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Terrein Biosynthesis in *Aspergillus terreus* and Its Impact on Phytotoxicity

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SUMMARY

Terrein is a fungal metabolite with ecological, antimicrobial, antiproliferative, and antioxidative activities. Although it is produced by *Aspergillus terreus* as one of its major secondary metabolites, not much is known about its biosynthetic pathway. Here, we describe an unexpected discovery of the terrein biosynthesis gene locus made while we were looking for a PKS gene involved in production of conidia coloration pigments common for Aspergilli. The gene, ATEG_00145, here named *terA*, is essential for terrein biosynthesis and heterologous production of TerA in *Aspergillus niger* revealed an unusual plasticity in the products formed, yielding a mixture of 4-hydroxy-6-methylpyranone, orsellinic acid, and 6,7-dihydroxymellein. Biochemical and molecular genetic analyses indicate a low extension cycle specificity of TerA. Furthermore, 6-hydroxymellein was identified as a key intermediate in terrein biosynthesis. We find that terrein production is highly induced on plant-derived media, that terrein has phytotoxic activity on plant growth, and induces lesions on fruit surfaces.

INTRODUCTION

Aspergillus terreus is a ubiquitous filamentous fungus frequently isolated from soil rhizospheres (He et al., 2004; Wijeratne et al., 2003), decaying organic matter (Reddy and Singh, 2002), and marine environments (Damare et al., 2006). *A. terreus* is able to produce potent lipases (Yadav et al., 1998) and cellulases (Narra et al., 2012) and secretes itaconic acid (Kuenz et al., 2012), which makes this fungus attractive for white biotechnology. In addition, *A. terreus* produces a variety of natural products, most notably the medically important HMG-CoA reductase inhibitor lovastatin (Alberts et al., 1980; Tobert 2003). Additionally, a range of metabolites has been extracted from *A. terreus* cultures, among them territrems, citreoveridin, and butyrolactones (Samson et al., 2011). Several gene clusters in *A. terreus* responsible for metabolite production have been analyzed, including lovastatin (Hendrickson et al., 1999), methylsalicylic acid (Fujii et al., 1996), atrochrysone (Awakawa et al., 2009), acetylaranotin (Guo et al., 2013), and terretonin (Guo et al., 2012), as well as the gene cluster for the PKS-NRPS hybrid metabolite isoflavipucine/dihydroisoflavipucine (Gressler et al., 2011). The latter belongs to the family of fruit rot toxins, implying that *A. terreus* could have specifically adapted to the decay of plant organic matter.

However, the origin of the conidia pigment in *A. terreus* remains to be elucidated. All Aspergilli analyzed so far employ a polyketide synthase to produce naphthopyrone precursors. *A. terreus* neither produces naphtropyrone (Slesiona et al., 2012) nor contains the highly conserved naphthopyrone synthase (Ahuja et al., 2012). This points to a separation of *A. terreus* from other *Aspergillus* species in terms of secondary metabolite production.

This is also highlighted by the fact that *A. terreus* is the only *Aspergillus* species producing terrein as one of its major secondary metabolites (Yin et al., 2013). Terrein was first described approximately 80 years ago (Raistrick and Smith, 1935), and its structure was elucidated in the 1950s (Barton and Miller 1955; Grove 1954). However, a series of recent studies revealed the ecological importance of terrein as an inhibitor of plant growth (Phattanawasin et al., 2007). Furthermore, a number of important biological activities have been attributed to terrein, such as antimicobial, antiproliferative, and antioxidative activities, among others (Arakawa et al., 2008; Demasi et al., 2010; Lee et al., 2010; Liao et al., 2012; Park et al., 2004).

Considering the great interest in terrein functions, it is striking that so little is known about its biosynthesis. Classical stable-isotope-labeling experiments revealed that terrein biosynthesis involves the condensation of five acetate units, whereas the molecular formula of $C_8H_{10}O_3$ implied that it is composed of four acetate units only (Birch et al., 1965). It was proposed that the cyclopentanone ring derived from an oxidative decarboxylation with ring contraction of a dihydroisocumarin (Hill et al., 1981). However, experimental support for this model has been lacking, and to date, the molecular basis for terrein biosynthesis has remained elusive.

Here, we report the serendipitous discovery of the terrein biosynthesis gene locus while searching for polyketide synthases that might be responsible for the pigmentation of *A. terreus* conidia. Through targeted mutations, heterologous expression of the terrein PKS, and analyses of pathway intermediates, we not only discovered the hydroxylated dihydroisocoumarin, which was predicted as a terrein precursor, but also found that the terrein PKS produces a variety of products. Finally, we describe that terrein and congeners exert a phytotoxic effect on radish seeds and fruits.

RESULTS

The PKS ATEG_00145 Is Uniquely Positioned in *Aspergillus* PKS Phylogeny

The color of asexual conidia of Aspergilli derives from polyketides that generally originate from the precursor naphthopyrone (Jørgensen et al., 2011; Langfelder et al., 1998; Mayorga and Timberlake, 1992; Watanabe et al., 1999). All these PKSs share the following in common: (1) they are nonreducing (NR), and (2) their domain structure shows tandem ACP domains (Fujii et al., 2001). Interestingly, in *A. terreus* no PKS with close homology to naphthopyrone synthases was identified (Slesiona et al., 2012). However, BLAST analyses revealed two PKSs with the same modular organization, namely, ATEG_00145 and ATEG_07500. To analyze the phylogenetic relation of these *A. terreus* PKSs with other nonreducing fungal PKS, a phylogenetic tree based on the ketosynthase (KS) domains was constructed. This analysis included naphthopyrone synthases, putative melanin, and orsellinic acid synthases. NRPS-PKS hybrids served as an outgroup (Figure 1). Whereas ATEG_07500 appeared at least very distantly related to naphthopyrone synthases, we found that ATEG_00145 does not belong to any of the selected groups of PKSs and seems unique for *A. terreus*. However, because neither ATEG_00145 nor ATEG_07500 closely clustered with naphthopyrone synthases, a contribution in conidia pigmentation remained in question.

ATEG_00145 and ATEG_07500 Do Not Contribute to Conidia Coloration

Deletion mutants of ATEG_00145 and ATEG_07500 were generated in *A. terreus* SBUG844Δ*akuB* (Gressler et al., 2011) to investigate their contribution to the development of the conidia color. When the resulting transformants, Δ00145 and Δ07500, were tested on various media for conidia pigmentation, none of the mutants showed a difference in comparison to the wild-type (as exemplified in Figure 2A). However, when grown on phosphate-buffered Sabouraud agar plates, the mutant Δ00145 completely lacked a typical secreted red pigment observed by all other strains (Figure 2B). Additionally, cultivation in liquid potato dextrose broth (PDB) resulted in a red culture broth with the wild-type and Δ07500 mutant, but not with Δ00145.

The Metabolic Profile of ATEG_00145 Deletion Mutant Lacks Terrein

To investigate the impact of ATEG_00145 on metabolite production, we cultivated the parental strain and Δ00145 on various media, such as glucose containing *Aspergillus* minimal medium (AMM), YPD, Sabouraud (Sab), and PDB. Culture supernatants were extracted with ethylacetate and were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figures 2C–2F). On AMM, no significant differences were observed. In contrast, YPD and PDB wild-type extracts contained a highly complex mixture of metabolites that largely lacked in the mutant. Furthermore, although the metabolic profile from Sabouraud medium showed a lower complexity than those from YPD and PDB, a major metabolite at 7.5 min (**1**) with an apparent molecular mass of 154 Da was completely absent from the mutant. This indicates that the deleted polyketide synthase ATEG_00145 is responsible for the production of various metabolites. However, because (**1**) was detected in significant amounts from all complex media, we isolated and purified the metabolite by crystallization. High-resolution mass determination revealed a molecular formula of $C_8H_{12}O_3$ indicative for the metabolite terrein. Comparing nuclear magnetic resonance (NMR) data with reported spectroscopic data (Hill et al., 1981) unequivocally confirmed this assumption. Therefore, we denoted the polyketide synthase encoded at locus ATEG_00145 TerA.

ORFs terA–terJ Constitute the Terrein Biosynthesis Gene Cluster

To confirm expression of *terA* in complex media and to analyze the genes coregulated with *terA*, we monitored expression of 25 genes (ATEG_00126–ATEG_00150) by semiquantitative RT-PCR analyses. Transcripts were analyzed after 36 and 48 hr from AMM (as negative control), YPD, Sab, and PDB media (Figure 3A). Expression of ATEG_00126 to ATEG_00134 and ATEG_00146 to ATEG_00150 did not correlate with expression of *terA* (ATEG_00145). Thus, these genes were excluded as constituents of a putative terrein biosynthesis gene cluster. In contrast, all genes spanning a region from ATEG_00135 to ATEG_00144 were coexpressed with ATEG_00145 and seemed to constitute the terrein biosynthesis gene locus (Figure 3B). The corresponding genes were denoted as *terA–J*, and a putative transcriptional regulator was termed *terR*.

