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Requirement of Two Acyltransferases for 4-*O*-Acylation during Biosynthesis of Harzianum A, an Antifungal Trichothecene Produced by *Trichoderma arundinaceum*

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Supporting Information

ABSTRACT: Trichothecenes are sesquiterpenoid toxins produced by multiple fungi, including plant pathogens, entomopathogens, and saprotrophs. Most of these fungi have the acyltransferase-encoding gene *tri18*. Even though its function has not been determined, *tri18* is predicted to be involved in trichothecene biosynthesis because of its pattern of expression and its location near other trichothecene biosynthetic genes. Here, molecular genetic, precursor feeding, and analytical chemistry experiments indicate that in the saprotroph *Trichoderma arundinaceum* the *tri18*-encoded acyltransferase (TRI18) and a previously characterized acyltransferase (TRI3) are required for conversion of the trichothecene biosynthetic intermediate trichodermol to harzianum A, an antifungal trichothecene analog with an octa-2,4,6-trienedioyl acyl group. On the basis of the results, we propose that TRI3 catalyzes trichothecene 4-*O*-acetylation, and subsequently, TRI18 catalyzes replacement of the resulting acetyl group with octa-2,4,6-trienedioyl to form harzianum A. Thus, the findings provide evidence for a previously unrecognized two-step acylation process during trichothecene biosynthesis in *T. arundinaceum* and possibly other fungi.

KEYWORDS: *trichothecenes, harzianum A, Trichoderma, gene disruption, gene clusters, secondary metabolite, acyltransferase*

INTRODUCTION

Trichothecenes are toxins produced by fungi, including some species of *Aspergillus*, *Fusarium*, *Isaria*, *Microcyclospora*, *Myrothecium*, *Peltaster*, *Stachybotrys*, *Trichothecium*, and *Trichoderma*.[1−5] These species have diverse lifestyles (plant pathogenic, entomopathogenic, or saprotrophic), and collectively, they have at least 18 trichothecene biosynthetic genes (*tri*) distributed across one or more loci.[4,6−8] The saprotroph *Trichoderma arundinaceum* has biocontrol activity against multiple plant pathogenic fungi[9,10] and has *tri* genes at three loci, which exemplifies the *tri*-gene organization in fungi.[4,7,11] The first *T. arundinaceum tri* locus consists of seven clustered *tri* genes, namely, three enzyme-encoding genes (*tri3, tri4*, and *tri22*), two transcription factor genes (*tri6* and *tri10*), one transporter gene (*tri12*), and one gene (*tri14*) of unknown function. The second *T. arundinaceum tri* locus includes the polyketide synthase gene *tri17*, an acyltransferase gene *tri18*, and a cytochrome P450 monooxygenase gene that appears to be unique to *T. arundinaceum*. The third locus includes only the terpene synthase gene *tri5*.

Trichoderma arundinaceum produces high levels of the trichothecene analog harzianum A (**1**),[2] which consists of (i) 12,13-epoxytrichothec-9-ene (**2**), the tricyclic molecule that is common to all trichothecenes, and (ii) a linear eight-carbon-long acyl substituent (octa-2,4,6-trienedioyl, **3**) at carbon atom 4 (C4) (Figure 1). The proposed biosynthetic pathway of **1** begins when the *tri5*-encoded enzyme, TRI5, catalyzes cyclization of farnesyl diphosphate to form the terpene trichodiene. TRI4, a cytochrome P450 monooxygenase, catalyzes three oxygenations of trichodiene that lead to formation of isotrichotriol, which can undergo spontaneous cyclization to form **2**. TRI22, also a cytochrome P450 monooxygenase, then catalyzes hydroxylation of **2** at C4 to form trichodermol (**4**). TRI17 catalyzes formation of the linear eight-carbon polyketide, octa-2,4,6-trienoic acid, which undergoes further modification(s) by an as yet unidentified enzyme(s) to form the CoA-activated derivative of **3**. TRI3, an acyltransferase, is hypothesized to catalyze the esterification of the CoA-activated derivative of **3** to the C4 hydroxyl of **4** to form **1** (Figure 1).[4] A related species of *Trichoderma*, *T. brevicompactum*, produces trichodermin (**5**), a trichothecene analog that has the same structure as **1** except that it has an acetyl group at C4 rather than the acyl group octa-2,4,6-trienedioyl. Likewise, the biosynthetic pathway for **5** is proposed to be the same as that of **1** except for the last step, where an acetyl group rather than octa-2,4,6-trienedioyl is esterified to the C4 hydroxyl group.

Homologous trichothecene biosynthetic enzymes usually have the same function in different fungi.[4,7,9,12,13] TRI3 is an exception; it is proposed to catalyze esterification (acylation) of the C4 hydroxyl in *T. arundinaceum* but esterification of the

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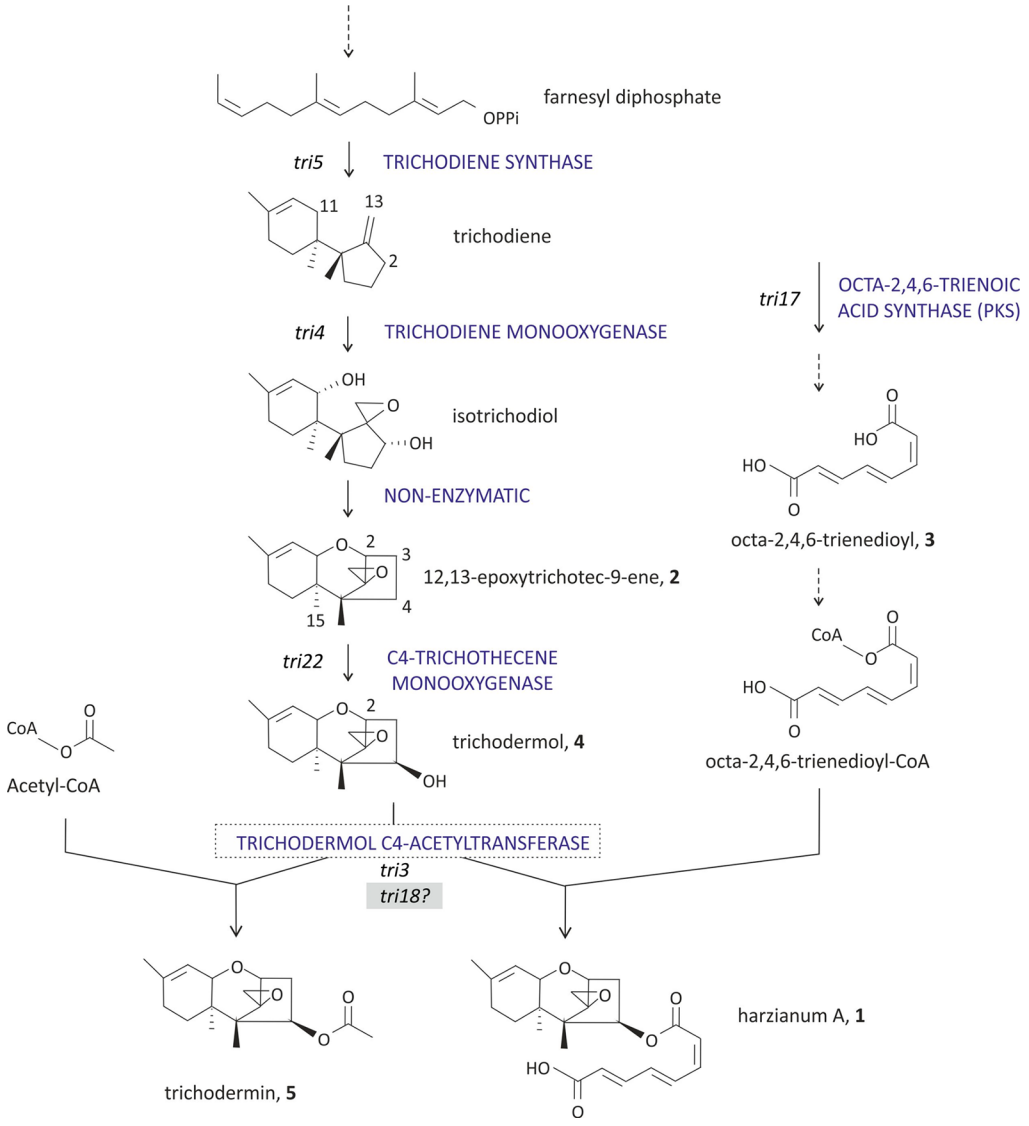

Figure 1. Biosynthetic pathway of harzianum A (**1**) and trichodermin (**5**) from farnesyl diphosphate in *Trichoderma* species. The *tri18* gene, encoding an acyltransferase, is indicated by a gray rectangle and is tentatively involved in the last step of the biosynthetic pathway. Genes and enzymes are indicated in black italic or blue capital letters, respectively.

