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Molecular Genetic Characterization of a Cluster in *A. terreus* for Biosynthesis of the Meroterpenoid Terretonin

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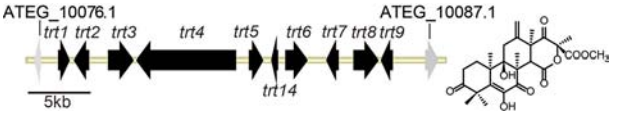
ABSTRACT



Meroterpenoids are natural products produced from polyketide and terpenoid precursors. A gene targeting system for *A. terreus* NIH2624 was developed, and a gene cluster for terretonin biosynthesis was characterized. The intermediates and shunt products were isolated from the mutant strains, and a pathway for terretonin biosynthesis is proposed. Analysis of two meroterpenoid pathways corresponding to terretonin in *A. terreus* and austinol in *A. nidulans* reveals that they are closely related evolutionarily.

Filamentous fungi are known to produce a wide variety of secondary metabolites. Genome sequencing of members of the genus *Aspergillus* revealed that there are more secondary metabolite gene clusters than known secondary metabolites, suggesting that more secondary metabolites could be discovered from these organisms. These metabolites display a broad spectrum of biological activity. One example is lovastatin from *Aspergillus terreus*, which became the first cholesterol-lowering drug of its class approved for human use in the United States.[1]

Terretonin, a mycotoxin identified from *A. terreus*, belongs to a structurally complex class of natural products called meroterpenoids (Figure 1).[2] Pioneering work by Simpson and Vederas in the 1980s using labeled precursors demonstrated that terretonin is produced by both polyketide and terpenoid biosynthetic pathways.[3−5]

Recently, the function of the polyketide synthase (PKS) gene, the prenyltransferase (PT) gene, and the epoxidase gene involved in terretonin biosynthesis were ascertained via expressing the above genes in *A. oryzae*.[6] Whereas the biosynthetic genes for terretonin are clustered in one discrete unit, we identified two separate clusters required for the formation of the meroterpenoid austinol in *A. nidulans*,

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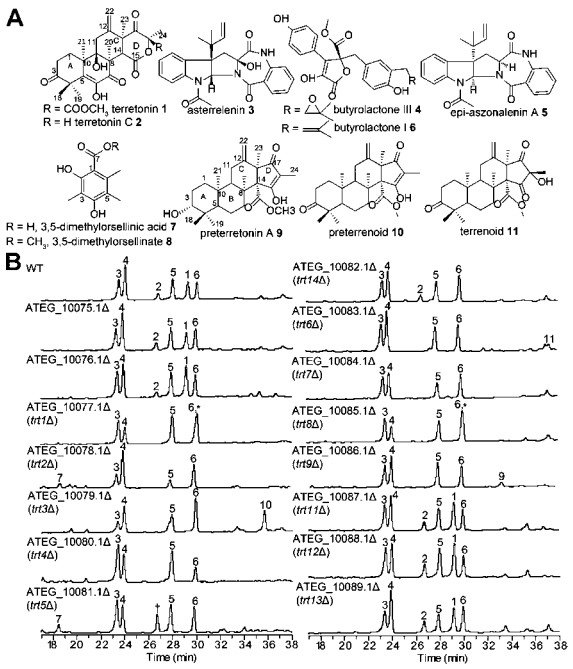

Figure 1. (A) Natural products isolated from this study. (B) HPLC profile of extracts of strains in the cluster as detected by UV at total scan. *This compound coelutes with **6** and decomposes to **8** upon isolation. †This compound decomposes to **7** upon isolation.

one containing four genes including the PKS *ausA*, and the other containing 10 additional genes including the PT gene *ausN*.[7] This case represents one of the few examples in fungi in which more than one cluster is responsible for the biosynthesis of a particular natural product.[7−9]

Herein we present a bioinformatic analysis of *A. terreus* NIH2624 and identified a putative gene cluster for terretonin biosynthesis. We identified six secondary metabolites from *A. terreus* NIH2624 including terretonin (**1**),[10] terretonin C (**2**),[11] asterrelenin (**3**),[11] butyrolactone III (**4**),[12] epi-aszonalenin A (**5**),[13] and butyrolactone I (**6**)[14] [Figure 1; NMR data available in Supporting Information (SI)]. We identified 31 PKS genes in *A. terreus*[15] and narrowed the search to the nine nonreducing PKSs (NR-PKS) that produce the aromatic polyketides.[16] Since secondary metabolite genes in Aspergilli are often clustered,[17] we examined genes surrounding these nine NR-PKS genes to locate an NR-PKS that is close to a PT gene. This bioinformatic analysis indicated that the NR-PKS ATEG_10080.1 and the adjacent putative PT ATEG_10078.1 are most likely involved in terretonin biosynthesis. To confirm their involvement, we selected these genes for deletion experiments.