To verify their specific contribution, all genes, except those of the MFS transporters (*terG* and *terJ*), were deleted in strain SBUG844Δ*akuB* (Gressler et al., 2011). Gene deletions were confirmed by Southern blot analyses (Figure S1 available online), and mutants were cultivated on PDB medium to monitor medium coloration, metabolite profiles, and terrein production level (Figure 4). As expected, the *terA* mutant did not produce any detectable levels of terrein, and most of the prominent metabolites found in the wild-type extract were absent. A similar profile was detected for the Δ*terR* mutant, implying that transcription of the gene cluster strictly depends on this putative transcription factor. Additionally, mutants Δ*terB*, Δ*terC*, Δ*terD*, Δ*terE*, and Δ*terF* did not produce terrein. However, several metabolites accumulated in culture broth of mutants, some of which could also be detected in wild-type extract (**1**–**5** in Figure 4B). Interestingly, although unable to produce terrein, the mutants Δ*terD* and Δ*terE* especially showed an enhanced coloration of the medium, implying that the colored substance does not depend on terrein production but seems to derive from TerA-derived intermediates. Although *terH* and *terI* were clearly coexpressed with *terA* (Figure 3A), both mutants still produced terrein, though the amount in relation to the biomass was reduced. In conclusion, TerA to TerF directly contribute to terrein synthesis with

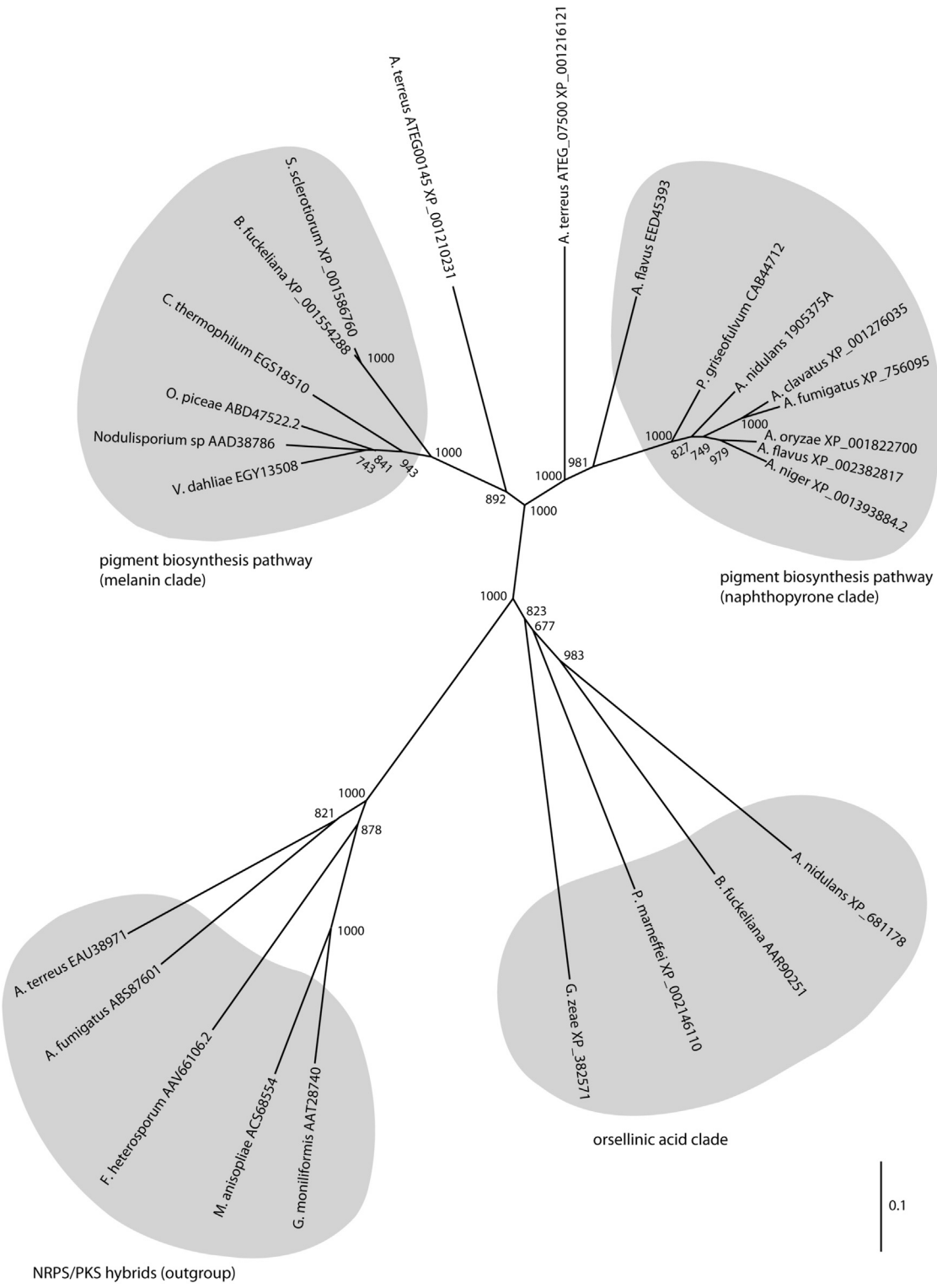


Figure 1. Phylogenetic Analysis of Fungal KS Domain Sequences

PKSs belonging to pigment biosynthesis pathways and the orsellinic acid clade were used for analysis. KS domains from fungal NRPS/PKS hybrids served as an outgroup. *A. terreus* ATEG_00145 and ATEG_07500 neither group with pigment biosynthesis KS domains nor with orsellinic acid synthases.
See also Figure S5.

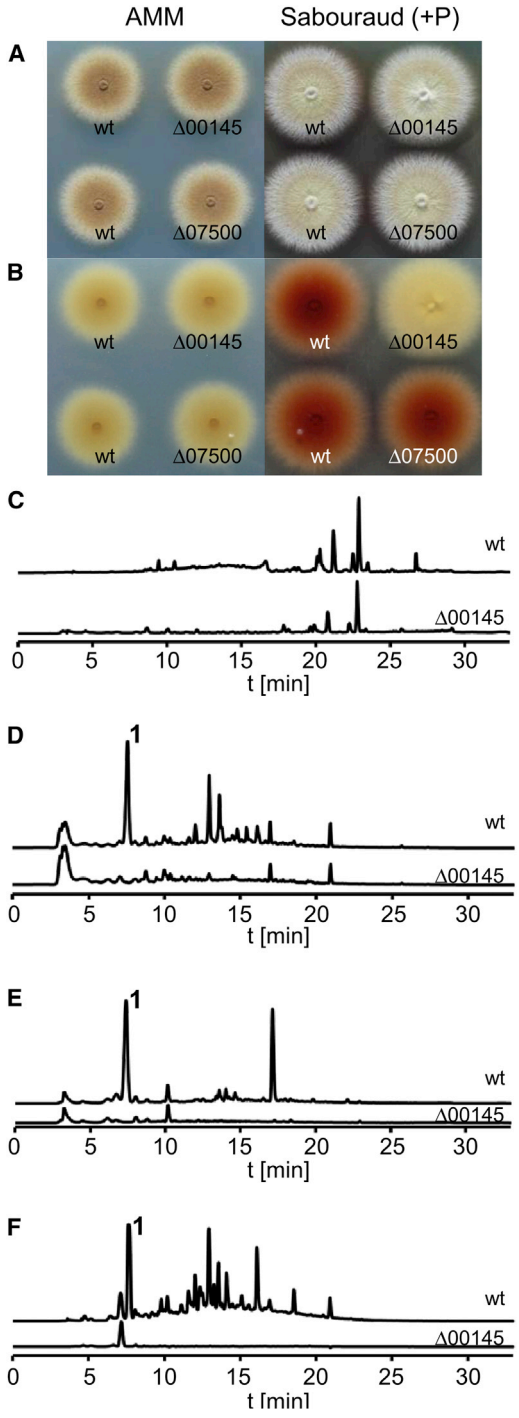


Figure 2. Phenotypic Characterization of ATEG_00145 and 07500 Deletion Mutants and Metabolic Profiling of Δ00145 in Comparison to the Parental Strain SBUG844Δ*akuB*

(A and B) Colony morphology of wild-type and deletion mutants on solid AMM and Sabouraud medium in (A) top and (B) bottom views. The Δ00145 mutant lacks the typical red pigmentation on Sabouraud medium.

(C–F) HPLC profiles (UV 254 nm) of culture extracts from strain Δ*akuB* (upper profiles) and Δ00145 mutant (lower profiles) cultivated on (C) AMM, (D) YPD, (E) Sab, and (F) PDB. "**1**" denotes the major metabolite terrein, which is lacking in the deletion mutant.

See also Figure S1.

TerR as an essential regulator, whereas TerH and TerI are dispensable. Therefore, the "minimal" gene cluster required for the synthesis of terrein spans the region from *terR–terA* and most likely uses one of the two MFS transporters (*terG* or *terJ*) for efficient secretion of metabolites.