C15 hydroxyl in *Fusarium*.[14,15] Although no *Fusarium* trichothecenes are reported to have an octa-2,4,6-trienedioyl substituent at C4, some have an acetyl substituent at this position. In *Fusarium*, trichothecene 4-*O*-acetylation is catalyzed by an acyltransferase encoded by *tri7*,[16,17] a gene that has not been reported in other trichothecene-producing genera.[4,7,11] Together, the distribution of *tri3* and *tri7* homologues among trichothecene-producing fungi, comparisons of trichothecene structures produced by these fungi, and phylogenetic relationships of *tri* homologues from these fungi suggest that the 4-*O*-acylation activity of TRI3 represents the ancestral condition, while 15-*O*-acetylation activity represents a derived condition.[4]

Of the six enzyme-encoding *tri* genes in *T. arundinaceum*, the acyltransferase gene, *tri18*, is the only gene whose role in trichothecene biosynthesis has not been determined.[4,7,9−11] Previous studies indicate that *tri18* may not be necessary for biosynthesis of **1** because all catalytic activities required for its formation can be accounted for by activities of enzymes (TRI5, TRI4, TRI22, TRI17, and TRI3) encoded by other *tri* genes (Figure 1).[4,7] Nevertheless, orthologs of *tri18* have been identified in trichothecene-producing species of *Trichoderma, Trichothecium, Myrothecium, Stachybotrys*, and *Spicellum*.[4] In addition, the pattern of expression of *tri18* is similar to that of other *tri* genes in *T. arundinaceum*.[11] Together, these findings indicate that *tri18* is involved in trichothecene biosynthesis. Therefore, the objective of the current study was to determine whether and how *tri18* functions in trichothecene biosynthesis in *T. arundinaceum*.

MATERIAL AND METHODS

Strains and Culture Conditions. *Trichoderma arundinaceum* IBT 40837 (Ta37) (IBT Culture Collection of Fungi, Lyngby, Denmark) was used as the wild-type strain. For heterologous expression experiments we used a previously described *tri3*-deletion mutant of *T. arundinaceum* (strain Δtri3.1)[4] and a previously described *tri3* mutant of *Fusarium sporotrichioides* (strain O2).[14] For complementation and/or heterologous expression experiments, we amplified *T. arundinaceum tri3* (*Ta-tri3*) and/or *tri18* (*Ta-tri18*) homologues from Ta37, *tri18* homologues *Tb-tri18* and *Mr-tri18* from *Trichoderma brevicompactum* IBT 40841 (Tb41) (IBT) and *Myrothecium roridum*

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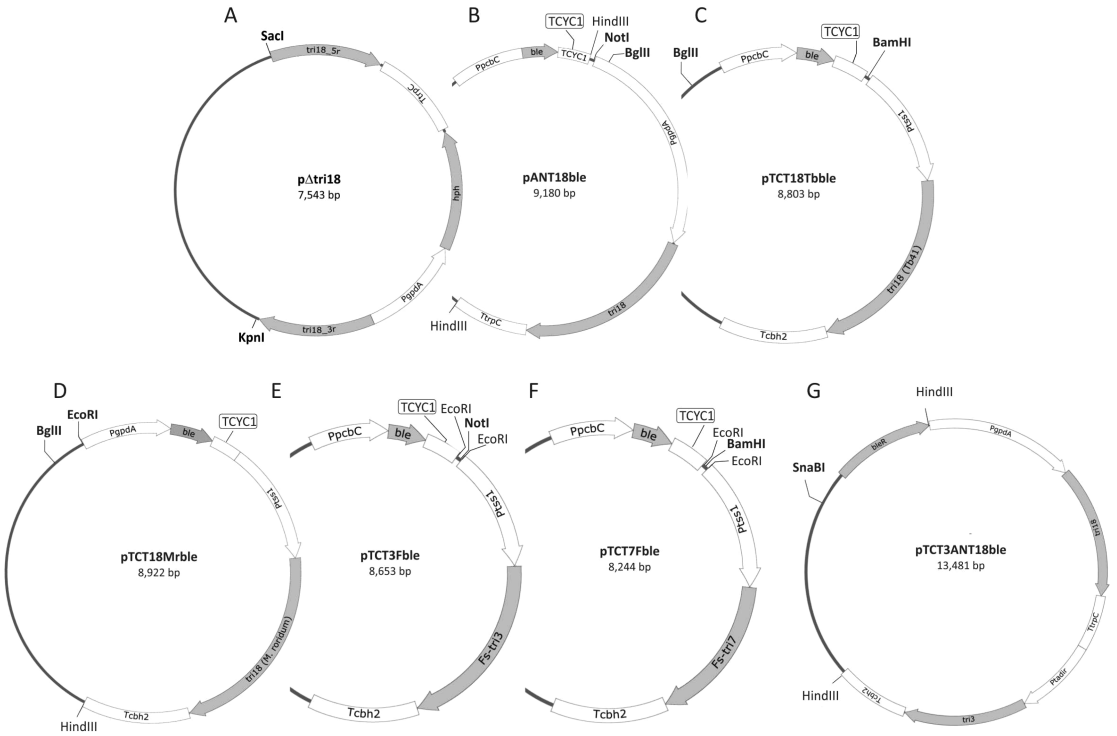

Figure 2. Plasmids designed for the present work: (A) plasmid pΔtri8, (B) pANT18ble, (C) pTCT18Tbble, (D) pTCT18Mrble, (E) pTCT3Fble, (F) pTCT7Fble, and (G) pTCT3ANT18ble. The genetic elements included in these plasmids are tri18, *T. arundinaceum tri18*; tri3, *T. arundinaceum tri3*; tri18 (Tb41), *T. brevicompactum tri18*; tri18 (*M. roridum*), *M. roridum tri18*; and Fs-tri3, *F. sporotrichioides tri3* gene coding region; tri18−5r and tri18−3r indicate the 5′ and 3′ adjacent regions to the *Ta-tri18* gene, respectively; PgpdA indicates the promoter region of the glyceraldehyde-3-phosphate dehydrogenase gene from *Aspergillus nidulans*; hph indicates the *E. coli* hygromycin resistance gene; TtrpC indicates the *A. nidulans trpC* terminator region; PpcbC indicates the promoter region of the *pcbC* gene of *Penicillium chrysogenum*; *ble* indicates the bleomycin/phleomycin resistance gene from *Streptoalloteichus hindustanus*; TCYC1 indicates the terminator region of the *Saccharomyces cerevisiae CYC1* gene; Ptss1 indicates the promoter region of the *T. harzianum tss1* gene; and Tcbh2 indicates the transcriptional terminator of the *T. reesei* cellobiohydrolase 2 encoding gene.

Table 1. Predicted Genes in the *tri17-tri18* Region of the *T. arundinaceum* Genome

gene[a]	protein length (aa)	predicted function[b]	*E*-value and GenBank accession for best BLAST hit[c]
TARUN_5171	637	peptidase	0.0; XP_013950476
TARUN_5172	195	unknown	$2e^{-128}$; XP_013950475.1
TARUN_5173	2424	polyketide synthase	0.0; XP_001910647.1
TARUN_5174	917	oligopeptide transporter	0.0; ETR96766.1
TARUN_5175	418	unknown	$1e^{-178}$; XP_013950472.1
TARUN_5176	527	feruloil esterase	0.0; XP_002151622.1
TARUN_5177 (*tri18*)	554	acetyltransferase	$2e^{-143}$; KFA68874.1
TARUN_5178	403	cytochrome P450 monooxygenase	$1e^{-109}$; XP_018078321.1
TARUN_5179 (*tri17*)	2355	polyketide synthase[c]	0.0; CRG92715.1

[a]Gene names are indicated with the locus tag designations as previously described for *T. arundinaceum* strain IBT 40837.[11] [b]Predicted functions are based on results of BLASTp analyses. [c]The polyketide synthase encoded by *tri17* is predicted to catalyze synthesis of octa-2,4,6-trienoic acid, the precursor of the octa-2,4,6-trienedioyl substituent, **3**, at C4 of harzianum A, **1**.[4]

NRRL 2183 (NRRL, Northern Regional Research Laboratory, Peoria, IL), respectively, and *tri3* (*Fs-tri3*) and *tri7* (*Fs-tri7*) homologues from *Fusarium sporotrichioides* strain NRRL 3299 (NRRL).

Trichoderma strains were grown on potato glucose agar (PPG) medium (2% mashed potatoes, 2% glucose, 2% agar) and incubated at 28 °C in the dark. *F. sporotrichioides* and *M. roridum* were grown on modified cornmeal agar (CMA) medium [5% cornmeal extract, 0.5% potato dextrose broth (PDB) (Difco, Detroit, MI), 2% agar] and incubated in the dark.

To assess production of **1**, 100 mL of complex-malt broth (CM; 0.5% malt extract, 0.5% yeast extract, 0.5% glucose) was inoculated with 2×10^6 spores/mL and incubated for 24 h at 28 °C and 250 rpm on an orbital shaker. Then, 20 mL of the resulting culture was transferred to 100 mL of PDB and incubated on an orbital shaker as described above.[7] After 48 h, mycelia were harvested by filtration through sterile Nytal filters (Sefar Maissa, Barcelona, Spain). Filtrates were stored at −80 °C until chemical analysis. For trichodermin (**5**) feeding experiments, 2×10^6 spores/mL of each analyzed strain were inoculated in 250 mL flasks containing 50 mL of PDB medium, amended with 0.1 mM of **5**, and incubated for 120 h. Then, 5 mL samples were harvested every 24 h from each culture until the end of the experiment and analyzed to determine production of compounds **1**, **4**, and **5**.

