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Differential Gene Expression Network in Terpenoid Synthesis of *Antrodia cinnamomea* in Mycelia and Fruiting Bodies

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Supporting Information

ABSTRACT: *Antodia cinnamomea*, a precious brown-rot fungus endemic to Taiwan, has pharmaceutical applications due to its diverse array of metabolites. The terpenoids found in *A. cinnamomea* contribute to its most important bioactivities. We identified several terpenoid compounds in *A. cinnamomea* and revealed that their content in mycelium and fruiting body were significantly different. Using next-generation sequencing and an in-house transcriptome database, we identified several terpene synthase (TPS) candidates. After sequence analysis and functional characterization, 10 out of 12 candidates were found to have single or multiple terpene synthesis functions. Most of the terpenoid compounds were found to confer important bioactivities. RT-PCR results showed a positive correlation between terpene synthase expression pattern and terpenoid content. In addition, we identified several modification enzyme candidates that may be involved in the postmodification of terpenoid compounds with a genomic DNA scaffold, and a putative genetic network.

KEYWORDS: *Antrodia cinnamomea, genetic network, next-generation sequencing, terpenoid synthase, transcriptome*

INTRODUCTION

Antrodia cinnamomea (Syn. *Antrodia camphorata*, *Taiwanofungus camphoratus*) also called "chang-chih", is a precious saprophytic brown-rot fungus that grows in the inner cavity of its only host *Cinnamomum kanehirai* Hayata, which is already an endangered native species in Taiwan.[1] The wild-type fruiting body of *A. cinnamomea* has been used as a folk medicine to cure diarrhea, alcohol poisoning, drug intoxification, abdominal pain, hypertension, and liver cancer for a long period of time and has a good reputation. It is colloquially named "ruby in the forest".[2] Research has revealed that *A. cinnamomea* has a diverse range of biological activities and possesses great pharmaceutical potential. It has been shown to have anticancer,[3,4] anti-inflammatory,[5,6] antioxidative,[7,8] antitumor,[9] antihepatitis,[10] hepatoprotective,[11,12] and vasorelaxation[13] activity.

With increasing evidence of the possible bona fide medical applications of *A. cinnamomea*, the demand for the wild-type fruiting body is increasing. However, *A. cinnamomea* can only grow on a specific plant host at an extremely slow rate and it is difficult to induce fruiting body formation in a laboratory culture system. The mycelium can easily be cultured in an artificial system, but it has been found to be deficient in certain specific, active compounds.[14−16] Therefore, many researchers have focused on the improvement of the *A. cinnamomea* culture system. However, the relative genes involved in the secondary metabolite biosynthesis pathway of *A. cinnamomea* have rarely been studied.

Many mushrooms have been used as a folk medicine because some produce a variety of bioactive compounds. Terpenoids, the largest and most diverse family of small molecular natural products, are the primary class of bioactive compounds isolated from many fungal species.[17,18] More than 30,000 different terpenoids have been identified.[19] Two important isoprene building blocks, namely, dimethylallyl pyrophosphate (DMAPP) and its isomer isopentenyl pyrophosphate (IPP), are generated by the mevalonate pathway. Terpenoid precursors (prenyl pyrophosphate) are generated by sequential condensation of DMAPP and IPP. Specific precursors catalyzed by different terpene synthases generate a huge variety of structurally diverse mono-, sesqui-, di-, triterpenoid, and other derivatives. Some higher basidiomycete fungi like *Omphalotus olearius* or *Coprinopsis episcopalis* can produce a series of sesquiterpenes and their derivatives called illudins, which exhibit cytotoxic activities and are a potential anticancer drug.[20,21] Others such as isovelleral from *Lactarius vellereus* and merulidial from *Merulius tremellosus* are also terpenoid compounds with specific bioactivities.[22] Diverse triterpenoid compounds contribute the most important bioactivities of *A. cinnamomea*. In addition to triterpenoids, a different kind of mono- and sesquiterpenoid has also been identified in the fruiting body of *A. cinnamomea*.[23,24] Major terpenoid compounds found from the volatile metabolites include *p*-cymene, a natural antioxidant containing anti-inflammatory activity[25] which has potential for development as an anticancer drug,[26] terpenen-4-ol, which can suppress inflammation[27] and also contains antibacterial and antifungal activities,[28] and isolongifolene, which has antioxidant activity.[29] Antrocin, a

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drimane-type sesquiterpenoid, is the only sesquiterpene lactone isolated from *A. cinnamomea* to date and has strong cytotoxic activity against several cancer cell lines such as breast cancer cell lines MDA-MB-231 and MCF-7, lung cancer cell line A549, liver cancer cell line Huh7, and colon cancer cell line HT-29.[16] We believe that besides triterpenoids the diverse mono- or sesquiterpenoid compounds found in *A. cinnamomea* also have significant bioactivities with potential for future pharmaceutical development. However, to date, no genetic information on terpenoid synthesis in *A. cinnamomea* has been published.

Next-generation sequencing techniques have been extensively used and improve the efficiency of discovering novel genes or further understanding the differential gene expression patterns within organisms.[30] In the previous study, we focused on the gene transcriptional features of different development stages of *A. cinnamomea* and all the NGS data were submitted to the National Center for Biotechnology Information Short Read Archive (NCBI SRA) database.[31]

We used published information to help us to improve the assembly and search for the terpenoid synthesis-related genes. In Figure 1, a putative synthesis pathway of antrocin was

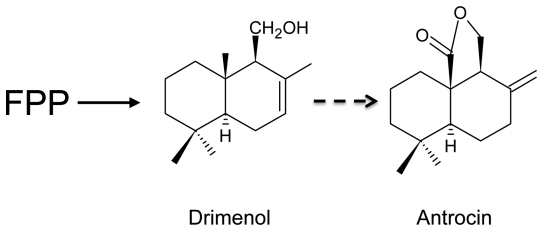

Figure 1. Putative synthesis pathway of antrocin. FPP: farnesyl pyrophosphate. Arrow with solid line represents the terpene synthase; dashed arrow represents the postmodification enzyme.

predicted. One or multiple terpene synthases help to convert the farnesyl pyrophosphate (FPP) into drimenol. Then finished the subsequent cyclization with postmodification enzymes such as cytochrome p450 to synthesize the sesquiterpene lactone antrocin. In this article, we use the published *A. cinnamomea* genomic DNA and transcriptome data to identify several terpene synthases and the possible precursors of antrocin or undiscovered terpenoids. A custom microarray DNA chip based on the transcriptome data was used as reference to create a genetic network of different developmental stages of *A. cinnamomea* to illustrate the gene correlation relationship and find the modification enzyme candidates.

MATERIALS AND METHODS

Fungal Strain and Culture Media. The fungus *A. cinnamomea* wild-type fruiting body (AT), fruiting body on solid plate of isolate B479 (AF), liquid culture mycelium of isolate B479 (AL), and mycelium before transform to fruiting body on a solid plate of isolate B479 (EAP) were provided by Dr. Tun-Tschu Chang (Taiwan Forestry Research Institute). The mycelium was maintained in MEA medium (2% malt-extract, 2% glucose and 2% phyto-agar if cultured in the solid state) in darkness at 24 °C without shaking.

RNA and Genomic DNA Preparation. The total RNA and genomic DNA preparation were as described by Lin.[24] Single strand cDNAs were generated with oligo-dT primer by Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA).

Terpene Synthase Isolation, Protein Expression, and Enzyme Activity Assay. Terpene synthases from other fungal species collected from NCBI were imported into the CLC Genomic Workbench to create a local database. The assembled unigene contigs from a previous study[31] were used to search the homologous hits using the BLAST function with E-value cutoff set to 1E−30.

The predicted terpene synthase contigs were cloned and sequenced, and then the 5′ and 3′ Rapid Amplification of cDNA Ends System (RACE) version 2.0 (Invitrogen, Carlsbad, CA) was used to amplify the 5′ and 3′ cDNA sequences separately. The full lengths of *AcTPS*s were submitted to CD-search to search the Conserved Domain Database (CDD) in NCBI to find the position-specific conserved domain to provide further insights into sequence/structure/function relationships. The molecular evolutionary phylogenetic tree of the *AcTPS* amino acid sequence was generated by MEGA software version 5.05[32] based on the neighbor-joining method using bootstrapping with 1,000 replicates.

For the recombinant protein expression, each full-length of terpene synthase coding sequence was constructed into pET21a protein expression vector (Merck, Darmstadt, Germany) and transformed into the recombinant protein expression host *Escherichia coli* BL21 (DE3). Overnight suspension culture was refreshed in fresh LB broth until OD_{595} reached 0.6, and the overproduction of recombinant protein was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16 °C for a further 20 h. The cell pellets were collected by centrifugation at 4 °C 8000*g* for 10 min. After interrupting the pellet the recombinant proteins were purified with TALON superflow (GE healthcare, Piscataway, NJ).

The terpene synthase enzymatic analysis was performed in a 500 μL reaction mixture containing 50 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM DTT, 10% glycerol, 30 μg of recombinant protein, and the substrate, 50 μM GPP or FPP, set up separately. The mixtures were then sealed with parafilm and kept in the 30 °C incubator for another hour. After 1 h reaction time the reaction mixtures were directly used for GC/MS analysis.

Octaprenyl pyrophosphate synthase activity analysis was performed in a 200 μL reaction mixture containing 100 mM HEPES pH 7.5, 50 mM KCl, 0.5 mM $MgCl_2$, 10% glycerol, 5 μM FPP, 50 μM IPP, and 10 μg of recombinant protein and kept at 25 °C for 16 h. Then the reaction mixture was analyzed by LC-MS/MS performed on a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA) equipped with a Phenomenex Luna 5 μm C18 (2) column (250 × 4.60 mm, Phenomenex, Torrance, CA) and the mass detector was performed on an amaZon speed (Bruker, Fremont, CA) ion trap. The ion source temperature was 250 °C, using N_2 as dry gas at 9.0 L min^{-1} with capillary voltage at 4,500 V and end plate offset at 500 V, with scan range 70 to 800 m/z in positive and negative ionization mode.

