



Functional characterization and evolutionary diversification of sesquiterpene synthases in *Cunninghamia konishii*

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ABSTRACT

Cunninghamia konishii is a precious coniferous species endemic to Taiwan and is highly valued for its superior timber quality and durability, which make it an important resource for construction and furniture manufacturing. Furthermore, its characteristic fragrance represents a valuable natural product with potential applications in essential oils, perfumes, and related aromatic industries. The characteristic aroma of *C. konishii* is mainly derived from volatile terpenes; however, the biosynthesis of these compounds remains poorly understood. In this study, six full-length sesquiterpene synthase genes were isolated and functionally characterized to elucidate the molecular basis of terpene biosynthesis in *C. konishii*. Functional analyses revealed that CkTPS1 and CkTPS2 primarily produced dauca-4(11),8-diene, CkTPS3 produced germacrene A, CkTPS4 produced β -caryophyllene, CkTPS5 produced cubebol, and CkTPS6 produced δ -cadinene. These findings provide new insights into volatile terpene biosynthesis in *C. konishii* and advance our understanding of the functional diversification and evolutionary trajectories of sesquiterpene synthases in conifers.

1. Introduction

Cunninghamia konishii (Cupressaceae) is an evergreen conifer which is widely distributed in northern and central Taiwan at elevations of 1300–2700 m. In taxonomy, previous studies have indicated that several populations are derived from *C. lanceolata*, thus *C. konishii* was widely considered an ecotype of *C. lanceolata* (*C. lanceolata* var. *konishii*) via long-distance seed dispersal and shaped by environmental plasticity and incomplete isolation (Chung et al., 2004; Zhang et al., 2025). Known for its fine texture, excellent wood durability and fragrance, *C. konishii* is an important conifer species in Taiwan, and is considered one of the five precious woods of Taiwan, along with *Taiwania cryptomerioides*, *Chamaecyparis formosensis*, *Chamaecyparis obtusa* var. *formosana*, and *Calocedrus macrolepis* var. *formosana*. (Cheng et al., 2012; Hussain et al., 2021; Hsieh et al., 2022; Wu et al., 2024). The wood of *C. konishii* has been used for the production of essential oil, which contains various natural compounds to exhibit antifungal and larvicidal activities, suggesting its potential applications as an environmentally friendly pesticide or antifungal agent (Cheng et al., 2012, 2013).

Terpenoids constitute a large and structurally diverse group of natural products, representing one of the major classes of plant secondary

metabolites (Whitehead et al., 2023). They are important constituents of plant volatile compounds and essential oils (Jin et al., 2025). Plants release volatile terpenoids as a defense mechanism in response to biotic and abiotic stresses, for instance, *Cucumis sativus* will release a mixture mainly composed of volatile terpenes such as (E,E)- α -farnesene, (E)- β -ocimene, and linalool to attract natural enemies of the pest for defense when they are invaded by the two-spotted spider mites (TSSM) (He et al., 2022). During *Ricinus communis* seedlings establishment, the expression levels of more than 30 TPS genes were significantly higher at 35°C than at 25°C and 30°C, indicating that these genes may be involved in high-temperature stress-coping mechanisms, enabling the plant to survive under high-temperature stress and maintain high yield (Silva de Oliveira et al., 2022). Besides, terpenoids also play an important role in the allelopathy effect in plants, *Pinus halepensis* can release β -caryophyllene in the roots to inhibit the growth of surrounding herbaceous plants, allowing plants to gain a growth advantage (Huang et al., 2021; Ninkuu et al., 2021). In addition, plant terpenoids can attract pollinators and seed dispersers and facilitate communication with other plants, for instance, the monoterpenoid β -ocimene produced during carrot flowering can attract pollinating bees to help with pollination; while *Arum maculatum* may emit bicyclogermacrene as a pheromone mimic as part

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of their deceptive pollination strategy (Szenteczki et al., 2022; Quarrell et al., 2023). Terpenoids also have a wide range of applications due to their diverse biological activities. The most famous among them is taxol isolated from *Taxus brevifolia*, known for its anticancer effects (Guchelaar et al., 1994). δ -Cadinene, a major cadinane-type sesquiterpene in a lot of plant essential oils (Setzer, 2008), is able to induce cellular contraction, nuclear condensation, and nuclear envelope breakdown, while ovarian cancer cell growth through caspase-dependent apoptosis and cell-cycle arrest (Hui et al., 2015). And the resins of *Commiphora myrrha* contain furanoeudesma-1,3-diene and curzarene, which can produce analgesic effects by interacting with opioid receptors in the brain, therefore, they are often used as the main components of the medicinal herb myrrh (Lesburg et al., 1997).

Terpenoids are assembled from isoprene (C_5H_8) units. Prenyl-transferases synthesize terpene precursors of varying carbon-chain lengths, which are subsequently converted by terpene synthases (TPSs) into structurally diverse acyclic and cyclic terpene compounds (Davies et al., 2015). Classified by carbon number, the common product, monoterpenoids, sesquiterpenoids, and diterpenoids can be catalyzed from geranyl pyrophosphate (GPP, C_{10}), farnesyl pyrophosphate (FPP, C_{15}) or geranylgeranyl pyrophosphate (GGPP, C_{20}) as long-chain precursor, respectively (Cheng et al., 2025). Among the enzymes involved in terpene biosynthesis, TPSs are considered the key determinants of terpene composition because they control the cyclization and rearrangement of prenyl diphosphate substrates into diverse terpene skeletons. The functional diversification of TPSs has therefore contributed substantially to terpenoid chemical diversity, particularly in conifers, where terpenoids constitute major components of volatile emissions (Alicandri et al., 2020). In order to realize the specific compound synthesis in *C. konishii*, we identified and functionally characterized six novel sesquiterpene synthases in this study. The new compounds were identified by NMR analysis, and the putative cyclization mechanisms catalyzed by these TPSs were proposed.

2. Materials and methods

2.1. Plant materials and RNA extraction

The cone, needles, twigs and phloem of 50-year-old *C. konishii* (Hayata) were collected from Lianhuachih Research Center of Taiwan Forestry Research Institute in 2023 and were identified by Dr. Jeng-Der Chung (Taiwan Forestry Research Institute). After collected from plants, all of the sample was immediately frozen in liquid nitrogen then was stored at $-80^{\circ}C$ until further use. The sample pictures were showed in Fig. 1. Total RNA from the selected tissues was isolated following the

method reported in previous study's protocol (Ma et al., 2019). RNA quality and concentration were assessed by RNA electrophoresis and the Nanodrop Lite spectrophotometer (Thermo, United States), respectively.

The cDNA libraries of cones, needles, phloem, and twigs from *C. konishii* (Ck_C, Ck_N, Ck_LP, Ck_MP, Ck_UP, and Ck_T) were prepared for sequencing using the Illumina HiSeq 4000 platform. The raw sequencing reads have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA1495996, with SRA accession numbers SRR39772529–SRR39772534, respectively. *De novo* transcriptome assembly was performed using Trinity (Grabherr et al., 2011). The assembled transcriptome was subsequently evaluated using BUSCO (Manni et al., 2021). The assembled transcriptome was imported into CLC Genomics Workbench v12 for sequence retrieval and downstream analysis.

2.2. Full-length cDNA cloning

Twelve sesquiterpene synthases (Accession number: MK404538~MK404546 and MK430066~MK430068) from *T. cryptomerioides* (Ma et al., 2019) were used as the bait to find the candidate genes in the transcriptome database (SRA # SRR39772529–SRR39772534) of *C. konishii*. After preliminary selection of candidate genes, fragments per kilobases per million (FPKM) value was used as the further screening criterion to select tissues with high expression levels for gene cloning. Gene-specific primers (Table S1) were then used to amplify complete open reading frames using Phanta Super-Fidelity DNA Polymerase (ZGene Biotech, Taipei, Taiwan).

