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Identification, Functional Characterization, and Seasonal Expression Patterns of Five Sesquiterpene Synthases in *Liquidambar formosana*

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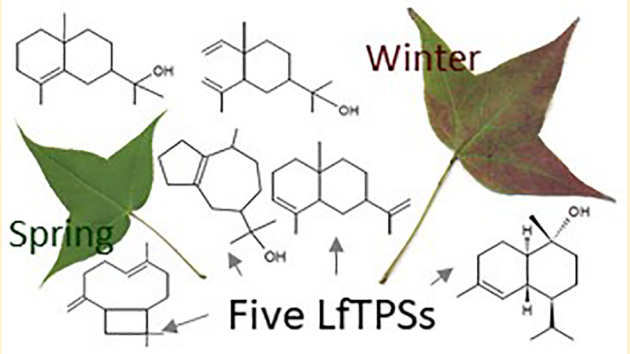
Supporting Information

ABSTRACT: Terpenoids are a large group of important secondary metabolites that are involved in a variety of physiological mechanisms, and many are used commercially in the cosmetics and pharmaceutical industries. During the past decade, the topic of seasonal variation in terpenoid biosynthesis has garnered increasing attention. Formosan sweet gum (*Liquidambar formosana* Hance) is a deciduous tree species. The expression of terpene synthase and accumulation of terpenoids in leaves may vary in different seasons. Here, four sesquiterpene synthases (i.e., *LfTPS01*, *LfTPS02*, *LfTPS03*, and *LfTPS04*) and a bifunctional mono/sesquiterpene synthase (*LfTPS05*) were identified from Formosan sweet gum. The gene expression of *LfTPS01*, *LfTPS02*, and *LfTPS03* showed seasonal diversification, and, in addition, expression of *LfTPS04* and *LfTPS05* was induced by methyl jasmonate treatment. The major products LfTPS01, LfTPS02, LfTPS04, and LfTPS05 are hedycaryol, α-selinene, *trans*-β-caryophyllene, α-copaene/δ-cadinene, and nerolidol/linalool, respectively. The data indicated that the sesquiterpenoid content in the essential oil of Formosan sweet gum leaves shows seasonal differences that were correlated to the sesquiterpene synthase gene expression.



Terpenoids are structurally diverse, multifunctional secondary metabolites. Some of these compounds directly protect plants from herbivores and pathogens, and others protect them indirectly by recruiting the predators of herbivores. Terpenoids also play important roles in plant−plant interaction.[1,2] Many terpenoids have been demonstrated to have medicinal properties; for example, the sesquiterpene lactones identified in *Artemisia annua* have become one of the most widely used antimalarial drugs,[3] and the terpenoids isolated from *Commiphora sphaerocarpa* showed antiproliferative effects against human cancer cells.[4] Because of the ecological and physiological roles of terpenoids in plants and their potential applications in medicine, the identification and characterization of more natural terpenoids in plants is of interest.[5] The diversity of terpenoids in plants means there is a large variation of terpene synthases that convert geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) into different structures of monoterpenes, sesquiterpenes, and diterpenes.[6,7] Thus, to identify and characterize the function of unknown terpene synthases in plants has become an important topic in both the pharmaceutical industry and plant biology.

Many plants used as traditional Chinese medicines (TCM) have been reported to contain multiple and high levels of terpenoids.[8] Formosan sweet gum (*Liquidambar formosana* Hance) is a well-known TCM; it is a woody plant with high commercial value for furniture manufacturing and mushroom cultivation, which is widespread in southern China and Taiwan.[9−11] It has been shown that the leaves can be used against rheumatoid arthritis and dermatosis,[10] while their balsam can be used as a remedy for blood stasis alleviation, analgesia, and inflammation.[11,12] The terpenoids isolated from the leaves of Formosan sweet gum exhibit in vitro antioxidant activity.[10] Some of these compounds have even been shown to strengthen nonspecific immunity and cellular immunological function in mice.[13] In addition to the medical applications, the antifungal activity shown in the balsam may be applied in a variety of industries.[11]

As major biogenic volatile organic compounds (BVOC), many studies have discussed the seasonal dynamics of violatile terpenoids. In Japan, the emissions of mono- and sesquiterpenes from *Cryptomeria japonica* and *Chamaecyparis obtusa* have seasonal variation, and the chemical contribution of sesquiterpenes also showed a seasonal variation.[14] Fourteen sesquiterpenes and oxygenated compounds were found in the emissions of Scots pine (*Pinus sylvestris*), and sesquiterpene

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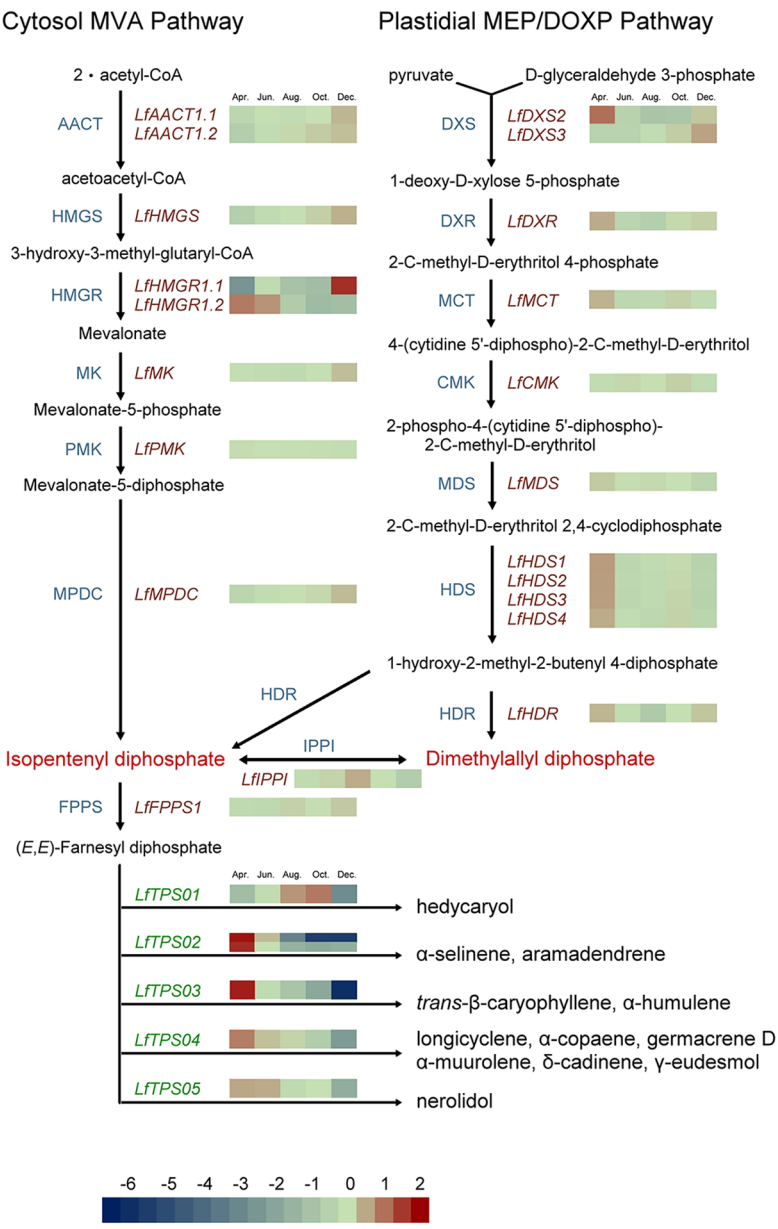

Figure 1. Gene expression heat map of the MVA and MEP pathways in Formosan sweet gum. Gene expression data from microarrays were used. The result is presented as average gene expression over the years 2011−2013, and a $\log_2$ fold change compared with an average over five months is shown. The MVA and MEP pathways in *Arabidopsis* were used as a reference.[18] Contigs LfGR21553 and LfGR12209 are two halves of LfTPS02, with one near 5′ and one near 3′, respectively. The sesquiterpene products of the five *Lf TPSs* were identified in this work.

content, in particular, changes dramatically across seasons.[15] The authors suggest that the seasonal variations in terpene contribution indicate that the temperature dependence of the terpene emission is linked to synthase activities and governed by the developmental state of the plant.[15] The seasonal variation of essential oil composition in one of the three *Leptospermum* spp. influences the antibacterial activity against Gram-positive *Bacillus cereus*[16] and highlights the importance of harvest time of particular species. In order to achieve higher production of objective compounds, knowledge of the best harvest time is useful.