Solid Phase Microextraction Absorption and Gas Chromatography Mass Spectrometry Analysis. A 100 μm polydimethylsiloxane (PDMS)-coated solid phase microextraction (SPME) fiber was used for further chemical compound absorption among different experiments.[33] To analyze the compound released to the medium by *A. cinnamomea* throughout the suspension culture, the fiber was directly inserted into the medium for 20 min to absorb chemical compounds. To analyze the volatile compounds of *A. cinnamomea* wild-type fruiting body, the basidiomes were cut into small pieces and placed in a 50 °C water bath for 20 min for SPME headspace absorption.

After absorption, the fiber was directly used for GC/MS analysis. The authentic standards are diluted with ethyl acetate to 100 ppm for directed injection. GC/MS analysis was performed by a Thermo Polaris Q ion-trap mass spectrometer coupled with a Thermo Finnigan Trace GC Ultra gas chromatograph (Thermo, Waltham, MA) equipped with a DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Folsom, CA). The temperature of the injector was set to 250 °C; the ion source was 230 °C with EI at 70 eV; the mass analyzer was set to scan from 50 to 400 amu, and the flow rate of the He carrier gas was 1 mL min^{-1}. The oven temperature started at 60 °C and increased at a rate of 5 °C min^{-1} to 210 °C, then at 30 °C min^{-1} to 290 °C and was held for 5 min.

Microarray DNA Chip Analysis and Genetic Network Construction. Sequence lengths below 300 bp were first filtered out from the contigs from *A. cinnamomea* transcriptome.[31] 11,984 contigs were kept and used to design the Agilent Custom Gene

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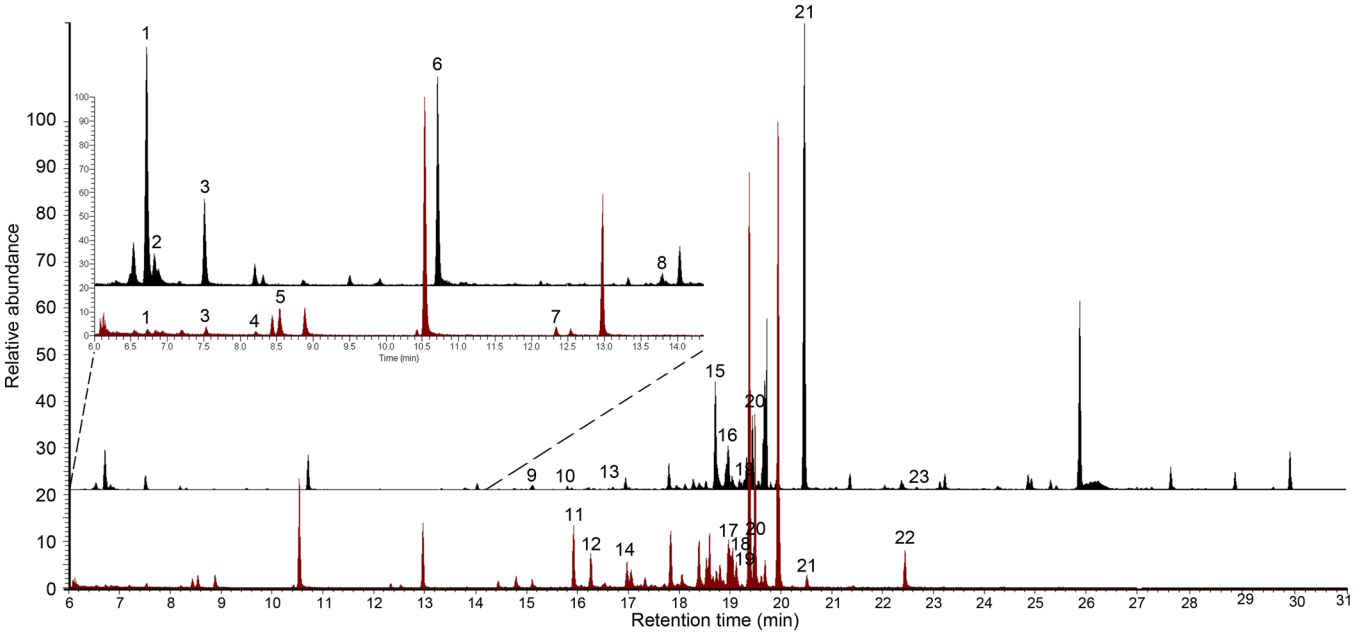

Figure 2. SPME headspace absorption GC/MS analysis of wild-type fruiting body and liquid cultured mycelium. Top, in black: wild-type fruiting body; bottom, in red: liquid cultured mycelium. 1: *p*-Cymene. 2: Limonene. 3: γ-Terpinene. 4: Terpinolene. 5: Linalool. 6: Terpineol. 7: Geraniol. 8: Thymol. 9: α-Cubebene. 10: α-Copaene. 11: Geranyl acetate. 12: β-Longipinene. 13: α-Guaiene. 14: γ-Amorphene. 15: Valencene. 16: α-Muurolene. 17: σ-Amorphene. 18: γ-Cadinene. 19: δ-Cadinene. 20: α-Cadinene. 21: Nerolidol. 22: T-Cadinol. 23: α-Cadinol.

Expression Microarray 4 × 44K microarray chip (Agilent Technologies). Total RNA from different development stages (AF, AP, EAP, and AT) of *A. cinnamomea* was purified and subjected to microarray analysis. The microarray experiment was performed according to Wen et al.[34] with slight modification. In brief, 200 ng of total RNAs was labeled with Cy3 using a Low Input Quick Amp Labeling kit (Agilent Technologies). The labeled cRNAs were hybridized with the microarray chip performed by the DNA Microarray Core Laboratory of the Institute of Plant and Microbial Biology, Academia Sinica. After 65 °C, 17 h hybridization the chip was scanned with an Agilent Microarray Scanner (Agilent Technologies) and the pixels were transformed into digital data with Feature Extraction ver. 10.7.1.1; then the data were further normalized with GeneSpring ver. 11.5 under percentile = 75.

Gene expression data from the microarray was imported into ConigViews (National Taiwan University). The genetic network was constructed using the algorithms based on the R program.[35] The procedures are summarized below: First, the variance was analyzed with ANOVA to pick up the differentially expressed genes with three times or more fold change in expression abundance. Second, the correlation coefficient was calculated among the retained genes, and only correlation coefficients of ≥0.9 or ≤−0.9 were kept. Lastly, the genetic network was visualized with Gephi ver. 0.8.2 beta.[36]

RESULTS

Terpenoid Synthesis Pathway and Isolation of Putative Terpene Synthases. To distinguish the different metabolites in liquid-cultured mycelium and wild-type fruiting body, an analysis was performed using headspace SPME absorption GC/MS, and the result is shown in Figure 2. The GC diagram showed that these two samples have significantly different composition. The mass fragmentation spectrum was compared with the Wiley/NBS Mass Spectral Library with NIST MS search software. Kovats index related to *n*-alkanes (C_9−C_{21}) was also determined, and most of the terpenoid compounds were also compared to authentic standards. Detailed information is listed in Supplemental Table S1. We could identify diverse terpenoid compounds in these two samples. Overall, there were 132 peaks in the GC diagram among these two development states of *A. cinnamomea*. Although several terpenoid compounds can be identified in both samples, the content was significantly different. Limonene (2), terpinolene (4), terpineol (6), thymol (8), α-copaene (10), valencene (15), α-muurolene (16), and α-cadinol (23) were only found in the fruiting body. There were several mono-, sesqui-, and even diterpenoids in wild-type fruiting body, but the major composition of liquid cultured mycelium was sesquiterpenoids. We could identify some of the terpenoid compositions; however, there were a lot of unknown volatile compounds.

To identify the terpene synthases from *A. cinnamomea*, we collected hundreds of terpene synthases from other fungal species from the NCBI protein library and then imported them into the CLC Genomic Workbench to set up a local database. The assembled unigenes were screened using the BLAST algorithm to the local database with a homology threshold at 1E−30. Fortunately, we obtained 12 putative terpene synthase candidates. The protein sequence similarities are listed in Supplemental Table S2. The most similar were M_Fcontig40411 and M_Fcontig47706 (64.67%); the most dissimilar were M_Fcontig62520 and M_Fcontig23363 (only 0.77%). The amino acid phylogenetic analysis and CD-search result is shown in Figure 3. In addition to M_Fcontig27792, other terpene synthase candidates contained one to two conserved DDxxD and NSE/DTE motifs that are important in terpene synthase for divalent ion binding.[37,38] The clustering according to the enzymatic functional domains was partially matched to the clustering with the phylogenetic tree. In addition, M_Fcontig13346 was clustered in the group of two function domains. This may be because the protein sequence similarity was much higher in this subgroup. M_Fcontig36944 and M_Fcontig23363 were clustered into a separate subgroup because their similarity was higher than that of other groups and their product was also a linear-form terpenoid. Finally,

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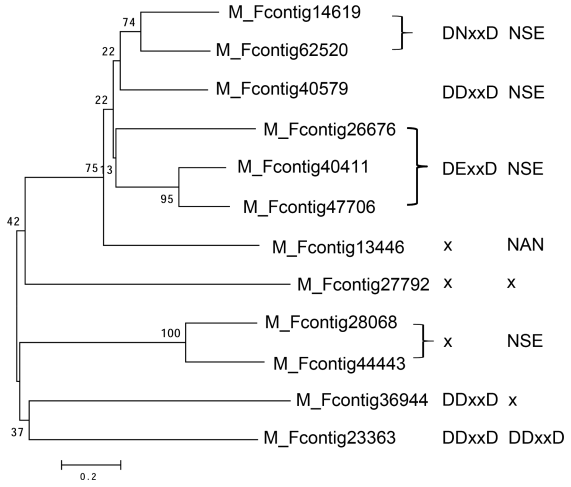

Figure 3. Phylogenetic analysis of terpene synthase candidates using amino acid sequences and the neighbor-joining method (replicate = 1,000). The conserved domain arrangement is also shown on the left in the figure.