6-hydroxymellein Is the Key Biosynthetic Precursor of Terrein

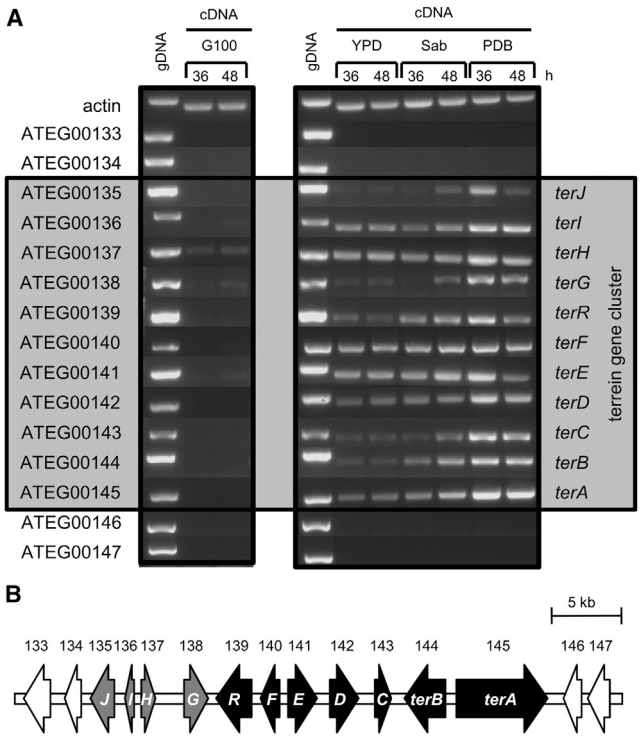
The accumulation of several metabolites in the mutants Δ*terB* to Δ*terF* implied the accumulation of putative terrein precursor molecules. Indeed, when culture broth extracts from Δ*terC*, Δ*terD*, Δ*terE*, or Δ*terF* were added to the culture medium of the Δ*terA* mutant, terrein (**1**) biosynthesis was restored (Figure 5A). However, Δ*terB* extracts did not restore terrein formation. Thus, metabolites (**3**) and (**4**) (Figure 4B), which are also produced by Δ*terB*, are not pathway intermediates, but are rather shunt or side products. To identify a true terrein precursor, we fractionated the Δ*terC* extract. We collected eight fractions by preparative high-performance liquid chromatography (HPLC) that baseline separated the major peaks (Figure 5B), including the major metabolite (**2**), and added these fractions to the Δ*terA* mutant. Interestingly, only fraction VII consisting of metabolite (**2**) restored terrein synthesis. Its characterization by high-resolution mass spectrometry (HR-MS) and NMR revealed a molecular mass of 194 Da and the molecular formula $C_{10}H_{10}O_4$. Structure elucidation by one-dimensional and two-dimensional NMR revealed the identity of **2** with 6-hydroxymellein (6-HM), a postulated intermediate en route to terrein (Hill et al., 1981).

To unequivocally prove that 6-HM (**2**) is an immediate terrein precursor, we added [1-^{13}C]-glucose to the Δ*terC* culture medium (2 × 100 ml cultures) and isolated 4 mg of labeled 6-HM (**2**). ^{13}C-NMR analysis revealed the expected ^{13}C-labeling pattern of an isocoumarin derivate with polyketide origin (Figure 5B). Next, we added labeled 6-hydroxymellein to the Δ*terA* mutant and succeeded in isolating ^{13}C-labeled terrein (Figure 5B). NMR experiments revealed two adjacent nonlabeled carbon atoms in the cyclopentanone ring of terrein (Figure S6), which is completely in line with the model involving the loss of carbon atoms during ring contraction of 6-HM (**2**) (Hill et al., 1981; Zamir and Chin, 1982).

Recombinant TerA Produces Polyketides of Different Chain Length

Because TerA is a NR-PKS, we assumed that 6-hydroxy-2,3-dehydromellein was the product of TerA. Therefore, we heterologously expressed *terA* in *Aspergillus niger* FGSC A1144. To test for suitable promoters, β-galactosidase fusions (Gressler et al., 2011) with *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (P*gpdA*) promoter and the *Aspergillus oryzae* α-amylase promoter (P*amyB*) were analyzed. Although P*gpdA* was constitutively active under all conditions, the P*amyB* allowed inducible expression on AMM and YM medium but was not induced on casamino acids (Figure S3). Thus, P*amyB* was selected to construct a promoter fusion with *terA* for heterologous expression in *A. niger*.

Transformants with different integration numbers were screened for metabolite profiles on casamino acids and AMM. Unexpectedly, instead of a single metabolite, three new metabolites that were absent from the parental *A. niger* strain were



gene	gene code	putative function
ATEG00135	*terJ*	major facility transporter (MFS)
ATEG00136	*terI*	protein of the superfamily of bleomycin resistance protein, glyoxalase I, and type I ring-cleaving dioxygenases
ATEG00137	*terH*	NAD dependent epimerase/dehydratase
ATEG00138	*terG*	major facility transporter (MFS)
ATEG00139	*terR*	Zn_2Cys_6 transcriptional regulator
ATEG00140	*terF*	protein with kelch motif
ATEG00141	*terE*	multicopper oxidase
ATEG00142	*terD*	FAD dependent monooxygenase
ATEG00143	*terC*	FAD dependent monooxygenase
ATEG00144	*terB*	DH-KR, multidomain protein with dehydratase and ketoreductase function
ATEG00145	*terA*	non-reducing polyketide synthase (NR-PKS)

Figure 3. Expression Analysis of the Putative Terrein Synthesis Locus and Gene Annotations

(A) Semiquantitative PCR analysis of ATEG_00133–ATEG_00147 from actin-normalized cDNA generated from mycelium grown for 36 and 48 hr on AMM (G100), YPD, Sab, and PDB medium. Genes from ATEG_00135–00145 (*terJ*–*terA*) show the same expression pattern and form a putative terrein synthesis cluster.

(B) Genome map of the terrein gene cluster (filled arrows). Putative functional domains of the encoded proteins are listed.

detected in the transformants on glucose minimal medium (Figures 5C and 5D), but not on casamino acids (data not shown). HR-MS analyses revealed that compound **4**, the most abundant metabolite, has a molecular mass of 168 Da ($C_8H_8O_4$). For congeners **3** and **5**, masses of 210 Da ($C_{10}H_{10}O_5$) and 126 Da ($C_6H_6O_3$) were inferred. Interestingly, the two major metabolites (**3** and **4**) showed the same retention time and UV spectra as metabolites (**3** and **4**) of the Δ*terB* mutant (Figure 4A), which did not restore terrein production (Figure 5A). Purification of the three metabolites with subsequent NMR-based structure elucidation revealed the structures of orsellinic acid (OA) for **4**, 6,7-dihydroxymellein (6,7-DHM) for **3**, and 4-hydroxy-6-methylpyranone (4-HMP) for the minor metabolite **5**. OA (**4**) and 4-HMP (**5**) represent typical products of an NR-PKS. In contrast, 6,7-DHM (**3**) lacks the expected double bond of 6-hydroxy-2,3-dehydromellein and has a hydroxyl-group on position C7, although there is no oxygenase domain within TerA.

Although 6,7-DHM (**3**) is closely related to 6-HM (**2**) and had been identified from different cluster mutants (Δ*terB*–Δ*terI*; Figure 4A), feeding of complete culture extracts from *A. niger* transformants or their purified metabolites did not restore terrein production in the Δ*terA* mutant strain (data not shown). Thus, we assumed that the unexpected hydroxylation at C7 was nonspecifically introduced by the host, thus hampering uptake of the compounds and/or its further conversion to terrein.

To test this hypothesis, we supplemented AMM media with 6-HM (**2**) and inoculated them with either *A. terreus* or *A. niger* wild-type strains. Subsequent analyses of culture extracts revealed metabolites corresponding to hydroxylated forms of 6-HM, among them 6,7-DHM (Figure S4). Thus, this particular hydroxylation takes place in the absence of terrein biosynthesis enzymes. Consequently, recombinant expression of *terA* in *A. niger* yields 6-HM, which is subsequently converted into 6,7-DHM by unspecific enzymes in the heterologous host.