In the current study, the seasonal expression of sesquiterpene synthase genes and the composition of major sesquiterpenes were investigated in an attempt to explain the dynamics of terpene biosynthesis. Five sesquiterpene synthases (*Lf TPSs*) from Formosan sweet gum leaves were identified and characterized. They were found to differ in expression across seasons in a microarray database. The gene expressions through the year 2012 were further confirmed by reverse transcript-polymerase chain reaction (RT-PCR). Two *Lf TPSs* showed no obvious gene expression pattern through the year in RT-PCR experiments, but were responsive to methyl jasmonate (MeJA) treatment. This indicates that some sesquiterpene synthase genes are expressed mainly according to growing season or leaf development, and the others are influenced by biotic or environmental events. Their active site sequence and products also showed a difference. By in vitro recombinant protein assay and GC/MS analysis, the products of the five sesquiterpene

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synthases were shown to be major contributors to the sesquiterpenoid content in the leaf essential oil. The result uncovered the seasonal fluctuation of gene expression and product accumulation for the sesquiterpene synthases. This knowledge will be useful for biotechnology applications or harvest time optimization.

RESULTS AND DISCUSSION

Five sesquiterpene synthases in Formosan sweet gum were identified and characterized. All of the identified synthases catalyzed different products. They showed different expression patterns throughout the years 2011 to 2013. Based on their sequences, these five *LfTPSs* could be categorized into two groups, namely, those catalyzing the production of cyclized sesquiterpnoids and of linear sesquiterpenoids. Otherwise, with different expression patterns through growing seasons and responses to MeJA treatment, the five *LfTPSs* are suggested to have different roles and may be regulated by different mechanisms. The different features of these five *LfTPSs* provide an example of the functional partition of sesquiterpene synthases in plants.

Expression of Terpene Biosynthetic Genes through the Growing Season. In order to demonstrate the expression dynamics of the terpene biosynthetic genes, microarray data throughout leaf development were obtained from a previous experiment using the leaves collected in April, June, August, October, and December of 2011−2013.[17] Genes from the mevalonic acid (MVA) and methylerythritol phosphate (MEP) pathways were extracted by BLAST from the Formosan sweet gum database. The precursors of sesquiterpene synthase largely come from the MVA pathway in the cytosol.[18] In Formosan sweet gum, gene expression data for the years 2011−2013 showed that most of the MVA pathway genes were slightly up-regulated throughout leaf development except for the rate-limiting enzyme *LfHMGR1.1* (Formosan sweet gum contig serial number: LfGR313) and *LfHMGR1.2* (LfGR8655) (Figure 1). *LfHMGR1.1* and *LfHMGR1.2* had opposite expression patterns, but due to lack of enzyme property data, the effect is currently unknown. The expression patterns of most of the genes in the MEP pathway were opposite from the genes in the MVA pathway, with MEP gene expression down-regulated throughout leaf development. Exceptions were the rate-limiting enzymes *LfDXS2* (LfGR13547) and *LfDXS3* (LfGR16378), both of which showed up-regulation in December, with *LfDXS2* showing a high expression level in April. In short, most of the genes in these two pathways had mild expression dynamics; the exceptions were the rate-limiting enzymes and terpene cyclase. Five putative Formosan sweet gum sesquiterpene synthase genes (*LfTPSs*) were acquired by a keyword search on TAIR annotation of the contigs and were named *LfTPS01−05*. Four of the five *LfTPSs* were expressed at high levels in April and were down-regulated throughout leaf development. The exception was *LfTPS01*, which was expressed at a high level around August and October, the time when leaves started to senesce.

Gene Expression of *LfTPSs* Showed Seasonal Variation. To confirm the gene expression of *LfTPSs*, leaf samples collected monthly in the growing seasons through the year 2012 were used for RT-PCR (Figure 2). The gene expression of the three *LfTPSs* showed seasonal variation and correlated with microarray data. In brief, *LfTPS01* was expressed mainly in the summer and autumn. Gene expression of *LfTPS02* was enhanced from the start of the growing season and declined

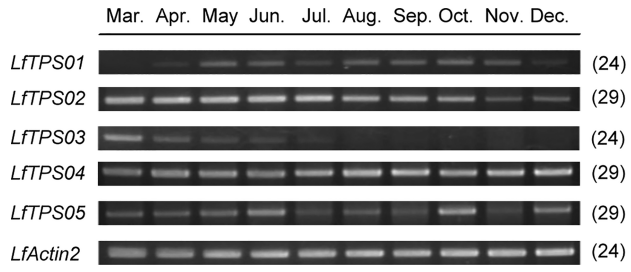

Figure 2. Gene expression of five *LfTPSs* through the year 2012. Numbers in parentheses represent PCR cycles. *LfActin2* was used as internal control gene.

after July. *LfTPS03* was expressed mainly in spring, and the expression gradually declined after March. The other two *LfTPSs* (*LfTPS04* and *LfTPS05*) showed no obvious expression pattern. The difference between the RT-PCR and microarray data of *LfTPS04* and *LfTPS05* may be due to the low expression difference in the microarray data or the position of the microarray probe. In addition to the seasonal effect, the other inducing factors were also considered as effectors which may influence the expression of *LfTPS04* and *LfTPS05*. MeJA has been reported to be an inducer of terpene biosynthesis;[19] thus a MeJA treatment was designed to explore the putative regulation of the *LfTPSs* (Figure 3). In MeJA-treated

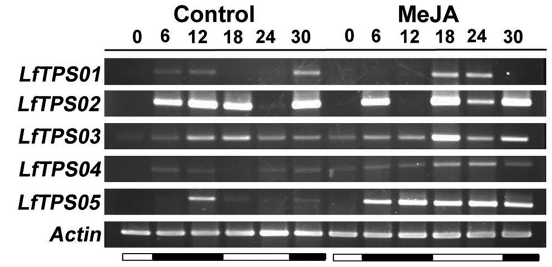

Figure 3. Transcript expression of the five *LfTPSs* after methyl jasmonate (MeJA) treatment. The expression of *LfTPS03*, *LfTPS04*, and *LfTPS05* was enhanced, and the expression of *LfTPS05* was prolonged. The bar with alternate black and white regions represents day (white) and night (black).

Formosan sweet gum seedlings, MeJA did not induce the gene expression of *LfTPS01* and *LfTPS02*, but did induce *LfTPS03*, *LfTPS04*, and *LfTPS05* expression. This suggests seasonal/leaf developmental regulation of *LfTPS03*. *LfTPS04* and *LfTPS05* may be regulated by other regulatory mechanisms different from *LfTPS01* and *LfTPS02*, and this regulatory mechanism can induce the expression of *LfTPS03*.