M_Fcontig27792 was in an individual group because it does not contain any typical terpene synthase functional domain.

Functional Characterization of AcTPSs. After isolation of each full-length of terpene synthase candidate, the coding sequences were used for recombinant protein expression followed by functional analysis by adding different enzymatic substrates (GPP or FPP). The sequence features and relative terpenoid products are listed in Table 1. The conserved domain prediction, SDS−PAGE of recombinant protein, and GC/MS diagrams of each candidate are shown in Supplemental Figures S1 to S12. Ten out of 12 candidates had terpene synthase activity and were named *AcTPS*. According to the functional domains and the protein functional analysis results, we were able to divide these candidates into 3 types.

Group 1: Enzymes with Two Functional Domains. M_Fcontig40579 (*AcTPS5*) has complete DDxxD and NSE motifs and almost only generates a single product when using GPP as substrate (geraniol); when using FPP as substrate it can generate a major product (T-cadinol) and several minor sesquiterpenoid products. M_Fcontig26676 (*AcTPS3*) contains DExxD and NSE motifs and can generate multiple monoterpene with GPP and EE α-farnesol with FPP, and most *AcTPS3* products are linear form terpenoids. M_Fcontig40411 (*AcTPS4*) and M_Fcontig47706 (*AcTPS9*) also contain DExxD and NSE motifs the same as *AcTPS3*, but these two enzymes generate multiple products when using GPP and FPP as substrate. M_Fcontig14619 (*AcTPS1*) and M_Fcontig62520 (*AcTPS10*) contain DNxxD and NSE motifs, but *AcTPS1* can only generate one unknown terpenoid and δ-cadinol and the content of both is very low. Otherwise *AcTPS10* generates mostly linear form terpenoids (geraniol and farnesol). In this group, most of the terpene synthases with two functional domains can generate multiple terpenoid compounds.

Group 2: Enzymes with a Partial Functional Motif. M_Fcontig13346 (*AcTPS2*) contains only one incorrect NST/DTE motif; the motif sequence became NAN. No product was generated with GPP, and a minor peak (farnesol) was seen with FPP. Although M_Fcontig28068 (*AcTPS6*) and M_Fcontig44443 (*AcTPS11*) contain a correct NSE motif, minor linear terpenoid products are generated. M_Fcontig36944 (*AcTPS7*) contains a correct DDxxD, but the NSE/DTE motif was absent, and one major product, linalool, was generated when adding GPP as substrate and nerolidol with FPP.

Group 3: Others. Although M_Fcontig27792 is listed as a terpene synthase candidate, it was found not to contain any conserved functional motif after CD-search analysis, and no product was generated. M_Fcontig23363 contains two DDxxD motifs, and no product was generated with GPP, FPP, or GGPP as a substrate. Further functional prediction showed that M_Fcontig27792 may function as a protein prenyl transferase and M_Fcontig23363 (*AcOPPs*) may function as an octaprenyl pyrophosphate synthase (OPPs) that leads the terpenoid backbone synthesis pathway into the ubiquinone synthesis pathway and may participate in the antroquinonol synthesis. So we combined the purified recombinant protein with FPP and IPP as a substrate and analyzed with LC-MS/MS. The result is shown in Supplemental Figure S13. Compared to the reaction mixture containing FPP and IPP but without any recombinant protein, there was a minor peak when adding *AcOPPs* recombinant protein. Since no standard is available, we suggest that the mass fragment at 734 *m*/*z* may represent the parent molecule of $C_{40}H_{65}O_7P_2$ (MW = 719) with one methyl group (MW = 15) modification or the mass fragment from C_{45} isoprenyl pyrophosphate when breaking into C_4H_6 + $C_{41}H_{67}O_7P_2$. 651 *m*/*z* may represent the molecular weight of the molecule $C_{35}H_{57}O_7P_2$. The two mass fragments shown in the *AcOPPs* reaction mixture did not appear in the reaction mixture without any recombinant protein.

Time Course Analysis of *A. cinnamomea* Suspension-Cultured Mycelium. Since the fruiting body formation of *A. cinnamomea* was unclear and unregulated, we could only monitor the terpenoid compounds in growing mycelium. The SPME headspace absorption and medium broth direct absorption GC/MS analysis results after the specified sampling periods (5, 10, 15, 20, 25, 30, and 40 days) are shown in Figures 4a and 4b, respectively. The RT-PCR result is shown in Figure 5.

With increasing culture time, the contents of the volatiles and the terpenoids increased. As shown in Figure 4a, the most abundant volatiles were two nonidentified sesquiterpenoids (RT around 19.38 and 19.95 min) and one benzaldehyde-type compound (RT around 19.69 min) with molecular weight 196 *m*/*z*. In Figure 4b, the most abundant compound in the medium was also a nonidentified sesquiterpenoid (RT around 19.95 min). The detailed information on the GC/MS analysis is also listed in Supplemental Table S1. According to the RT-PCR result in Figure 5, different *AcTPS* genes have different expression patterns at different suspension culture times and in different states of *A. cinnamomea*. The RT-PCR showed that *AcTPS4*, *5*, *7*, *9*, and *10* have higher expression on different sample harvest days. This result was matched to the terpenoid compounds generated by *AcTPS4*, *5*, *7*, *9*, and *10*. But there are still several terpenoid compounds for which the related terpene synthases could not be identified, and this may be caused by the postmodification of other enzymes. *AcOPPs* is upregulated in mycelium compared to the wild-type fruiting body, and this may also be related to the fact that the antroquinonol can only be identified in mycelium.

Drimenol Synthase Identification. To date, only one drimenol synthase gene sequence from plant *Valeriana officinalis* has been published.[39] We also take this gene sequence as a blasting target, but no homologous hit can be found in *A. cinnamomea* transcriptome library.

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Table 1. General Features of the Terpene Synthases Isolated from RNA-seq[a]

contig name	name	CDS (bp)	substrate	area (%)	KI	rKI	product	identification
M_Fcontig14619	AcTPS1	1,017	GPP	–	–	–	–	
			FPP	*	1578	–	?	
				*	1645	–	δ-cadinol	MS, ST
M_Fcontig13346	AcTPS2	924	GPP	–	–	–	–	
			FPP	*	1703	1697	ZE α-farnesol	MS, KI, ST
M_Fcontig26676	AcTPS3	999	GPP	5.01	977	975	β-pinene	MS, KI, ST
				2.38	1019	1011	3-carene	MS, KI, ST
				4.28	1031	1037	β-ocimene	MS, KI, ST
				4.07	1090	1097	linalool	MS, KI, ST
				77.35	1251	1252	geraniol	MS, KI, ST
			FPP	*	1725	1722	EE α-farnesol	MS, KI, ST
M_Fcontig40411	AcTPS4	1,158	GPP	37.27	979	975	β-pinene	MS, KI, ST
				36.36	1022	1011	3-carene	MS, KI, ST
				3.51	1032	1037	β-ocimene	MS, KI, ST
				8.34	1088	1097	linalool	MS, KI, ST
				0.35	1092	–	?	
				1.63	1118	–	?	
				0.31	1122	–	?	
				0.42	1193	1189	α-terpineol	MS, KI, ST
				2.17	1219	1229	nerol	MS, KI
				9.64	1250	1252	geraniol	MS, KI, ST
			FPP	16.67	1357	1348	α-cubebene	MS, KI
				0.66	1388	–	?	
				10.38	1401	1400	sibirene	MS, KI
				0.68	1412	–	?	
				0.9	1444	–	?	
				2.15	1464	–	?	
				4.12	1487	–	?	
				2.22	1497	1495	γ-amorphene	MS, KI
				5.34	1508	1513	γ-cadinene	MS, KI, ST
				46.44	1530	1529	zonarene	MS, KI
				7.14	1547	–	?	
				0.66	1588	–	?	
				0.12	1599	–	?	
				0.41	1638	–	?	
				0.79	1700	1697	ZE α-farnesol	MS, KI, ST
				0.37	1716	1722	EE α-farnesol	MS, KI, ST
M_Fcontig40579	AcTPS5	1,014	GPP	0.62	1088	1097	linalool	MS, KI, ST
				0.24	1121	–	?	
				0.15	1217	–	?	
				1.06	1221	1229	nerol	MS, KI
				97.21	1248	1255	geraniol	MS, KI, ST
				0.71	1268	1267	E-citral	MS, KI, ST
			FPP	0.38	1357	1351	α-cubebene	MS, KI
				0.75	1388	–	?	
				0.93	1402	–	?	
				0.2	1444	–	?	
				0.1	1460	–	?	
				0.14	1464	–	?	
				0.07	1474	–	?	
				0.08	1476	–	?	
				0.57	1489	1479	γ-muurolene	MS, KI
				0.69	1496	1495	γ-amorphene	MS, KI
				0.1	1507	–	?	
				1.09	1512	–	?	
				10.51	1527	1513	γ-cadinene	MS, KI
				2.58	1531	1523	δ-cadinene	MS, KI
				3.59	1550	–	?	
				1.23	1589	–	?	
				0.33	1631	–	?	
				74	1651	1640	T-cadinol	MS, KI, ST