TerA Is Exclusively Primed with Acyl-CoA Starter Units

TerA is an unusual NR-PKS that produces polyketides of different chain length. This may be rationalized by the loading of starter units of different chain lengths or by a different number of elongation cycles. The first scenario would require a "relaxed" specificity of the starter acyl transferase (SAT) domain that may not only load acetyl-CoA but may also load other short-chain acyl-CoA starter units. In SAT domains, a catalytic diad formed by a conserved cysteine and histidine residue initiates the loading process (Crawford et al., 2006). However, inspection of the annotated protein sequence of TerA revealed no conserved cysteine in the *N*-terminal region. Further analysis of the proposed open reading frame (ORF) revealed an unusual large intron sequence of 223 bp. To rule out an incorrect intron prediction, we generated cDNA from *A. terreus* and sequenced the 5′ region. Indeed, our analysis revealed that the intron only spanned a region of 49 bp (accession KF647874). The resulting corrected SAT domain contained the expected cysteine residue (Figure S5). This indicates that an acyl-CoA unit is loaded, but it gave no direct clue on the kind of acyl-CoA ester utilized. Because OA (**4**) was one of the products of TerA, we investigated a possible increase in product specificity by exchanging the TerA SAT domain with that of the orsellinic acid synthase OrsA from *A. nidulans* (Sanchez et al., 2010; Schroeckh et al., 2009). Yet *A. niger* expressing the SAT_{orsA}:*terA* construct completely lacked synthesis of both 6,7-DHM (**3**) and OA (**4**) (data not shown). This finding suggests that the OrsA SAT domain and TerA are not compatible, although both PKS produce at least one common polyketide.

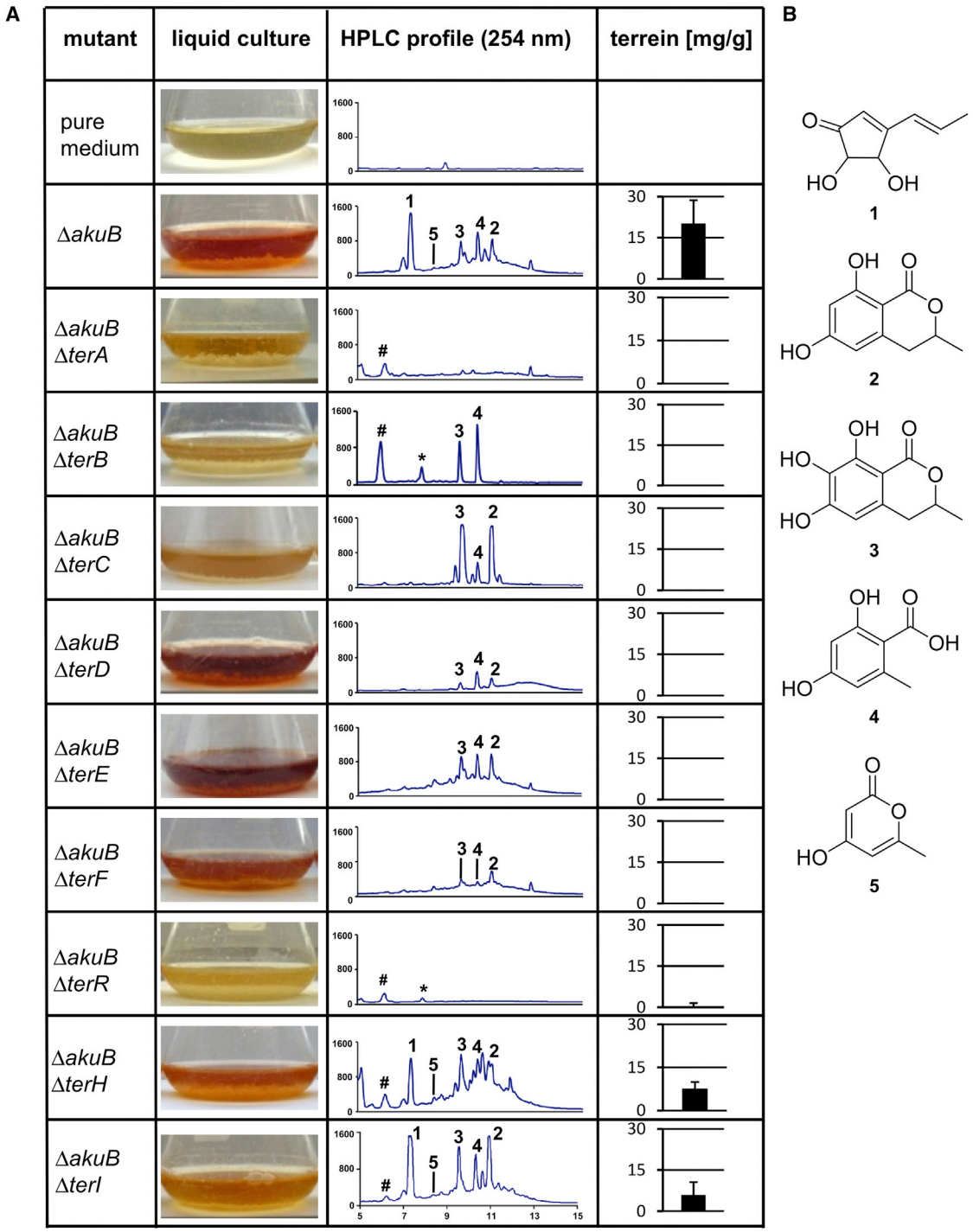


Figure 4. Metabolic Profiling and Terrein Quantification from Terrein Cluster Deletion Mutants

(A) Photographs of cultures were taken after 72 hr of growth in PDB medium at 30°C (column 2). Metabolite profiles of culture supernantants from control, wild-type, and mutants recorded at 254 nm (column 3). Specific terrein (**1**) concentrations per gram of mycelial dry weight were determined from three independent cultures (column 4). The metabolite peaks annotated with # (156 Da; molecular formula $C_7H_7O_4$) and * (154 Da; molecular formula $C_7H_6O_4$) are present in all mutants at varying extent and not related to terrein biosynthesis. Error bars denoted ± SD.

(B) Structures of metabolites representing the numbered peaks in the HPLC profile: terrein (**1**), 6-hydroxymellein (**2**), 6,7-dihydroxymellein (**3**), orsellinic acid (**4**), and 4-hydroxy-6-methylpyrone (**5**).

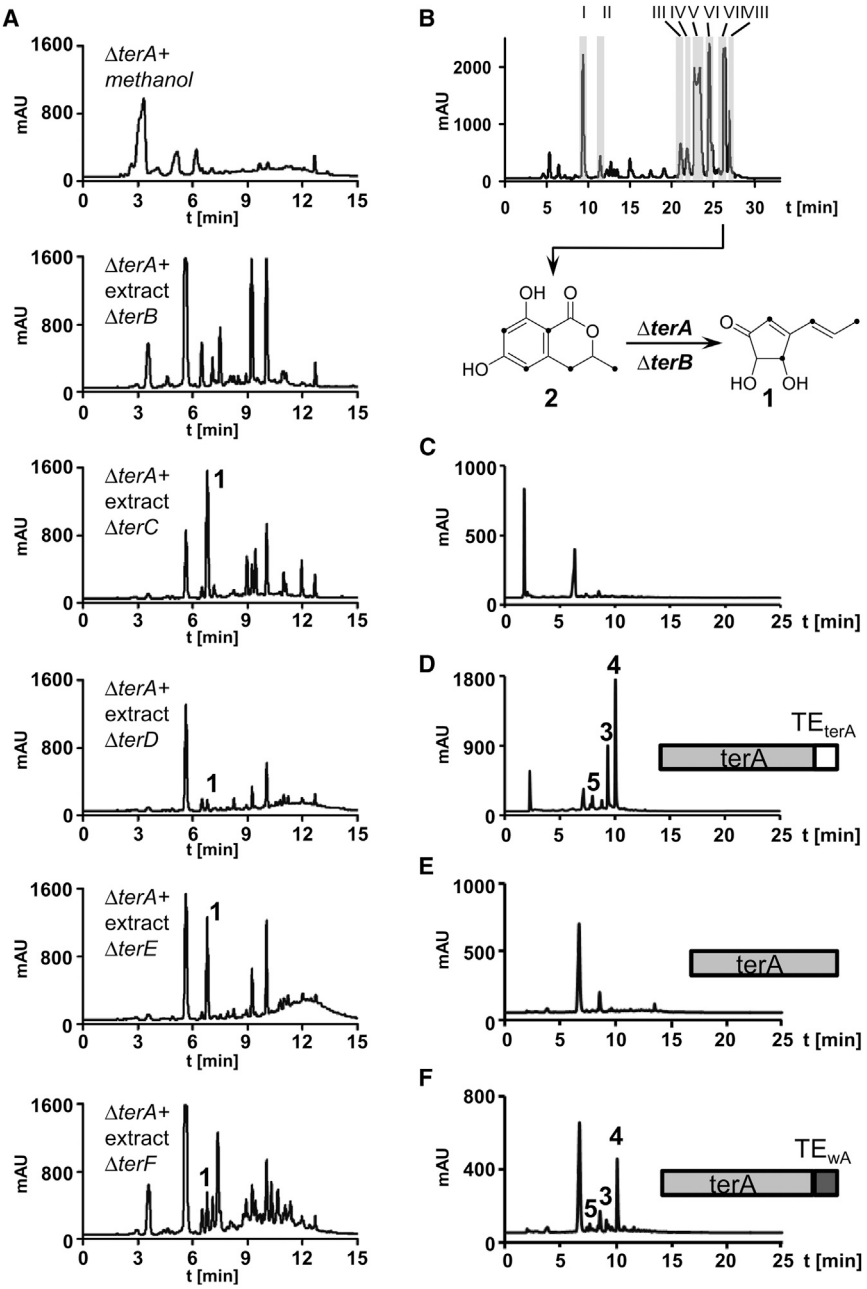


Figure 5. HPLC Profiles of Culture Supernatants from Cross-Feeding Experiments and from Recombinant *A. niger* Strains Expressing Different Modified *terA* Gene Versions

(A) HPLC profiles (UV 254 nm) of culture extracts from *A. terreus* Δ*terA* with or without cross-feeding of extracts from other cluster mutants. Feeding of Δ*terB* cannot restore terrein production, whereas extracts from Δ*terC* to *F* lead to varying amounts of terrein.