Gene Expression of *LfTPSs* and Weather. The Formosan sweet gum sample tree is on the campus of National Taiwan University; thus statistical data on the climate for 2011−2013 were collected from the Taipei weather station to compare with gene expression and meteorological factors (Figure 4). During the years 2011 to 2013, the temperature showed no obvious difference, but the rainfall and hours of sunshine differed between the years [Figure 4(A) to (E)]. In northern Taiwan the rainy season is from May to June. There may be typhoons bringing much rainfall in the summer and fall, and occasional monsoons (Northeast Monsoon) may bring rainfall in winter. The expression of most genes in the MVA and MEP pathways showed a gradual increase or decrease through the year (Figure 1). These genes may be regulated

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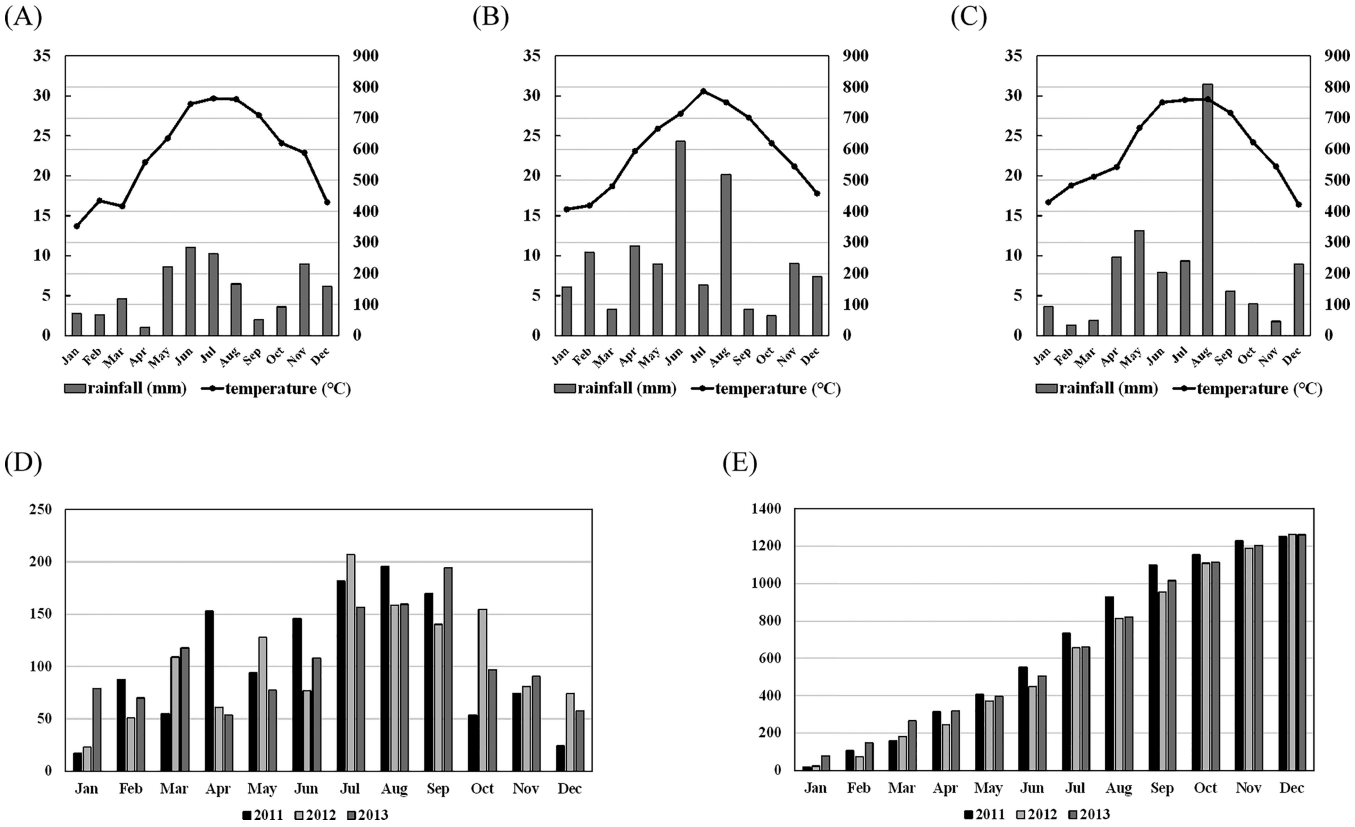

Figure 4. Climate statistics for the years 2011−2013 from the Taipei weather station. (A) Rainfall and temperature for the year 2011. (B) Rainfall and temperature for the year 2012. (C) Rainfall and temperature for the year 2013. (D) Monthly sunshine hours for the years 2011, 2012, and 2013. (E) Accumulated sunshine hours for the years 2100, 2012, and 2013. Data acquired from the Web site of the Central Weather Bureau (Taipei, Taiwan).

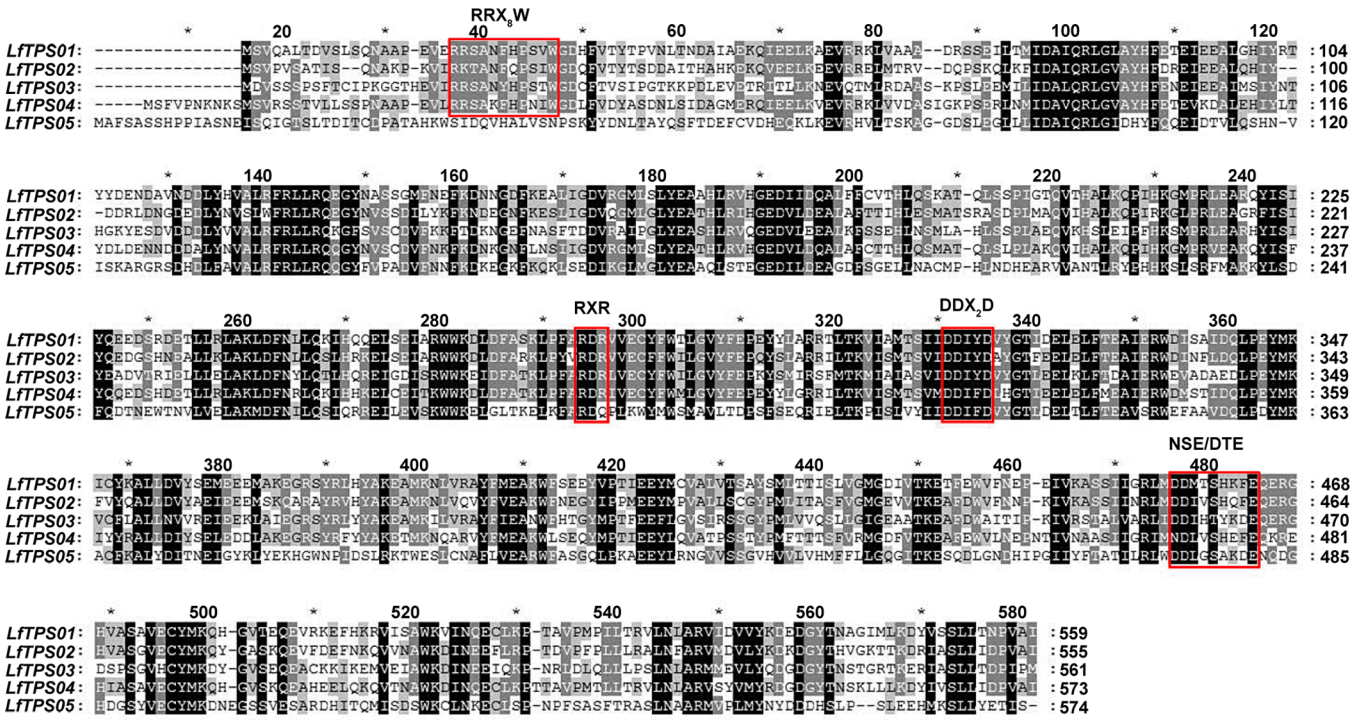

Figure 5. Alignment of five Formosan sweet gum sesquiterpene synthases. The red boxes in the figure were designated the domains labeled above them. Amino acid sequence alignments were generated using the CLUSTALW computer program (Larkin et al., 2007). The degree of conservation between the sequences is shown by black (100%), dark gray (70%), and light gray (60%) shading.