2.3. Phylogenetic analysis

All cloned TPSs with complete coding sequences were translated into amino acid sequences and compared with functionally characterized terpene synthases reported in previous studies (Table S2). For gymnosperms, reference TPSs were selected primarily from the Cupressaceae and Pinaceae families, whereas functionally characterized TPSs representing diverse angiosperm lineages were included for comparison. Reference sequences were selected based on their reported biochemical functions and availability of complete coding sequences. Multiple sequence alignment of the full-length TPS protein sequences was performed using MUSCLE implemented in MEGA12. The resulting alignment was used for subsequent phylogenetic analysis without additional trimming. Phylogenetic relationships were inferred using the Maximum Likelihood method implemented in MEGA12 with the Jones–Taylor–Thornton (JTT) substitution model and 1000 bootstrap replicates to assess branch support (Kumar et al., 2024).

2.4. Heterologous expression of TPSs in *E. coli*

Using 5'- and 3'-specific primers containing restriction endonuclease recognition sites, the target genes were amplified by PCR, and the resulting full-length TPS genes were cloned into the pET21a(+) expression vector (Novagen, USA). Then the plasmid was transformed into *E. coli* strain BL21DE3-C41 (Lucigen, Middleton, WI, USA), incubated in 750 ml LB at $37^{\circ}C$ until OD_{600} value between 0.6 and 0.8. Then, 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the medium, and suspended at $16^{\circ}C$ for 24 h to let cells to grow. After that, cells were centrifuged at $4^{\circ}C$, 7000 rpm, then discarded the supernatant and resuspended the pellet with protein binding buffer (50 mM HEPES & 300 mM NaCl, pH 7.3), and the suspension was disrupted by sonication, and centrifuged at $4^{\circ}C$, 8000 rpm. The soluble protein was subsequently purified with TALON® Superflow™ Metal Affinity Resin (GE Healthcare Life Sciences, USA).

2.5. In vitro assay enzyme characterization

To characterize the enzymatic activity of the target protein with



Fig. 1. A: timber which has phloem; B: twigs; C: cones; D: needles.

farnesyl pyrophosphate (FPP, Sigma, St Louis, MO, USA, 1 mg/ml), *in vitro* assays were performed in a 500 μ L reaction mixture containing 100 μ g of protein, 300 mM HEPES (pH 7.0), 0.1 M dithiothreitol, 1 M MgCl_2 , 1 M KCl, 10% Glycerol and 10 mM Vitamin C, and 11 μ L FPP (57.6 μ M). Then put the mixture at 30°C for 1 h to react. After reaction, the product was extracted by using the solid-phase microextraction (SPME) method. The SPME fiber (CAR/PDMS, 75 μ m) absorbed the compounds produced by *in vitro* reaction for 30 min at 50°C, and inserted into injection port of gas chromatography-mass spectrometry (GC-MS, Bravo GC matching MAESTRO- α MS) for 3 min at 250°C or 150°C for compound analysis. Rxi®-5ms (crossbond®5% diphenyl/95% dimethyl polysiloxane, 30 m $\times$ 0.25 mm $\times$ 0.25 μ m) column was used, and the carrier gas was helium, which has a constant flow rate of 1 ml/min. All enzyme assays were performed twice with consistent results, and one representative dataset is presented. The relative abundance of each detected product was calculated as the percentage of its peak area relative to the summed peak areas of all detected products in the corresponding chromatogram, which were presented as mean $\pm$ SD. The major product was defined as the product exhibiting the highest mean relative abundance among the detected products for each CkTPS enzyme.

2.6. *In vivo* assay co-expression of TPSs in *E. coli*

The *in vivo* co-expression assays were carried out following procedures revealed in previous literature (Chen et al., 2025; Hong and Chu, 2026). The constructs were co-transformed into *E. coli* C41 (DE3) (Lucigen, Middleton, WI, USA) together with the following pathway plasmid: pET21a (+)—truncated CkTPSs, has six histidine at C-terminal as protein fusion tag (his-tag) to bind the antibody; pMVA2 (Addgene plasmid # 119951; RRID: Addgene_119951)—heterologous mevalonate (MVA) pathway; pCTD—truncated AtGPPS (GenBank: NP_850234.1) and TcFPPS (GenBank: QHZ00912.1). *E. coli* cells harboring the three aforementioned heterologous plasmids were cultured in 50 ml Super Optimal Broth (SOB) containing 5 μ g/ml tetracycline (for pCTD), 25 μ g/ml kanamycin (for pMVA2), and 25 μ g/ml carbenicillin (for pET21a (+)) at 37°C until the OD₆₀₀ reached 0.6 and 0.8. Using IPTG which had adjusted final concentration to 1 mM to induce the foreign genes' expression, and also adding 20% glucose to final concentration of 20 mM to facilitate the terpene synthesis. After 72 h of induction, an equal volume of n-hexane was added and stirred to extract the sesquiterpenoids for overnight. After extraction, the extract was concentrated using a rotary evaporator and subsequently analyzed by GC-MS. Meanwhile, authentic standards, essential oils, or products obtained from other genes with known functions were co-injected with the samples, and the retention times and mass spectra were compared to confirm the identities of the products. Unknown terpenoids were further characterized by NMR analysis using products accumulated from multiple 500-mL *E. coli* co-expression cultures. The negative control was processing at the same condition, the only distinction was the pET21a (+) vector hasn't reconstructed with sesquiterpene synthase gene. The number of independent biological replicates were 3, and the relative abundance was calculated as described above.

2.7. HPLC separation of unknown compounds

Compound separation was performed using a normal high-pressure liquid chromatography (HPLC) system (AGILENT/1100) with a semi-preparative grade silica column (5 μ m, 10.0 $\times$ 250 mm, Phenomenex, USA). An HPLC-grade solution of n-hexane and dichloromethane in a 2:1 ratio was used as the mobile phase at a flow rate of 3 mL/min, with an injection volume of 100 μ L and UV detection at 254 nm.

2.8. Nuclear magnetic resonance (NMR) analysis

Following HPLC purification, the collected compounds were verified

and quantified by GC-MS, and at least 2 mg of each product was subjected to NMR analysis. The product was dissolved in deuterated chloroform (CDCl_3 , Sigma-Aldrich) and analyzed using a Bruker Avance 400 MHz FT-NMR spectrometer (Bruker, Billerica, MA, USA) to acquire various NMR spectra, including ^1H , ^{13}C , Heteronuclear Multiple Bond Correlation (HMBC), Heteronuclear Single Quantum Coherence (HSQC), ^1H - ^1H Correlation Spectroscopy (COSY), and Nuclear Overhauser Effect Spectroscopy (NOESY) spectra to analyze each structure of unknown terpenoids.

3. Results

3.1. Volatile compound analysis of *C. konishii*

Volatile compounds were extracted from three *C. konishii* tissues—leaves, branches, and phloem—using ethyl acetate (EA) and subsequently analyzed by GC-MS. Compound identification was based on comparisons with the NIST 2020 mass spectral library and Kovats retention indices (KIs) reported in the literature (Adams, 2007). A total of 61 volatile compounds were detected across the three tissues. Of these, 41 were tentatively identified as known compounds, whereas 20 remained unidentified. The detected compounds included nine monoterpenes, 30 sesquiterpenes, 15 diterpenes, and seven non-terpene compounds (Fig. 2; Table 1). The volatile profiles differed among tissues. In the leaves, the predominant monoterpenes were α -thujene, α -pinene, and limonene. In contrast, branches and phloem contained relatively high levels of α -pinene but showed lower monoterpene diversity than leaves. Sesquiterpenes were also more diverse in leaves, where β -elemene, germacrene D, and β -selinene were detected at relatively high levels. By comparison, phloem exhibited the lowest sesquiterpene diversity, with β -caryophyllene as its predominant component.