To assess production of other trichothecenes as well as other secondary metabolites,[18] each strain was grown on V8 juice agar medium.[19] Two 0.5 cm^2 plugs cut from the resulting cultures were added to 20 mL of YEPD medium (5% g of dextrose, 0.1% peptone, 0.1% yeast extract). Cultures were grown at 28 °C on a rotary shaker at 200 rpm in the dark. After 7 d, cultures were extracted with 8 mL of ethyl acetate. The extracts were dried under a nitrogen stream, and residues were resuspended in 1 mL of ethyl acetate.

Genetic Nomenclature. In this study, all gene and protein designations follow conventions of *Trichoderma* genetic nomenclature.[20,21] That is, genes are designated with three italicized lowercase

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letters and a number (e.g., *tri3*, *tri18*), and proteins are designated with three unitalicized uppercase letters and a number (e.g., TRI3, TRI18).

Plasmid Construction. *tri18 Deletion Plasmid.* Regions adjacent to *T. arundinaceum tri18* were PCR amplified using oligonucleotides T18−3F/T18−3R and T18−5R/T18−5F to obtain the fragments corresponding to the *tri18* 3′- (957 bp) and 5′-flanking (991 bp) regions. The fragments were digested with *Xho*I-*Eco*RV and *Eco*RV-*Sac*I, respectively, gel purified with the GFX gel band purification kit (GE Healthcare, Chicago, IL), and ligated to a *Xho*I-*Sac*I digested pBluescript KS+ vector (Stratagene, La Jolla, CA). The resulting plasmid, pBT18−3r5r was linearized with *Eco*RV, dephosphorylated with alkaline phosphatase, and ligated to a 2710-bp fragment containing the hygromycin resistance cassette (*hph*), which was isolated from pAN7−1[22] by *Ecl*136II-*Hin*dIII digestion and treatment with the Klenow fragment (Thermo Scientific, Waltham, MA). This ligation yielded *tri18* deletion plasmid pΔtri18 (Figure 2A), which was linearized with *Kpn*I and then transformed into wild-type strain Ta37.

pANT18ble, Plasmid with T. arundinaceum tri18. The *Ta-tri18* coding region without the start codon was amplified using Q5 High-Fidelity DNA Polymerase (NE Biolabs, Ipswich, MA), phosphorylated primers T18F and T18R, and genomic DNA of Ta37. The resulting amplicon was gel purified and ligated to plasmid pAN52−1,[22] which was previously digested with *Nco*I, treated with the Klenow fragment (Thermo Scientific), and dephosphorylated with the FastAP enzyme (Thermo Scientific). The resulting plasmid was cut with *Bgl*II and then ligated to the 1537-bp fragment carrying the phleomycin resistance cassette (*ble*), which was isolated from plasmid pJL43[23] by *Bst*XI-*Xho*I digestion and treatment with Klenow. The resulting plasmid, pANT18ble (Figure 2B), was linearized with *Bgl*II and used to transform *T. arundinaceum tri18* deletion mutant strain Δtri18.5.

pTCT18Tbble, T. brevicompactum tri18 Expression Plasmid. A 1930-bp fragment, corresponding to the *Tb-tri18* gene without the start codon, was amplified using Q5 High-Fidelity DNA Polymerase (NE Biolabs), phosphorylated primers Tb41−T18F and Tb41−T18R, and genomic DNA of strain Tb41. The resulting amplicon was cloned into plasmid pTAcbh2,[24] which was previously digested with NcoI, treated with the Klenow, and dephosphorylated. The resulting plasmid was cut with *Eco*RI and ligated with the *ble* cassette, which was prepared as indicated above. The resulting plasmid, pTCT18Tbble (Figure 2C), was linearized with *Bgl*II and used to transform the Δtri18.5 strain.

pTCT18Mrble, M. roridum tri18 Expression Plasmid. The *M. roridum tri18* gene without the start codon (1985 bp) was amplified as indicated above using primers Mr-T18F and Mr-T18R from genomic DNA of *M. roridum* NRRL 2183. The amplicon was cloned into plasmid pTAcbh2[24] following procedures described above. The resulting plasmid was cut with *Eco*RI and ligated with the 1589-bp *ble* cassette, previously isolated from plasmid pJL43b1[25] by digestion with *Ecl*136II-*Hin*dIII and treatment with Klenow. The resulting plasmid, pTCT18Mrble (Figure 2D), was digested with *Bgl*II prior transformation Δtri18.5 strain.

Construction of F. sporotrichioides tri3 and tri7 Expression Plasmids. The *Fs-tri3* and *Fs-tri7* coding regions (without start codon) were amplified from genomic DNA of *F. sporotrichioides* strain NRRL 3299 with primer pairs T3FUSF/T3FUSR and T7FUSN5/T7FUSC3, respectively, using Q5 polymerase (NE Biolabs). The resulting fragments were phosphorylated using polynucleotide kinase (EURx, Gdansk, Poland), gel-purified, and ligated to plasmid pTAcbh2,[24] which was prepared as described above. The resulting plasmids were digested with *Eco*RI, filled in, dephosphorylated, and then ligated to the *ble* cassette, which was prepared from plasmid pJL43[23] as indicated above. The *Fs-tri3* expression plasmid, pTCT3Fble (Figure 2E), and the *Fs-tri7* expression plasmid, pTCT7Fble (Figure 2F), were linearized with *Eco*RI prior to transformation into *T. arundinaceum tri3* and *tri18* deletion mutants.

T. arundinaceum tri3−tri18 Expression Plasmid. Plasmid pANT18ble (Figure 2B) was digested with *Hin*dIII and *Bgl*II and then was treated with Klenow to produce a 4867 bp fragment containing the *Ta-tri18* expression cassette. The fragment was gel purified and ligated to pTCT3ble,[4] which was previously digested with *Eco*RI, filled with Klenow, and dephosphorylated. The resulting plasmid, pTCT3ANT18ble (Figure 2G), included the *T. arundinaceum tri3* coding region fused to the *T. harzianum tss1* promoter region and the *T. arundinaceum tri18* coding region fused to the *A. nidulans gpdA* promoter region.[22,24] The plasmid was linearized with *Sna*BI prior to transformation into protoplasts of the *F. sporotrichioides tri3* deletion mutant.[14]

Fungal Transformation. Protoplasts of *T. arundinaceum* were transformed as previously described.[25−28] Transformants from deletion or complementation procedures were selected on regeneration medium[27] amended with 100 μg/mL hygromycin (Sigma-Aldrich, St. Louis, MO) or 40 μg/mL phleomycin (InvivoGen, Toulouse, France), respectively. Transformants were transferred to potato dextrose agar medium (PDA) (Difco) without antibiotic, and after 2 days, transformants were transferred to tryptic-soy agar medium (Difco) supplemented with 150 μg/mL hygromycin or 40 μg/mL phleomycin. Transformants were then analyzed by Terra PCR (Takara Bio, Kusatsu, Japan).

For deletion of *T. arundinaceum tri18*, transformants were analyzed with PCR primer pairs T18−5/T18−3 to detect *tri18* and Db741/Db742 to detect *hph*. Transformants that yielded the *hph* amplicon but not the *tri18* amplicon were further analyzed to assess the juxtapositions of the 3′ and 5′ flanking regions of *tri18* with *hph*, which were predicted to occur if the *tri18* coding region was replaced by the resistance cassette via double homologous recombination between the *tri18* flanking regions and the deletion plasmid pΔtri18. For the PCR analyses, primer pair T18−3rr/PgpdA-d was used to detect juxtaposition of *hph* and the 3′ flanking region, and primer pair T18−5rr/TtrpC-d was used to detect the juxtaposition of *hph* and the 5′ flanking region.

For complementation of *T. arundinaceum tri18* deletion mutant strain Δtri18.5 with different *tri18* homologues, transformants were analyzed with primer pairs T18F/T18R, Tb41-T18F/Tb41-T18R, and Mr-T18F/Mr-T18R to detect *tri18* homologues from *T. arundinaceum, T. brevicompactum,* and *M. roridum*, respectively. The presence of the *ble* gene was assessed with PCR primer pair Phleo-3/Phleo-4.

For complementation of *T. arundinaceum* mutant strains Δtri18.5 and Δtri3.1 with *Fs-tri3* (plasmid pTCT3Fble), transformants were analyzed with PCR primer pairs T3FUSF/T3FUSR to detect *Fs-tri3* and with Phleo-3/Phleo-4 to detect *ble*. For complementation of the *T. arundinaceum* mutant strain Δtri3.1 with *Fs-tri7* (plasmid pTCT7Fble, Figure 2F), transformants were analyzed with PCR primer pairs T7FUSN5/T7FUSC3 to detect *Fs-tri7* and with Phleo-3/Phleo-4 to detect *ble*.

For complementation of the *F. sporotrichioides* Δ*tri3* mutant strain O2 with *Ta-tri3* and *Ta-tri18*, protoplasts of strain O2 were prepared and transformed as previously described.[27] Transformants were assessed for the presence of plasmids carrying *Ta-tri3* and/or *Ta-tri18* using PCR primer pairs Tarun-T3int3/Tarun-T3int5 to detect *Ta-tri3*,[4] T18-5/T18-3 to detect *Ta-tri18* and Phleo-3/Phleo-4 to detect *ble*.