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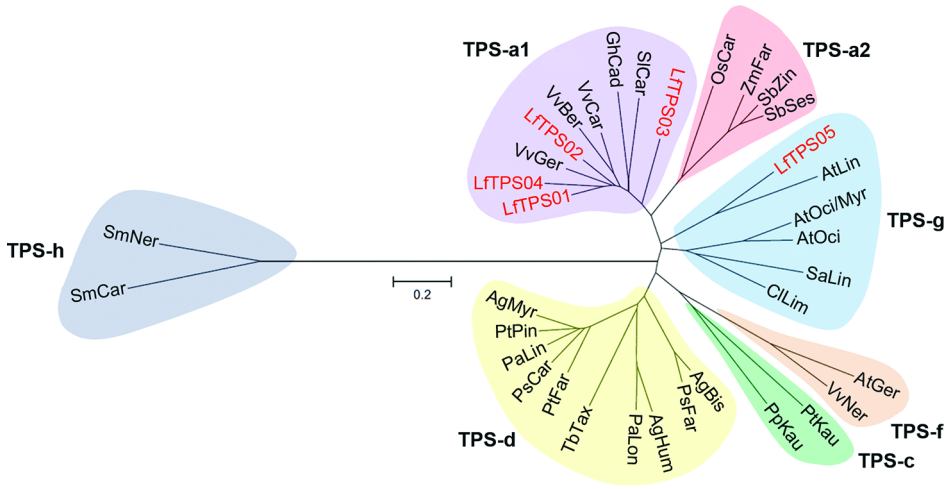

Figure 6. Phylogenetic analysis of five Formosan sweet gum sesquiterpene synthases. Plant terpene synthases were categorized into TPS-a1, TPS-a2, TPS-g, TPS-f, TPS-c, TPS-d, and TPS-h according to Chen et al., 2011. The *LfTPS01*, *LfTPS02*, *LfTPS03*, and *LfTPS04* were all categorized into the TPS-a2 subfamily. Only *LfTPS05* was categorized into the TPS-g subfamily. Sequence relatedness was analyzed using the neighbor-joining method, and the unrooted tree was visualized using MEGA5.1.[53]

mainly by leaf developmental programs but not meteorological factors on a monthly scale. The exception was *LfTPS01*, whose gene expression was roughly correlated with temperature. In 2012 the gene expression slightly declined in July (Figure 2), this may be caused by drought stress due to high temperature combined with lower rainfall. Although the monthly sunshine hours differed across years, the accumulated sunshine hours reached about 1100 h around October in these three years [Figure 4(E)], which is also the time when Formosan sweet gums enter autumnal leaf senescence.

Identification of *LfTPSs*. The full-length cDNA sequences of *LfTPS01*-05 are 1680 nucleotides in the open reading frame (ORF) for *LfTPS01* (LfGR10688), 1668 nucleotides for *LfTPS02* (LfGR11842), 1686 nucleotides for *LfTPS03* (LfGR21553 and LfGR12209), 1722 nucleotides for *LfTPS04* (LfGR28969), and 1725 nucleotides for *LfTPS05* (LfGR24115). The number of nucleotides in the untranslated region and the ORF are listed in Table S1 of the Supporting Information. All five LfTPSs contain the common terpene synthase aspartate-rich motif DDXXD, which interacts with divalent metal ions such as Mg^{2+} in metal-dependent ionization (LfTPS01: $D^{312}D^{313}IYD^{316}$, LfTPS02: $D^{308}D^{309}IYD^{312}$, LfTPS03: $D^{314}D^{315}IYD^{318}$, LfTPS04: $D^{324}D^{325}IFD^{328}$, LfTPS05: $D^{328}D^{329}IFD^{332}$) labeled in Figure 5. The less conserved motif NSE/TDE[20] is also present in all five LfTPSs (LfTPS01: $D^{456}DMTS^{460}HKFE^{464}$, LfTPS02: $D^{452}DIVS^{456}HQFE^{460}$, LfTPS03: $D^{458}DIHT^{462}YKDE^{466}$, LfTPS04: $N^{469}DLVS^{473}HEFE^{477}$, LfTPS05: $D^{473}DLGS^{477}AKDE^{481}$). With the exception of LfTPS05, the other four LfTPSs all contain the N-terminal tandem arginine motif $RR(X)_8W$ (LfTPS01: $R^{21}R^{22}SANFHPSVW^{31}$, LfTPS02: $R^{19}K^{20}TANFQPSIW^{29}$, LfTPS03: $R^{22}RSANYHPSTW^{32}$, LfTPS04: $R^{31}R^{32}SAKFHPNIW^{41}$).

The phylogenetic analysis was performed using the neighbor joining method with protein sequences of 18 plants obtained from the NCBI. *LfTPS01*, *LfTPS02*, *LfTPS03*, and *LfTPS04* were all categorized into the TPS-a1 subfamily with sesquiterpene synthases from other eudicots according to Chen et al. (2011).[21] Only *LfTPS05* was categorized into the TPS-g subfamily with linear monoterpene and sesquiterpene synthases that lack the RRX_8W motif (Figure 6).[21] The highest identity was between *LfTPS01* and *LfTPS04*. The identities and similarities between each *LfTPS* and selected representative terpene synthases from different species are listed in Table S2 of the Supporting Information. The sequences, from different taxonomies, were chosen from NCBI and are listed in Table S3 of the Supporting Information.

Functional Characterization of the *LfTPSs*. To characterize the function of the five putative TPSs, the coding sequences of the five *LfTPSs* from cDNA were constructed, subcloned into the IMPACT-CN system (NEB), and expressed as a fusion protein in *E. coli*. After purification and removal of the fusion tag, the purified LfTPSs were incubated with different precursors (FPP or GPP) individually. The product of the enzyme reaction was analyzed by GC/MS. In GC/MS analysis, the reaction products of four of the five *LfTPSs* were the 15 major sesquiterpenoids in Formosan sweet gum leaf essential oil (Figure 7 and Table 1, MS plot list in Figure S1 of the Supporting Information); the exception was LfTPS05. The major products of LfTPS01 are compounds with hydroxy groups, including β-elemol (peak 10), guaiol (peak 12), T-cadinol (peak 15), and α-cadinol (peak 16). As β-elemol has been reported to be an authentic compound through Cope rearrangement,[22] the injection temperature was adjusted to 150 and 200 °C. The signal of β-elemol was indeed lower when injected at lower temperature, but in this heating program the precursor hedycaryol was not found (Figure S2 of the Supporting Information).