(B) Fractionation of the Δ*terC* crude extract by preparative HPLC and collection of the major peaks in separate fractions. Isolated fractions were used to supplement the medium of the Δ*terA* mutant. Fraction VII restored terrein production and was identified as 6-hydroxymellein (2). When the Δ*terC* mutant was supplemented with [1-^{13}C]-glucose, NMR analysis revealed ^{13}C-labeled carbon atoms (marked with black dots) in 6-hydroxymellein that were reidentified in terrein (see also Figure S6).

(C–F) HPLC profiles of culture extracts from *A. niger* wild-type (C), *A. niger* expressing the native *terA* gene (D), the truncated *terA* gene that lacks the TE domain (E), and the *terA* gene with the native TE domain substituted by the TE domain from *A. nidulans wA*. Numbers denote metabolites OA (4), 6,7-DHM (3), and 4-HMP (5). Metabolite peaks not annotated by numbers are also present in *A. niger* wild-type extracts and therefore are not related to *terA* expression. Note that metabolites (3, 4, and 5) were not observed in UV spectra from the truncated *terA* version but were detectable by mass spectrometry (see also Figures S2 and S3).

We next tested alternative PKS priming. First, we added [1-^{13}C]-acetate to the culture broth of *A. niger* A1144_P*amyB*:*terA* and analyzed the labeling pattern of the metabolites. Analysis confirmed the condensation of three acetyl units in 4-HMP (**5**), four acetyl-units in OA (**4**), and five acetyl-units in 6,7-DHM (**3**). Although the incorporation rate in 6,7-DHM (**3**) was rather weak, the labeling pattern was already confirmed by addition of labeled [1-^{13}C]-glucose to the *A. terreus terC* mutant (Figure 5B). These data indicated that, in principle, all three metabolites derived from acetyl-CoA units. Nonetheless, we could not exclude that short-chain fatty acids served as starter units to generate the products of different chain length. Thus, we added [1,2,3,4-^{13}C]-3-hydroxybutyrate as an alternative starter unit to *A. niger* A1144_P*amyB*:*terA* and investigated the labeling pattern in 6,7-DHM (**3**). Analysis by ^{13}C-NMR revealed 6,7-DHM with an equal distribution of ^{13}C on all carbon atoms, indicating that 3-hydroxybutyrate was cleaved into acetyl units prior to chain elongation and thus did not specifically serve as a starter unit (data not shown). We additionally tested for a relaxed specificity of the SAT domain by adding the *N*-acetylcysteamine-esters of butyrate, 3-hydoxybutyrate, 3-hydroxypentanoate, and 3-hydroxyhexanoate. LC-MS/MS analysis neither revealed metabolites with altered or extended carbon skeleton nor an altered relative quantity of one of the three metabolites (data not shown). From these results, we conclude that TerA only utilizes acetyl-CoA starter and malonyl-CoA extender units, and the metabolites of different chain length seem to derive from low extension cycle specificity.

Interestingly, in all experiments, we exclusively detected the reduced 6,7-DHM (**3**) rather than a 6,7-dihydroxy-2,3-dehydromellein. This implies that, independent from the gene cluster, *A. niger* enzymes reduce 6,7-hydroxy-2,3-dehydromellein to 6,7-DHM (**3**). Similarly, this reduction also seems to occur nonspecifically in *A. terreus*, because all cluster mutants, except

the Δ*terA* and the Δ*terR* strain, accumulated 6,7-DHM (**3**) or 6-HM (**2**). However, the cluster also contains a putative ketoreductase (TerB) that may be essential for the correct timing of this reduction step during terrein production, but coexpression of *terB* together with *terA* in *A. niger* resulted in the same product pattern as observed for the strain solely expressing *terA* (data not shown).

The TerA Thioesterase Domain Is Exchangeable without Alteration of Product Formation

Bioinformatics and labeling studies imply that different numbers of elongation cycles are responsible for the different chain lengths of the TerA products. Thioesterase (TE) domains have been shown to contribute to chain length specificity and product release (Watanabe and Ebizuka, 2004). In the *A. nidulans* naphthopyrone synthase WA, the TE domain acts as a Claisen cyclase involved in closure of the "B-ring" in the heptaketide naphthopyrone that is also the sole product of this PKS (Fujii et al., 2001). When deleted, ring closure occurs via nonenzymatic lactonization, resulting in an isocoumarin heptaketide. In contrast, PKS1 from *Colletotrichium lagenarium* produces the tetraketide OA (10%), the pentaketides α-acetylorsellinic acid (25%), tetrahydroxynaphthalene (50%), and the hexaketide 2-acetyl-1,3,6,8-tetrahydroxynaphthalene (15%) (Watanabe and Ebizuka, 2004). Removal of the TE domain resulted in >95% production of an isocoumarin hexaketide. Thus, it was concluded that PKS1 generally produces a hexaketide but that the Claisen cyclase domain interferes with chain growth (Watanabe and Ebizuka, 2004), which could also be true for TerA. Thus, two different constructs were generated: (1) a truncated version of TerA lacking the TE domain (called *terA*ΔTE) and (2) a construct in which the C-terminal part next to the phosphopantetheine binding site was replaced by the TE domain from *A. nidulans* WA (called *terA:*TE_{wA}). Both constructs were produced in *A. niger*, and Southern blot analyses confirmed complete genomic integration of the *terA*ΔTE construct. Whereas the HPLC profile did not reveal production of any prominent product (Figure 5E), molecular masses of OA (**4**) and 6,7-DHM (**3**) were still detected by high-resolution mass spectrometry. In contrast, the exchange of the TE domain in the *terA*:TE_{wA} construct reduced the total amount of OA and DHM, but both metabolites were produced in significant amounts and in similar ratios as seen from the native TerA protein (compare Figures 5D and 5F). Thus, we cannot attribute the formation of different polyketides specifically to the TerA TE domain. We conclude that the thioesterase domain in TerA is of general importance for the efficient release of polyketides. Furthermore, the thioesterase domain of TerA is exchangeable, but at least by using a WA-type Claisen cyclase, the same polyketides are produced. Thus, TerA behaves different to PKS1 from *Colletotrichum lagenarium* and WA from *A. nidulans*.

The Terrein Pathway Produces Phytotoxic Compounds

Although terrein (**1**) is likely the major product of the cluster, OA (**3**) and 6,7-DHM (**4**) that both cannot be converted to terrein were also present in substantial quantities in *A. terreus* wild-type and mutant strains. As we noted high terrein production rates on potato dextrose broth, we assumed that under natural conditions biologic activities of terrein might be associated with plant interactions. Therefore, we investigated the phytotoxic potential of terrein and TerA-derived metabolites.

First, we investigated the phytotoxic effect of terrein on radish (*Raphanus sativus*) seeds in a range between 1 and 100 μg/ml. As shown in Figures 6A and 6B, terrein inhibits root and shoot elongation in a concentration-dependent manner. Whereas 100 μg/ml nearly completely abolished seed germination, 1 μg/ml only showed a very weak inhibitory effect. Because 25 μg/ml resulted in approximately 50% reduced root elongation after 5 days of incubation, purified OA (**4**), 6-HM (**2**), and 6,7-DHM (**3**) were also tested at 25 μg/ml. As shown in Figure 6C, OA (**4**) and 6-HM (**2**) did not inhibit root elongation. 6,7-DHM (**3**) showed a low, but not substantial, inhibition at the applied concentration.

In a second series of biological activity assays, we investigated the ability of terrein and TerA-derived metabolites to cause lesions on fruit surfaces, which has not been tested before. Terrein was highly effective in causing surface lesions, with the highest activity on bananas (2.5–5 μg effective concentration), but was also effective on other fruits, such as pears (12.5 μg effective concentration; Figure 6D). However, when OA (**4**), 6-HM (**2**), and 6,7-DHM (**3**) were tested in the banana-surface assay, none of the metabolites generated lesions at concentrations of up to 50 μg (Figure 6E). These experiments indicate that transformation of primary metabolites from TerA into terrein is required to harm fruit surfaces and may support the nutrient acquisition of *A. terreus* in its natural habitat.