3.2. Identification of the sTPS gene family in the *Cunninghamia konishii*

Initially, *C. konishii* transcriptome databases were searched using the nucleotide sequences of TcTPS1–TcTPS12 as queries, identifying a total of 79 candidate terpene synthase genes, each exceeding 700 bp in length. According to the FPKM value, six *C. konishii* TPSs (CkTPSs) were selected from cDNA of twigs and phloem of *C. konishii*, which had higher expression level in different tissues among another candidate genes (Fig. 3). After cloning and gene function identification, they were named CkTPS1 to CkTPS6 with the lengths of open reading frames ranged from 1737 to 1821 bp, which translated into 578 to 606 amino acids (Table 2, Table S3), with similarities in the amino acid sequences ranging from 56 to 99% (Table S4). The 5' terminal untranslated region (UTR) of them are ranged from 54 to 94 bp, while 3' terminal UTR are ranged from 109 to 492 bp (Table S3). These TPS genes identified from *C. konishii* lacked plastid-targeting signal peptides, suggesting that they belong to the sesquiterpene synthase class. This prediction was subsequently confirmed by GC-MS analysis of their enzyme products.

The six synthases in this study were classified as Class I TPSs and contained the conserved RRX₅W, RXR, DDXXD, and NSE/DTE motifs characteristic of this enzyme class (Keeling and Bohlmann, 2006; Alicandri et al., 2020) (Fig. S1). Among them, DDXXD and NSE/DTE motif are particularly important for the catalytic activity of sesquiterpene synthases, they're around the active site of protein structure, and has function of chelating the divalent ion Mg^{2+} to catalyze the substrate to form sesquiterpenoids and coordinate the stabilization of the active site (Jiang et al., 2019). And we further aligned the putative active-site regions and compared the amino acid substitutions among CkTPS1–CkTPS6. Although several sequence differences were identified, none of the substitutions could be confidently assigned, based on currently available evidence, as a determinant of the observed product differences. These observations suggest that the product profiles of CkTPS1–CkTPS6 may not be explained by a single active-site residue alone and could involve the combined effects of multiple residues or

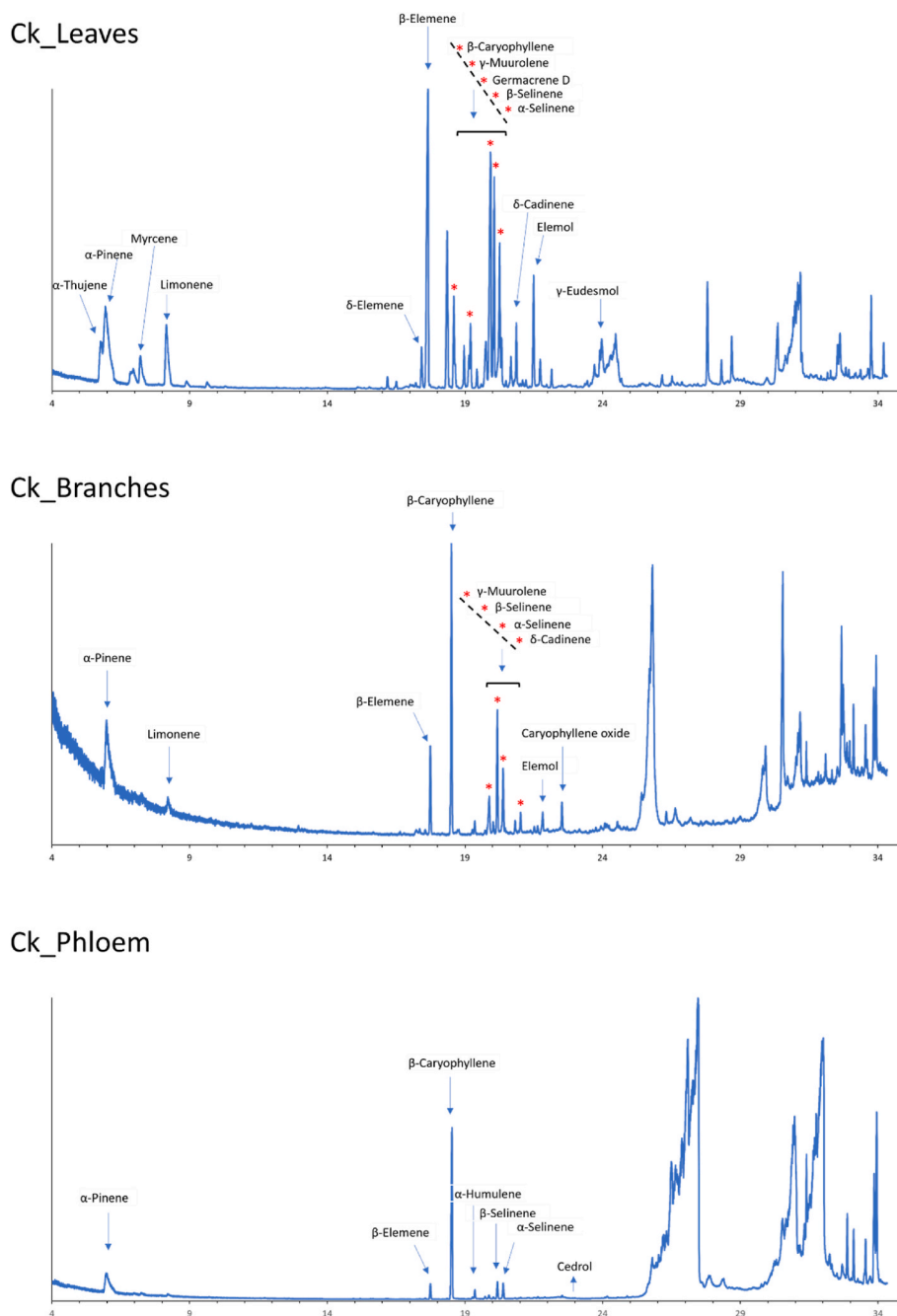


Fig. 2. Volatile compound analysis of leaves, branches, and phloem in *C. konishii*.

other structural features of the enzymes instead.

The six gene in this study had shown the second DDXXD motif as *G. arboreum*, consistent with previous findings on *T. cryptomeroides*, *C. formosensis* and *C. obtusa* var. *formosana*, indicated the phenomenon that NSE/DTE motifs are usually replaced by a second DDXXD can be traced back to the origin of Cupressaceae family (Ma et al., 2019; Hong et al., 2022; Chen et al., 2025). This situation also has found in the δ -cadinene synthase of *Gossypium arboreum*, this region presents another aspartic acid-rich DDXXD motif, rather than the conserved NSE/DTE sequence as seen in typical isoprene transferases (Gennadios et al., 2009). In addition to cloning of coding region, we also obtained the genomic DNA sequences of some of genes in this study, which had a wide range of length from 2910 to 6507 bp (Table S3). We identified a genomic DNA sequence showing 99% sequence similarity to both *CkTPS1* and *CkTPS2*, suggesting the presence of multiple highly similar TPS gene sequences in

C. konishii. However, it remains unclear whether *CkTPS1* and *CkTPS2* represent two alleles of the same gene or paralogous genes resulting from gene duplication. Further genomic and functional analyses would be required to distinguish between these possibilities. In addition, the genes could be classified into two groups based on their intron numbers, *CkTPS1*, *CkTPS2* and *CkTPS3* have nine introns in gene structure as normal type of most genes in Cupressaceae, while *CkTPS5* only has eight introns in gene structure (Table S3). An interesting fact is the longer sequence of *CkTPS5* was due to a large intron XI, supposed to cause by a transposable element insert event. This event doesn't influence the translation and expression of *CkTPS5*, it still remains its function. However, this is not a common situation for sesquiterpene synthases of Cupressaceae, it is more prevalent in pine trees instead, due to the gene accumulated.