The major product of LfTPS01, β-elemol, is present in a wide range of plant essential oils. For example the leaf essential oil of *Coptotermes formosanus* is rich in β-elemol and contributes to the excellent antitermitic activity of this oil.[23] As the expression of *LfTPS01* showed an opposite pattern to that of the control upon MeJA treatment (Figure 3), *LfTPS01* may contribute to the antipathogen activity in the tree. A hedycaryol synthase (*CbTPS1*) was identified from *Camellia brevistyla*, which generated only one product.[24] Besides hedycaryol, LfTPS01 also possesses the ability to produce a trace amount of guaiol, T-cadinol, and α-cadinol. These three compounds were found in the leaf essential oil, but under high-temperature conditions, there was only a trace of hedycaryol heat forming compound β-elemol (Figure 7). A similar situation is found in

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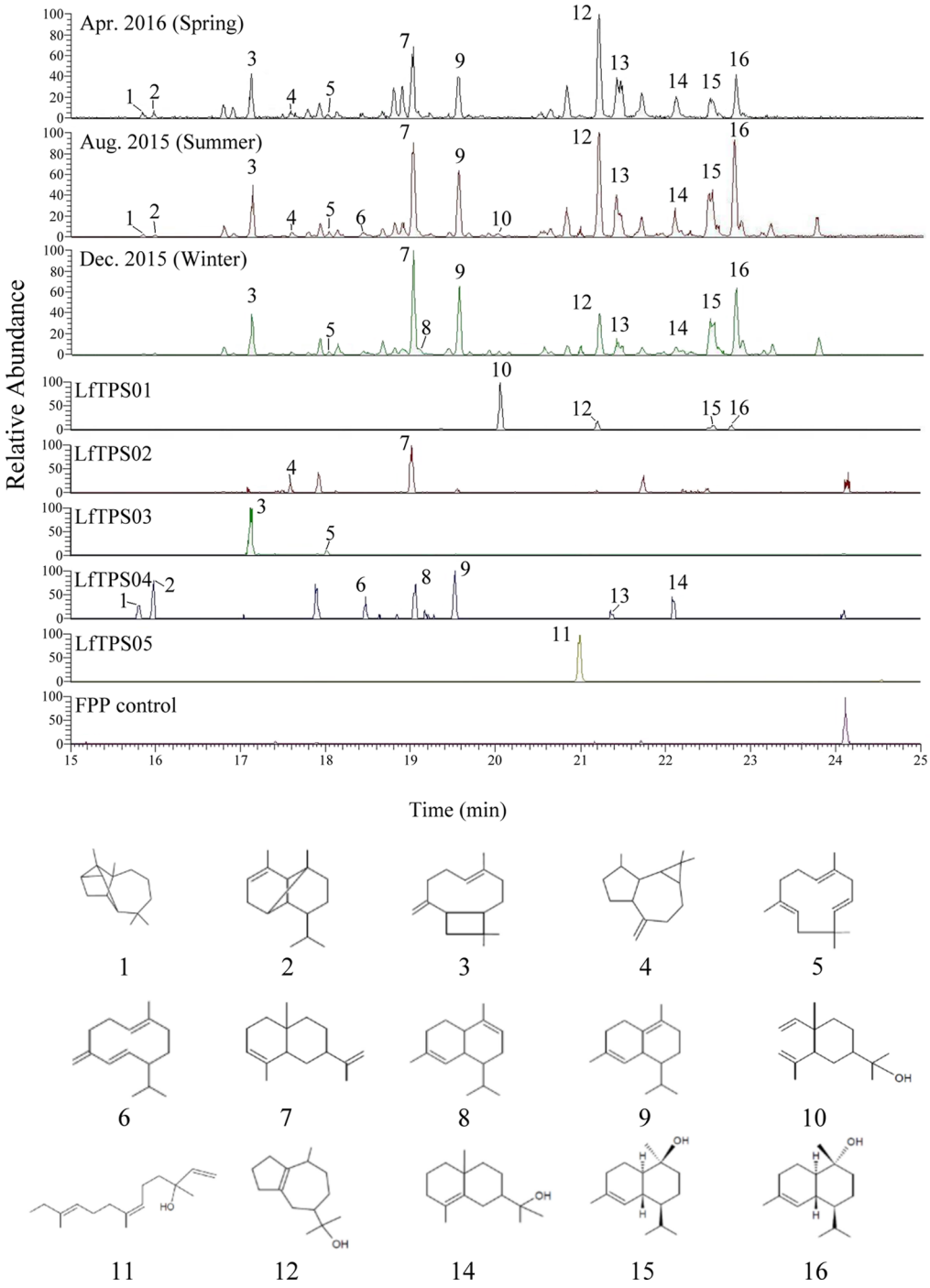

Figure 7. GC of Formosan sweet gum essential oil and in vitro enzyme reaction product of the five LfTPSs. The numbers represent major compounds that appear in both the enzyme reaction and essential oil. The only exception is LfTPS05, with peak 11 not being found in the essential oil. Number order was based on retention time (RT), and the corresponding chemical structures are listed below the GC. The MS plot for each peak is provided in Figure S1 of the Supporting Information. The most intense ion detected in the spectrum was set to 204, and total ion current chromatogram of the essential oil is provided in Figure S3 of the Supporting Information. Compound names are listed in Table 1.

chicory (*Cichorium intybus*); a germacrene A synthase was responsible for the constitution of costunolide, but no trace of germacrene A has ever been found in chicory due to modification by germacrene A oxidase.[25] If hedycaryol was the main product of LfTPS01, it is speculated that it is modified into an unidentified derivate. A study using recombinant (+)-δ-cadinene synthase from *Gossypium arboretum* illustrated the possibility of multiple biosynthetic pathways to synthesize (+)-δ-cadinene, including forming a germacradienyl cation as intermediate.[26] It is likely that LfTPS01 uses a similar mechanism, forming hedycaryol and guaiol through a 1,10-cyclization and producing T-cadinol and α-cadinol as by-products.

LfTPS02 has multiple products, two of which appear in the essential oil. The major product (peak 7) has an MS spectrum most similar to α-selinene, and the minor product is aramadendrene (peak 4). The second major product of LfTPS02 is an unidentified sesquiterpene, which was not

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Table 1. LfTPS Products Compared with Formosan Sweet Gum Essential Oil

			essential oil				
no.	compound	TPS	Aug.	Dec.	KI[a]	reference KI[b]	identification[c]
1	longicyclene	TPS04	√		1384	1374	KI, MS
2	α-copaene	TPS04	√		1389	1377	KI, MS, NMR
3	*trans*-β-caryophyllene	TPS03	√	√	1437	1428	KI, MS, NMR
4	aromadendrene	TPS02	√		1456	1441	KI, MS, NMR
5	α-humulene	TPS03	√	√	1474	1454	KI, MS, NMR
6	germacrene D	TPS04	√		1490	1485	KI, MS, NMR
7	α-selinene	TPS02	√	√	1515	1494	KI, MS, NMR
8	α-muurolene	TPS04		√	1518	1499	KI, MS, NMR
9	δ-cadinene	TPS04	√	√	1537	1523	KI, MS, NMR
10	β-elemol	TPS01	√		1556	1549	KI, MS, NMR
11	nerolidol	TPS05			1600	1563	KI, MS
12	guaiol	TPS01	√	√	1601	1595	KI, MS
13	unknown	TPS04	√	√	1614		
14	γ-eudesmol	TPS04	√	√	1643	1632	KI, MS, NMR
15	T-cadinol	TPS01	√	√	1659	1640	KI, MS
16	α-cadinol	TPS01	√	√	1671	1653	KI, MS, NMR

[a]Kovats index. [b]The reference KIs were acquired from The Pherobase (http://www.pherobase.com).[57] [c]NMR: ^{13}C NMR in $CDCl_3$ experiments used summer essential oil as sample and used Kubeczka and Formáček 2002 as reference.[52] ^{13}C NMR ($CDCl_3$) δ of these seven compounds are listed in Table S4 of the Supporting Information.

found in leaf essential oil (Figure 7, peak at 17.95 min). A sesquiterpene synthase that has not been reported before synthesizes both α-selinene and aromadendrene. The δ-selinene synthase in grand fir (*Abies grandis*) has multiple products, and it is speculated that δ-selinene is formed through germacrene A.[27] LfTPS02 may use a similar mechanism to form such multiple products through forming a germacene structure. The minor product of LfTPS02, aromadendrene, is a sesquiterpene that is commonly found in plants. In *Aristolochia longa* root essential oil, aromadendrene is found in April but not in August, September, or March.[28] The expression of LfTPS02 shows a seasonal dynamic, and so does the leaf essential oil, as aromadendrene was not identified in the winter sample. In the essential oil of *Eucalyptus globulus*, aromadendrene has been identified as the major contributor to its antibiotic properties against Gram-positive bacteria.[29] As aromadendrene showed seasonal variation, the results outlined here may be applied to decisions about optimum harvesting times for obtaining different compositions of leaf essential oil.