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Table 1. continued

contig name	name	CDS (bp)	substrate	area (%)	KI	rKI	product	identification
				1.14	1663	1653	α-cadinol	MS, KI, ST
				0.11	1667	–	?	
				0.17	1699	1697	ZE α-farnesol	MS, KI, ST
				0.16	1716	1722	EE α-farnesol	MS, KI, ST
				0.11	2014	–	?	
M_Fcontig28068	AcTPS6	990	GPP	*	1088	1097	linalool	MS, KI, ST
				*	1248	1255	geraniol	MS, KI, ST
				*	1277	–	?	
			FPP	*	1568	1563	nerolidol	MS, KI, ST
M_Fcontig36944	AcTPS7	999	GPP	*	1090	1097	linalool	MS, KI, ST
				*	1251	1255	geraniol	MS, KI, ST
			FPP	1.5	1357	1348	α-cubebene	MS, KI
				1.71	1400	–	?	
				64.31	1573	1563	nerolidol	MS, KI, ST
				10.05	1715	1722	EE α-farnesol	MS, KI, ST
M_Fcontig47706	AcTPS9	1,014	GPP	44.43	977	975	β-pinene	MS, KI, ST
				0.67	1015	1011	3-carene	MS, KI, ST
				1.56	1020	–	?	
				1.43	1031	1037	β-ocimene	MS, KI, ST
				4.14	1086	1097	linalool	MS, KI, ST
				0.96	1192	1189	α-terpineol	MS, KI, ST
				8.6	1218	–	?	
				37.74	1250	1255	geraniol	MS, KI, ST
			FPP	8.86	1357	1348	α-cubebene	MS, KI
				16.47	1400	1400	sibirene	MS, KI
				1.08	1444	–	?	
				1.37	1463	–	?	
				1.03	1487	–	?	
				2.45	1496	1495	γ-amorphene	MS, KI
				7.66	1510	1515	cubebol	MS, KI
				15.72	1531	–	?	
				1.65	1538	–	?	
				1.99	1546	–	?	
				2.65	1589	–	?	
				2.07	1598	–	?	
				22.68	1637	1628	1-epi-cubenol	MS, KI
				3.7	1700	–	?	
				8.03	1716	1722	EE α-farnesol	MS, KI, ST
M_Fcontig62520	AcTPS10	1,170	GPP	1	975	975	β-pinene	MS, KI, ST
				0.65	1084	1097	linalool	MS, KI, ST
				0.33	1118	–	?	
				0.76	1217	–	?	
				1.01	1220	1229	nerol	MS, KI
				94.96	1249	1255	geraniol	MS, KI, ST
			FPP	3.24	1465	–	?	
				0.75	1503	–	?	
				0.35	1512	–	?	
				0.27	1527	–	?	
				0.25	1531	–	?	
				0.96	1572	1563	nerolidol	MS, KI, ST
				0.33	1589	–	?	
				2.37	1655	1654	α-cadinol	MS, KI, ST
				0.66	1695	1697	ZE α-farnesol	MS, KI, ST
				89.82	1716	1722	EE α-farnesol	MS, KI, ST
M_Fcontig44443	AcTPS11	1,032	GPP	88.52	1250	1255	geraniol	
			FPP	*	1724	1722	EE α-farnesol	

[a]CDS: coding sequence. *: The percentage was too low or could not be measured. –: Not available. KI: Kovats index determined related to *n*-alkanes (C_9–C_{21}). rKI: reference KI based on Adams.[40] "?": Peak with significant terpenoid mass fragmentation features but cannot be identified in database. Identification based on mass spectrum (MS), Kovats index (KI), authentic standard (ST).

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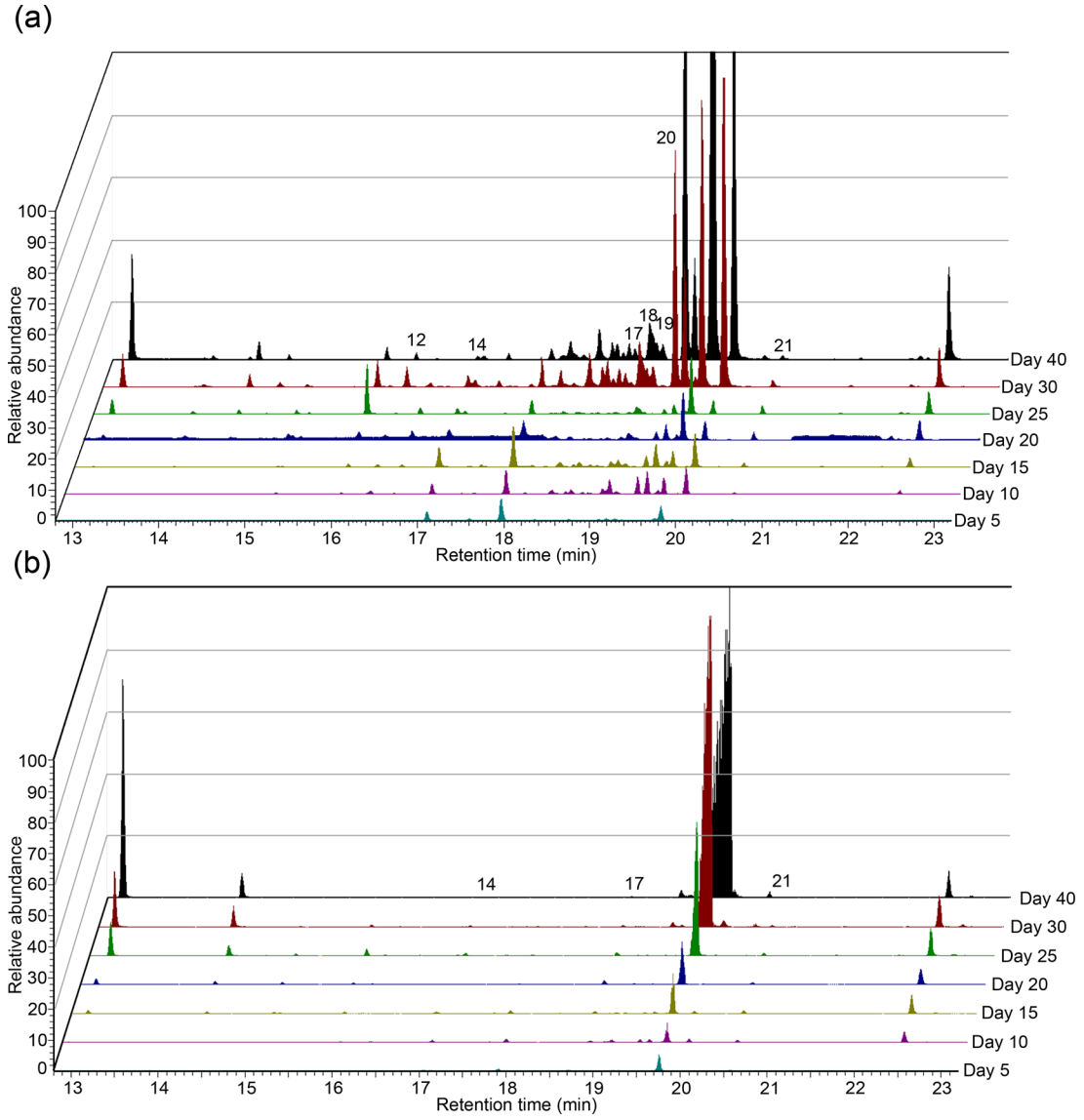

Figure 4. Diagram of GC/MS analysis from mycelium. (a) SPME headspace absorption. (b) The SPME fiber was directly inserted into the liquid broth for absorption. The numbers represented here are the same as in Figure 2.

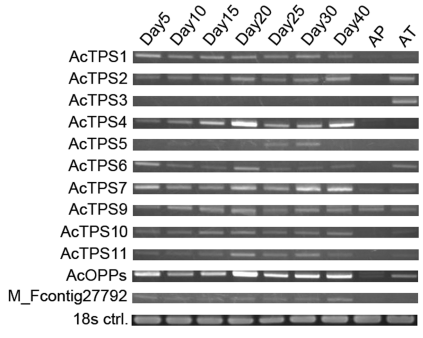

Figure 5. RT-PCR analysis of *A. cinnamomea* across different culture conditions. AP: Mycelium culture on solid MEA plate. AT: Wild-type fruiting body.

Since no drimenol standard is available, we can only use the mass fragmentation record in the NIST Standard Reference Database (mainlib) and the published Kovats index.[40] As shown in Supplemental Figure S14a, drimenol has a significant major mass fragmentation peak at 109 *m*/*z*, and this differs from most of the terpenoids found in *A. cinnamomea* (most are 91 or 105 *m*/*z*). Only the headspace analysis from wild-type fruiting body had a similar peak with base peak set at 109 *m*/*z* (shown in Supplemental Figure S14b, RT = 22.21 min, KI = 1,642, and RT = 24.93 min, KI = 1753). At the same retention time, we can find a minor peak with closer Kovats index only in *AcTPS4*, *AcTPS5*, and *AcTPS9* GC/MS analysis, but the mass fragment differs. Unfortunately, we could not identify a terpene synthase which directly synthesizes drimenol from these candidates. It may contain an uncharacterized terpene synthase or go through a different intermediate to generate drimenol.