Analytical Methods. **1** was quantitated by HPLC analysis of ethyl acetate extracts of 48-h liquid PDB cultures as previously described.[7,29] In brief, 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 were applied to a reversed-phase C18 column of 150 mm × 4.6 mm i.d., 4 μm, [octadecyl-silica (ODS)] (YMC Europe GmbH, Dinslaken, Germany), and eluted with a 1 mL/min flux of mobile phase [(60% water plus 0.1% trifluoroacetic acid)/40% acetonitrile] for 50 min to reach 100% of acetonitrile in 30 min, held for 15 min, and then returned to 40% acetonitrile at the 50 min time point. Using these conditions, **1** was eluted at 22.9 min and quantitated by comparison of the peak area with a standard curve.

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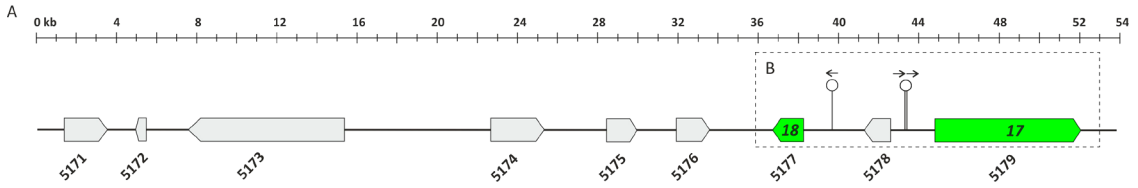

Figure 3. (A) Genomic organization of the *T. arundinaceum* region containing the *tri17* (***17***) and *tri18* (***18***) genes; arrows indicated predicted position and direction of transcription of genes. Numbers below arrows are locus tag numbers assigned to each gene as previously described.[11] A detailed description of each gene is included in Table 1. (B) Positions of the putative TRI6-binding motifs in the promoter regions of *T. arundinaceum tri18* and *tri17* genes, according to the binding sequence described by Hohn and co-workers (TNAGGCC)[36] (white circles). Arrows above the symbols indicate the strand where these motifs were found.

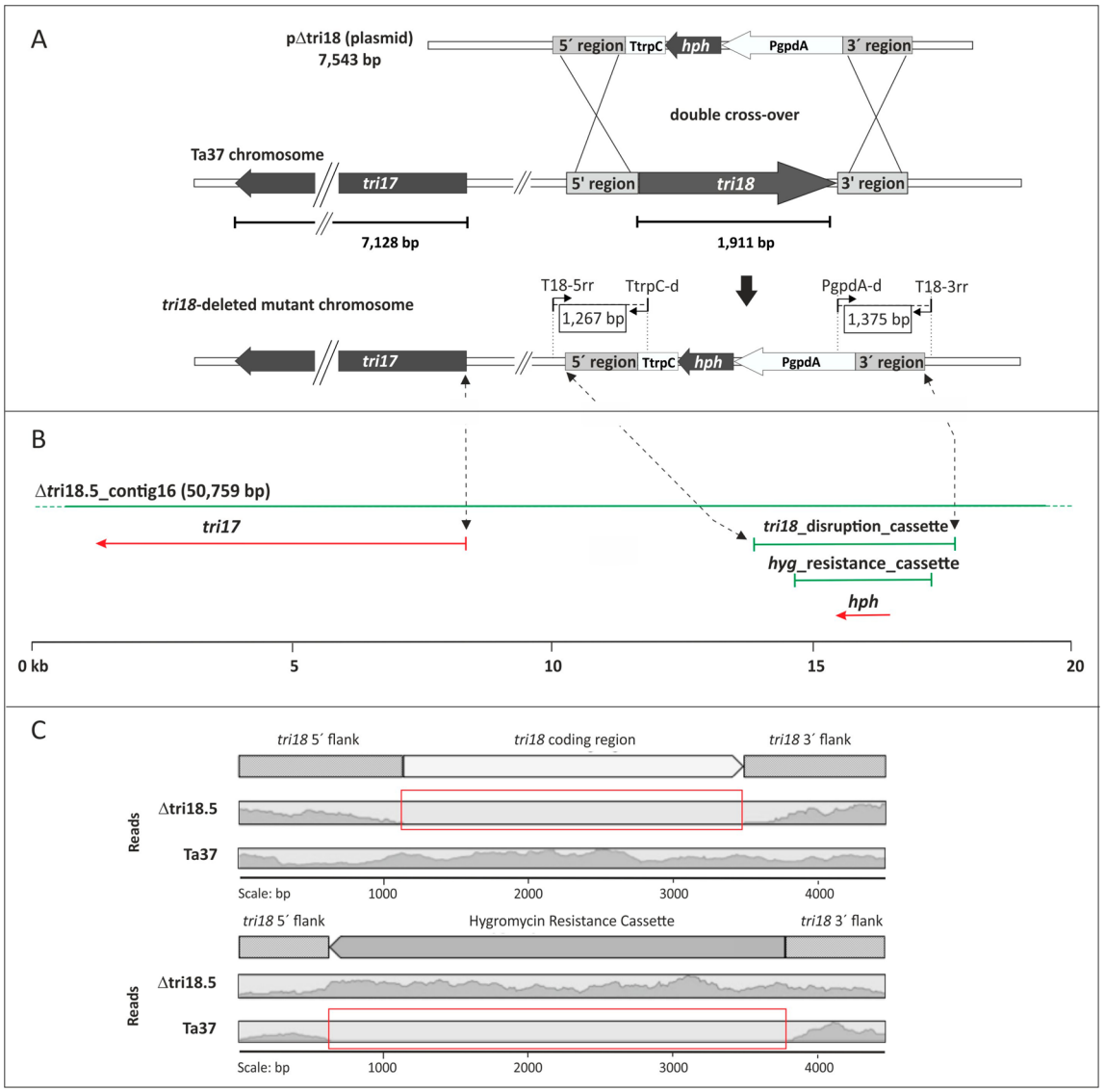

Figure 4. (A) Strategy used for *tri18* deletion in *T. arundinaceum* using plasmid pΔtri18. The regions labeled as 1267 bp and 1375 bp shown in the lower panel of (A) were amplified by PCR and sequenced to confirm *tri18* deletion. (B) Analysis of the *tri17-tri18* genomic region in the *tri18* mutant strain Δtri18.5. (C) Mapping of the genome sequence reads to the wild-type (Ta37) *tri18* region (lower panels) and the *tri18* deletion construct (upper panels). The gray areas indicate the number of reads for a given region of the reference sequence. The higher the gray area, the greater the coverage of that region. The absence of gray in a given region of the reference sequence indicates that no reads cover that region (red rectangles).

For ergosterol and squalene analysis, mycelia obtained by filtration of the PDB cultures used for analysis of **1** were extracted with *n*-heptane. Ergosterol and squalene levels in the extracts were determined by spectrophotometric analysis as previously described.[30,31] Samples were assayed in duplicate, and data were analyzed by the IBM SPSS Statistics 24 software, using analysis of variance with a post-hoc analysis (Tukey test).

Gas chromatography−mass spectrometry (GC-MS) analysis was used to assess production of trichothecene analogs other than **1**. *T. arundinaceum* and *F. sporotrichioides* strains grown for 7 d in 20 mL of

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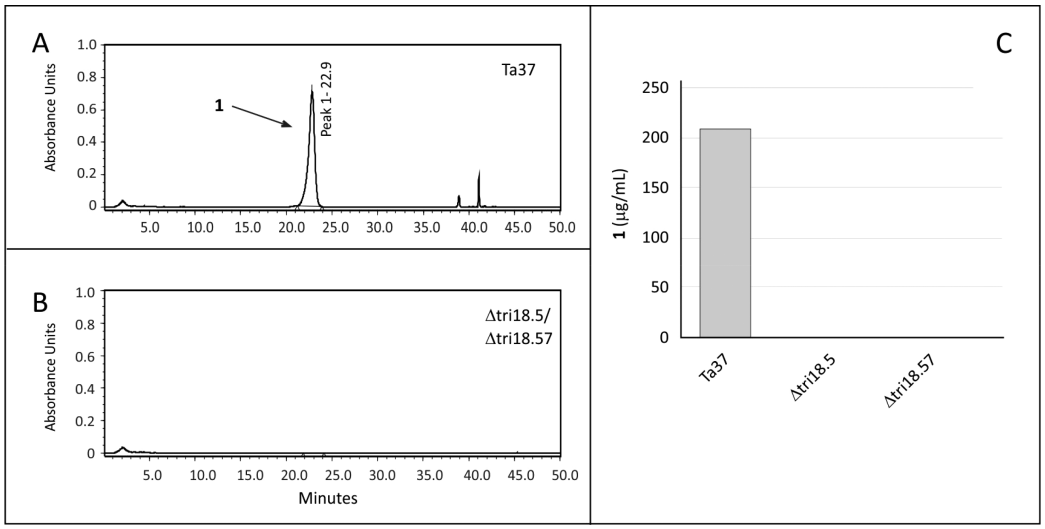

Figure 5. HPLC chromatograms showing levels of harzianum A (**1**) produced by (A) wild-type strain Ta37 and (B) *tri18* deletion mutants Δtri18.5 and Δtri18.57 grown for 48 h in PDB medium. (C) Production of **1** by Ta37 and the two *tri18* mutants.