Table 1
Volatile compound analysis of different *C. konishii* tissues.

RT ^a	Compounds	Formula	RI ^b	Identification	Tissues
5.77	α -Thujene	C ₁₀ H ₁₆	924	RI, MS	L
5.97	α -Pinene	C ₁₀ H ₁₆	932	RI, MS	L B P
6.85	Sabinene	C ₁₀ H ₁₆	969	RI, MS	L
7	β -Pinene	C ₁₀ H ₁₆	974	RI, MS	L B P
7.23	Myrcene	C ₁₀ H ₁₆	988	RI, MS, STD	L B P
8.2	Limonene	C ₁₀ H ₁₆	1024	RI, MS, STD	L B P
8.88	γ -Terpinene	C ₁₀ H ₁₆	1054	RI, MS	L
9.64	Terpinolene	C ₁₀ H ₁₆	1086	RI, MS, STD	L
12.95	Verbenone	C ₁₀ H ₁₆	1204	RI, MS	B
16.19	δ -Elemene	C ₁₅ H ₂₄	1335	RI, MS	L
16.65	α -Cubebene	C ₁₅ H ₂₄	1345	RI, MS	L
17.35	α -Copaene	C ₁₅ H ₂₄	1374	RI, MS	L
17.74	β -Elemene	C ₁₅ H ₂₄	1389	RI, MS, STD	L B P
18.34	Unknown 1	C ₁₅ H ₂₄	-	-	L
18.5	β -Caryophyllene	C ₁₅ H ₂₄	1417	RI, MS, STD	L B
18.57	β -Copaene	C ₁₅ H ₂₄	1430	RI, MS	L
18.63	γ -Elemene	C ₁₅ H ₂₄	1434	RI, MS	L
18.96	Unknown 2	C ₁₅ H ₂₄	-	-	L
19.12	Unknown 3	C ₁₅ H ₂₄	-	-	L
19.35	α -Humulene	C ₁₅ H ₂₄	1452	RI, MS, STD	B P
19.42	Unknown 4	C ₁₅ H ₂₄	-	-	L
19.75	γ -Gurjunene	C ₁₅ H ₂₄	1475	RI, MS	L
19.87	γ -Murolene	C ₁₅ H ₂₄	1478	RI, MS	L B P
20.04	germacrene D	C ₁₅ H ₂₄	1484	RI, MS	L B P
20.17	β -Selinene	C ₁₅ H ₂₄	1489	RI, MS	L B P
20.38	α -Selinene	C ₁₅ H ₂₄	1498	RI, MS	L B P
20.65	γ -Cadinene	C ₁₅ H ₂₄	1513	RI, MS	L B P
20.85	δ -Cadinene	C ₁₅ H ₂₄	1522	RI, MS	L B P
21.09	<i>trans</i> -Cadin-1,4-diene	C ₁₅ H ₂₄	1533	RI, MS	L
21.47	Elemol	C ₁₅ H ₂₆ O	1548	RI, MS	L B P
21.7	(<i>E</i>)-Nerolidol	C ₁₅ H ₂₆ O	1561	RI, MS	L P
22.15	Germaecene D-4-ol	C ₁₅ H ₂₆ O	1574	RI, MS, STD	L
22.52	Caryophyllene oxide	C ₁₅ H ₂₄ O	1582	RI, MS	B P
22.98	Cedrol	C ₁₅ H ₂₆ O	1600	RI, MS	P
23.34	1,10-di- <i>epi</i> -Cubenol	C ₁₅ H ₂₆ O	1618	RI, MS	L
23.43	10- <i>epi</i> - γ -Eudesmol	C ₁₅ H ₂₆ O	1622	RI, MS	L
23.70	Unknown 5	C ₁₀ H ₁₄ O ₃	-	-	L
23.89	γ -Eudesmol	C ₁₅ H ₂₆ O	1630	RI, MS	L B P
23.97	β -Acorenol	C ₁₅ H ₂₆ O	1636	RI, MS	L B
24.17	Selin-11-en-4 α -ol	C ₁₅ H ₂₆ O	1658	RI, MS	L
24.47	Unknown 6	C ₂₀ H ₃₄ O ₂	-	-	L
25.8	Unknown 7	C ₂₀ H ₃₄ O ₂	-	-	B
26.15	Unknown 8	C ₁₄ H ₂₈ O ₂	-	-	L
26.52	Unknown 9	C ₁₉ H ₂₄	-	-	L B
27.47	Unknown 10	C ₂₀ H ₃₂ O	-	-	P
27.8	Unknown 11	C ₂₀ H ₃₈	-	-	L
28.32	Unknown 12	C ₂₀ H ₃₂	-	-	L
28.68	Unknown 13	C ₂₀ H ₄₀ O	-	-	L
30.36	Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	1959	RI, MS	L B
31.17	Sclareol	C ₂₀ H ₃₆ O ₂	2222	RI, MS	B
31.18	Manoyl oxide	C ₂₀ H ₃₄ O	1987	MS	B P
31.24	Unknown 14	C ₂₀ H ₃₂	-	-	L
31.95	Communic acid	C ₂₀ H ₃₀ O ₂	2365	RI, MS	P
32.63	Linoleic acid	C ₁₈ H ₃₀ O ₂	2132	RI, MS	L
32.75	Oleic acid	C ₁₈ H ₃₄ O ₂	2141	RI, MS	B
32.95	Unknown 15	C ₂₀ H ₃₂	-	-	L
33.13	Unknown 16	C ₂₀ H ₃₂	-	-	B P
33.55	Unknown 17	C ₁₈ H ₂₄ O ₂	-	-	L B P
33.75	Unknown 18	C ₂₀ H ₃₀ O	-	-	L B P
33.93	Unknown 19	C ₂₀ H ₃₀ O	-	-	B P
34.24	Unknown 20	C ₂₀ H ₃₄ O ₂	-	-	L

RT^a indicates the retention time of each compound, expressed in minutes (min). RI^b indicates the Kovats retention index of each compound. Identification indicates the method used to identify each compound, including mass spectral comparison (MS), comparison of Kovats retention indices (RI), and comparison with authentic standards (STD). L, B, and P represent volatile compounds detected in leaves, branches, and phloem, respectively.