DISCUSSION

In this study, the molecular basis for terrein biosynthesis in *A. terreus* was elucidated. The discovery of this gene cluster was unexpected, because we were initially searching for secondary metabolites responsible for coloration of asexual conidia. In all related *Aspergillus* species, the conidia color is naphthopyrone derived (Langfelder et al., 1998; Watanabe et al., 1999) and essential to inhibit phagolysosome acidification and allows for escape from macrophages (Slesiona et al., 2012; Thywißen et al., 2011). As putative candidates for conidial pigment synthesis in *A. terreus*, we selected two nonreducing polyketide synthases that revealed the same domain structure (SAT-KS-AT-PT-ACP-ACP-TE) as naphthopyrone synthases. When deleted, both mutants showed normal coloration of conidia, and phylogenetic analyses revealed that, despite the same domain structure, none of the two PKSs clustered with PKSs for which products had been described. Especially the ketoreductase domain of ATEG_00145 (subsequently denoted as TerA) formed a distinct phylogenetic branch. This observation was in agreement with a recent study, in which aromatic PKSs from Aspergilli were phylogenetically investigated, and ATEG_00145 (TerA) could not be explicitly assigned to any particular clade (Ahuja et al., 2012).

We showed that the *terA* gene encodes for a functional polyketide synthase. When *terA* was expressed in *A. niger*, it produced the triketide 4-hydroxy-6-methylpyranone (**5**), the tetraketide orsellinic acid (**4**), and a pentaketide, which is 6,7-dihydroxymellein (**3**). Although these products were also identified from *A. terreus* wild-type and some of the cluster mutants, the production of 6,7-DHM (**3**) especially did not fit to the domain structure of a NR-PKS. Subsequent analyses revealed that the

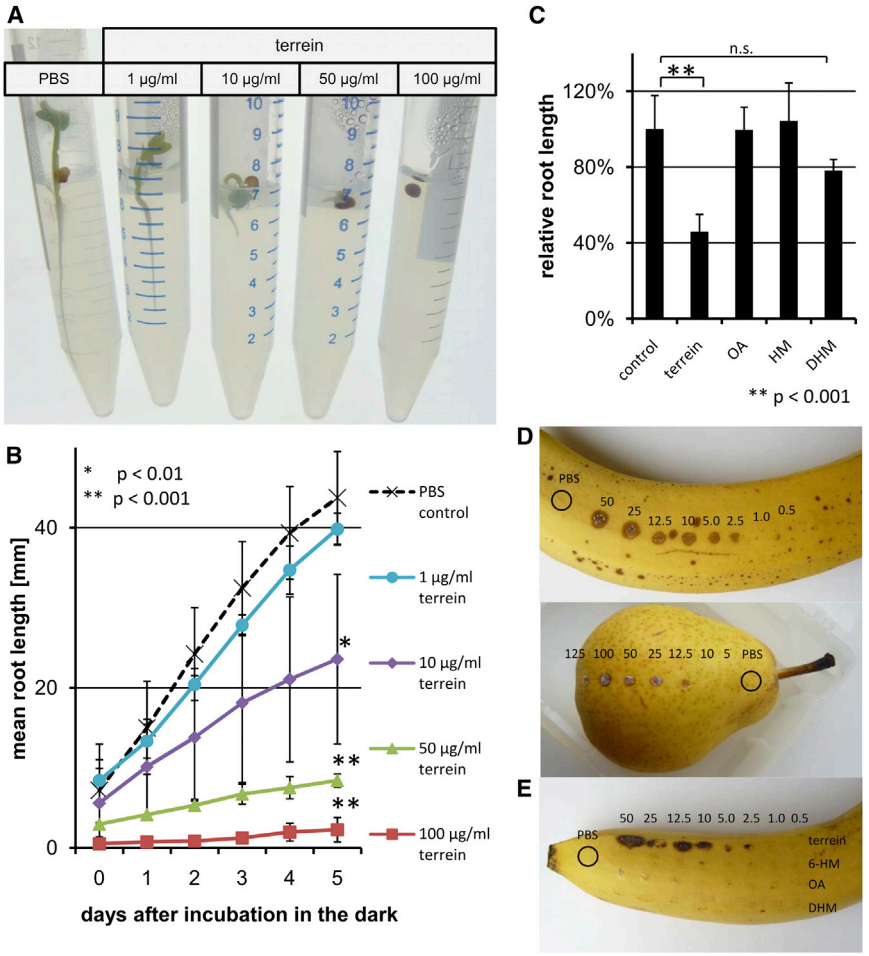


Figure 6. Effect of Terrein and Terrein Cluster Metabolites on Radish Seed Germination and Fruit-Surface Damage

(A) Root growth inhibition by terrein. Terrein was added in concentrations of 0 (PBS), 1, 10, 50, and 100 µg/ml. Pictures were taken 2 days after incubation in the light.

(B) Graphical presentation of time response analysis of root growth inhibition. Each data point represents the average values from three independent experiments each containing 12 individual seedlings. Error bars represent ± SD. Significance against control was calculated by the two-tailed t test.

(C) Root length determination of radish seedlings at day 5 in light in the presence of 25 µg/ml terrein, OA, 6-HM (HM), and 6,7-DHM (DHM). The assay was carried out four times with at least ten individual seedlings. Significance was calculated by the two-tailed t test. Error bars represent ± SD.

(D) Surface damage of bananas and pears inoculated with 0.5 to 50 µg and 5 to 125 µg terrein, respectively, and incubated at room temperature. PBS was used as control. Pictures were taken at days 4 and 6.

(E) Banana surface damage by 0.5 to 50 µg terrein, orsellinic acid (OA), 6-hydroxymellein (HM), or 6,7-dihydroxymellein (DHM). PBS + 0.1% Tween 80 served as control.

hydroxylation at position 7 of 6,7-DHM (**3**) occurs independent from TerA, implying that a derivative of 6-HM (**6** or **2**) rather than of 6,7-DHM (**3**) is the pentaketide produced by TerA. In this respect, one would have expected that 2,3-dehydro-6-HM (**6**) is produced because of a lacking ketoreductase domain in TerA. Indeed, this product was identified by an independent investigation (Ahuja et al., 2012), in which different NR-PKS were expressed in an *A. nidulans* strain deleted for several intrinsic secondary metabolite gene clusters. Thus, although 2,3-dehydro-6-HM (**6**), rather than 6-HM (**2**), is the pentaketide produced by TerA, 6-HM restored terrein production in the *terA* and *terB* mutants and acts as a direct precursor for terrein as shown by feeding studies with ^{13}C-labeled 6-HM (**2**). In contrast, triketide (**5**) and tetraketide (**4**) did not serve as precursors for terrein in any of the mutants. Thus, we were interested in the reason for the low chain-length specificity of TerA, especially because we discovered that the recombinant PKS produced the tetraketide (**4**), rather than the pentaketide (**3**), as major product.

For other PKSs, such as the norsolorinic acid synthase, it was shown that the SAT domain accepts various starter units (Crawford et al., 2006), resulting in products of different chain length, whereby under natural conditions the main substrate is provided by a coregulated fatty acid synthase (Brown et al., 1996; Watanabe et al., 1996). In contrast, our feeding studies showed that in TerA the low specificity for a product with a specific chain length is independent from the utilization of alternative starter units. A respective SAT-domain-independent mechanism has previously been described for PKS1 from *C. lagenarium* (Crawford et al., 2006), in which the TE domain causes low product specificity. In *C. lagenarium*, the deletion of the TE domain strongly altered the product spectrum in the direction of the product with the highest chain length (Watanabe and Ebizuka, 2004). This is unusual, because in other polyketide synthases, such as the naphthopyrone synthase WA from *A. nidulans*, the TE domain is important for product chemistry but does not direct chain-length specificity (Fujii et al., 2001). In the case of TerA, neither deletion nor exchange of the TE domain altered the product spectrum, but a TE domain was essentially required for high product yields. This implies that the TE domain is not directly involved in chain-length determination but is essential for efficient product release and thus overall activity of the PKS.

These data suggest that the low specificity is a trait inherent to the KS and likely the product template (PT) domain. This PT domain limits the pocket size and thus the possible substrate chain length and substrate orientation prior to the cyclization step (Crawford et al., 2009). It is remarkable that the KS domain of TerA did not fall into any KS clades in the phylogenetic tree. Thus, this part of the PKS may indeed cause the low product specificity. In this context, it should also be noted that fungal HR-PKS have been shown to produce polyketides of different chain lengths when taken out of the biosynthetic context (Kennedy et al., 1999). It is very conceivable that accessory enzyme contribute to the fidelity of the PKS.

