Kuan-Feng Huang, Yi-Ru Lee, Yen-Hsueh Tseng, Sheng-Yang Wang and Fang-Hua Chu*

Cloning and functional characterization of a monoterpene synthase gene from *Eleutherococcus trifoliatus*

Abstract: *Eleutherococcus trifoliatus* also known as the three-leaved *Eleutherococcus*, a member of the Araliaceae (ginseng) family, is commonly used in traditional Chinese medicine. Recently, many studies have demonstrated the bioactivities of the secondary metabolites in *E. trifoliatus*. In this study, a monoterpene synthase from *E. trifoliatus* has been characterized. A pair of degenerate primers was designed and a fragment with conserved region of terpene synthase (TPS) was obtained. After 5′- and 3′-rapid amplification of cDNA ends (RACE), the full-length cDNA was obtained. The gene designated *EtLIM* contains an open reading frame of 1752 bp with a predicated molecular mass of 67.3 kDa. It was expressed in young leaves, stems, and drupes. The product of *EtLIM* has been identified by gas chromatography/mass spectrometry (GC/MS) as limonene.

Keywords: Araliaceae, *Eleutherococcus trifoliatus*, limonene, monoterpene synthase

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Introduction

Terpenoids are one of the largest groups of natural products. Tens of thousands of terpenoid compounds have been identified. Accordingly, an abundance of terpene synthase (TPS) genes and enzymes can be found in plants, bacteria, and fungi (Collado et al. 2007; Kuo et al. 2012; Smanski et al. 2012; Wen et al. 2012; Ma et al. 2013). There are several major classes of terpenoids, among which the monoterpenes (C_{10}), sesquiterpenes (C_{15}), and diterpenes (C_{20}) are derived from geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), respectively. These terpene precursors are produced from a basic five-carbon unit, isopentenyl diphosphate (IPP), and its isomer dimethylallyl diphosphate (DMAPP) via the cytosolic mevalonate (MVA) pathway or the plastidial 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Terpenoid compounds are responsible for the odor, fragrance, and flavor associated with plants. They also attract pollinators and defend against herbivores. Commercially, they are widely used as natural flavor additives for food or fragrances in perfumery. In addition, some terpenes are used as medicines, for example, myrcene, which has analgesic activity, and linalool, which has anti-inflammatory activity (Gershenzon and Dudareva 2007).

Eleutherococcus trifoliatus (L.) S.Y. Hu var. *trifoliatus* (Syn. *Acanthopanax trifoliatus*) is a member of the Araliaceae (ginseng) family and is a climbing shrub with ternately compound leaves. It is a medicinal plant in China, Taiwan, Vietnam, and the Philippines and is purported to have ginseng-like activity (Lischewski et al. 1985). It is utilized in folk medicine for bruising, neuralgia, gout, and impotence (Yook et al. 1999). Many volatile compounds have been identified from the essential oil of the Araliaceae plants, most of them belonged to monoterpenes or sesquiterpenes, and showed different kinds of bioactivities (Garneau et al. 2006; Li et al. 2009; Verma et al. 2010). In order to realize the synthesis of terpene in *E. trifoliatus*, we designed pairs of degenerate primers to obtain the monoterpene synthase gene in this study.

Materials and methods

Plant materials: The plants were collected from a field on Chiufenershan Mountain in Nantou County, Taiwan, and identified by Professor Yen-Hsueh Tseng. The plants were incubated in a greenhouse at 25°C with natural light before the experimentation. The plant tissue was harvested and frozen immediately in liquid nitrogen and then stored at -80°C until RNA preparation.

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RNA isolation: Fresh plant tissue was weighed and ground into fine powder in liquid nitrogen. The total RNA extraction followed the protocol provided with the Plant Total RNA Miniprep Purification Kit (Hopegen). Two micrograms of total RNA were used for reverse transcription (RT) using SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Isolation of partial and full-length TPS cDNA: To isolate the conserved region of the monoterpene synthase, we designed a pair of degenerate primers. Then, the rapid amplification of cDNA ends (3′- and 5′-RACE system; Invitrogen) technique was applied to obtain the full-length cDNA sequence. The full-length open reading frame was amplified by Phusion Hot-Start High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) with specific primers 5′-ATGTTTTTTTCACCTCTCACAGTCTCTC-3′/5′-CTAGTCGAGGGTAAA GGGTTCGACCAG-3′. The polymerase chain reaction (PCR) program was designed as follows: an initial denaturing step at 98°C for 30 s followed by 35 cycles of 98°C for 15 s, 60°C for 20 s, 72°C for 1 min 30 s, and finally 72°C for 7 min. After gel electrophoresis analysis, the PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) following the standard procedure and transformed into *Escherichia coli* strain DH5α (Lee et al. 2006).