Genome Analysis of the *AcTPS* Gene Clusters. As we predicted, the synthesis of antrocin may go through drimenol during a postmodification process. In some cases, the genes involved in metabolite metabolism in fungi may be arranged in a gene cluster. So we used the assembly genomic DNA data previously published[31] coupled with the sequences of *AcTPS* and *AcOPPs* to search the possible modification enzyme candidates. The result is shown in Figure 6. There were several cytochrome p450s (marked with red) and putative modification

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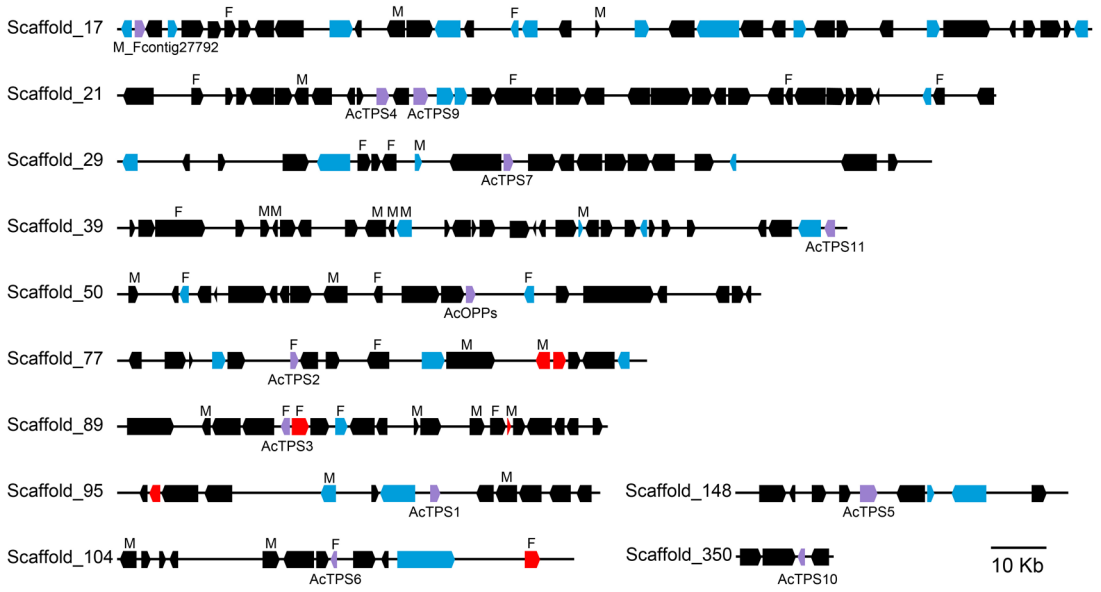

Figure 6. Terpene synthase gene cluster of *A. cinnamomea*. F: Upregulated expression in wild-type fruiting body. M: Upregulated expression in liquid cultured mycelium. Purple: *AcTPS* and *AcOPPs*. Red: Cytochrome p450. Blue: Candidate for the modification enzyme. Black: Others and unknown.

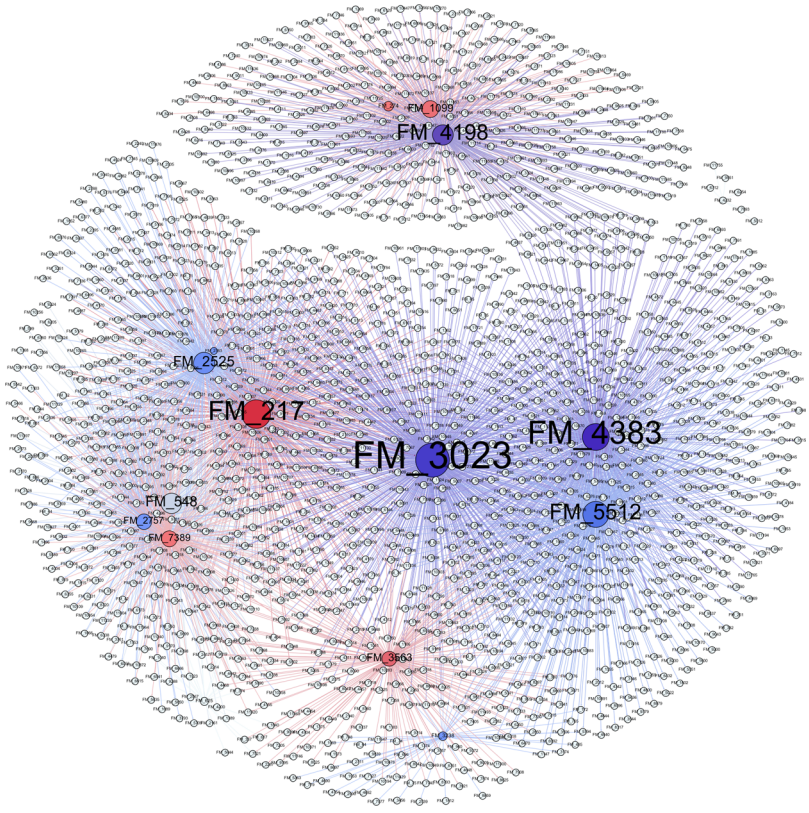

Figure 7. Putative genetic network among mycelium (AL) compared to fruiting body (AT) of *A. cinnamomea*. Red: Transcription factor or regulator. Blue: Terpene synthase and related genes. The node size represents the degree of centrality.

enzymes such as hydroxylase, oxidase, dehydroxylase, transferase, etc. (marked with blue) among these genomic scaffolds. All the genes on the scaffold also underwent differentially expressed gene (DEG) analysis, and the genes with significantly differential expression are marked with F (upregulated in fruiting body) and M (upregulated in mycelium). Only *AcTPS3* and *AcTPS6* were significantly upregulated in fruiting body, and there were also modification enzymes arranged near these two *AcTPS* with the same expression pattern. These enzyme candidate genes may be involved in terpenoid synthesis or modification. Detailed information about Figure 6 is listed in the Supporting Information. These modification enzyme candidates may explain why the terpenoids identified from the wild-type fruiting body and mycelium are larger than all the terpenoids generated from *AcTPS*s, and further analysis is

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needed to elucidate the whole picture of terpenoid compound metabolism in *A. cinnamomea*.

Microarray and Putative Genetic Network. NGS techniques can generate massive amounts of genetic data and help us to identify the expressed genes, and we indeed found several terpene synthases in *A. cinnamomea*. But we also suggest that postmodification may be involved in the terpenoid biosynthesis of *A. cinnamomea*. So we attempted to build up a specific genetic network to identify which enzyme or specific transcription factors may be involved in terpenoid synthesis.

A total of 11,984 unigene contigs were used for custom microarray chip design. Three different sites on each unigene were chosen from probe design, and 35,923 specific probes were generated on the chip. Total RNA from different development stages including liquid cultured mycelium (AL), mycelium on agar plate before transforming to fruiting body (EAP), fruiting body on agar plate (AF), and wild-type fruiting body (AT) were extracted and purified for further hybridization. After image capture and raw data transformation, the digital raw data was processed by GeneSpring, and the hieratical clustering is shown in Supplemental Figure S15. The four development stages of *A. cinnamomea* had different gene expression patterns. AL had the most dissimilar development stage compared to AT and AF, and EAP may represent the transition stage of the fruiting body formation. The microarray data combined into the terpenoid synthesis pathway is shown in Supplemental Figure S16. The mevalonate pathway, where the terpenoid building block C_5 isoprenoid compounds are generated, seems to be more active in mycelium in addition to diphosphomevalonate decarboxylase.

The genetic regulation and networks of terpenoid synthesis in fungal species are not well understood. In this study we used gene expression patterns at different developmental stages to create a putative genetic correlation network. At the beginning we set up a whole spectrum of genetic networks between AL and AT. Of 11,984 unigenes, 2,221 were found to be differentially expressed after ANOVA calculation and three times fold-change filtering. After correlation calculations, 309,959 lines (also called edges in genetic networks) were created, but the relation map was too complicated to illustrate. Then a list containing terpene synthesis genes, related modification enzymes, and transcription factors or regulators from the *A. cinnamomea* transcriptome library was used to filter the genetic network. After image visualization with Gephi software the network contained 1,605 nodes and 3,807 edges as shown in Figure 7. The sizes of the nodes represent the nodal centrality in the network, which is an index to quantify the relationship in the network.[41] In Figure 7, several nodes with higher nodal centrality were annotated as transcription factors/regulators (represented in red) and terpene synthase and related enzymes (represented in blue). The annotation features are listed in Table 2. A subnetwork extracted from the total genetic network only with the list used before is present in Figure 8. Several terpene synthesis pathway-related enzymes and terpene synthases were found to be putatively regulated with different transcription factors. Different developmental stages of *A. cinnamomea* were also compared with the same procedures as used before. The general features of nodes and edges are listed in Supplemental Table S3, and the putative network is shown in Supplemental Figure S17. After genetic network screening, several important nodes with higher centrality are also shown in the figure, such as those enzymes involved in terpene backbone synthesis, terpene synthases, and terpenoid modification enzymes: acetyl-CoA acetyltransferase, HMG-CoA reductase, diphosphomevalonate decarboxylase, IPP isomerase, AcTPS3, AcTPS5, cytochrome p450, and AcCYP51. Transcription factors or regulators: transcription factor PacC, transcription factor IIIC, TFIIH basal transcription factor, transcription initiation factor IIF, C_2H_2 transcription factor, transcription factor *Gf*. BMR1, and jumonji superfamily. All this information provides a putative genetic network in *A. cinnamomea* terpenoid biosynthesis and may be helpful in modifying the artificial culture conditions in further experiments.

Table 2. Annotation of the Nodes with Higher Centrality from Genetic Network of AL Compared to AT in Figure 7

ID	accession no.	annotation
Transcription Factors (Red)		
FM_217	XP_001829114	transcription factor PacC
FM_274	EIW63316	transcription regulator
FM_1099	EUC66281	RNA PolIII transcription factor (TF) IIIC subunit
FM_3563	XP_001829114	transcription factor PacC
FM_7389	EJF58635	transcription initiation factor IIF beta subunit
Terpene Related (Blue)		
FM_2525		AcTPS5
FM_2757	XP_007363597	HMG-CoA reductase
FM_2783		AcTPS2
FM_3023	XP_008033662	phosphomevalonate kinase
FM_3238	EIW52730	squalene monooxygenase
FM_4198	EJF55832	diphosphomevalonate decarboxylase
FM_4383		AcTPS3
FM_5512	ABV66226	AcCYP51

DISCUSSION

A. cinnamomea is a precious fungus with many potent pharmaceutical activities. As mentioned above, *A. cinnamomea* is a host-specific saprophyte fungus with an extremely slow growth rate. Many important bioactive compounds are only present in the wild-type fruiting body and rare in the artificial cultured mycelium. Much research has focused on the improvement of the culture system, but benefits so far have been limited. The only host, *C. kanehirai* Hayata, is an endangered tree species in Taiwan which faces a severe illegal logging problem. For the sustainable management of forests and the development of medicine, it is important to increase basic genetic knowledge and knowledge about the secondary metabolite synthesis pathway.