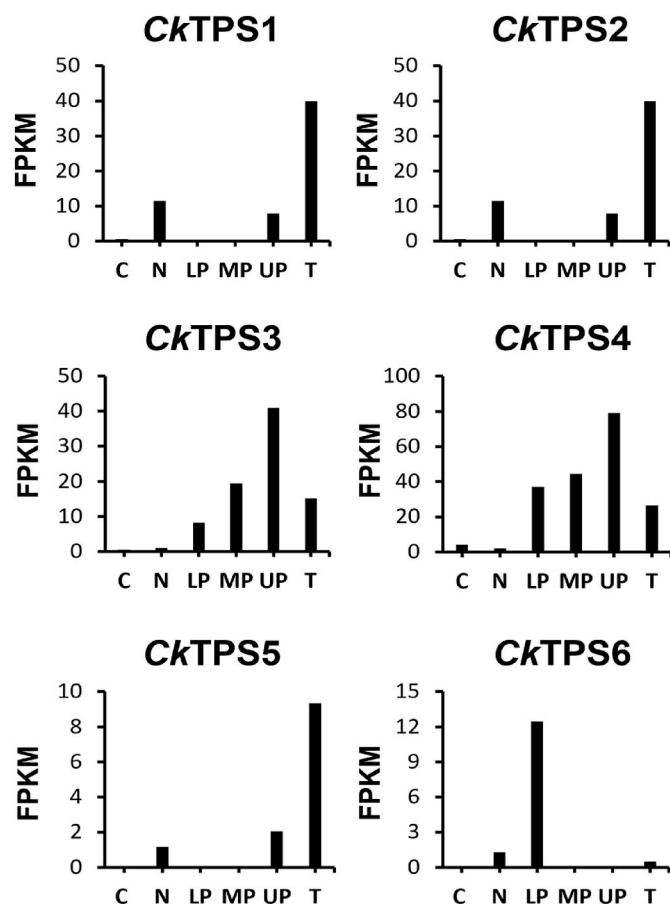


Fig. 3. The expression levels of six candidate genes in six tissues in *C. konishii*, including cones (C), needles (N), lower, middle and upper phloem (LP, MP and UP) and twig (T). “Low phloem” refers to phloem collected from the lower part of the trunk, whereas “upper phloem” refers to phloem collected from the upper part. These terms are designated according to the upper, middle, and lower positions along the trunk. The expression level is assessed using FPKM values.

Table 2
Summary of CkTPSs.

Enzyme	Contig	Protein length	Major product	Identification
Ck TPS1	155_c0_g3_i1	602	dauca-4 (11),8-diene	RI, MS, NMR
Ck TPS2	155_c0_g3_i1	602	dauca-4 (11),8-diene	RI, MS, NMR
Ck TPS3	609_c0_g1_i6	606	germacrene A	RI, MS, STD
Ck TPS4	983_c0_g1_i13	578	β -caryophyllene	RI, MS, STD
Ck TPS5	9508_c0_g2_i4	579	cubebol/ δ -cadinene	RI, MS, STD
Ck TPS6	13859_c0_g1_i18	592	δ -cadinene	RI, MS, STD

3.3. Functional characterization of the putative sesquiterpene synthases in *E. coli*

The result of Western blot analysis showed that all of the reconstructed genes can translate into amino acid and produce sufficient soluble protein (Fig. S2). Functional characterization was carried out in two ways, one of them was the *in vivo* assay, a co-expression system of the recombinant protein in *E. coli*, which can produce a large amount of target product; other was the *in vitro* assay, utilizing FPP as substrate, used as a gene enzymatic function confirm in the neutral environment (Fig. 4, Fig. S4). GC-MS analysis revealed that the compound profiles of the six CkTPSs were largely consistent between the *in vitro* and *in vivo*

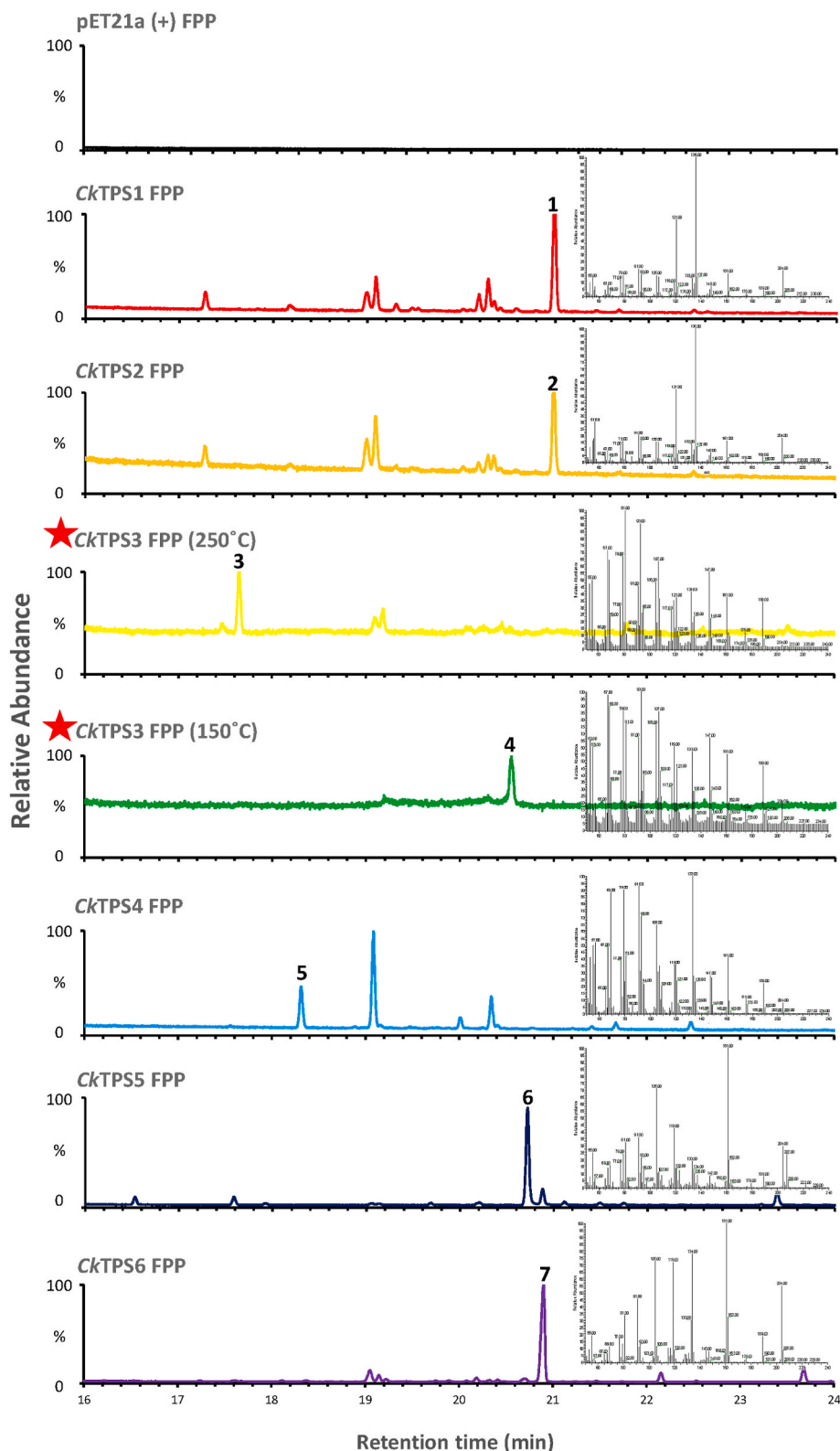


Fig. 4. Gas chromatography analysis of catalytic products extracted from *in vivo* assays of *CkTPSs* in *E. coli*, and only sesquiterpenoid products with the highest content of each gene are shown here. From top to bottom: control group, *CkTPS1* to *CkTPS6*. Numbered peaks are as follows: 1, unknown 1; 2, unknown 2; 3, β -elemene; 4, germacrene A; 5, β -caryophyllene; 6, cubebol; 7, δ -cadinene. *CkTPS3* is the exception, it is the result of *in vitro* assay, labeled by red star.