The full-length cDNA was sequenced, named as *EtLIM* according to the result of gas chromatography/mass spectrometry (GC/MS) analysis afterward. The deduced amino acid sequence was aligned with other TPS genes from GenBank and neighbor-joining analysis was used to construct a phylogenetic tree. The alignment was created by MEGA 4.1 software (Tamura et al. 2007) and edited with GeneDoc software (Nicholas et al. 1997).

Molecular modeling: The 3D structure of *EtLIM* was predicted by SWISS-MODEL (http://swissmodel.expasy.org/; Arnold et al. 2006). The protein structure was constructed with the identified crystal structure of (4S)-limonene synthase from *Mentha spicata* (PDB code: 2ONGA; Hyatt et al. 2007) as a template. The tertiary structure and interaction prediction was viewed and edited using the visualization system UCSF Chimera (Pettersen et al. 2004).

Protein expression and purification: Plastid targeting signal peptides are known to influence the expression of most monoterpene synthases in vitro. We therefore predicted it by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) in advance. The results suggested that *EtLIM* does not have a plastidial signal peptide. The full-length *EtLIM* full-length gene was fused to a self-cleavable intein tag with the bacterial expression vector pTYB12 with the IMPACT-CN protein fusion and purification system (New England Biolabs, Ipswich, MA, USA) by different cloning sites (*Nde*I and *Xho*I). The resultant construct was expressed in *E. coli* BL21 (DE3; Novagen, Madison, WI, USA). The transformed *E. coli* cells were cultured overnight at 37°C in Luria-Bertani medium and induced with 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) at 16°C for 20 h. Soluble fusion proteins were purified by a chitin-bead resin (New England Biolabs). The intein underwent specific self-cleavage when 1 mM dithiothreitol (DTT) was added for 20 h at 16°C. The soluble protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein concentration was determined with a Bio-Rad assay kit (Bio-Rad, Hercules, CA, USA).

Functional characterization of EtLIM: The activity of *EtLIM* was measured in vitro GC/MS analysis as described by Chang and Chu (2011) with slight modifications. The purified protein (20 μg) was added to monoterpene synthase buffer [25 mM HEPES (pH 7.2), 100 mM KCl, 10 mM $MgCl_2$, 10% (v/v) glycerol, 5 mM DTT] with 30 μM GPP (Sigma). The reaction mixture was then covered with 1 ml pentane to trap volatile products and incubated at 30°C for 1 h. The pentane extracts were combined and passed through a silica gel column (3 cm=5 mm i.d.). After the pentane extract was obtained, it was analyzed by an ion trap GC/MS system (Polaris Q Ion trap/Trace GC system; Thermo Finnigan, San Jose, CA, USA) with a DB-5 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The oven temperature was held at 60°C for 1 min, then increased from 60°C to 130°C at a rate of 5°C min^{-1}, and then increased from 130°C to 290°C and held for 5 min. Injector temperature was 250°C; ion source temperature was 230°C; EI was 70 eV; flow rate of the carrier gas He was 1 ml min^{-1}; split ratio 1:10; mass range 50–400 m/z. The structure of the major compounds was confirmed by a comparison with the mass spectral fragmentation based on the Wiley/NBS Registry of Mass Spectral Data and a NIST Mass Search. Kovats retention index (KI) (Kovats 1965), which is a parameter calculated in reference to n-alkanes that convert retention times into system-independent constants, was also confirmed.