Terpene Synthase and Functional Domains. In this study we isolated 12 putative terpene synthase candidates through homology searching with BLAST against the previously constructed RNA-seq library. We further conducted molecular cloning and CD search. We sorted the *AcTPS* into 3 groups according to the DDxxD and NSE/DTE functional motifs. After functional characterization, 10 out of 12 candidates were found to have terpene synthesis activity and be able to generate terpenoids when a specific substrate was added (Table 1).

Group 1: AcTPS with Two Functional Domains. Only *AcTPS5* was found to have two complete and correct functional domains (DDxxD and NSE). Therefore, *AcTPS5* has a major synthesis product, a monoterpenol (geraniol) and a sesquiterpenol (T-cadinol) with GPP and FPP as a substrate, respectively. But there are still several minor sesquiterpenes like

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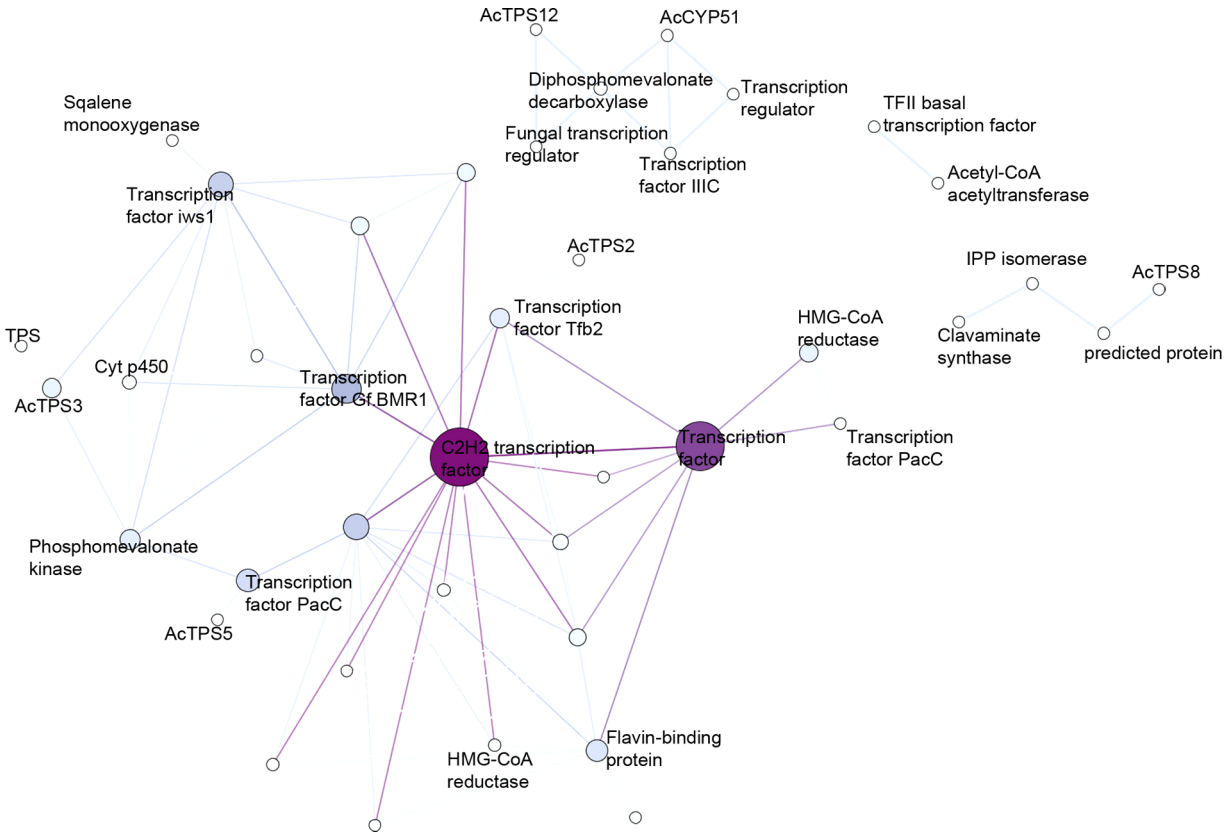

Figure 8. Simplified putative genetic network of the terpene synthesis pathway enzymes, terpene synthases, and transcription factor of *A. cinnamomea*.

γ-cadinene and δ-cadinene, which have conformation similar to T-cadinol except a hydroxyl group on carbon 1 (Supplemental Figure S18). These sesquiterpenes may be the intermediate compounds through T-cadinol synthesis.

AcTPS1 and *AcTPS10* contained DNxxD and NSE motifs, which are not typical DDxxD motifs. In *AcTPS1* δ-cadinol was the only product. In *AcTPS10* only linear form terpenoid could be generated. Although it has been proved that an important tryptophan located in the bottom of the reaction center from *Laurus nobilis* can affect the cyclic terpenoid synthesis,[42] the homologous amino acid of AcTPS10 is also a tryptophan (W357) and without any cyclic terpenoid compounds. This may also, because of the amino acid asparagine (N), which has a positively charged side chain which may affect the divalent ion bonding between substrate and the functional motif on the enzyme's reaction pocket, reduce the enzyme activity and not able to proceed with the following cyclic step (see Supplemental Figure S19a).

AcTPS3, *4*, and *9* have DExxD and NSE motifs, which were also not typical DDxxD motifs. But the amino acid aspartate (D) and glutamate (E) all contained a negatively charged side chain and may not affect the terpene synthase activity but bring out the multiple products on *AcTPS4* and *AcTPS9*.

AcTPS3 was similar to *AcTPS4* and *9* and grouped in the same subclade (Figure 3). But *AcTPS3* does not have multiple products instead of acyclic terpenoid (linalool, geraniol, and nerolidol). The *AcTPS3* amino acid sequence was similar to *AcTPS4* (29.52%) and *9* (30.12%), but the product was totally different. The key points might be that the amino acids next to the functional motifs point into the reaction pocket but have different amino acid side chain structure, polarity, or charge property. Comparison to the homology model constructed by Swiss model suggests that several amino acid candidates might affect the enzyme activity (Supplemental Figures S19b and S19c). M78, I218 in *AcTPS3* and V80, and C219 in *AcTPS4* and *9* were in the bottom of the reaction pocket. Although methionine, valine, and isoleucine were nonpolar amino acids, the lengths of the side chains were different. And cysteine was a polar amino acid that might affect the prenyl group of the substrate. On the top of the reaction pocket, D234 in *AcTPS3* and H235 in *AcTPS4* were totally different in the predicted protein structure model. Aspartate in *AcTPS3* may take shape as a lid, block the cyclization of the prenyl group, and make *AcTPS3* only able to generate acyclic terpenoid. López-Gallego[43] conducted a similar study using site-directed mutagenesis and sequence swapping to examine the amino acids around the conserved functional motifs. They were focused on a similar region to our study and found potent targets to increase the diversity of the terpene synthase.

Group 2: AcTPS with a Partial Functional Domain. *AcTPS7* has a DDxxD motif but no NSE/DTE motif. *AcTPS7* can generate linalool and geraniol when GPP is added as substrate and nerolidol when FPP is a substrate. Although linalool and nerolidol are common terpenoids among fungal species, *AcTPS7* was the first isolated and characterized terpene synthase with bifunctional activity in the NCBI database. The bifunctional activity may be because of the flexible capacity or extensive selection of the reaction center against the substrate on the terpene synthase, and there were several bifunctional linalool/nerolidol synthases in plant species.[33] As mentioned before, *AcTPS7* does not have NSE/DTE motif according to CD search. But the protein 3D

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homology modeling shown in Supplemental Figure S19d indicated that a potential amino acid aspartate opposite to the DDxxD motif might participate in the divalent ion binding. This finding may provide a new aspect of metabolic function domain of terpene synthase in fungi.

AcTPS2 does not contain a DDxxD motif, and the NSE/DTE motif has been changed to NAN in which alanine belongs to a nonpolar aliphatic amino acid and asparagine was a positively charged amino acid, which may affect the divalent ion chelating through the negatively charged side chain provided by the aspartate. According to the replacement of the amino acid, *AcTPS2* cannot provide a suitable reaction pocket conformation, no product is generated with GPP, and there is very weak farnesol production. *AcTPS6* has a complete NSE motif but does not contain a DDxxD motif that is also important in the terpene synthase reaction pocket and causes the minor activity of the enzymes.

Group 3: Others. M_Fcontig27792 does not contain any terpene synthase functional motifs (DDxxD and NSE/DTE) but does contain a protein prenyl-transferase domain (PTase) after CD-search analysis. M_Fcontig23363 (*AcOPPs*) contains two DDxxD motifs but does not generate any product.

Antrocin, the only sesquiterpene lactone isolated from *A. cinnamomea* to date, was one of the targets for which we were trying to characterize the synthase gene or the modification enzyme. Although we did not identify the exact terpene synthase that generates antrocin directly, some unidentified terpenoid compounds possess similar mass fragmentation features, and these may be the possible precursors of antrocin through terpenoid biosynthesis. In addition to antrocin, several terpenoid compounds identified in this analysis also showed significant bioactivities and may contribute to the pharmaceutical activity of *A. cinnamomea*.

Modification Enzymes in Terpene Synthase Gene Clusters. Since the amounts of terpenoid compounds found in the fruiting body and mycelium were much larger than *AcTPS*s can possibly generate, we predict that more modification enzymes or novel terpene synthases are involved in the terpenoid synthesis pathway. Wawrzyn[20] used the draft genome sequence of *O. olearius* to predict the framework involved in sesquiterpenoid biosynthesis. They found different modification enzymes in the gene clusters and proposed a cyclization pathway leading to diverse sesquiterpene products.

There may also be undiscovered novel classes of terpene synthase. Chooi[44] identified a first example of a cytochrome P450 that serves as a terpene cyclase and provides new insights into meroterpenoid biosynthesis in fungi. Itoh[45] also found a novel family of terpene cyclases in *Aspergillus fumigatus*. They identified a gene cluster from the genome sequence. This terpene synthase also does not have an aspartate-rich motif such as DDxxD or DxDD normally found in terpene synthases. Bromann[46] identified a novel diterpene gene cluster in *Aspergillus nidulans* by genome mining. They not only found a gene cluster for biosynthesis of *ent*-pimaradiene but also found a transcription factor PbcR that upregulated the transcription of the relative genes. Many of the studies began with genome mining, and the next step of our study we continue in our draft genome sequence completeness for mining more information. At the same time this information from other fungal species also allowed homology search against our local database for novel terpene synthases or relative regulation transcription factors.