We then developed a transformation system based on methods developed for *A. niger*.[18] A knock out cassette was constructed using a fusion PCR approach,[19] and ATEG_10080.1 and ATEG_10078.1 were replaced with the *hph* marker via homologous recombination. Indeed, only the production of terretonin (**1**) and terretonin C (**2**) was eliminated in ATEG_10080.1 and ATEG_10078.1 deletant strains (Figure 1). Only 3,5-dimethylorsellinic acid (DMOA, **7**), which is the polyketide precursor in terretonin biosynthesis, accumulated in ATEG_10078.1Δ (Figure 1). Our results are consistent with previous results shown by Itoh et al.[6] For consistency we have used the same gene nomenclature as set forth by Itoh et al. and labeled ATEG_10080.1 and ATEG_10078.1 as *trt4* and *trt2*, respectively (Table 1).

Table 1. *Trt* Gene Cluster and Gene Function Prediction[a]

Gene ATEG_100XX.1	BLASTP homologs	Putative function
77 (*trt1*)	AN9257.4 (*ausL*)	Terpene cyclase
78 (*trt2*)	AN9259.4 (*ausN*)	Aromatic prenyltransferase
79 (*trt3*)	AN8379.4 (*ausB*) AN8381.4 (*ausC*)	Monooxygenase
80 (*trt4*)	AN8383.4 (*ausA*)	Polyketide synthase
81 (*trt5*)	AN8384.4 (*ausD*)	Methyltransferase
82 (*trt14*)	AN9252.4 AN9247.4 (*ausF*) AN11214.4 (*ausJ*) AN9249.4 (*ausH*)	Hypothetical protein
83 (*trt6*)	AN9248.4 (*ausG*) AN9251.4 AN9253.4 (*ausI*)	Cytochrome P450 monooxygenase
84 (*trt7*)	AN9246.4 (*ausE*)	Phytanoyl-CoA dioxygenase
85 (*trt8*)	AN11206.4 (*ausM*) AN8378.4	Epoxidase
86 (*trt9*)		Short chain dehydrogenase
87 (*trt11*)		Not involved
88 (*trt12*)		Not involved
89 (*trt13*)		Not involved

[a] The protein sequence similarity between genes in the *aus* cluster and the corresponding genes in the *trt* cluster is at least 50%.

To explicitly characterize this cluster, an additional 13 genes from ATEG_10075.1 to ATEG_10089.1 that are in

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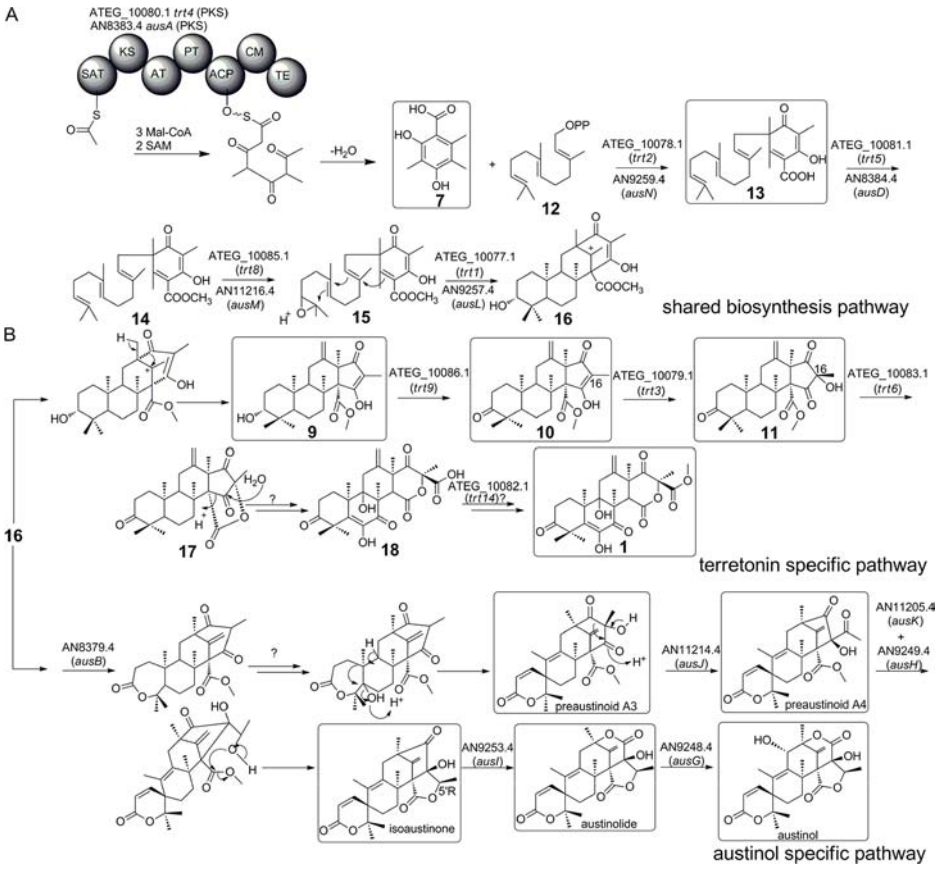