The major product of LfTPS03 was *trans*-β-caryophyllene (Figure 7, peak 3), with trace amounts of α-humulene (Figure 7, peak 5). β-Caryophyllene is one of the most widespread and common volatile sesquiterpenes found in floral scent.[30] In nonfloral tissue, caryophyllene directly decreases the survival of insects feeding on cotton[31] and also indirectly protects maize (*Zea mays*) from attracting parasitic wasps to lay eggs on lepidopteran larvae feeding on its leaves.[32] In *Arabidopsis thaliana*, it serves as a constitutively present antimicrobial substance that directly protects flowers from bacterial pathogens.[33] The expression of *LfTPS03* in the leaves of Formosan sweet gum reaches the highest level in spring, while the young leaves are still weak and vulnerable to aphids, with LfTPS03 expression decreased after MeJA treatment. This implies that LfTPS03 may be involved in defense of predators in young leaves in Formosan gum. Three other sesquiterpene synthases that also produce β-caryophyllene and α-humulene have been studied: ZSS1 (GenBank: BAG12020.1) from shampoo ginger (*Zingiber zerumbet* Smith), QHS1 (GenBank: AAL79181) from *Artemisia annua*, and TPS21 (At5g23960, Refseq: NP_001190374.1) from *Arabidopsis thaliana*. ZSS1 catalyzes α-humulene formation as the major product, whereas the other two also catalyze β-caryophyllene formation as the major product like LfTPS03. The amino acid sequence identities of LfTPS03 and these TPSs are 45%, 37%, and 43% for QHS1, TPS21, and ZSS1, respectively. However, the highest identity between LfTPS03 and the functionally characterized genes in the NCBI nonredundant protein sequence database (Nr database) is a (−)-germacrene D synthase from wine grape (*Vitis vinifera*) (GenBank: AAS66357.1).[34] Thus, in the case of LfTPS03, the enzyme activity cannot be predicted simply by sequence identity.

LfTPS04 also has multiple products that make up most of the sesquiterpenes in leaf essential oil. The products of LfTPS04 are longicyclene (Figure 7, peak 1), α-copaene (peak 2), germacrene D (peak 6), α-muurolene (peak 8), δ-cadinene (peak 9), an unknown sesquiterpene (peak 13), and γ-eudesmol (peak 14). *LfTPS04* has the highest identities and similarities to *LfTPS01*; both synthesized germacrene skeleton compounds. LfTPS04 has multiple products. According to the hypothetical pathway of δ-selinene synthase in grand fir,[27] it is speculated that α-copaene, α-muurolene, and δ-cadinene may be synthesized by 1,10-cyclization to form a germacrene structure as intermediate. γ-Eudesmol might be formed through a germacradienol structure, but longicyclene is speculated to be formed by 1,11-cyclization in the pathway described for grand fir γ-humulene.[27] The products of LfTPS04 contribute to most of the sesquiterpene compounds in Formosan sweet gum leaf essential oil, and most of them do not exhibit seasonal dynamic change. The constitutive expression through all seasons and responding to MeJA imply that LfTPS04 may be involved in biotic stress defense in Formosan sweet gum.

LfTPS05 has a single product, nerolidol (peak 11), which was not found in the leaf essential oil. As predicted, when providing GPP to LfTPS05 as a substrate, the linear product linalool was produced (data not shown). Based on the cDNA sequence, *LfTPS05* reveals the lowest similarity to the other four terpene synthases and is categorized into the TPS-g family, which produces mainly noncyclic terpenoids.[21] LfTPS05 was

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predicted to have bifunctional nerolidol/linalool synthase activity converting both FPP and GPP into sesquiterpenoids (nerolidol) and monoterpenoids (linalool). Nerolidol and linalool can be synthesized by nerolidol synthase and linalool synthase, respectively, or synthesized by a bifunctional nerolidol/linalool synthase.[35] In snapdragon flower (*Antirrhinum majus*), AmNES/LIS-1 (GenBank: ABR24417.1) is localized in the cytosol and is responsible for nerolidol biosynthesis, whereas AmNES/LIS-2 (GenBank: ABR24418.1) is located in the plastids and accounts for linalool formation.[35] The protein sequence of LfTPS05 shares more sequence identity with AmNES/LIS-1 (51%) than AmNES/LIS-2 (49%) and was predicted not to contain the chloroplast signal peptide (predicted by ChloroP v. 1.1), thus suggesting a role in nerolidol synthesis in the cytosol. Like the close correlation between increases in (*E*)-nerolidol synthase activity and DMNT emission upon herbivore damage in maize,[36] the enhanced expression after MeJA treatment of LfTPS05 may imply the involvement of its role in the defense against biotic stress.

Seasonal Variation of Sesquiterpenoids in Formosan Sweet Gum Essential Oil. A comparison of the sesquiterpenoids in leaf essential oil acquired from summer and winter in the same year showed that most of the compounds appear in both (Figure 7 and Table 1). The compounds that appeared in both seasons include *trans*-β-caryophyllene (peak 3), α-humulene (peak 5), α-selinene (peak 7), δ-cadinene (peak 9), guaiol (peak 12), an unknown sesquiterpene (peak 13), a trace amount of γ-eudesmol (peak 14), T-cadinol (peak 15), and α-cadinol (peak 16). Longicyclene (peak 1), α-copaene (peak 2), aramadendrene (peak 4), germacrene D (peak 6), and β-elemol (peak 10) were only found in summer, roughly correlating with the expression of *LfTPS01* and *LfTPS02*, as LfTPS01 is mainly expressed in summer and LfTPS02 is mainly expressed in spring to summer. α-Muurolene (peak 8) was found only in winter.

Seasonal Expression of Sesquiterpene Biosynthesis Genes and Putative Regulation. Seasonal variation of terpenoid emission from forest trees has been studied in different species,[37,38] but the seasonal expression of terpene synthase genes and the difference in the composition of sesquiterpenoids are barely mentioned in the literature. As Formosan sweet gum is a subtropical deciduous tree, its leaves have an annual life cycle. Leaves sprout in March and fall in February the following year.[17] The expression of the two isoprenoid biosynthesis pathway genes differs. This might be because the two pathways participate in different metabolic processes. The final product of the MEP pathway contributes to the precursor of chlorophyll;[39] thus, it is reasonable that genes of the Formosan sweet gum MEP pathway are mainly expressed in spring when the leaf is expanding (Figure 1). In *Arabidopsis*, genes from the MVA and MEP pathways showed opposite expression patterns in organs and showed a diurnal dynamic.[18] The annual opposite expression pattern is illustrated for the first time here in a deciduous plant.