Detection of EtLIM transcript in different tissues: The transcript of *EtLIM* was detected in different tissues by RT-PCR. One microgram of total RNA from each tissue was isolated. SuperScript™ III reverse transcriptase (Invitrogen) and oligo(dT) primer (Invitrogen) was used according to the manufacturer's protocol to obtain cDNA. To amplify *EtLIM*, forward and reverse primers were applied: 5′-AAAGACAAT-GTGATCTCATTACT-3′ and 5′-TTACGATTTGGATAGGTTTGTTAT-3′. The forward primer was 22 bp upstream from the stop codon and the reverse primer was located at 3′-untranslated region (3′-UTR), 104 bp downstream from the stop codon. cDNA from the stems, drupes, young leaves, and old leaves was prepared as previously described. Because the seeds could not be completely removed, whole drupes were used. The PCR conditions were as follows: 2 min denaturation at 94°C followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, and then 72°C for 7 min. An actin gene was amplified simultaneously as a control with the following forward and reverse primers: 5′-CATGTATGTTGCTATCCAGGCTGTG-3′ and 5′-GTTGTTTCGTGAATTC-CAGCAGATTC-3′.

Results and discussion

Cloning and identification of *EtLIM*

A fragment that exhibited sequence similarity to known monoterpene synthase genes was obtained by means of degenerate primers. RACE was applied to isolate the 5′ and 3′ ends of the gene. The full-length cDNA of the putative monoterpene synthase was obtained. This gene has an open reading frame containing 1752 bp and encodes for 583 amino acids; before the start codon, there is a 5′-UTR of 11 bp, and after the stop codon, there is a 3′-UTR of 175 bp (GenBank accession no. KJ126717). *EtLIM* has a predicted molecular weight of 67.26 kDa and its theoretical isoelectric point is 5.55. The amino acid sequence of *EtLIM* shows a high degree of sequence similarity with a linalool synthase (Bc-LIS; GenBank accession no. BAG82825) from

lemon myrtle (*Backhousia citriodora*) and a β-bisabolene (Sa-BIS; GenBank accession no. ADO87003) synthase from *Santalum austrocaledonicum* (56% and 54%, respectively; Table 1).

The *EtLIM* amino acid sequence contained the common TPS family motifs, RR(x)8W (R^{48}RSANYPPSFW^{58}), RxR ($R^{298}DR^{300}$), DDxxD ($D^{335}DIYD^{339}$), and NSE/DTE (N^{479}DLGTSSGE^{487}; Figure 1). RR(x)8W is a highly conserved TPS motif that may influence TPS cyclization (Williams et al. 1998); the aspartate-rich motif DDxxD is another highly conserved motif. In addition, DDxxD and NSE/DTE are typically included in most TPS and are the important activity sites in the TPS. DDxxD and NSE/DTE are associated with metal ion (e.g., Mg^{2+} and Mn^{2+}) binding. Mutation of DDxxD may even result in the loss of all TPS activity (Seemann et al. 2002; Köksal et al. 2010). The RxR motif has also been reported to be a conserved TPS deduced amino acid sequence that is involved in terpenoid catalysis (Aubourg et al. 2002).

Protein structure predictions

The protein structure of *EtLIM* was predicted by SWISS-MODEL with a (4S)-limonene synthase from *M. spicata* (PDB code: 2ONGA; Hyatt et al. 2007) as a template (Figure 2a). This enzyme was selected as a template because it has the highest sequence similarity (44.6%) with *EtLIM* in the SWISS-MODEL database. As seen in Figure 2b, the $D^{335}DIYD^{339}$ motif and N^{479}DLGTSSGE^{487} motif are just located around the metal ion Mg^{2+} and substrate, and the side chains all point to the active site. Based on the work of Köllner et al. (2006), the side chains of these two motifs may affect the secondary structure of the protein. A change of protein structure may lead to a change in product specificity or the increase/decrease of protein activity.

Functional characterization

According to the results from NCBI Protein BLAST, *EtLIM* is predicted to function as a monoterpene or sesquiterpene synthase (Table 1). To determine the function of this gene, the *EtLIM* full-length cDNA was subcloned into the pTYB12 expression vector, and the encoded proteins were then expressed in *E. coli* BL21 (DE3). The yield of *EtLIM* recombination protein that was cultured in Luria-Bertani medium to O.D. ratio 0.5 is 30 mg/l. The protein was then extracted and analyzed by SDS-PAGE (Appendix Figure 1), and the molecular weight of *EtLIM* is just as we have predicted previously. Then, we assayed the protein for in vitro enzyme activity. The activity of the full-length recombinant *EtLIM* protein was tested with the monoterpene substrates GPP and sesquiterpene substrates FPP. After reaction at 30°C for 1 h, the pentane extract was analyzed by GC/MS to detect the product. *EtLIM* was active with GPP but not with FPP. The major product of *EtLIM* was identified as limonene (Figure 3), based on comparison of the retention time, KI value, and mass spectra from a study that investigated the composition of the essential oil extract from *Acanthopanax brachypus* (Hu et al. 2009).