Figure 2. Comparison of terretonin and austinol biosynthetic pathways. The pathway shown in A is assumed to be shared by both terretonin and austinol biosynthesis. The double arrows indicate multiple proteins are involved in this conversion. Boxed compounds are the natural products that have been isolated in this or a previous study.[6,7,20]

proximity to *trt4* were individually deleted. Examination of the LC/MS profiles revealed that ATEG_10077.1Δ (*trt1*Δ), ATEG_10079.1Δ (*trt3*Δ), ATEG_10081.1Δ (*trt5*Δ), ATEG_10082.1Δ (*trt14*Δ), ATEG_10083.1Δ (*trt6*Δ), ATEG_10084.1Δ (*trt7*Δ), ATEG_10085.1Δ (*trt8*Δ), and ATEG_10086.1Δ (*trt9*Δ) impaired the production of either terretonin (**1**) or terretonin C (**2**) or both (Figure 1). ATEG_10082.1 was not included in the *trt* cluster predicted by Itoh et al.[6] Since this gene was shown to be involved, we labeled it as *trt14*. ATEG_10087.1 (*trt11*), ATEG_10088.1 (*trt12*), and ATEG_10089.1 (*trt13*) were predicted by Itoh et al. to be involved although no experimental data were shown to verify the prediction.[6] Terretonins (**1** and **2**) were produced in the *trt11*Δ, *trt12*Δ, and *trt13*Δ mutant strains we generated (Figure 1), indicating that these genes are not involved and they define one end of the *trt* cluster. The other end of the *trt* cluster is deciphered by examining the metabolite profiles of the mutants ATEG_10075.1Δ and ATEG_10076.1Δ that continue to produce terretonins (**1** and **2**, Figure 1).

From the *trt3*Δ, *trt5*Δ, *trt6*Δ, *trt8*Δ, and *trt9*Δ strains, the intermediates were purified by semipreparative HPLC. The intermediate from the *trt5*Δ strain decomposed to compound **7** (Figure 1). Its molecular formula was $C_{25}H_{38}O_6$ (*m*/*z* calcd for $C_{25}H_{39}O_6$ $[M + H]^+$: 435.2741; found: 435.2747) which is identical to dihydroxyfarnesyl-DMOA, a shunt product in terretonin biosynthesis. This natural product was identified by Itoh et al. when *trt4*, *trt2*, and *trt8* were coexpressed in *A. oryzae*.[6] The *trt8*Δ strain accumulated one new metabolite that can also be identified in *trt1*Δ. However, it decomposed to compound **8** (Figure 1, NMR data shown in SI) during purification. The molecular formula of the unstable precursor was $C_{26}H_{40}O_6$ (*m*/*z* calcd for $C_{26}H_{41}O_6$ $[M + H]^+$: 449.2898; found: 449.2908). This molecule contains one more CH_2 fragment compared to the unstable intermediate from *trt5*Δ, probably because the carboxylic acid group in the intermediate from *trt5*Δ has not been methylated. Itoh et al. also noticed the decomposition of **13** (Figure 2) back to **7** upon long exposure to solvents, indicating that **13** is less thermodynamically stable than **7**. This may explain why the unstable intermediates decomposed to **7** or **8** in our study.[6]

From the *trt9*Δ, *trt3*Δ, and *trt6*Δ deletant strains, three tetracyclic intermediates **9**, **10**, and **11** were isolated. Comparison of the ^1H and ^{13}C NMR spectra with the published data revealed that **9** is a known compound preterretonin A.[20] The structures of two new natural products **10** and **11** were determined by 1D and 2D NMR

spectroscopy (Figure 1). Comparison of ^{13}C NMR and HMBC spectra of both **9** and **10** revealed that a secondary hydroxyl carbon C3, which correlates with the C18 and C19 methyl groups in the HMBC spectrum of **9**, is oxidized to a carbonyl group in **10** (Tables S3 and S4). Compound **11** has a similar backbone compared to **10**, only the D ring partial structure of **11** is different from that of **10** (Figure 1). We named **10** and **11** as preterrenoid and terrenoid, respectively.