Different seasonal expression patterns were also found in the *LfTPS*s. In particular, *LfTPS01* differed from the others in that it was mainly expressed in August and October (Figures 1 and 2). The expression pattern of *LfTPS01* was correlated to large-scale BVOC studies in Japan and Europe, which showed that the rate of sesquiterpenoid emission can be high in summer or autumn.[14,40] *LfTPS02* and *LfTPS03* were mainly expressed in the spring (Figure 2). Sesquiterpenoids were found to be emitted at higher levels in the spring than in the summer in some of the sesquiterpenoid compounds in Scots pine, but the highest emission rate can occur in different months for different compounds.[15] The emission composition of Formosan sweet gum is currently unknown, but the composition of leaf essential oil did not show much difference between summer and winter. The major difference occurs in the minor products of the multiple product TPSs LfTPS02 and LfTPS04, but the seasonal effect can be different between these two *LfTPS*s. In LfTPS04, since the expression is almost constitutive, the difference of each product in essential oil may be influenced by other compounds that contribute to the total amount of essential oil so the minor product cannot be identified. The possibility of further modification may also influence the composition of the sesquiterpenoid compounds in leaf essential oil. Otherwise, for LfTPS02, it may be caused by the decreasing expression of the *TPS* gene.

As well as seasonal dynamics, diurnal expression dynamics were observed in the *LfTPS*s. In the MeJA treatment experiment, most of the *LfTPS*s in Formosan sweet gum expressed their transcripts at night. This result is different from previous research that suggested most sesquiterpenoids are emitted at maximum levels during the day.[41] The diurnal expression patterns of the terpene synthases and terpenoid emission have been investigated in many species.[5] In rice (*Oryza sativa*) seedling leaves, the mRNA level of *OsTPS3* was shown to oscillate across light/dark conditions, reaching a high level at noon.[42] Because the sesquiterpenoids in maize are released almost immediately after transcription, it was assumed that the transcripts were also expressed at a high level at noon.[43] The different diurnal gene expression patterns seen in Formosan sweet gum terpene synthase and rice terpene synthase may be due to the diversity between species; that is, rice is a monocot and Formosan sweet gum is a woody dicot. In addition, the terpene synthases with different functions in plants may result in different expression times.

The seasonal difference in Formosan sweet gum sesquiterpene synthase gene expression implies a dynamic in the content and the regulation of sesquiterpenoids, which has rarely been mentioned before. Seasonal differences in terpenoid content and emission have been studied in many species, and the variation can be seen within compounds in different plants or organs. In a study of *Abies sachlinensis* and *Thuja occidentalis*, the leaf essential oil content did not change much through the year with regard to terpenoid emission.[44] In contrast, a study of seven Mediterranean woody species showed that the terpenoid emission rate did not correlate with essential oil content and varied between species.[45] In Formosan sweet gum, the five *LfTPS*s differ in expression throughout the growing season. Although the major compounds did not show much difference between the seasons, the proportions were different and the minor compounds showed seasonal variation. Thus, to acquire higher sesquiterpene production in Formosan sweet gum, the strategy will be different for each compound.

Recently, transcriptome data for Formosan sweet gum have been reported and gene coexpression network analysis has been employed to predict the regulators of key enzymes in biosynthesis pathways.[17] For putative regulation of *LfTPS*s, a gene coexpression network was formed and the correlation coefficient threshold was set at ≥ 0.9 (or ≤ -0.9). Three *LfTPS*s, five terpene biosynthesis genes, and 29 putative transcription factors were included in this simplified network (Figure 8). In brief, *LfTPS02* and *LfTPS03* formed a network,

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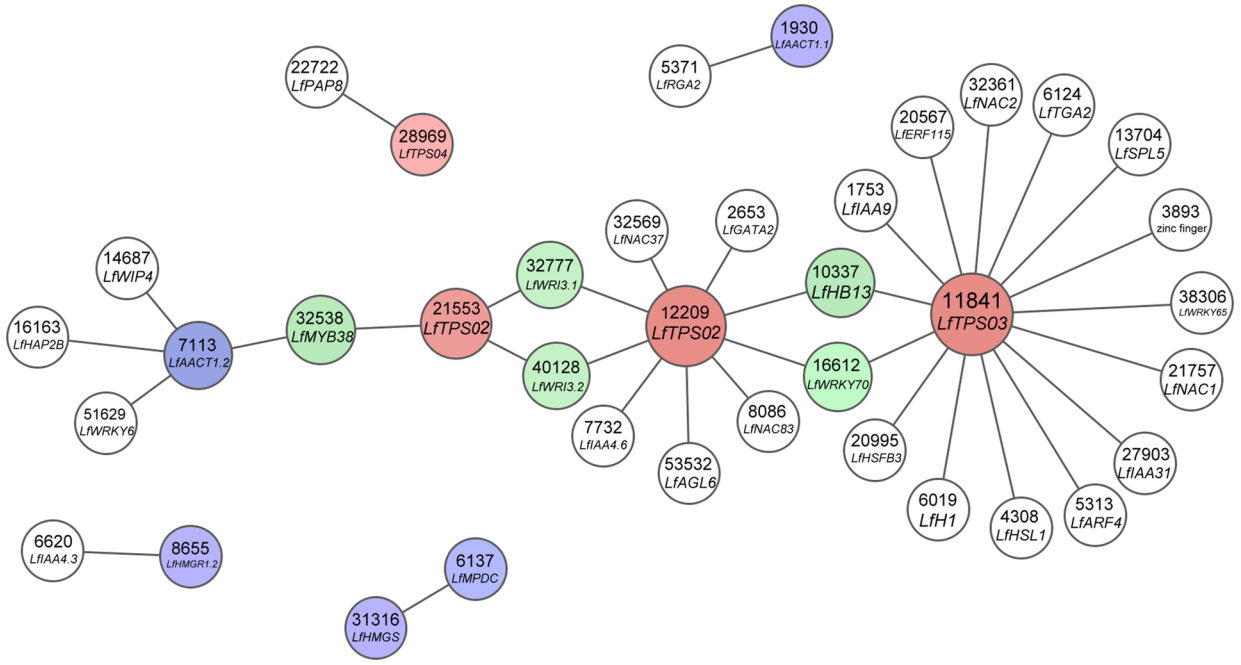

Figure 8. Simplified gene coexpression network of the sesquiterpene biosynthesis genes and transcription factors in Formosan sweet gum. Every node represents a contig. The numbers shown in the nodes are the serial numbers of the contigs. Below the number is the name of the contig given according to best hit in TAIR. Blue nodes are genes in the isoprene biosynthesis pathway, pink nodes are sesquiterpene synthases, and the remaining nodes are transcription factors: those colored green attached to two other nodes and those colored white attached to one other node.

and *LfTPS04* was connected to *LfPAP8* (LfGR22722). In the major network, *LfAACT1.2* was linked to *LfWRKY6* (LfGR51629), a transcription factor related to senescence, wounding, pathogens, and nutrition.[46] In addition, transcription factors related to plant hormones showed a link to *LfTPS02* or *LfTPS03*, including *LfIAA9* (LfGR1753), *LfIAA31* (LfGR27903), *LfERF115* (LfGR20567), and *LfARF4* (LfGR5313), which were connected to *LfTPS02* and *LfWRI3.1* (LfGR32777), and *LfWRI3.2* (LfGR40128) and *LfIAA4.6* (LfGR7732), which were connected to *LfTPS03*. *LfWRKY70* (LfGR16612) and *LfHB13* (LfGR10337) made a link between *LfTPS02* and *LfTPS03*, thus indicting a putative regulatory effect on these two *LfTPSs*. As an increase in aromadenrene and β-caryophyllene formation has been reported in *Sambucus ebulus* leaf essential oil after indole-3-acetic acid (IAA) treatment,[47] the expression of *LfTPS02* and *LfTPS03* may be regulated by IAA in a similar manner.

From the point of view of agriculture and silviculture, improvement in best practice and harvest time to acquire ideal compounds is the ultimate goal. In the present study, we characterized the enzyme function of five newly identified sesquiterpene synthase genes in Formosan sweet gum and presented the seasonal expression patterns of sesquiterpene biosynthesis genes. This is the first study of the sesquiterpene synthase genes in a subtropical deciduous tree across a seasonal time series. It was illustrated that the relative amounts of sesquiterpenoids in Formosan sweet gum differ by season.