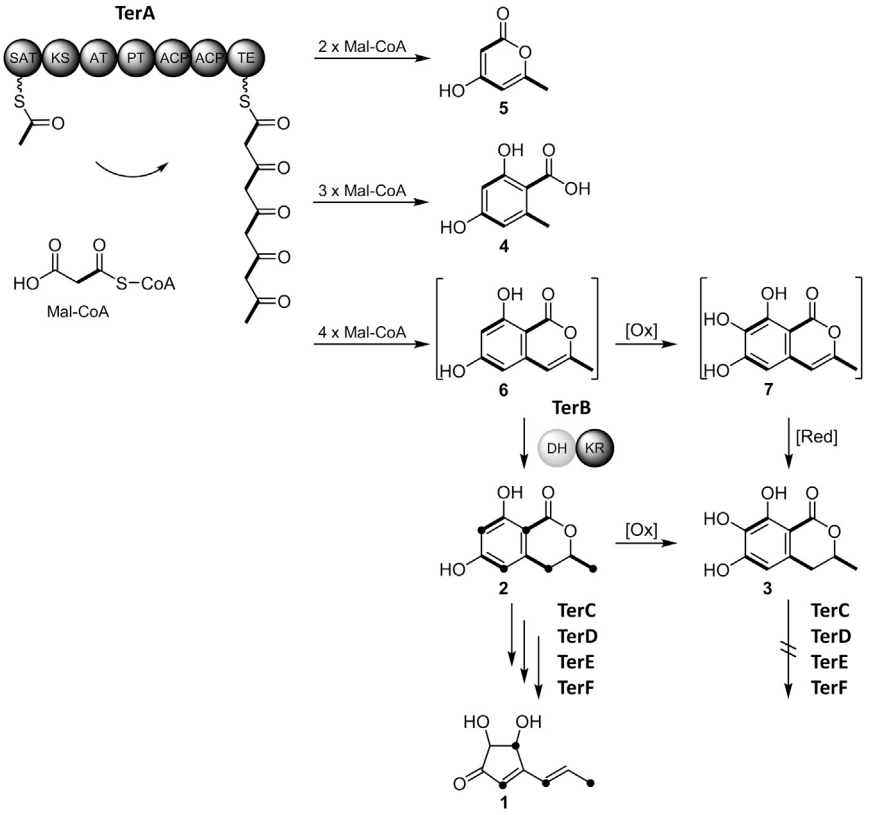


Figure 7. Proposed Scheme for Terrein Biosynthesis

Initially, TerA produces compounds **5** (4-HMP), **4** (OA), and **6** (2,3-dehydro-6-HM) by condensing acetyl-CoA with two, three, or four malonyl-CoA units. Only 6-HM serves as precursor for terrein production. For a detailed explanation of the figure, refer to the discussion section and also see Figure S4.

In this study, we showed that TerA generates 2,3-dehydro-6-HM (**6**) as a precursor of terrein (Figure 7). The timing of hydroxylation at position C7 and hydrogenation between C2 and C3 appears important. When both reactions are performed on 2,3-dehydro-6-HM (**6**), 6,7-DHM (**3**) is formed, which is not converted into terrein in any of the mutants. However, the hydrogenation of 2,3-dehydro-6-HM (**6**) to 6-HM (**2**) yields an appropriate pathway intermediate for the Δ*terA* and Δ*terB* strains. Thus, we conclude that the specific reduction of 2,3-dehydro-6-HM (**6**) to 6-HM (**2**) without hydroxylation at position C7 is performed by TerB, which is in agreement with an *N*-terminal PKS dehydratase domain that could open and close the lactone ring to enable the *C*-terminal ketoreductase domain the hydrogenation at positions C2 and C3. However, because 6,7-DHM (**3**) is also found in the Δ*terB* strain, unspecific ketoreductases may also perform this reduction on a substrate hydroxylated at position C7.

Although our analyses revealed that *terC*, *terD*, *terE*, and *terF* are essential for terrein production, it remains difficult to assign a specific function to each of the enzymes. One can assume that at least two additional monooxygenase reactions are required. One reaction could involve the hydroxylation at position C9 (Zamir and Chin, 1982) to set the stage for the subsequent decarboxylation at the lactone ring. Another hydroxylation would be required at position C7 or C5 (Hill et al., 1981) to allow for the ring contraction. Because *terC*, *terD*, and *terE* encode either FAD-dependent monooxygenases or multicopper oxidases, all three enzymes are putative candidates for these reactions. Despite many attempts, potential downstream products evaded isolation and structure elucidation because of their instability and/or reactivity.

Terrein has been described to exhibit antibacterial, antifungal, anti-inflammatory, antioxidative, antiproliferative, and proapoptotic properties (Arakawa et al., 2008; Lee et al., 2010; Park et al., 2004; Porameesanaporn et al., 2013). However, according to the high expression levels on the plant-derived PDB medium (>1.1 g/l without further optimization), we focused on possible phytotoxic effects. Previous studies showed that terrein (**1**) and 6-HM (**6**) inhibit root and shoot growth of *Mimosa pigra* and *Echinochloa crus-galli* (Phattanawasin et al., 2007). Similarly, in our studies, terrein (**1**), but not 6-HM (**6**), showed a concentration-dependent germination inhibition of radish seedlings. On the contrary, 6-HM (**2**) inhibits pollen development in *Arabidopsis thaliana* (Shimada et al., 2002). We also found that terrein, but none of the other compounds produced, causes lesions on fruit surfaces. Thus, in its natural habitat terrein may be specifically required during the interaction with plants and may trigger release of nutrients from the interaction partners. However, various intermediates of terrein synthesis display different beneficial or phytotoxic effects on plant cells, which may explain the high diversity of products released during terrein synthesis.

SIGNIFICANCE

***Aspergillus terreus* is an important fungus that plays a pivotal role in biotechnology, not only for the production of enzymes and fine chemicals but also for pharmaceutically relevant compounds. Moreover, *A. terreus* has been implicated in various diseases and is regarded as an emerging fungal pathogen. Thus, detailed knowledge about its biosynthetic potential is essential to improve metabolite production and to identify and study potential risk factors. Terrein is a famous, characteristic *A. terreus* metabolite that is endowed with a variety of biological activities, such as antimicrobial and antiproliferative activities. Yet little is known about terrein biosynthesis, and the genes involved remain fully unknown. In this study we unveil the molecular basis for the biosynthesis of this important natural product and gain insights into regulation and mechanisms of the terrein pathway. The identified terrein PKS is unusual as it yields three compounds of different sizes and shapes. Functional and mutational analyses contribute to a better understanding of terrein**

assembly. Most notably, we identify a key intermediate of the terrein pathway and unequivocally show that it is transformed into the rearranged polyketide metabolite. Beyond biosynthetic studies, we also report an additional role—which has been overlooked thus far—for terrein, in which it forms lesions on fruit surfaces. This finding is particularly noteworthy as terrein biosynthesis is upregulated in organic media that may mimic the natural habitat. Taken together, our studies not only shed light on the biosynthesis of an important fungal metabolite but may also unveil an additional role for the terrein pathway in environmental competition and phytotoxin production.

EXPERIMENTAL PROCEDURES

Reconstruction of the Phylogenetic Tree

Protein sequences of known and predicted PKSs belonging to pigment biosynthesis pathways and the orsellinic acid clade were collected from the National Center for Biotechnology Information. KS domains from fungal NRPS/PKS hybrids were used as an outgroup. The KS domains were extracted and aligned with Muscle, and the tree was inferred with the neighbor-joining (NJ) method using the Phylip package, with the number of bootstrap trials set to 1,000. Numbers at the nodes indicate the bootstrap support for each clade.

Strains, Cultivation Conditions, and Metabolite Extraction

All strains and mutants used and generated in this study are listed in the Supplemental Experimental Procedures. Minimal media are based on *Aspergillus* minimal medium (AMM; http://www.fgsc.net/methods/anidmed.html). The following media were used: AMM (containing 100 mM glucose as standard medium), AMM-CA1% (nitrate and glucose replaced by 1% casamino acids), YPD (20 g/l peptone, 20 g/l glucose, and 10 g/l yeast extract), Sabouraud (Sigma Aldrich; buffered in solid media with sodium phosphate buffer [pH 6.5] to a final concentration of 150 mM), potato dextrose broth (PDB; Sigma Aldrich), and yeast extract/malt extract medium (YM; 5 g/l peptone, 3 g/l malt extract, and 3 g/l yeast extract). Liquid cultures were inoculated with a final concentration of 1×10^6 conidia/ml and incubated at 30°C and 200 rpm. When required, plates were supplemented with 140 μg/ml hygromycine B (Roth) or 0.1 μg/ml pyrithiamine (Sigma Aldrich). Conidia were harvested from solid media in water and filtered over 40 μm cell strainers (VWR). Culture broth was extracted by adding 50 ml ethylacetate to 50 ml broth, and the extraction was repeated once. Evaporated residues were solved in 1 ml methanol and filtered. Standard extract analyses were performed on an Agilent 1100 series HPLC-DAD system coupled with a MSD trap (Agilent Technologies) operating in alternating ionization mode (Gressler et al., 2011).

