichoderma Tri3* likely functions as an acetyltransferase because of (i) the relatively high level of amino acid sequence identity between *TaTri3* and *FsTri3* and (ii) *Trichoderma Tri3* has the amino acid sequence motif, HXXXDG, that is indicative of acetyl-transferases (43). Given these data, a possible function of the *Trichoderma Tri3* is acetylation of the oxygen at C-4 of the trichothecene skeleton. This putative function is consistent with the presence of an acetyl moiety at the C-4 oxygen in trichodermin, the predominant trichothecene produced by *T. brevicompactum* strain IBT 40841. Some acetyltransferases utilize acyl groups other than acetyl as substrates (54). If *Trichoderma Tri3* utilizes such a substrate, then the enzyme might also be able to catalyze esterification of octatriendioic acid to the C-4 oxygen to yield HA in *T. arundinaceum*. This hypothesis can be tested by gene inactivation, i.e., if *Tri3* is responsible for the esterification of an octatriendioyl or acetyl moiety to the C-4 oxygen in *T. arundinaceum* or *T. brevicompactum*, respectively, inactivation of *tri3* should result in production of trichodermin (i.e., C-4-hydroxylated EPT) in both species.

The *Trichoderma* trichothecenes trichodermin and HA have simpler structures than the *Fusarium* trichothecenes DON, NIV, and T-2, suggesting that fewer genes are involved. The results of molecular and functional characterization of *tri* genes in *Trichoderma* are consistent with this relative structural simplicity and suggest a biosynthetic pathway for HA that requires as few as five biosynthetic enzymes: *Tri5* for formation of trichodiene; *Tri4* for oxygenation of trichodiene, which leads to the formation of EPT; *Tri11* for the oxygenation of EPT at C-4; and the acylation of the C-4 oxygen, possibly by *Tri3* (Fig. 6). Formation of HA would most likely require another enzyme, possibly a polyketide synthase (PKS), for synthesis of the octatriendioyl moiety that is esterified to the C-4 oxygen (Fig. 6). The absence of a PKS gene within or adjacent to the *Trichoderma tri5* or *tri* cluster suggests that there could be a third *tri* locus in *T. arundinaceum* with a PKS gene responsible for synthesis of octatriendioic acid. Production of trichodermin would not require any other enzymes besides *Tri5*, and those encoded by the *tri* cluster since a cellular pool of acetyl coenzyme A (acetyl-CoA) would be available for *Tri3* acetylation of the C-4 oxygen.

The results of this study have provided insight into the trichothecene toxin biosynthetic pathway in *Trichoderma* and into complexities of the evolutionary history of the *tri/TRI* cluster in the fungal order *Hypocreales*. These complexities include gene loss and/or gain as well as divergence and convergence of gene function. The relocation of *tri5* outside the *Trichoderma tri*

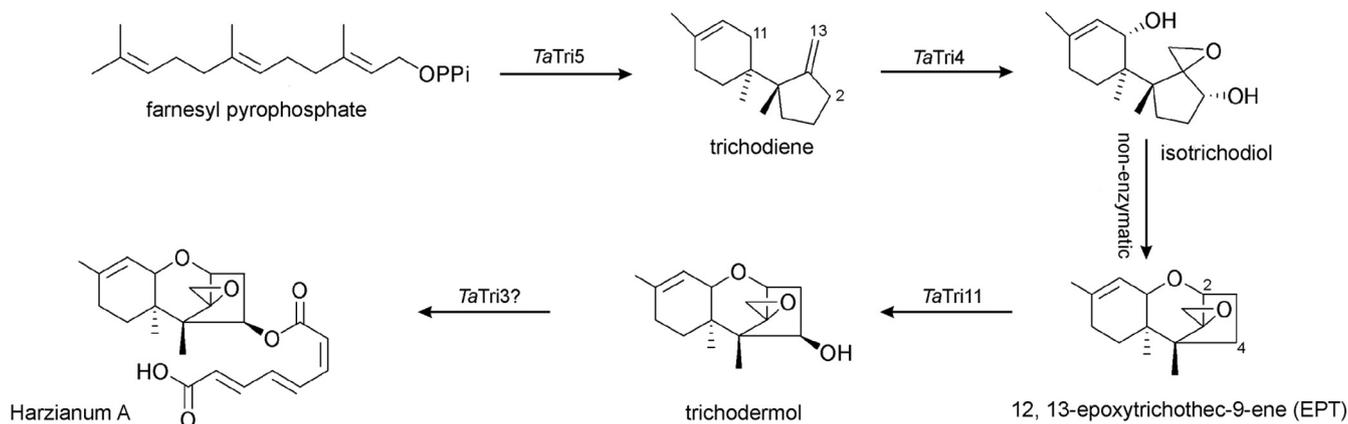


FIG. 6. Hypothetical HA biosynthetic pathway deduced by heterologous expression of the *TaTri5*, *TaTri4*, and *TaTri11* genes in *S. cerevisiae* and *F. verticillioides* and the identification of the products of the feeding experiments with the corresponding substrate for each enzyme.

cluster contrasts the situation in some *Fusarium* species, where genes (e.g., *TRI1* and *TRI101*) have relocated into the core cluster from smaller *TRI* loci (49). This contrast suggests that different forces have acted to affect movement of genes into and out of the *tri/TRI* cluster during evolution of trichothecene-producing genera. Thus, elucidation of the function of trichothecenes in the ecology of the fungi that produce them may provide insight into factors that affect assembly, growth, and disassembly of secondary metabolite biosynthetic gene clusters.

In conclusion, eight *tri* genes involved in the trichothecene biosynthesis by *T. arundinaceum* and *T. brevicompactum* have been cloned. *TaTri5* produce trichodiene from FPP in a fashion similar to that of *Tri5* from other trichothecene producers. *TaTri4* carried out three consecutive oxygenations at C-2, C-13, and C-11 to give isotrichodiol, similar to *Myrothecium* and *Trichothecium* species but different from *Fusarium* *Tri4*, that oxygenates trichodiene at four different positions to produce isotrichotriol. Finally *TaTri11* carried out a hydroxylation at the C-4 position, which differs substantially from *Fusarium* *Tri11* that hydroxylates at C-15. All these results, together with the possible role of *TaTri3* in the trichothecene biosynthesis, allowed us to propose a hypothetical biosynthetic pathway of HA and trichodermin, two mycotoxins with antibiotic and antitumor activities that may also be important agricultural contaminants.

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