EXPERIMENTAL SECTION

General Experimental Procedures. GC/MS analysis was performed using a Thermo Finnigan Trace GC Ultra equipped with a DB-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) and coupled with an ITQ 900TM mass spectrometers (Thermo Fisher Scientific, Waltham, MA, USA). The NMR spectra were obtained using a Bruker ASCEND-400 in $CDCl_3$ with tetramethylsilane as internal control. The chemicals and culture medium used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA).

NGS Sequencing. Leaves of Formosan sweet gum were collected on the campus of National Taiwan University on December 13, 2010 (*LfTPS01*), and April 8, 2011 (*LfTPS02*, *LfTPS03*, *LfTPS04*, *LfTPS05*), respectively. Total RNA was extracted by Concert Plant RNA Reagent (PureLink, Invitrogen Life Technologies, USA), treated with DNase I (TURBO DNA-free kit, Ambion, Life Technologies, Grand Island, NY, USA), and purified by EtOH precipitation.

The samples used in NGS, RNA isolation, library construction, and data analysis were as reported.[17] The raw transcriptome reads have been submitted to the NCBI Short Read Archive under the accession numbers SRR1514949 and SRR1514913. The published transcriptome contig sequences are available in the ContigViews database (www.contigviews.bioagri.ntu.edu.tw).[48]

Construction and Identification of Full-Length cDNA Clones. The primers were designed based on the partial sequences obtained from the NGS library. The resultant fragments were cloned into the vector pGEM-T Easy (Promega, Madison, WI, USA) and confirmed by DNA sequencing. Rapid amplification of cDNA ends (3′RACE and 5′RACE) (Invitrogen, Life Technologies) was carried out to obtain full-length cDNA sequences. The full-length cDNA sequences were confirmed by Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland), cloned, and sequenced.

Seasonal Gene Expression of the Five *LfTPS*s. Samples collected monthly through 2012 in the growing season were used for time series gene expression experiments. Total RNA of each sample was extracted using the Pine Tree Method.[49] DNase I (2 μg; TURBO DNA-free kit, Ambion, Life Technologies)-treated total RNA from each sample was used for reverse-transcription to cDNA (SuperScript III First Strand Synthesis System, Thermo Fisher Scientific, USA). The RT-PCR conditions for each *LfTPS* gene were as follows: *LfTPS01*: 95 °C for 3 min, followed by 24 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min; *LfTPS02*: 95 °C for 3 min, followed by 29 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min; *LfTPS03*: 95 °C for 3 min, followed by 24 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min;

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LfTPS04: 95 °C for 3 min, followed by 24 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min; *LfTPS05*: 95 °C for 3 min, followed by 29 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min. *LfActin2* was used as a loading control. The PCR conditions were as follows: 95 °C for 3 min, followed by 24 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min.

Methyl Jasmonate Treatment. For MeJA treatment, two-year-old Formosan sweet gum seedlings were sprayed with a 0.1% solution of 95% MeJA (w/w) (Sigma-Aldrich, Munich, Germany) dissolved in 0.1% polyoxyethylene-20 solution, and the control seedlings were sprayed with 0.1% polyoxyethylene-20.[50] The leaves of the seedlings were harvested during a 30 h period at 6 h intervals under controlled growth conditions (12 h light, 12 h dark at 27 and 25 °C, respectively). Total RNA from treated and untreated leaves was extracted by Concert Plant RNA Reagent (PureLink, Invitrogen Life Technologies), and DNA contamination was eliminated by DNase treatment for 30 min at 37 °C (TURBO DNA-free kit, Ambion, Life Technologies). The DNA-free total RNA was reverse transcribed into cDNA as described previously.[51]

The RT-PCR conditions for each *LfTPS* gene were as follows: *LfTPS01*: 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min; *LfTPS02*: 95 °C for 3 min, followed by 27 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min; *LfTPS03*: 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min; *LfTPS04*: 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min; *LfTPS05*: 95 °C for 3 min, followed by 27 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, ending with 72 °C for 7 min.

Phylogenetic Analysis. Amino acid sequence alignments were generated using the CLUSTALW computer program.[52] Sequence relatedness was analyzed using the neighbor-joining method, and an unrooted tree was visualized using MEGA5.1.[53]

Heterologous Expression of the Five LfTPSs. After confirming the sequence of *LfTPSs* by DNA sequencing, the resultant fragments were subcloned into the vector pTYB12 (*LfTPS01*), pTYB22 (*LfTPS03*, *LfTPS04*) (IMPACT-CN system, New England BioLabs, Ipswich, MA), and pET21A (*LfTPS02* and *LfTPS05*) (Novagen, Darmstadt, Germany) for protein expression in *Escherichia coli* strain BL21. The transformed *E. coli* was cultured at 37 °C and induced with isopropyl-β-D-thiogalactoside as described by Wen et al.[48]

Enzyme Characterization and Product Detection. The functions of the LfTPSs were determined in vitro by GC/MS analysis according to Martin et al.[54] The purified protein was added into sesquiterpene synthase buffer (25 mM HEPES, 0.1 M KCl, 10 mM $MgCl_2$, 10% glycerol, 10 mM dithiothreitol) and 50 μM substrate geranyl diphosphate or farnesyl diphosphate substrate (Sigma-Aldrich, Munich, Germany). The reaction was performed at 30 °C for 1 h, and the mixture was extracted by solid-phase microextraction for 20 min and analyzed by GC/MS (Polaris-Q mass spectrometer with Trace GC, Thermo Finnigan). The injection temperature was 250 °C, and the low injection temperature was 150 °C. Oven temperature was programmed to 100−170 °C at 5 °C/min and then 30 °C/min up to 290 °C. The identification of the compounds was assigned by comparison of their mass spectra with those in the Wiley/NBS Mass Spectral Library using NIST MS search.

Formosan Sweet Gum Leaf Essential Oil Analysis. About 150 g (fresh weight) of leaf samples was collected in August and December 2015, and essential oil was obtained by hydrodistillation for 6 h using a Clevenger-type apparatus. The essential oil was diluted 1000× with EtOAc before injecting into a GC/MS, and the same program as enzyme product detection was used. For ^{13}C NMR analysis in $CDCl_3$, 100 μL of August essential oil was used as the sample. The experimental procedure was according to Kubeczka and Formáček,[55] and analysis was done on a Bruker Ascend 400 MHz spectrometer.

Isoprene Biosynthesis Pathway Gene Expression and Gene Coexpression Network Analysis. The microarray experiment was as described by Wen et al.[17] To search for isoprene biosynthesis pathway genes, genes in *Arabidopsis* were used as queries[18] and a built-in BLAST program in ContigViews[48] was used. The names of genes in Formosan sweet gum were given according to their orthologues in *Arabidopsis*. Gene coexpression network analysis was carried out with all the isoprene biosynthesis genes and the five *LfTPSs* including their homologue contigs combined with the contigs that were annotated with GO:0003700 (sequence-specific DNA binding transcription factor activity), and expression showed a 2-fold change between April and December on average for the years 2011−2013. The ANOVA threshold was set to $P \leq 0.05$, and the threshold for the correlation coefficient was set to ≥ 0.8 (or ≤ -0.8). For a more restricted network, the correlation coefficient was set to ≥ 0.9 (or ≤ -0.9) and the nodes linking to isoprene biosynthesis genes or *LfTPSs* were extracted. The network analysis program was built in ContigViews.[48] The network images were generated in Gephi.[56]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00773.

Additional information (PDF)

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Notes

The authors declare no competing financial interest.

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