assays (Tables S5 and S6). No products were detected when GPP was used as the substrate. In the GC chromatograms, only the sesquiterpene with the highest mean relative abundance was labeled as the major product for each gene. Hydrocarbon and oxygenated products generated

by the recombinant enzymes were analyzed by GC-MS. The analysis showed that in the *in vivo* assay, *CkTPS1* and *CkTPS2* produced multiple sesquiterpenes with a daucene-type skeleton, including daucene (RT = 17.28 min) and dauca-4(11),8-diene (RT = 20.99/21.00 min),

with the latter being the major product. The unknown compound 1 was initially tentatively identified as dauca-4(11),8-diene by GC-MS analysis, and the molecular formula was determined to be $C_{15}H_{24}$, corresponded to four degrees of unsaturation. The structure was subsequently elucidated by the proton nuclear magnetic resonance (^1H NMR) (Fig. S10), the carbon-13 nuclear magnetic resonance (^{13}C NMR) (Fig. S11), heteronuclear multiple bond correlation (HMBC) (Fig. S12), heteronuclear single quantum coherence (HSQC) (Fig. S13), ^1H - ^1H correlation spectroscopy (COSY) (Fig. S14), and nuclear overhauser effect spectroscopy (NOESY) (Fig. S15). Analysis of the ^{13}C NMR spectrum exhibited fifteen carbon resonances, and the ^1H NMR spectrum revealed the presence of four methyl groups, including one tertiary methyl group [δ H 0.98 (3H, s, H-15)] and three olefinic methyl groups [δ H 1.77 (3H, br s, H-12), 1.85 (3H, br q, H-13), and δ H 1.88 (3H, br s, H-14)], suggesting a sesquiterpene framework. Among the four quaternary carbons, three were sp^2 carbons, indicating the presence of two trisubstituted double bonds, which accounted for two of the four degrees of unsaturation (Table 3). The observed spectroscopic data were consistent with those reported for dauca-4(11),8-diene, confirming the identity of Unknown 1 (Fig. S4). By comparing their mass spectra, Unknown compound 2 was found to be identical to compound 1, indicating that both corresponded to the same compound dauca-4(11),8-diene (Table 3). Dauca-4(11),8-diene is a relatively rare natural compound and has only been reported in a few species, such as *Omphalotus olearius*, *Rhaponticum carthamoides*, and *Bazzania trilobata* (Warmers and König, 1999; Wawrzyn et al., 2012; Skala et al., 2016) (Fig. 4, Fig. S4). To our knowledge, this is the first TPS reported in the Cupressaceae family that is capable of synthesizing dauca-4(11),8-diene as its major product. CkTPS3 was detected the primary product β -elemene (RT: 17.65 min) at 250°C injector temperature through comparison with an authentic standard. However, previous study showed that β -elemene was a cope rearrangement product of germacrene A, a reaction transforming the ten-membered ring structure into a six-membered ring structure (Degenhardt et al., 2009). Actually, after decrease the temperature of the injector to 150°C, the major product was changed into germacrene A (RT: 20.55 min) in contrast (Fig. 4, Fig. S5). Thus, CkTPS3 was identified as a germacrene A synthase. Interestingly, CkTPS3 only has function in the *in vitro* assay, which may reflect differences in the heterologous expression context. However, the underlying causes cannot be determined from the present data. Functional characterization of CkTPS4 as a β -caryophyllene synthase, producing major product β -caryophyllene (RT: 18.45 min) and it also showed different activity in the different assay, it has a minor byproduct α -humulene (RT: 8.91 min) in the *in vitro* assay, and both of α -humulene and β -caryophyllene are tricyclic

structure sesquiterpene (Fig. 4, Fig. S3 and S6). CkTPS5 produced cubebol (RT: 20.72 min) as major product in the *in vivo* assay, and δ -cadinene (RT: 20.88 min) was a low content byproduct, both of their frame formation are bicyclic. And again, this gene also can regulate the enzymatic activity in the different environment. In the *in vitro* enzyme assay, δ -cadinene would be the principal product, however, cubebol would take the biggest part of the product when CkTPS5 react in *E. coli* (Fig. 4, Fig. S7). CkTPS5 products compared with Piper cubeba essential oil as a reference standard (cited in text now), with the base peak set at m/z 93 (Fig. S7). We further found that this is not simply due to pH levels. Experimental results show that adjusting the pH from 6 to 9 in the *in vitro* experiments did not significantly change the product composition ratio. However, when the pH was lowered to 5, the product completely disappeared, indicating that the enzyme activity was inactivated and the compound could not be produced in acid environment. And the temperature also was not the main factor causing changes in the product ratio (Fig. S8). Therefore, we hypothesize that the change in the product ratio is caused by partial enzyme modification in *E. coli* or other external reactions. CkTPS6 was a multifunction gene in *E. coli* system, it showed the diverse product which could be detected by GC-MS. The major was δ -cadinene (RT: 20.90 min), and accompanied with two minor product, germacrene D-4-ol (RT: 22.13 min) and T-murolol (RT: 23.66 min), while it only exhibits an activity that produce δ -cadinene (RT: 9.51 min) in the *in vitro* enzyme assay (Fig. 4 Fig. S3 and S9). Although this study revealed the diversity of sesquiterpene synthases in *C. konishii*, the biosynthetic mechanisms underlying many natural volatile compounds remain to be elucidated, providing opportunities for further investigation in future studies. For example, cedrol, one of the major volatile compounds in *C. konishii*, was not associated with any of the sesquiterpene synthases characterized in this study, and the mechanism underlying its biosynthesis remains to be elucidated. In contrast, β -elemene and β -caryophyllene, which have previously been reported as constituents of *C. konishii* essential oil, were successfully associated with the corresponding sesquiterpene synthase genes identified in this study. These findings also highlight the need for further investigation of the biosynthetic pathways underlying the remaining unidentified volatile compounds in *C. konishii*.

3.4. Phylogenetic tree

The phylogenetic tree was constructed with six volatile sesquiterpene synthases and several amino acid sequence data of other gymnosperm and angiosperm TPSs which had been identified and made public on National Center for Biotechnology Information (NCBI) (Fig. 5A). The published sequences used for alignment included the TPS-d1 to d3 subfamilies. Members of the TPS-a subfamily were also included to observe the phylogenetic relationships of sesquiterpene synthases in gymnosperms and angiosperms. The bifunctional gene *PpCPS/KS* of *Physcomitrium patens* from the TPS-c family was used as the root of the phylogenetic tree.

The TPS-d family comprises gymnosperm terpene synthases and can be further divided into three groups: TPS-d1, TPS-d2, and TPS-d3. TPS-d1 primarily contains monoterpene synthases and several sesquiterpene synthases, whereas TPS-d2 includes other gymnosperm sesquiterpene synthases. TPS-d3 is composed predominantly of gymnosperm diterpene synthases (Chen et al., 2011). Within TPS-d family, sesquiterpene synthases which have tridomain in protein structure such as *GbBis* (α -bisabolene synthase) from *Ginkgo biloba* and *PmBis* (γ -bisabolene synthase) from *Pseudotsuga menziesii* were first differentiated into the TPS-d3 subfamily. Then, the sesquiterpene synthases of Pinaceae and Cupressaceae, which only have two domains, were further differentiated within the TPS-d2 subfamily. All of the sesquiterpene synthases of *C. konishii* in this study belong to TPS-d2 group, and they are more phylogenetically related to TPSs from *T. cryptomeroides*. This result match with our expectations for using *TcTPS* as bait to screen candidate genes in the transcriptome database of *C. konishii*. The CkTPSs are

Table 3

^1H and ^{13}C data for unknown product of CkTPS1 and CkTPS2.