Putative Genetic Network of *A. cinnamomea* in Terpene Synthesis. We found that several important genes are involved in the putative genetic network shown in Figure 8. Acetyl-CoA acetyltransferase, HMG-CoA reductase, diphosphomevalonate decarboxylase, and IPP isomerase belong to the terpene backbone synthesis pathway. Enzyme activity of HMG-CoA reductase affects the quantity of terpenoid compounds and is believed to be the key factor in terpene backbone synthesis.[47] *AcTPS3* and *AcTPS5* were characterized as terpene synthases and were related to volatile compounds generated in mycelium development. Cytochrome p450s and *AcCYP51* are postmodification enzymes and may participate in triterpenoid compound synthesis and contribute to the diversity of triterpenoids in *A. cinnamomea*.

Transcription factor PacC is a regulator in sensing the ambient pH and regulates downstream gene expression via a conserved signaling cascade, and this transcription factor is also related to the virulence of many fungal species.[48] Transcription factor PacC has also been demonstrated to regulate several gene clusters related to secondary metabolite synthesis.[49] C_2H_2 transcription factor is highly conserved in different filamentous fungal species and acts as an ambient stress detection regulator. Deletion of C_2H_2 transcription factor also affects the sporulation processes.[50] C_2H_2 transcription factor also affects asexual and sexual development through secondary metabolism.[51] The transcription factor *Gf*. BMR1 has been proved to be induced by near-ultraviolet and blue light and regulate the fruiting body formation.[52] In *Bipolaris oryzae*, BMR1 regulated 3 melanin biosynthesis genes.[53] Several transcription factors involved in secondary metabolite biosynthesis or fruiting body formation have been identified in the putative genetic network.

In summary, in this study we identified diverse terpenoid compounds in wild-type fruiting body and liquid cultured mycelium of *A. cinnamomea*. These terpenoids may result in the specific bioactivities of *A. cinnamomea*. To further discuss the terpene synthese activity and gene expression patterns, NGS was used and a reliable database for study of *A. cinnamomea* gene expression was constructed. Using the database, we isolated several terpene synthases with different enzyme activities. We discovered that the functional domains of terpene synthases did not always contain DDxxD and NSE/DTE motifs, and may be a new location of the functional motif in fungal terpene synthases. Although we did not identify the antrocin synthase directly, some of the unidentified terpenoid compounds with similar mass spectral features may be the possible precursors of antrocin. Putative genetic networks provide several important candidate genes that regulate or participate in terpenoid biosynthesis. This is the first report that combines NGS, proteomics, bioinformatics, and metabolomics to illustrate the physiological properties of the terpenoid compound biosynthesis in medicinal fungus *A. cinnamomea*. It can be expected that improving the pharmaceutical and industrial usage of *A. cinnamomea* will help maintain sustainable management of the forest, and benefit the development of medicines.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b05386.

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Modeling methods, protein sequence similarity, genetic network features, SDS−PAGE, CD-search, GC/MS, and LC-MS/MS results, and additional figures (PDF)
Detailed information about Figure 6 (XLSX)

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Chang, T. T.; Chou, W. N. *Antrodia cinnamomea* reconsidered and *A. salmonea* sp. nov. on *Cunninghamia konishii* in Taiwan. *Bot. Bull. Acad. Sin.* **2004**, *45* (4), 347−352.

(2) Wu, S. H.; Ryvarden, L.; Chang, T. T. *Antrodia camphorata* ("niu-chang-chih"), new combination of a medicinal fungus in Taiwan. *Bot. Bull. Acad. Sin.* **1997**, *38* (4), 273−275.

(3) Chen, C. H.; Yang, S. W.; Shen, Y. C. New steroid acids from *Antrodia cinnamomea*, a fungal parasite of *Cinnamomum micranthum*. *J. Nat. Prod.* **1995**, *58* (11), 1655−1661.

(4) Hsu, Y. L.; Kuo, Y. C.; Kuo, P. L.; Ng, L. T.; Kuo, Y. H.; Lin, C. C. Apoptotic effects of extract from *Antrodia camphorata* fruiting bodies in human hepatocellular carcinoma cell lines. *Cancer Lett.* **2005**, *221* (1), 77−89.

(5) Shen, Y. C.; Chou, C. J.; Wang, Y. H.; Chen, C. F.; Chou, Y. C.; Lu, M. K. Anti-inflammatory activity of the extracts from mycelia of *Antrodia camphorata* cultured with water-soluble fractions from five different *Cinnamomum* species. *FEMS Microbiol. Lett.* **2004**, *231* (1), 137−143.

(6) Liu, D. Z.; Liang, H. J.; Chen, C. H.; Su, C. H.; Lee, T. H.; Huang, C. T.; Hou, W. C.; Lin, S. Y.; Zhong, W. B.; Lin, P. J.; Hung, L. F.; Liang, Y. C. Comparative anti-inflammatory characterization of wild fruiting body, liquid-state fermentation, and solid-state culture of *Taiwanofungus camphoratus* in microglia and the mechanism of its action. *J. Ethnopharmacol.* **2007**, *113* (1), 45−53.

(7) Song, T. Y.; Yen, G. C. Antioxidant properties of *Antrodia camphorata* in submerged culture. *J. Agric. Food Chem.* **2002**, *50* (11), 3322−3327.

(8) Hsiao, G.; Shen, M. Y.; Lin, K. H.; Lan, M. H.; Wu, L. Y.; Chou, D. S.; Lin, C. H.; Su, C. H.; Sheu, J. R. Antioxidative and hepatoprotective effects of *Antrodia camphorata* extract. *J. Agric. Food Chem.* **2003**, *51* (11), 3302−3308.

(9) Nakamura, N.; Hirakawa, A.; Gao, J. J.; Kakuda, H.; Shiro, M.; Komatsu, Y.; Sheu, C.; Hattori, M. Five new maleic and succinic acid derivatives from the mycelium of *Antrodia camphorata* and their cytotoxic effects on LLC tumor cell line. *J. Nat. Prod.* **2004**, *67* (1), 46−48.

(10) Lee, I. H.; Huang, R. L.; Chen, C. T.; Chen, H. C.; Hsu, W. C.; Lu, M. K. *Antrodia camphorata* polysaccharides exhibit anti-hepatitis B virus effects. *FEMS Microbiol. Lett.* **2002**, *209* (1), 63−67.

(11) Ao, Z. H.; Xu, Z. H.; Lu, Z. M.; Xu, H. Y.; Zhang, X. M.; Dou, W. F. Niuchangchih (*Antrodia camphorata*) and its potential in treating liver diseases. *J. Ethnopharmacol.* **2009**, *121* (2), 194−212.

(12) Song, T. Y.; Yen, G. C. Protective effects of fermented filtrate from *Antrodia camphorata* in submerged culture against CCl_4-induced hepatic toxicity in rats. *J. Agric. Food Chem.* **2003**, *51* (6), 1571−1577.

(13) Wang, G. J.; Tseng, H. W.; Chou, C. J.; Tsai, T. H.; Chen, C. T.; Lu, M. K. The vasorelaxation of *Antrodia camphorata* mycelia: Involvement of endothelial Ca^{2+}-NO-cGMP pathway. *Life Sci.* **2003**, *73* (21), 2769−2783.

(14) Lin, Y. L.; Wen, T. N.; Chang, S. T.; Chu, F. H. Proteomic analysis of differently cultured endemic medicinal mushroom *Antrodia cinnamomea* T.T. Chang et W.N. Chou from Taiwan. *Int. J. Med. Mushrooms* **2011**, *13* (5), 473−481.

(15) Shen, Y. C.; Wang, Y. H.; Chou, Y. C.; Chen, C. F.; Lin, L. C.; Chang, T. T.; Tien, J. H.; Chou, C. J. Evaluation of the anti-inflammatory activity of zhankuic acids isolated from the fruiting bodies of *Antrodia camphorata*. *Planta Med.* **2004**, *70* (4), 310−314.

(16) Lu, M. C.; El-Shazly, M.; Wu, T. Y.; Du, Y. C.; Chang, T. T.; Chen, C. F.; Hsu, Y. M.; Lai, K. H.; Chiu, C. P.; Chang, F. R.; Wu, Y. C. Recent research and development of *Antrodia cinnamomea*. *Pharmacol. Ther.* **2013**, *139* (2), 124−156.

(17) Erkel, G.; Anke, T. Products from *Basidiomycetes*. In *Biotechnology*; Wiley-VCH Verlag GmbH: 1997; pp 489−533.

(18) Schüffler, A.; Anke, T. Secondary metabolites of Basidiomycetes. In *physiology and genetics*, 1st ed.; The Mycota; Springer Berlin Heidelberg: 2009; Vol. *15*, pp 209−231.

(19) Buckingham, J. *Dictionary of natural products on DVD*; Chapman and Hall/CRC: 1993.

(20) Wawrzyn, G. T.; Quin, M. B.; Choudhary, S.; López-Gallego, F.; Schmidt-Dannert, C. Draft genome of *Omphalotus olearius* provides a predictive framework for sesquiterpenoid natural product biosynthesis in Basidiomycota. *Chem. Biol.* **2012**, *19* (6), 772−783.

(21) Gonzalez Del Val, A.; Platas, G.; Arenal, F.; Orihuela, J. C.; Garcia, M.; Hernandez, P.; Royo, I.; De Pedro, N.; Silver, L. L.; Young, K.; Vicente, M. F.; Pelaez, F. Novel illudins from *Coprinopsis episcopalis* (syn. *Coprinus episcopalis*), and the distribution of illudin-like compounds among filamentous fungi. *Mycol. Res.* **2003**, *107* (10), 1201−1209.