Table 1 Similarity and identity analysis of *EtLIM* with other known TPS.

Enzyme	Species	Accession number	Similarity
Monoterpene			
Linalool synthase	*B. citriodora*	BAG82825	56%
(E)-β-ocimene/myrcene synthase	*Vitis vinifera*	ADR74206	51%
α-Thujene/sabinene synthase	*Litsea cubeba*	AEJ91556	51%
α-Thujene synthase	*L. cubeba*	AEJ91555	50%
Linalool/myrcene synthase	*Coffea arabica*	CCM43929	49%
Pinene synthase	*Quercus ilex*	CAK55186	48%
α-Phellandrene synthase	*V. vinifera*	ADR74201	46%
Limonene synthase	*C. arabica*	CCM43927	46%
β-Ocimene synthase	*Phaseolus lunatus*	ABY65110	46%
(E)-β-ocimene synthase	*Malus domestica*	AGB14628	46%
γ-Terpinene synthase	*Citrus unshiu*	BAD27259	45%
Sesquiterpene			
β-Bisabolene synthase	*S. austrocaledonicum*	ADO87003	54%
β-Bisabolene synthase	*Santalum album*	ADP37189	53%
9-Epi-caryophyllene synthase	*Lavandula* x *intermedia*	AGU13712	47%