C. No.	δ_C	δ_H	J
1	42.8	-	
2	40.5	a: 1.53 (1H, ddd) b: 1.42 (1H, ddd)	11.6, 7.5, ~1 Hz 11.6, 11.3, 9.0 Hz
3	29.9	a: 2.28–2.35 (1H, br m) b: 2.39–2.47 (1H, br m)	
4	138.5	-	
5	57.7	2.32 (1H, br m)	
6	25.2	a: 1.58 (1H, dddd) b: 2.33–2.36 (1H, m)	13.7, 12.1, 11.7, 2.6 Hz
7	34.4	a: 2.11 (1H, ddd) b: 2.18–2.25 (1H, br m)	14.7, 5.2, 2.6 Hz
8	138.6	-	
9	122.4	5.38 (1H, br m)	
10	42.0	a: 2.15 (1H, br d) b: 2.20 (1H, dd)	15 Hz 15.1, 8.1 Hz
11	122.6	-	
12	23.6	1.77 (3H, br s)	
13	20.8	1.85 (3H, br q)	1.7 Hz
14	27.5	1.88 (3H, br s)	
15	19.4	1.26 (3H, s)	

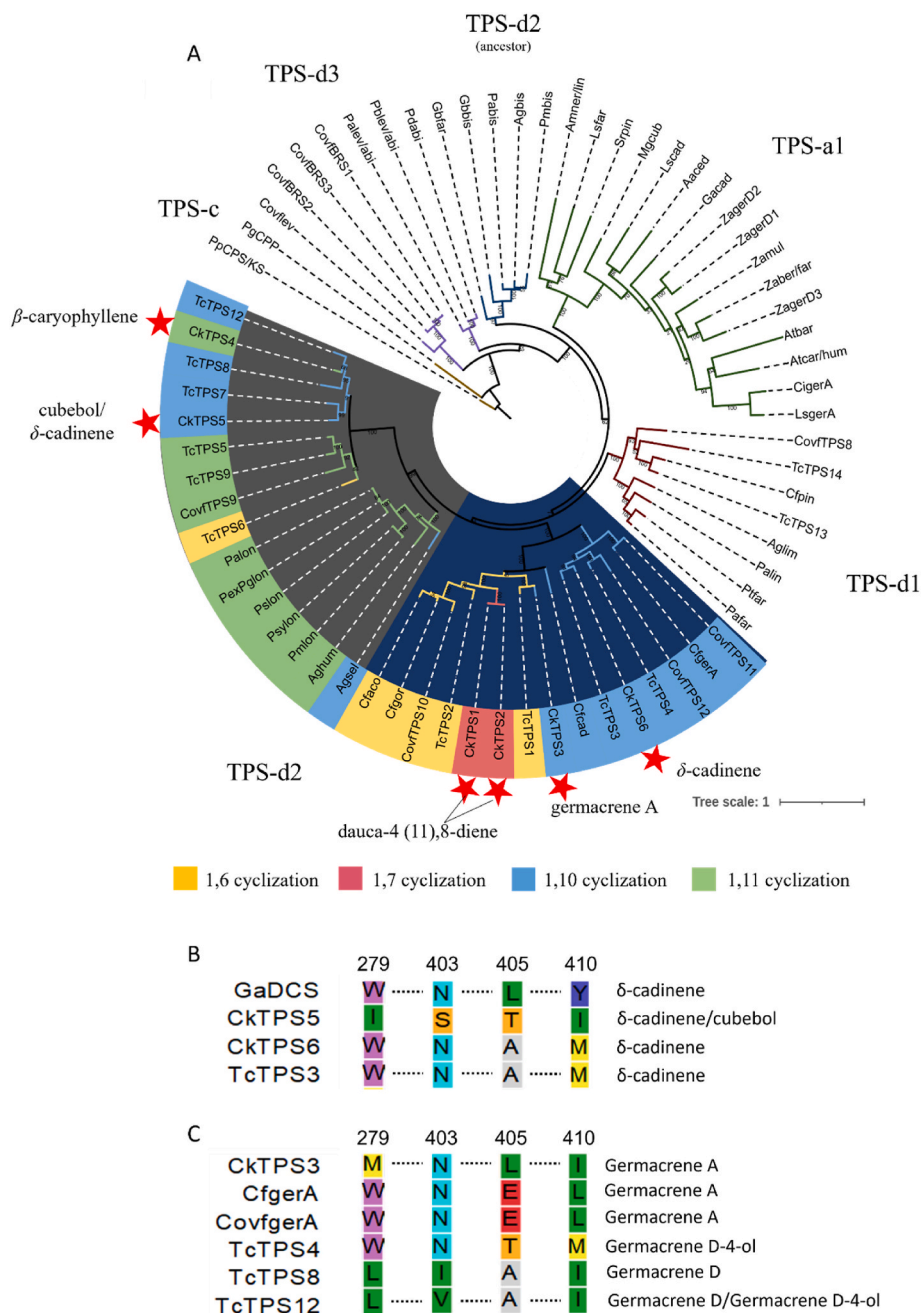


Fig. 5. (A) Phylogenetic tree of gymnosperm terpene synthase amino acid sequences from NCBI. The branch color represent the classification of TPSs, the color of outer ring represent cyclization mechanism of sesquiterpene synthases, and CkTPSs were labeled by red stars. The background color represent different intron number of each gene in TPS-d2 subfamily, dark gray part represent genes have eight introns, while dark blue part represent genes have nine introns. (B) The alignment of key amino acid residue in the active site of δ-cadinene synthases. (C) The alignment of key amino acid residue in the active site of germacrene A synthases.

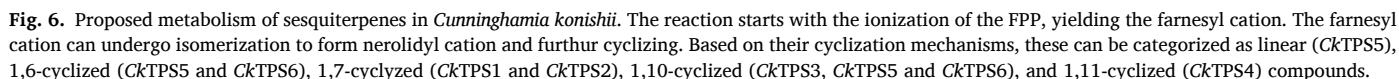
divided between two distinct clades as observed in other Cupressaceae species. The same phenomenon has been observed in other Cupressaceae species, it can be clearly observed that the sesquiterpene synthases of Cupressaceae plants are divided into two categories on the phylogenetic tree. One category differentiates out first, while the other further differentiates with the sTPS of Pinaceae later.

4. Discussion

4.1. The cyclization pathway of the CkTPSs

In this study, the sesquiterpene synthase could react with FPP to catalyze the formation of various sesquiterpenoids, exhibiting multiple

biosynthetic pathways including 1,7, 1,10 and 1,11 cyclization, among them, δ-cadinene can be generated not only through 1,10-cyclization but also had been predict as 1,6 cyclization enzymes in previous study (Faraldos et al., 2012) (Fig. 6). CkTPS1 and CkTPS2 are the first founded synthases whose main function was producing sesquiterpene with daucene type skeleton in Cupressaceae's terpene synthase family. They can produce three different sesquiterpenes with daucene frame, but since the main product are both dauca-4(11),8-diene, they are inferred to be dauca-4(11),8-diene synthase. For both enzymes, the proposed cyclization mechanism begins with pyrophosphate ionization of FPP, followed by isomerization to a nerolidyl cation. Subsequent 1,7-cyclization generates a cycloheptanyl cation intermediate. Then form different types of sesquiterpene, for instance, isodaucene, dauca-4(11),8-diene



with pyrophosphate ionization of FPP to generate a farnesyl cation. Subsequent 1,11-cyclization yields a humulyl cation intermediate. Next, second cyclization (2,10 closure) is going to produce caryophyllenyl cation, and it will be deprotonated to form β -caryophyllene (Degenhardt et al., 2009). CkTPS5 and CkTPS6 were identified as cubebol and δ -cadinene synthase, respectively, and CkTPS5 can form acyclic sesquiterpenoid, nerolidol at the same time. Both CkTPS5 and CkTPS6 are proposed to initiate catalysis through pyrophosphate ionization of FPP, followed by isomerization to a nerolidyl cation. A subsequent 1,10-cyclization generates a (Z,E)-germacradienyl cation, which undergoes further 1,6-cyclization to form a cadinyl cation, a key intermediate in cadinane-type sesquiterpene biosynthesis. Finally, the cadinyl cation

undergoes different quenching methods lead to the formation of different products. *CkTPS5* further occurs the third cyclization (2,6 closure) to yield cubebolyl cation, and be quenched by nucleophilic attack of water to produce cubebol, while *CkTPS6* can deprotonate cadinyl cation directly to form δ -cadinene. However, *CkTPS6* also produces germacrene D-4-ol and T-murolol through water-mediated reaction. Similar to cubebol formation, these oxygenated sesquiterpenes are generated by nucleophilic quenching of carbocation intermediates by water. (Yoshikuni et al., 2006). These mechanisms are presented as plausible biosynthetic routes rather than experimentally demonstrated intermediates.