YEPD were extracted with 8 mL of ethyl acetate. Extracts were dried under a stream of nitrogen, and the residue was resuspended in 1 mL of ethyl acetate. GC-MS analysis of the ethyl acetate extracts was performed on a 6890 Gas Chromatograph and a 5973 mass detector (Agilent, Wilmington DE) as described previously.[11] The column used was 30 m × 0.25 mm i.d., 0.25 μm, (5%-phenyl)-methylpolysiloxane (HP-5MS, Agilent, Wilmington DE). Some strains were also analyzed for production of trichothecenes by liquid chromatography−mass spectrometry (LC-MS) as previously described.[4]

Genome Sequence of Δ*tri18.5* Mutant. Genome sequence data for *tri18* mutant strain Δtri18.5 was obtained using a MiSeq Illumina platform (Illumina, Inc., San Diego, CA). The mutant was grown in YEPD medium for 2 d at room temperature with shaking at 200 rpm. Mycelia were harvested by filtration, lyophilized, and ground to a powder. Genomic DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, CA). A DNA sequencing library was prepared using the Nextera XT DNA library preparation kit (Illumina). Sequence data were processed using CLC Genomics Workbench (Qiagen, Hilden, Germany). Mapping of the genome sequence reads to reference sequences was done using the Read Mapping function in the CLC Genomics Workbench. Genomic sequence reads for Ta37 were generated previously.[4,11]

qPCR Analysis. qPCR primers for *T. arundinaceum tri3*, *tri18*, and the actin gene were as described previously,[32] and their amplification efficiencies were 104%, 105%, and 92%, respectively. RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) from mycelia harvested from a 48-h PDB culture of Ta37. RNA was treated with RNase-free DNase and purified through Zymo-Spin columns (Zymo Research). cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) using a blend of oligo(dT) and random hexamer primers and was quantified using a Nanodrop ND-1000 (Thermo Scientific). qPCR reactions were carried out as described previously[9] using the Step One system (Applied Biosystems, Foster City, CA) and the Express SYBR green qPCR Super-Mix Universal (Invitrogen) following the manufacturer's instructions. The resulting data were analyzed with REST 2009 software[33] to determine expression levels for each gene/condition normalized to amplification efficiencies of each primer pair and to expression of the actin gene. Each measurement was done in triplicate.

Sequence of *T. arundinaceum tri18* cDNA and Accession Numbers. The *tri18* coding region was amplified with Q5 high-fidelity DNA polymerase (NE Biolabs) and primer pair T18F/T18R from cDNA prepared from a 48-h PDB culture of Ta37. DNA sequence of the resulting amplicon was obtained by Sanger sequencing and compared to the genomic sequence of *tri18*, which was retrieved from genome sequence data of Ta37 (GenBank accession no. PXOA00000000).

Proteins deduced from the genes located in the genomic region that contains the *tri17-tri18* locus were annotated with the accession numbers RFU77057 to RFU77065, which correspond to the ORFs TARUN_5171 to TARUN_5179, respectively (Table 1).

RESULTS AND DISCUSSION

In Silico Analysis of *T. arundinaceum tri18*. Analysis of genomic and cDNA sequence data indicated that the *T. arundinaceum tri18* ORF is 1910 bp long and includes 4 introns of 71, 62, 51, and 61 bp. *tri18* is located upstream of *tri17*, in a genomic region where most of the other predicted ORFs are unlikely to be involved in trichothecene biosynthesis, based on their lack of significant homology with previously described *tri* genes (Figure 3A, Table 1). Analysis of its predicted amino acid sequence with HMMTOP software[34] indicated that TRI18 does not include transmembrane domains, and analysis with the SignalP 4.0 server[35] indicated that TRI18 does not include a signal peptide. Analysis of the *tri18*-promoter region led to the detection of a putative binding site for the trichothecene regulatory protein TRI6[36] (Figure 3B). Comparison with other known *tri18* homologues revealed that the DNA sequence identity of the *T. arundinaceum tri18* ranges from 64% for *Spicellum ovalisporum tri18* to 91% for the *T. brevicompactum tri18*,[4] which are similar values to those observed previously when comparing other *T. arundinaceum tri* genes to orthologous *tri* genes from other trichothecene producers.[7]

***tri18* Deletion.** Transformation of wild-type strain Ta37 with the *tri18* deletion plasmid pΔtri18 (Figure 2A) yielded 78 hygromycin resistant transformants. In PCR analyses, only two transformants, strains Δtri18.5 and Δtri18.57, yielded the amplicon pattern expected to result from deletion of *tri18*. That is, neither of the two transformants yielded an amplicon for the *tri18* coding region. Both transformants yielded an amplicon for *hph*; and both transformants yielded the amplicons indicative of the juxtapositions of the *tri18* 5′ and 3′ flanking regions to *hph* (Figure 4A). On the basis of these data, we concluded that the *tri18* coding region was deleted in transformants Δtri18.5 and Δtri18.57.

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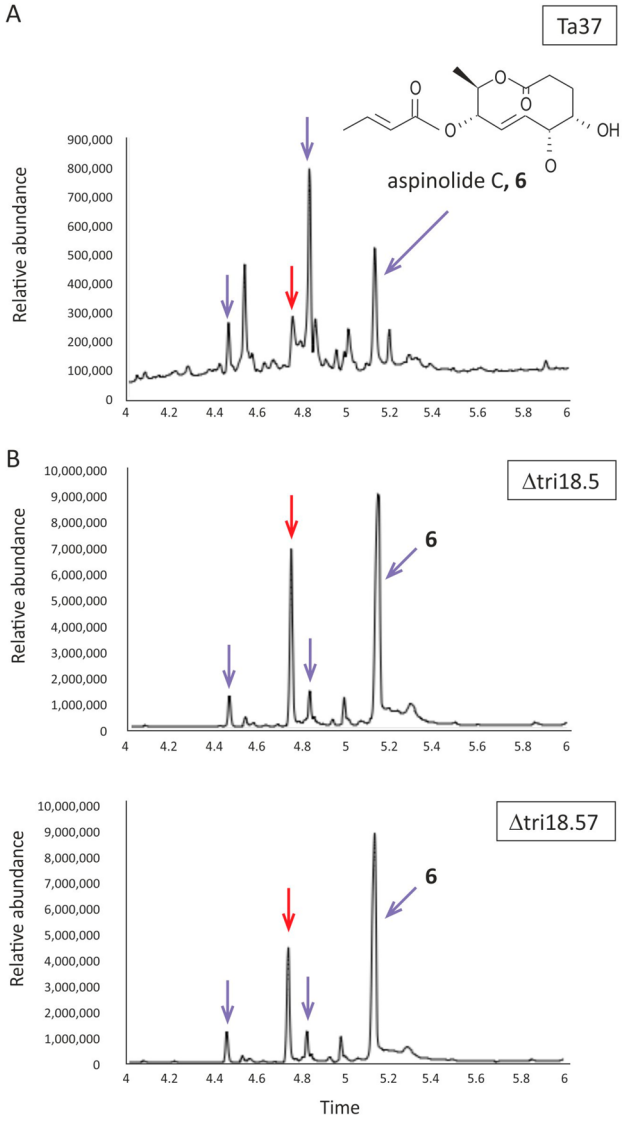

Figure 6. (A) Gas chromatography–mass spectrometry analysis of Ta37 and (B) *tri18* mutants Δtri18.5 and Δtri18.57, grown for 7 d in liquid YEPD medium. Red arrows indicate trichodermol (**4**), and blue arrows indicate aspinolides.[18] Aspinolide C (**6**) had a retention time of approximately 5.2 min. Note, the scale of the *Y*-axis is not the same for panels A and B.