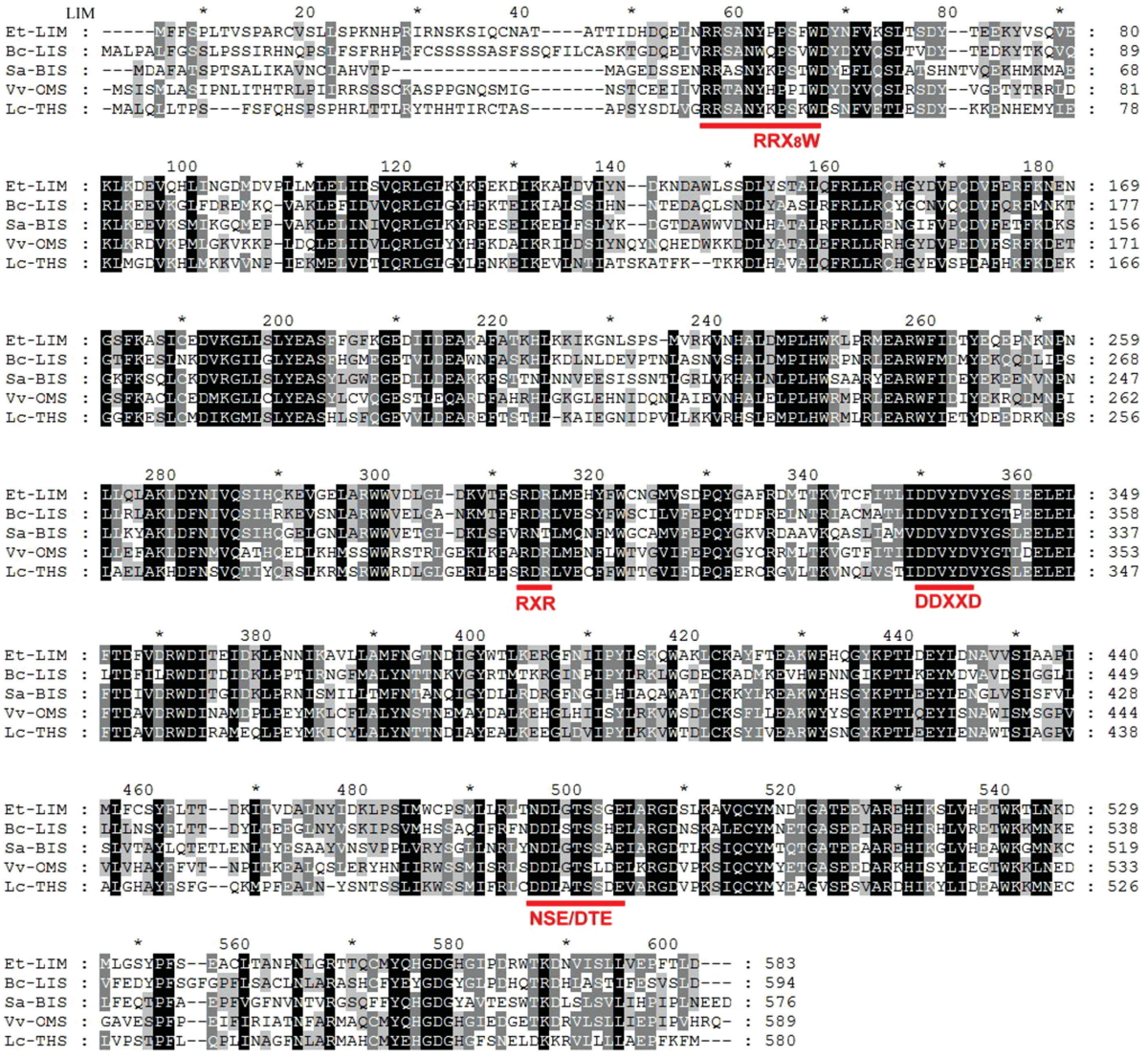

Figure 1 Alignment of amino acid sequence of *EtLIM* with four functionally characterized monoterpenes.
BcLIS is a linalool synthase from *B. citriodora* (BAG82825), *SaBIS* is a β-bisabolene synthase from *S. austrocaledonicum* (ADO87003), *VvOMS* is a (E)-β-ocimene/myrcene synthase from *V. vinifera* (ADR74206), and *LcTHS* is an α-thujene/sabinene synthase from *L. cubeba* (AEJ91556). RR(x)8W, RxR, DDxxD, and NSE/DTE motifs are shown. Black shading represents 100% conserved similarity, dark gray represents 70%, and light gray represents 60%.

Limonene is a monoterpene that is widely distributed in plants including other Araliaceae plants (Richter et al. 2007; Setzer et al. 2008). It is most well known as the major compound in citrus peel oil (Shaw 1979), and many other plants, both angiosperms and gymnosperms, contain limonene (Savage et al. 1995). Limonene is the most odor-active volatile in lemon and other citrus fruits, showing several tens of times more activity than the other volatiles in many plant tissues including leaves, peel, and pulp (Arena et al. 2006). It is the main odor constituent of citrus in cosmetic products and cleaners and is also a common flavoring in food manufacturing (Hener et al. 1990). Because of its anti-insect activities, it is also used as a botanical insecticide (Hebeish et al. 2008). In traditional folk medicine systems, limonene is usually used to relieve gastrointestinal disorders such as gastroesophageal reflux disease and heartburn (Werbach 2008). Recently, Miller et al. (2013) showed that limonene might have the potential to cure breast cancer.

Phylogenetic analysis

Based on sequence similarity, as well as by functional assessment, the TPS family in plants has been classified into different subfamilies (Bohlmann et al. 1998). The most detailed classification system divides the TPS family to eight