4.2. Phylogenetic analysis of *CkTPSs*

Phylogenetic can be analysis through different perspectives, such as compound types, tree species, gene structure and even the cyclization mechanism of compounds or the catalytic mechanism of sTPSs. First, the differentiation of TPSs are not absolutely related to its terpene products. In the current analysis of the phylogenetic tree of terpene synthases, even though the functions of various TPSs in gymnosperms differ greatly, they are still classified into the TPS-d family, which quite different from the TPSs in angiosperms. For instance, *CkTPS4* from *C. konishii* and TPS from *Zea perennis* are able to utilize FPP as substrate and convert it into β -caryophyllene, but *CkTPS4* in TPS-d2 group while TPS from *Z. perennis* in the TPS-a group, which is mainly composed of sesquiterpene synthases of angiosperm, indicate that although they share the same activity, they are significantly different in classification due to their distant plant phylogenetic relationships. These findings suggest that sesquiterpene synthases may produce identical products through distinct catalytic mechanisms. Therefore, phylogenetic analysis alone cannot reliably predict TPS product specificity, but it remains valuable for interpreting their evolutionary relationships (Durairaj et al., 2019).

Besides, it can be observed that gene classification does not fully depend on tree species classification. TPS of the same tree species will not completely cluster in the same clade. On the contrary, sTPSs are observed to prefer to group those with similar or identical functions in the same cluster in Cupressaceae family, for instance, *CkTPS5* and *TcTPS7* have the main function of cubebol, and as a neighbor in phylogenetic tree, which can be predicted that they are homologous to each other, while another *CkTPSs* are in different clade, implied they have heterologous phylogenetic relationship.

From the perspective of cyclization, it can be found that sesquiterpene synthases that synthesize sesquiterpenes via different cyclization pathways appear to be found in different branches of the phylogenetic tree (Fig. 5A). For instance, *CkTPS1* and *CkTPS2*, which have 1,7-cyclization ability, were clustered with *TcTPS2*, of 1,6-cyclization enzymes, all of them are cyclized through small cyclization. In this study, it was the first time that found the 1,7-cyclization enzyme in Cupressaceae seems to be a unique extension from later stages of 1,6-cyclization evolution according to the phylogenetic relationship analysis. While *CkTPS6*, which produced δ -cadinene through 1,10-cyclization, clustered with *CovfTPS12* and *TcTPS3*, which were germacrene D-4-ol synthase and δ -cadinene synthase catalyzed 1,10-cyclization of FPP substrate, cyclized through large cyclization. Previous studies have proposed that gymnosperm sesquiterpene synthases evolved from enzymes producing acyclic products to those forming small-ring structures and, subsequently, larger-ring structures. Hong et al. (2022) further suggested that, in Cupressaceae, 1,6-cyclizing enzymes may have acquired 1,10- and 1,11-cyclization activities during evolution. (Liang et al., 2021; Hong et al., 2022). In this study, the clade including *CkTPS1* and *CkTPS2* has another sTPSs that can produce variate yield through different cyclization mechanisms, such as *TcTPS2* and *CovfTPS10* can respectively produce β -bisabolene and acoradiene through 1,6-cyclization, while *Cfgor* can produce (5R,6R,10S)- α -gorgonene through 1,10/1,11-cyclization (Ma et al., 2019; Hong et al., 2022; Chen et al., 2025). In this study,

similar situations had been found. *CkTPS1* and *CkTPS2* were enzymes which could produce sesquiterpene compounds through 1,7-cyclization, showed a closer relationship to 1,6-cyclization enzymes than to 1,10/1,11-cyclization enzymes, and further differentiated from 1,6-cyclization enzymes, highlighting their uniqueness. Besides, *CkTPS3* was clustered with *TcTPS1*, which represent the 1,10-cyclization and 1,6-cyclization, respectively. It probably was a hint that sTPSs in this cluster was following the pattern of evolution from small rings to large rings, and the *CkTPS3* and *TcTPS1* where are typical examples of the transition period. To sum up, we can't determine the evolutionary relationship of TPS solely based on the cyclization mechanism so far, more experiments are needed to prove the correlation between them.

On the other hand, phylogenetic tree can be analyzed with residues in active site of enzymes. Previous studies have indicated that replacing W279 or Y410, located opposite N403 and L405 at the active site, with different small amino acids can alter the product (Yoshikuni et al., 2006; Loizzi et al., 2018). Sequence alignment of four sites between the sesquiterpene synthase with 1,10-cyclization activity in *C. konishii* and related enzymes in other species revealed that *CkTPS6* and *TcTPS3* share the same residues, suggesting similar catalytic processes. Their adjacent positions on the phylogenetic tree further suggest a close evolutionary relationship, indicating they are homologous genes with the same function (Fig. 5B). However, *CkTPS5* shows a completely different residue pattern from *CkTPS6*, suggested that it use another way to synthesis δ -cadinene, and this might be the reason that it can also produce cubebol as one of the major products (Fig. 5B). The phylogenetic analysis shown that they're distributed in two clades, indicated their relationship are not close as the one between *CkTPS6* and *TcTPS3* (Fig. 5A). Another instance is *CkTPS3*, which biosynthesizes Germacrene A and possesses distinct catalytic residues compared with other enzymes that catalyze germacrene-type sesquiterpenoids formation, such as Germacrene A synthases of *C. formosensis* and *C. obtusa* var. *formosana*, with N403 as an only conserved residue, indicating a unique catalytic mechanism (Fig. 5C). This differentiation could be observed on the phylogenetic tree, *CkTPS3* and other Germacrene A synthase also belong to different clades on the phylogenetic tree, indicating that the biosynthesis of Germacrene A in Cupressaceae plants is an independently evolving event, with different plants synthesizing Germacrene A in different ways, catalyzed by different residues. Nevertheless, further site-directed mutagenesis experiments will be required to determine whether these amino acid residues are critical for enzyme function and contribute to the functional divergence observed among phylogenetically distinct enzymes.

The last but not the least, there shows the differentiation of sesquiterpene synthases within Cupressaceae, which were based on the intron number in gene structure. One of them had nine introns, which like most of gene structure of sTPSs in Pinaceae, while the other had additionally lost intron XII, made it only had eight introns. Interestingly, the clade with nine introns in Cupressaceae had differentiated first, and the clade with eight introns in Cupressaceae was clustered to the clade of Pinaceae, which was divided later. It seems to be a hint of evolution that showed Cupressaceae and Pinaceae might have different evolution way and reflect on gene structure (Fig. 5A).

Author contributions

T.W. Lin: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. J.D. Chung: Resources. S.Y. Wang: Validation. F.H. Chu: Writing – Conceptualization, review & editing, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2026.111855>.

Data availability

Data will be made available on request.

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