(22) Bräse, S.; Encinas, A.; Keck, J.; Nising, C. F. Chemistry and biology of mycotoxins and related fungal metabolites. *Chem. Rev.* **2009**, *109* (9), 3903−3990.

(23) Chen, C. Y.; Chien, S. C.; Tsao, N. W.; Lai, C. S.; Wang, Y. Y.; Hsiao, W. W.; Chu, F. H.; Kuo, Y. H.; Wang, S. Y. Metabolite profiling and comparison of bioactivity in *Antrodia cinnamomea* and *Antrodia salmonea* fruiting bodies. *Planta Med.* **2016**, *82* (3), 244−249.

(24) Lin, Y. L.; Lee, Y. R.; Tsao, N. W.; Wang, S. Y.; Shaw, J. F.; Chu, F. H. Characterization of the 2,3-oxidosqualene cyclase gene from *Antrodia cinnamomea* and enhancement of cytotoxic triterpenoid compound production. *J. Nat. Prod.* **2015**, *78*, 1556−1562.

(25) de Santana, M. F.; Guimarães, A. G.; Chaves, D. O.; Silva, J. C.; Bonjardim, L. R.; Lucca Júnior, W. d.; Ferro, J. N. d. S.; Barreto, E. d. O.; Santos, F. E. D.; Soares, M. B. P.; Villarreal, C. F.; Quintans, J. d. S. S.; Quintans-Júnior, L. J. The anti-hyperalgesic and anti-inflammatory profiles of *p*-cymene: Evidence for the involvement of opioid system and cytokines. *Pharm. Biol.* **2015**, *53* (11), 1583−1590.

(26) Aman, F.; Hanif, M.; Siddiqui, W. A.; Ashraf, A.; Filak, L. K.; Reynisson, J.; Söhnel, T.; Jamieson, S. M. F.; Hartinger, C. G. Anticancer ruthenium(η^6- *p* -cymene) complexes of nonsteroidal anti-inflammatory drug derivatives. *Organometallics* **2014**, *33* (19), 5546−5553.

(27) Ninomiya, K.; Hayama, K.; Ishijima, S. A.; Maruyama, N.; Irie, H.; Kurihara, J.; Abe, S. Suppression of inflammatory reactions by terpinen-4-ol, a main constituent of tea tree oil, in a murine model of oral candidiasis and its suppressive activity to cytokine production of macrophages *in vitro*. *Biol. Pharm. Bull.* **2013**, *36* (5), 838−844.

(28) Mondello, F.; De Bernardis, F.; Girolamo, A.; Cassone, A.; Salvatore, G. *In vivo* activity of terpinen-4-ol, the main bioactive component of *Melaleuca alternifolia* Cheel (tea tree) oil against azole-susceptible and -resistant human pathogenic *Candida* species. *BMC Infect. Dis.* **2006**, *6*, 158.

(29) Rangasamy, K.; Namasivayam, E. *In vitro* antioxidant and free radical scavenging activity of isolongifolene. *Asian J. Biol. Sci.* **2014**, *7* (1), 13−23.

(30) MacLean, D.; Jones, J. D. G.; Studholme, D. J. Application of "next-generation" sequencing technologies to microbial genetics. *Nat. Rev. Microbiol.* **2009**, *7* (4), 287−296.

(31) Lin, Y. L.; Ma, L. T.; Lee, Y. R.; Lin, S. S.; Wang, S. Y.; Chang, T. T.; Shaw, J. F.; Li, W. H.; Chu, F. H. MicroRNA-like small RNAs

DOI: 10.1021/acs.jafc.6b05386
J. Agric. Food Chem. 2017, 65, 1874−1886

pediction in the development of *Antrodia cinnamomea*. *PLoS One* **2015**, *10*, e0123245.
(32) Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28* (10), 2731−2739.
(33) Lin, Y. L.; Lee, Y. R.; Huang, W. K.; Chang, S. T.; Chu, F. H. Characterization of *S*-(+)-linalool synthase from several provenances of *Cinnamomum osmophloeum*. *Tree Genet. Genomes* **2014**, *10* (1), 75−86.
(34) Wen, C. H.; Lin, S. S.; Chu, F. H. Transcriptome analysis of a subtropical deciduous tree: Autumn leaf senescence gene expression profile of Formosan gum. *Plant Cell Physiol.* **2015**, *56* (1), 163−174.
(35) Liu, L. Y. D.; Tseng, H. I.; Lin, C. P.; Lin, Y. Y.; Huang, Y. H.; Huang, C. K.; Chang, T. H.; Lin, S. S. High-throughput transcriptome analysis of the leafy flower transition of *Catharanthus roseus* induced by peanut witches'-broom phytoplasma Infection. *Plant Cell Physiol.* **2014**, *55* (5), 942−957.
(36) Bastian, M.; Heymann, S. Gephi: An open source software for exploring and manipulating networks. *Int. AAAI Conf. Weblogs Soc. Media* **2009**, 361−362.
(37) Starks, C. M.; Back, K.; Chappell, J.; Noel, J. P. Structural basis for cyclic terpene biosynthesis by tobacco 5-epi-aristolochene synthase. *Science* **1997**, *277* (5333), 1815−1820.
(38) Whittington, D. A.; Wise, M. L.; Urbansky, M.; Coates, R. M.; Croteau, R. B.; Christianson, D. W. Bornyl diphosphate synthase: structure and strategy for carbocation manipulation by a terpenoid cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99* (24), 15375−15380.
(39) Kwon, M.; Cochrane, S. A.; Vederas, J. C.; Ro, D. K. Molecular cloning and characterization of drimenol synthase from valerian plant (*Valeriana officinalis*). *FEBS Lett.* **2014**, *588* (24), 4597−4603.
(40) Adams, R. P. *Identification of essential oil components by gas chromatography/mass spectrometry*, 4th ed.; Allured Publising Corporation: Carol Sream, IL, 2007.
(41) Scott, J. *Social network analysis*; SAGE: 2012.
(42) Yahyaa, M.; Matsuba, Y.; Brandt, W.; Doron-Faigenboim, A.; Bar, E.; McClain, A.; Davidovich-Rikanati, R.; Lewinsohn, E.; Pichersky, E.; Ibdah, M. Identification, functional characterization, and evolution of terpene synthases from a basal dicot. *Plant Physiol.* **2015**, *169* (November), 1683−1697.
(43) López-Gallego, F.; Wawrzyn, G. T.; Schmidt-Dannert, C. Selectivity of fungal sesquiterpene synthases: role of the active site's H-1 alpha loop in catalysis. *Appl. Environ. Microbiol.* **2010**, *76* (23), 7723−7733.
(44) Chooi, Y. H.; Hong, Y. J.; Cacho, R. A.; Tantillo, D. J.; Tang, Y. A cytochrome p450 serves as an unexpected terpene cyclase during fungal meroterpenoid biosynthesis. *J. Am. Chem. Soc.* **2013**, *135* (45), 16805−16808.
(45) Itoh, T.; Tokunaga, K.; Matsuda, Y.; Fujii, I.; Abe, I.; Ebizuka, Y.; Kushiro, T. Reconstitution of a fungal meroterpenoid biosynthesis reveals the involvement of a novel family of terpene cyclases. *Nat. Chem.* **2010**, *2* (10), 858−864.
(46) Bromann, K.; Toivari, M.; Viljanen, K.; Vuoristo, A.; Ruohonen, L.; Nakari-Setälä, T. Identification and characterization of a novel diterpene gene cluster in *Aspergillus nidulans*. *PLoS One* **2012**, *7* (4), e35450.
(47) Ram, M.; Khan, M. A.; Jha, P.; Khan, S.; Kiran, U.; Ahmad, M. M.; Javed, S.; Abdin, M. Z. HMG-CoA reductase limits artemisinin biosynthesis and accumulation in *Artemisia annua* L. plants. *Acta Physiol. Plant.* **2010**, *32*, 859−866.
(48) Caracuel, Z.; Roncero, M. I. G.; Espeso, E. A.; González-Verdejo, C. I.; García-Maceira, F. I.; Di Pietro, A. The pH signalling transcription factor PacC controls virulence in the plant pathogen *Fusarium oxysporum*. *Mol. Microbiol.* **2003**, *48*, 765−779.
(49) Brakhage, A. A. Regulation of fungal secondary metabolism. *Nat. Rev. Microbiol.* **2013**, *11* (1), 21−32.
(50) Bok, J. W.; Wiemann, P.; Garvey, G. S.; Lim, F. Y.; Haas, B.; Wortman, J.; Keller, N. P. Illumina identification of RsrA, a conserved C2H2 transcription factor coordinating the NapA mediated oxidative stress signaling pathway in *Aspergillus*. *BMC Genomics* **2014**, *15* (1), 1011.
(51) Ramamoorthy, V.; Dhingra, S.; Kincaid, A.; Shantappa, S.; Feng, X.; Calvo, A. M. The putative C_2H_2 transcription factor MtfA is a novel regulator of secondary metabolism and morphogenesis in *Aspergillus nidulans*. *PLoS One* **2013**, *8* (9), e74122.
(52) Kurahashi, A.; Shimoda, T.; Sato, M.; Fujimori, F.; Hirama, J.; Nishibori, K. A putative transcription factor Gf.BMR1 in *Grifola frondosa*, the homolog of BMR1 in *Bipolaris oryzae*, was strongly induced by near-ultraviolet light and blue light. *Mycoscience* **2015**, *56* (2), 177−182.
(53) Kihara, J.; Moriwaki, A.; Tanaka, N.; Tanaka, C.; Ueno, M.; Arase, S. Characterization of the BMR1 gene encoding a transcription factor for melanin biosynthesis genes in the phytopathogenic fungus *Bipolaris oryzae*. *FEMS Microbiol. Lett.* **2008**, *281*, 221−227.

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J. Agric. Food Chem. 2017, 65, 1874−1886