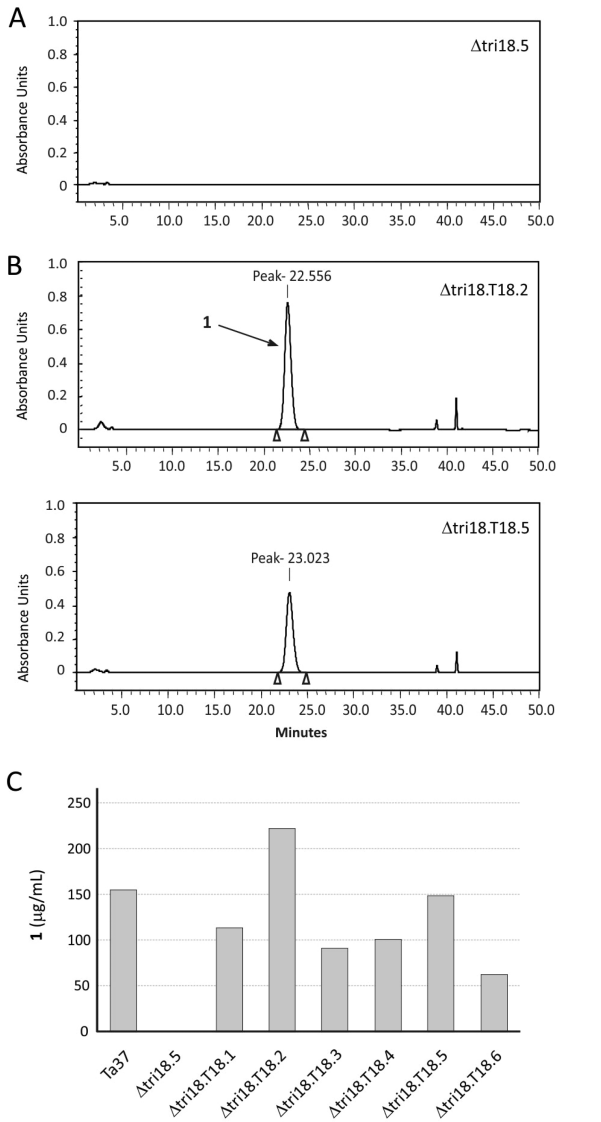

Figure 7. (A) HPLC chromatograms showing production of harzianum A (**1**) by *tri18* mutant strain Δtri18.5 and (B) two *Ta-tri18* complementation transformants Δtri18.T18.2 and Δtri18.T18.5, grown for 48 h in PDB medium. (C) Quantitation of **1** produced by the wild type, Δtri18.5, and six *tri18*-complementation transformants.

Genome sequence analysis indicated that the *tri18* coding region was absent in strain Δtri18.5 and that only one copy of *hph* had integrated into the genome at the *tri18* locus (Figure 4B). In addition, no sequences corresponding to the *tri18*-deletion construct were detected outside the *tri18* locus. Thus, both PCR and genome sequence analyses indicated that strain Δtri18.5 is a *tri18* deletion mutant (hereafter *tri18* mutant) (Figure 4C).

Metabolite Analysis of *tri18* Mutants. HPLC analysis of 48-h PDB cultures revealed that *tri18* mutants did not produce detectable levels of **1** (Figure 5). In contrast, the wild-type progenitor strain produced **1** at 206 μg/mL under the same conditions. GC-MS analysis of YEPD culture extracts indicated

Table 2. Quantitation of Ergosterol and Squalene Production by Ta37 (control) and the *tri18* Mutants Δtri18.5 and Δtri18.57 in Samples from 48-h Cultures Grown in PDB Medium[a]

	dry weight (g)	squalene (mg S/g DW)[c]	percent variation[b]	ergosterol (mg E/g DW)[d]	percent variation[b]
Ta37 48 h	0.13 ± 0	2.39 ± 0.09 a		70.62 ± 1.93 a	
Δtri18.5 48 h	0.13 ± 0	2.26 ± 0.18 a	−5.72%	95.81 ± 2.70 b	+35.67%
Δtri18.57 48 h	0.11 ± 0.02	1.93 ± 0.3 b	−19.30%	88.44 ± 1.87 c	+25.23%

[a] $n = 2$, ANOVA. Tukey test. On each column and time point, values followed by different letters (a, b, and c) are significantly different ($p < 0.05$). [b]Percent of variation compared to the control strain are given. [c]Units of mg S/g DW represent milligrams of squalene per gram of dry weight. [d]Units of mg E/g DW represent milligrams of ergosterol per gram of dry weight.

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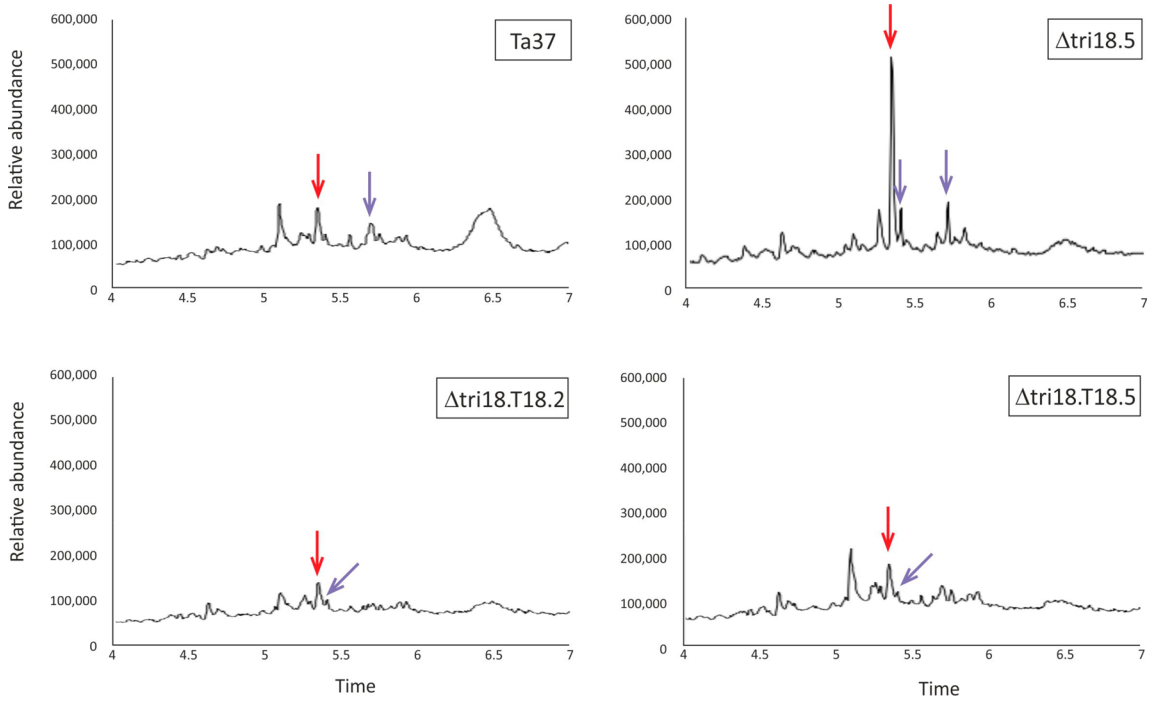
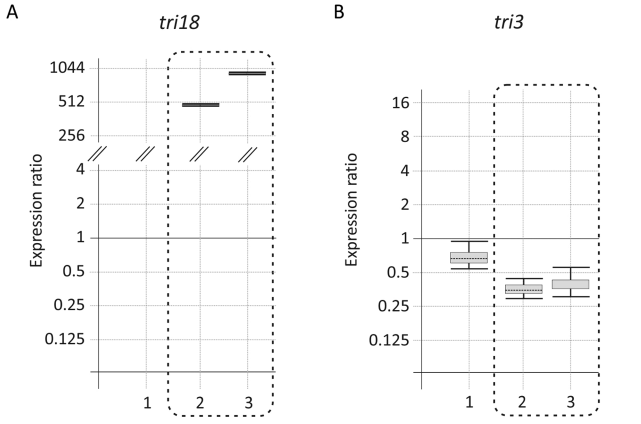


Figure 8. GC-MS analysis of the wild type (Ta37), Δtri18.5 mutant, and *tri18*-complementation transformants Δtri18.T18.2 and Δtri18.T18.5, grown for 7 d in liquid YEPD medium. Red arrows indicate trichodermol (**4**), and blue arrows indicate aspinolides.



Gene	Expression ratio	p(H1)
1. Δtri18.5 *vs* Ta37	nd	-
2. Δtri18.T18.2 *vs* Ta37	504.291*	0.000
3. Δtri18.T18.5 *vs* Ta37	930.348*	0.000

Gene	Expression ratio	p(H1)
1. Δtri18.5 *vs* Ta37	0.641	0.062
2. Δtri18.T18.2 *vs* Ta37	0.348*	0.000
3. Δtri18.T18.5 *vs* Ta37	0.432*	0.000

Figure 9. Analysis by quantitative PCR of (A) *tri18* and (B) *tri3* transcription levels in Δtri18.5 mutant and *tri18*-complementation transformants Δtri18.T18.2 and Δtri18.T18.5 grown for 48 h in PDB medium. Transcription levels are ratios calculated relative to the wild type. Statistically significant values ($p(H1) < 0.05$) are indicated with an asterisk in the lower part of the figure and outlined by a broken line in the graphical representation. nd = not detected.

that *tri18* mutants produced levels of **4** that were higher than those produced by the wild type (Figure 6A, B). These results, together with its location near other *tri* genes,[4,8] and its pattern of expression,[11] indicate that *tri18* is required for biosynthesis of **1**. Furthermore, the absence of **1** and increased levels of **4** produced by the mutants, combined with the fact that the TRI18 protein is predicted to be an acyltransferase, indicate that TRI18 catalyzes esterification of octa-2,4,6-trienedioyl (**3**) to the C4 hydroxyl of **4**. As noted above, previous analyses indicated that TRI3 catalyzes the same trichothecene biosynthetic reaction.[4]

GC-MS analysis also revealed that *tri18* mutants produced levels of the polyketide-derived metabolites aspinolides[18] that were higher than those produced by the wild type (Figure 6A, B). In the mutants, the levels of aspinolide C (**6**) were higher than those of other aspinolide analogs. In addition, analysis of mycelia extracts from PDB cultures indicated that the *tri18* mutants produced ergosterol, a triterpenic sterol that is also synthesized from farnesyl diphosphate, at levels that were 25−36% higher than the levels produced by the wild-type strain. In contrast, the mutants produced the ergosterol biosynthetic intermediate squalene at levels that were slightly less than or within the same range as levels produced by the wild type (Table 2).