Generation of Deletion Mutants in *A. terreus*

For generation of deletion mutants, *A. terreus* SBUG844/Δ*akuB* (Gressler et al., 2011) was used as the parental strain. Briefly, between 0.5 and 1.3 kbp 5′- and 3′-flanking regions were amplified from genomic DNA and fused with the pyrithiamine resistance cassette *ptrA* (Fleck and Brock, 2010). *A. terreus* was transformed by protoplast fusion and regenerated on osmotically balanced medium with pyrithiamine as a selection marker. Transformants deriving from single-haploid conidia were analyzed by Southern hybridization with digoxygenin-labeled probes directed against upstream- or downstream-flanking regions (Figure S1) (Gressler et al., 2011). For details on oligonucleotides used for fragment amplification and generation of labeled probes, refer to the Supplemental Experimental Procedures.

Generation of β-galactosidase Reporter Strains

Details on oligonucleotides and cloning strategies are found in the Supplemental Experimental Procedures. Briefly, the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter (An*PgpdA*, locus ANID_08209.1) amplified from genomic DNA of *A. nidulans* FGSC A4 or the *A. oryzae* α-amylase B promoter (P*amyB* from *A. oryzae* FGSC A815; Kanemori et al., 1999) was used to generate *lacZ* reporter strains in *A. niger* FGSC A1144. For selection of transformants, the *ptrA* resistance cassette (Fleck and Brock, 2010) was included in the reporter constructs. Transformation of *A. niger* FGSC A1144 and Southern hybridization (Figure S3) was performed as described previously (Gressler et al., 2011).

Generation of *terA*, *terA*ΔTE, and *terA*:TE_{wA} Expression Strains in *A. niger* A1144

Expression of *terA*, *terA*ΔTE, and *terA*:TE_{wA} was controlled by the *A. oryzae amyB* promoter as described above. All constructs were additionally fused with terminator sequences to avoid run-through events during transcription. Either the *ptrA* or the *hph* resistance cassette was used as a selection marker for *A. niger* A1144 transformation. Transformants were checked by PCR and Southern hybridization to confirm the complete integration of the constructs (Figure S2). For details concerning oligonucleotides used for fragment amplification, cloning strategies, and generation of labeled probes, refer to the Supplemental Experimental Procedures.

Semiquantitative PCR on Terrein Cluster Genes

RNA from *A. terreus* SBUG844 grown for 36 and 48 hr on AMM, Sabouraud, YPD, and PDB media was isolated using TRIsure (Bioline). After DNase treatment, cDNA was generated using anchored oligo_dT primers, and cDNA amounts were normalized against the actin gene (Gressler et al., 2011). Oligonucleotides P61–P114 (Supplemental Experimental Procedures) were used for semiquantitative PCR analyses on ATEG_00126–00150. Genomic DNA from SBUG844 was used as a positive control during PCR amplifications.

β-galactosidase Assay

As described above, β-galactosidase assays were performed on cell-free extracts from *A. niger* transformants harboring *lacZ* fusion constructs. Mycelia were harvested from cultures grown for 48 hr in AMM, YM, or AMM-CA1% media at 30°C. Mycelia were ground to a fine powder under liquid nitrogen and resuspended in 50 mM MOPS buffer (pH 7.5) with 2 mM $MgCl_2$ and 10 mM β-mercaptoethanol. After centrifugation at 21,000 × *g*, the supernatant was used for β-galactosidase assays as described (Gressler et al., 2011).

Isolation and Structure Elucidation of Metabolites

Purified terrein was obtained by recrystallization from crude extracts with ethylacetate (Demasi et al., 2010). Details for the isolation of 6-hydroxymellein, 6,7-dihydroxymellein, orsellinic acid, and 4-hydroxy-6-methylpyrone by solid-phase extraction and preparative HPLC are provided in the Supplemental Experimental Procedures. High-resolution electrospray ionisation mass spectrometry (HR-ESIMS) was carried out on an Accela UPLC-system combined with an Exactive mass spectrometer (Thermo Scientific) operating in positive ionization mode. Separation was carried out on a Betasil C18 column (2.1 × 150 mm; 3 μm; Thermo Scientific) using water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, as binary solvent system. A flow rate of 250 μl/min and the following gradient was used: 0–1 min = 5% B, 1–16 min = 5%–98% B, 16–19 min = 98% B, and 19–20 min = 98%–5% B. NMR spectra were recorded on a Bruker Avance III 500 and a Bruker Avance III 600 spectrometer (Bruker BioSpin GmbH) equipped with a cryoprobe head using DMSO-d_6 and methanol-d_4 as solvents and internal standards.

Quantification of Terrein

Extraction of 50 ml cultures with ethylacetate was performed as described above, and 80 ml of extracts were evaporated under reduced pressure, solved in 1 ml methanol, and diluted in a ratio of 1:20. Each sample was applied to an Agilent 1260 modular HPLC system (Agilent Technologies) equipped with DAD. As the stationary phase, a bifunctional-phenylpropyl-modified C18 column (Macherey-Nagel Sphinx RP, 4.0 × 250 mm; 5 μm) with a binary solvent system consisting of methanol (solvent B) and water containing 0.1% formic acid (solvent A) was used. The following gradient with a flow rate of 0.8 ml/min was applied: 0.5 min = 10% B, 0.5–20 min = 10%–70% B, 20–25 min = 70%–100% B, 25–28 min = 100% B, and 28–33 min = 100%–10% B. Quantification was performed from a calibration curve of known terrein concentrations. For dry-weight correlation the mycelia from the cultures were dried for 48 hr at 37°C and balanced. From these values, the terrein concentrations per gram

of dried mycelium were calculated. All quantifications were carried out in biological triplicates and technical duplicates.

Metabolite Supplementation Experiments

A. niger strains were cultivated for 48 hr on AMM, and *A. terreus* wild-type and mutant strains were cultivated for 72 hr on PDB medium. In precursor feeding experiments, media were supplemented with unlabeled SNAC derivatives of 3-hydroxy butyric acid, 3-hydroxy pentanoic acid, 3-hydroxy hexanoic, and labeled [1,2,3,4-^{13}C] 3-hydroxy butyric acid (Cortecnet). Cultures of *A. niger* A1144_P*amyB*:*terA* were supplemented at 0 hr and after 24 hr with 2.5 mM of the respective molecules (solved in DMSO). For labeling of terrein, *A. terreus* SBUG844 was cultivated for 72 hr at 30°C in a 4 l Biostat B-DCU II fermenter (Sartorius Stedim Biotech) in PDB medium supplemented with 5 mM [1-^{13}C] sodium acetate (Cortecnet). The culture was constantly stirred at 500 rpm, and air was sparged at 2 l/min. Labeled molecules were isolated from *A. terreus* strain SBUG844Δ*akuB*Δ*terC* cultivated in 100 ml PDB medium supplemented with 1 g [1-^{13}C]-D-glucose (Cortecnet). In cross-feedings, 100 μl culture extracts from the deletion mutants Δ*terB*, Δ*terC*, Δ*terD*, Δ*terE*, and Δ*terF* were added to cultures of SBUG844Δ*akuB*Δ*terA*. After 72 hr, culture supernatants were extracted and applied to LC-MS/MS, preparative HPLC, and NMR analysis as described.

Root Growth Inhibition and Fruit Surface Spot Dilution Assays

Details on root growth inhibition are provided in the Supplemental Experimental Procedures. Briefly, single sanitized radish seeds were transferred to 15 ml tubes with 7 ml of modified solid Hoagland medium and overlaid with 500 μl medium. Tested compounds were terrein, OA, 6,7-DHM, and 6-HM that were added to sterile media in final concentrations of 100, 50, 25, 10, or 1 μg/ml. Tubes were incubated for 3 days in the dark (1 day, 4°C; 2 days 20°C) and finally exposed to a constant 12 hr light/dark rhythm, at which time root and hypocotyl length were determined on a daily basis. Triplicates with 12 biological replicates were tested. To test the effect on fruit surfaces, organic bananas (type Bio) or pears were cleaned with water, and 5 μl of the metabolites OA, 6-HM, 6,7-DHM, and terrein (solved in PBS with 0.1% Tween 20; range: 2.5 mg/ml to 1 μg/ml) were applied. Photographs were taken after 2 and 7 days of incubation at room temperature in the dark.

Statistical Analysis

Error bars in figures represent SD (±SD). Statistical analyses were performed by applying the two-tailed Student's t test. Data were denoted as significantly different with p values $\leq$ 0.01 (*) or $\leq$ 0.001 (**).

ACCESSION NUMBERS

The DDBJ/EMBL/GenBank nucleotide sequence database accession number for the 5′ coding region spanning intron 1 of the *terA* gene (locus ATEG_00145) reported in this paper is KF647874.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.03.010.

AUTHOR CONTRIBUTIONS

C.Z. isolated and characterized metabolites, analyzed data, and contributed in writing the manuscript; M.G. constructed mutants, performed biological activity tests, analyzed transcription profiles, and contributed in writing the manuscript; E.S. performed phylogentic analyses; E.G. contributed in metabolite isolation and structure elucidation; and C.H. and M.B. designed the study, interpreted data, and wrote the manuscript.

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