Elucidation of the above intermediates in their respective mutant backgrounds enabled us to propose a biosynthetic pathway for terretonin (Figure 2). The first step of the pathway is the production of DMOA (**7**) by NR-PKS Trt4. The subsequent step is the prenylation of **7** catalyzed by the PT Trt2. Compound **7** (but no terretonins or any other intermediates) was identified from the *trt2*Δ strain (Figure 1). Coexpressing *trt2* and *trt4* in *A. oryzae* allowed Itoh et al. to isolate the prenylated intermediate **13**.[6] Thus, combination of the above two pieces of data provides solid evidence for the prenylation step in terretonin biosynthesis.

The prenylated precursor **13** is then modified via methylation by Trt5 to yield **14**. Trt5 possesses a conserved methyltransferase domain, and its sequence is 77% identical to AusD (Table 1). However, the function of AusD was not specified, because no UV-active intermediates were identified from the *ausD*Δ strain.[7] Modifications of precursor **14** include the epoxidation by Trt8 to **15** and cyclization by Trt1 to give the tetracyclic intermediate **16** (Figure 2). Only **7** accumulated in the *trt2*Δ and *trt5*Δ strains (Figure 1), suggesting that the methylation of **7** occurs after prenylation. Compound **8** was purified from the decomposition of an unstable intermediate identified in the *trt1*Δ and *trt8*Δ strains (Figure 1), indicating that the carboxylic acid group in **13** has been esterified before epoxidation and cyclization (Figure 2). Our speculation is also in accord with a recent study in which researchers showed that methylation of precursor **13** is an essential step for cyclization of **15** to **16**.[20]

Gene deletion experiments allowed us to identify several genes involved in the formation of terretonin (**1**) via intermediate **16** (Figure 2). Previous labeling studies suggest that the modifications of **16** involve an acyl shift to generate the olefinic moiety at C22, followed by hydroxylation and intramolecular lactonization to yield a terretonin precursor **17** (Figure 2).[5] Our study suggests that three genes, *trt9*, *trt3*, and *trt6*, are involved in this process. A BLAST search revealed that Trt9 belongs to the short chain dehydrogenase family (Table 1). Given that the 3-hydroxyl carbon in **9** is oxidized to a carbonyl in **10**, this indicates that *trt9* codes for a dehydrogenase that converts **9** to **10** (Figure 2). For Trt3, deletion of *trt3* accumulates **10**, indicating that *trt3* is required for the C-hydroxylation at C16 of **10** to yield **11** (Figure 2). We isolated **11** from the *trt6*Δ strain. We deduce that Trt6 is involved in converting **11** to **17** (Figure 2). The protein sequence of Trt6 is 57% similar to that of SmP450-2, a P450 monooxygenase that mediates the lactone formation of GA9 and GA4 in gibberellins biosynthesis in *Sphaceloma manihoticola*.[21]

We were able to identify terretonin C (**2**) but not terretonin (**1**) from the *trt14*Δ mutant. This implies that Trt14 is likely to be involved in the transformation of **18** to terretonin (**1**), and removal of *trt14* may accumulate **18** that converts into terretonin C (**2**) via spontaneous decarboxylation (Figures 1 and 2). Finally the *trt7*Δ mutant is unable to produce either terretonin (**1**) or terretonin C (**2**). A homology search reveals that the amino acid sequence of Trt7 has a conserved phytanoyl-CoA dioxygenase domain (Table 1). The phytanoyl-CoA dioxygenase catalyzes the initial α-hydroxylation of phytanoyl-CoA and converts it into 2-hydroxylphytanoyl-CoA.[22] The function of its homologue suggests that Trt7 might be involved in the conversion from precursor **17** to **18**, but elucidation of its specific function requires further examination. In addition, genes in different loci in *A. terreus* may be involved in this conversion.

In our study, we characterized one compact cluster for terretonin biosynthesis in *A. terreus*, and protein homology analysis indicates that this cluster is closely related evolutionarily to the two austinol clusters in *A. nidulans* (Table 1). In our previous work, we identified a sequence that was located between AN11205.4 (*ausK*) and AN9256.4 (nucleotides 76655 to 77031 on linkage group VIII) which possesses a high nucleotide identity with a portion in the SAT domain of AN8383.4 (*ausA*) ($P = 6.7 \times 10^{-44}$) and proposed that the two austinol clusters have originated from a single contiguous one.[7] In this work, characterization of the *trt* cluster in *A. terreus* for terretonin biosynthesis provides a piece of evidence for the hypothesis that the *trt* cluster and *aus* clusters may share a common ancestor (Figure S1).

In conclusion, we have identified a cluster of 10 genes that is responsible for the biosynthesis of terretonin. Aided by bioinformatic analysis and a series of targeted gene deletions, LC/MS profile analysis, and intermediate isolation and characterization, we have proposed a biosynthetic pathway for terretonin.

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Supporting Information Available. General methods, compounds characterization and spectral data, diagnostic PCR results and Southern blot figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.