The increased levels of ergosterol and aspinolides,[18] especially **6**, produced by *tri18* mutants were similar to those previously reported for some other *tri*-gene deletion mutants of *T. arundinaceum*.[10,11] In the previous studies, increased levels of ergosterol were attributed to an increased intracellular pool of farnesyl diphosphate resulting from the loss of trichothecene production.

Complementation of *tri18* Mutant. We recovered six transformants following transformation of mutant strain Δtri18.5 with the *Ta-tri18* complementation plasmid pANT18ble (Figure 2B). PCR analyses confirmed the presence of both *tri18* and *ble* in all six transformants. HPLC analysis of the transformants indicated that they were restored in their ability to produce **1**, at levels that ranged from 40−143% of the levels produced by the wild type (Figure 7). GC-MS analysis of YEPD culture extracts revealed that the levels of **4** produced by complementation transformants Δtri18.T18.2 and Δtri18.T18.5 were markedly lower than levels produced by *tri18* mutant strain Δtri18.5 but similar to levels produced by

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Table 3. Complementation of *T. arundinaceum* (*Ta*) and *F. sporotrichioides* (*Fs*) *tri* Gene-Deletion Mutants

recipient deletion mutant	complementing DNA/plasmid	major trichothecene produced	successful complementation[a]
Ta-Δ*tri18*	none	**4**	NA
	Ta-tri18/pANT18ble	**1**	+
	Tb-tri18[b]/pTCT18Tbble	**1**	+
	Mr-tri18[c]/pTCT18Mrble	**1**	+
	Fs-tri3/pTCT3Fble	**4**	−
Ta-Δ*tri3*	none	**4**	NA
	Ta-tri3/pTCT3ble	**1**	+[d]
	Fs-tri3/pTCT3Fble	**1**	+
	Fs-tri7/pTCT7Fble	**4**	−
Fs-Δ*tri3*[14]	none	15-decalonectrin[14]	NA
	Ta-tri3/pTCT3ble	15-decalonectrin	−
	Ta-tri18/pANT18ble	15-decalonectrin	−
	Ta-tri3 + *Ta-tri18*/pTCT3ANT18ble	15-decalonectrin	−

[a]"+" indicates that the complementing DNA changed trichothecene production of the recipient. "−" indicates that the complementing DNA did not change trichothecene production in the recipient. NA indicates not applicable, because the recipient strain did not have any complementing DNA. [b]*Tb-tri18* = *Trichoderma brevicompactum tri18* gene. [c]*Mr-tri18* = *Myrothecium roridum tri18* gene. [d]Previously reported result.[4]

the wild type (Figure 8). These results confirmed that the altered trichothecene production phenotype of *tri18* mutants was caused by deletion of *tri18*.

In the current study, results from analyses of *tri18* mutants and complementation transformants confirm that *tri18* is involved in trichothecene biosynthesis, but the results were also surprising because the phenotype of *tri18* mutants was almost the same as previously reported for *tri3* mutants. That is, both *tri3* and *tri18* mutants were blocked in production of **1** and instead accumulated **4**. The only notable phenotypic difference was that *tri3* mutants produced **1** at 6−8% of wild-type levels,[4] while *tri18* mutants produced no detectable **1**. The similar phenotypes of *tri3* and *tri18* mutants indicate that both genes, *tri3* and *tri18*, are required for conversion of **4** to **1**.

Furthermore, these findings raised the question of whether *tri3* and *tri18* can affect each others' expression. To address this, we used qPCR analysis to measure *tri3* expression in the wild type, *tri18* mutant strain Δtri18.5, and the complementation transformants strains Δtri18.T18.2 and Δtri18.T18.5. The results of the analysis indicated that *tri18* deletion did not significantly affect *tri3* gene expression. However, complementation of the *tri18* mutant by the method employed here significantly reduced *tri3* expression. qPCR data also showed that the *tri18* gene was expressed at a high level in the two complemented transformants analyzed (Figure 9).

Heterologous Complementation. To date, all trichothecene-producing species of *Myrothecium*, *Spicellum*, *Stachybotrys*, *Trichothecium*, and *Trichoderma* that have been examined have homologues of *tri18*.[4,8] All these fungi produce trichothecenes with a C4 acyl group that is two, four, six, or eight carbons long. It is not known, however, whether TRI18 has 4-*O*-acylation activity in these other fungi. To begin to address this issue, we attempted to complement *tri18* mutants of *T. arundinaceum* with *tri* homologues from *T. brevicompactum* and *M. roridum*. Transformation of *T. arundinaceum tri18* mutant strain Δtri18.5 with plasmids carrying either *Tb-tri18* (pTCT18Tbble) (Figure 2C) or *Mr-tri18* (pTCT18Mrble) (Figure 2D) restored production of **1**, although the levels produced were only 10−39% of wild-type levels and 14−56% of the levels produced by *tri18*-complementation transformant Δtri18.T18.2 (Table 3, Figure 7C). This result indicates that the *T. brevicompactum* and *M. roridum tri18* homologues function in a manner similar to the *T. arundinaceum* homologue. However, the low levels of **1** produced as a result of these heterologous complementations suggests that TRI18 homologues from the other fungi do not function as efficiently as the *T. arundinaceum* homologue.

In a second experiment, *T. arundinaceum tri3* mutant strain Δtri3.1 was transformed with a plasmid (pTCT3Fble) carrying *Fs-tri3* (Figure 2E). All six transformants recovered in this experiment produced **1** (Table 3) but at levels that were only 0.5−32% of wild-type levels (Figure 10). However, the levels of **1** produced by four of the transformants (Δtri3.T3F.1, .3, .6, and .7) were significantly higher than levels produced by the *tri3* mutant. These results indicate that the *F. sporotrichioides tri3* homologue can partially complement the block in trichothecene 4-*O*-acylation in the *T. arundinaceum tri3* mutant. This result is intriguing because the *F. sporotrichioides* TRI3 and *T. arundinaceum* TRI3 have different substrates and they affect different positions of **2**.[4,14] Previous analyses suggest that this 4-*O*-acylation activity is the ancestral condition of TRI3, while trichothecene 15-*O*-acylation exhibited by *Fusarium* TRI3 homologues is a derived condition.[4] If this is the case, the results of the *Fs-tri3* complementation study reported here indicate that *Fusarium* TRI3 has retained low levels of the ancestral TRI3 activity. These data are consistent with the previously reported finding that the *F. graminearum* TRI3 homologue has a low level of trichothecene-4-*O*-acylation activity.[37]

In three other experiments, heterologous expression of a *tri* gene did not result in complementation of *tri*-gene deletion mutants. Thus, transformation of the *T. arundinaceum* Δ*tri18* mutant strain Δtri18.5 with plasmids carrying the *Fs-tri3* (pTCT3Fble) (Figure 2E) or *Fs-tri7* (pTCT7Fble) (Figure 2F) did not restore production of **1** (Table 3). In addition, transformation of *F. sporotrichioides tri3* mutant (strain O2)[14] with the *Ta-tri3* (pTCT3ble),[4] *Ta-tri18* (pANT18ble) (Figure 2B), or both *Ta-tri3* and *Ta-tri18* (pTCT3ANT18ble) (Figure 2G) did not alter the trichothecene production phenotype of the mutant (Table 3).

Precursor Feeding with Trichodermin. A possible explanation for the requirement of both *tri3* and *tri18* in conversion of trichodermol (**4**) to harzianum A (**1**) is that TRI3 and TRI18 catalyze sequential esterification reactions: 4-*O*-acetylation of **4** to form trichodermin (**5**), and subsequently, replacement of the resulting acetyl group with octa-2,4,6-

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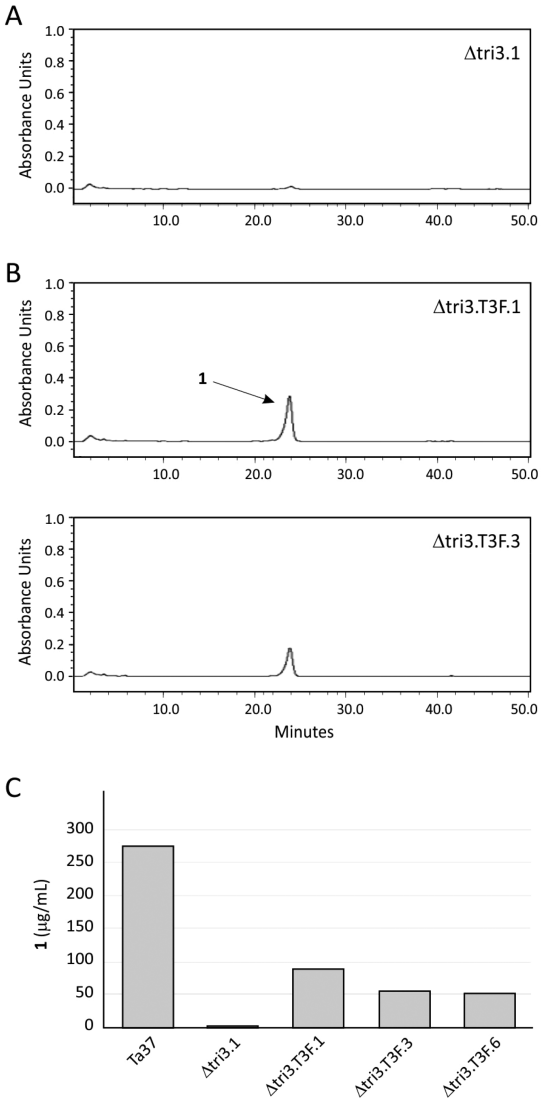

Figure 10. HPLC chromatograms showing harzianum A (**1**) production in 48-h PDB grown cultures by (A) the *tri3* mutant Δtri3.1 and (B) Δtri3.1 carrying the *F. sporotrichioides tri3* homologue (strains Δtri3.T3F1 and Δtri3.T3F3). (C) Quantitation of **1** production in the wild type (Ta37), the mutant Δtri3.1, and three transformants of Δtri3.1 carrying *F. sporotrichioides tri3*.

trienedioyl (**3**) to form **1**. To test this hypothesis, we did precursor feeding experiments in which we added **5** to cultures of either the *tri3* mutant strain Δtri3.1 or the *tri18* mutant strain Δtri18.5. HPLC analysis indicated that if **5** is not added to cultures the *tri3* mutant produced low levels of **1**, as previously described.[4] Addition of **5** to cultures of the *tri3* mutant resulted in increased production of **1**, indicating that the mutant could convert **5** to **1**. In contrast, addition of **5** to cultures of the *tri18* mutant did not result in production of detectable levels of **1**, indicating that the *tri18* mutant could not convert **5** to **1**. The feeding experiments with **5** were done using two different growth media (PDB and YEPD), and the experiment with each medium was done twice. The results were the same in all experiments. In addition, in the experiments with YEPD, trichothecenes were analyzed using a combination of GC-MS and LC-MS as described in the Materials and Methods (Figure 11).

The interpretation of the results of the **5** feeding experiments were complicated by the presence of an esterase activity in *T. arundinaceum* that can remove the C4-acetyl group of **5** to form **4**. This activity was evident in feeding experiments with both the *tri3* and *tri18* mutants. **5** added to cultures of the *tri3* mutant disappeared as the concentration of **1**, as well as **4**, increased (Figure 11B). Likewise, **5** added to cultures of the *tri18* mutant disappeared as the concentration of **4** increased. The levels of **4** produced accounted for the amount of **5** added to cultures and for the levels produced by the mutant when **5** was not added to the cultures. Esterase activities that remove the acetyl group from the C4 position of trichothecenes have been reported in two *Fusarium* species, namely, *F. sporotrichioides* and *F. verticillioides*.[38,39] Given the presence of this activity in both *T. arundinaceum* and two *Fusarium* species, it is possible that such activity occurs widely in fungi in a manner similar to trichothecene 3-*O*-acetylation activity.[40] The esterase activity in *T. arundinaceum* indicates that the **4** production phenotype of the *tri3* and *tri18* mutants may have different causes in the absence of exogenously added **5**. In *tri3* mutants, **4** likely accumulates because the trichothecene biosynthetic pathway is blocked at **4** in the absence of 4-*O*-acetyltranferase activity. By contrast, *tri18* mutants have the 4-*O*-acetyltranferase activity and, therefore, can convert **4** to **5**. However, because *tri18* mutants lack octa-2,4,6-trienedioyl transferase activity, the esterase activity likely coverts **5** back to **4**.

Despite the esterase activity in *T. arundinaceum*, the results of the feeding experiments with **5** as described above are consistent with the hypothesis that the conversion of **4** to **1** is a two-step process that involves **5** as an intermediate. According to this hypothesis, TRI3 catalyzes 4-*O*-acetylation of **4** to form **5**, and then, TRI18 catalyzes replacement of the resulting C4 acetyl group with octa-2,4,6-trienedioyl to form **1**. If the hypothesis is correct, we rationalize that *tri3* mutants produce low levels of **1** because they have a functional TRI18 that can use **4** as substrate to form **1**, but it does so much less efficiently than when it uses **5** as a substrate.

Concluding Statements. In a previous study, gene deletion and complementation analyses indicated that TRI3 was responsible for trichothecene 4-*O*-acylation during conversion of **4** to **1**. On the basis of the results of the previous study, the conversion was proposed to be a single-step process in which TRI3 catalyzed esterification of octa-2,4,6-trienedioyl to the C4 hydroxyl of **4**. However, results from the current study provide evidence that the conversion is a two-step process in which TRI3 catalyzes 4-*O*-acetylation, transforming **4** to **5**, and then TRI18 catalyzes replacement of the acetyl group with octa-2,4,6-trienedioyl. This two-step conversion process constitutes a heretofore unrecognized complexity in trichothecene biosynthesis. Furthermore, the two-step process explains why there are two acyltransferase-encoding genes (i.e., *tri3* and *tri18*) in the trichothecene biosynthetic gene clusters of *T. arundinaceum*, *Spicellum roseum*, and *Trichothecium roseum* even though trichothecenes produced by these fungi have, at most, only one acyl group.[4]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b05564.

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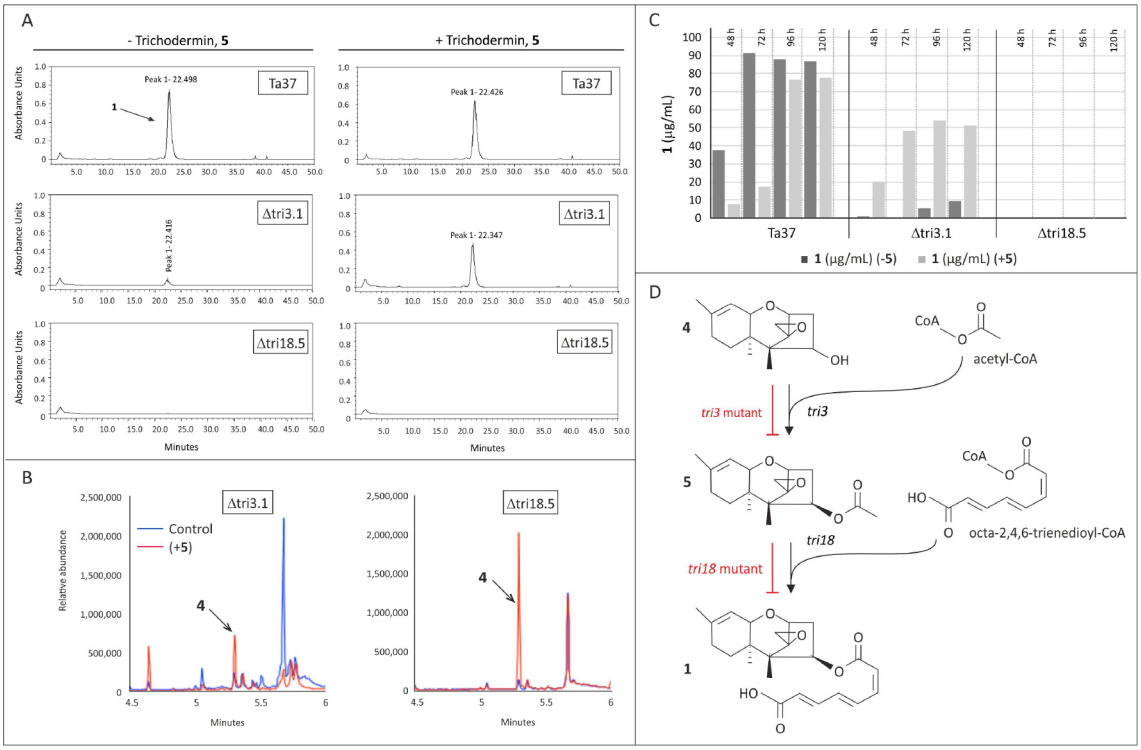

Figure 11. (A) HPLC chromatograms showing production of **1** by wild-type strain Ta37, *tri3* mutant strain Δtri3.1, and *tri18* mutant strain Δtri18.5 grown for 96 h in PDB medium with and without addition of **5**. The elution time for **1** ranged from 22.3 to 22.5 min. (B) GC-MS chromatograms of strains Δtri3.1 and Δtri18.5 after 96 h in YEPD cultures with and without addition of **5**. (C) Quantitation of **1** production by the wild type (Ta37), Δtri3.1, and Δtri18.5 grown for 48 to 120 h in PDB medium with and without **5**. (D) Pathway showing the putative sequential activity of the acyltransferase enzymes TRI3 and TRI18 during the conversion of **4** to **1**. The symbol ⊥ indicates that the pathway is partially or completely blocked at the step indicated in the *tri3* and *tri18* mutants.

Primer list, *tri18* intron sequences, PCR analysis of complemented transformants with *T. arundinaceum tri18* gene, heterologous complementation studies, and summary of trichothecene pathways of *Fusarium* and *Trichoderma*, including the results of the heterologous expression studies performed in the present work (PDF)

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Notes

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