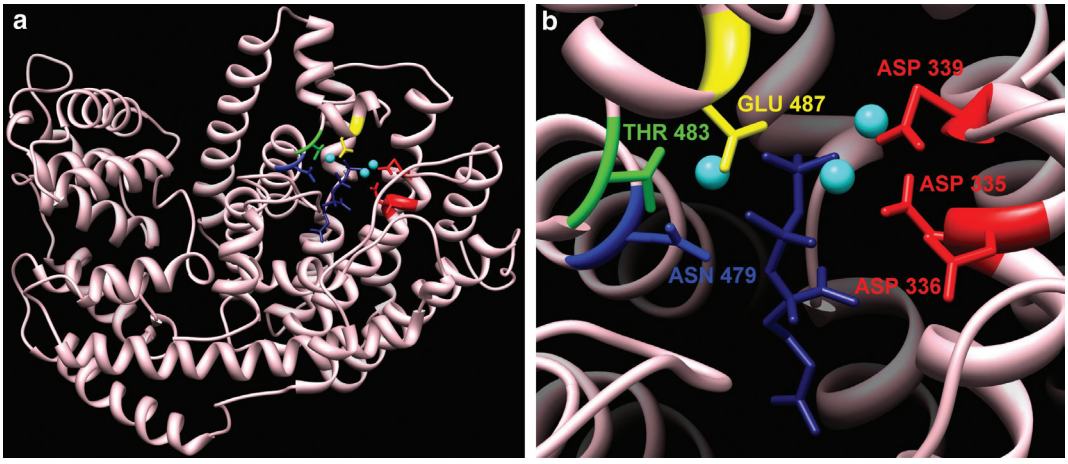

Figure 2 Protein structure prediction and the active site of *EtLIM*.
(a) Whole *EtLIM* structure with the active site in the center in which the substrate FGPP is catalyzed (navy blue color). (b) Relative positions of substrate-binding residue $D^{335}D^{336}IYD^{339}$ and $N^{479}DLGT^{483}SSGE^{487}$ (red: aspartic acid; yellow: glutamic acid; green: threonine; blue: asparagine). Mg^{2+} ions are shown as small cyan spheres and the FGPP is shown in navy blue.

subfamilies TPS-a to TPS-h (Chen et al. 2011). A phylogenetic tree was constructed with the *EtLIM* amino acid sequence as shown in Figure 4. *EtLIM* appears in the cluster with the TPS-b subfamily, which is specific to flowering plants. All the characterized TPS contained in the TPS-b subfamily are monoterpene synthases or isoprene synthases.

A comparison of the amino acid sequences similarity among the limonene synthases from different plants (Table 2 and Figure 5) revealed that *EtLIM* is obviously closer to angiosperm limonene synthases. The similarity of *EtLIM* and currently characterized angiosperm limonene synthases is 40–46%, in comparison with only 33% similarity

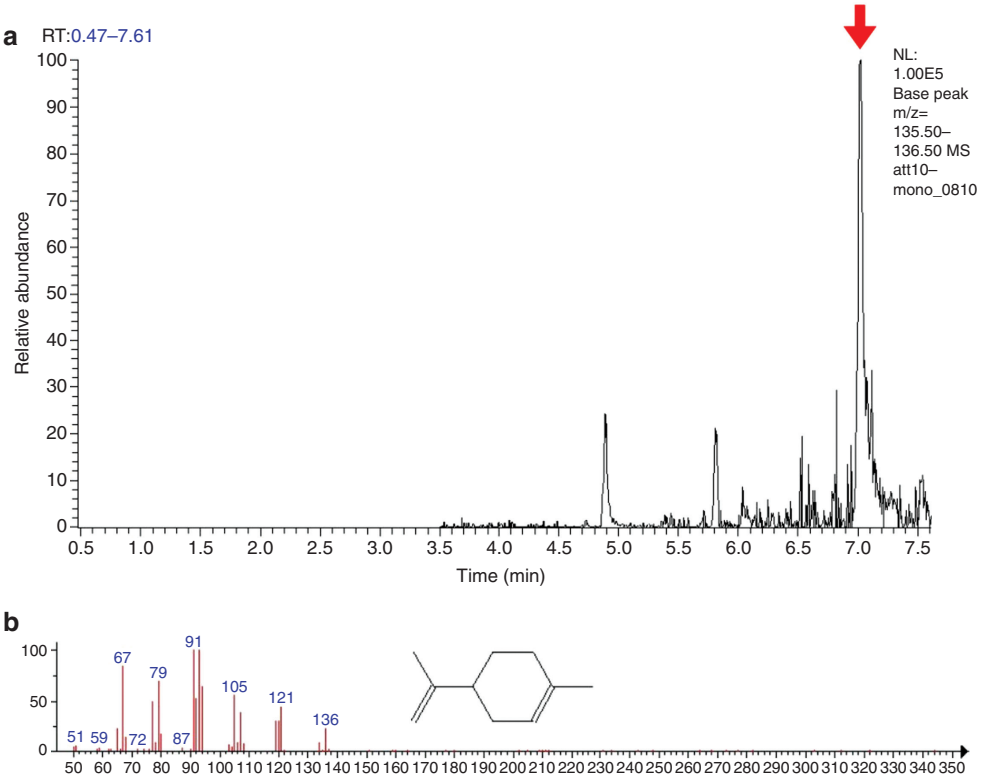

Figure 3 (a) GC/MS analysis of monoterpene products of recombinant protein product of *EtLIM* with GPP as the substrate. The red arrow point to the peak represents the product of the *in vitro* enzyme activity. (b) MS of the product of *EtLIM* is limonene.

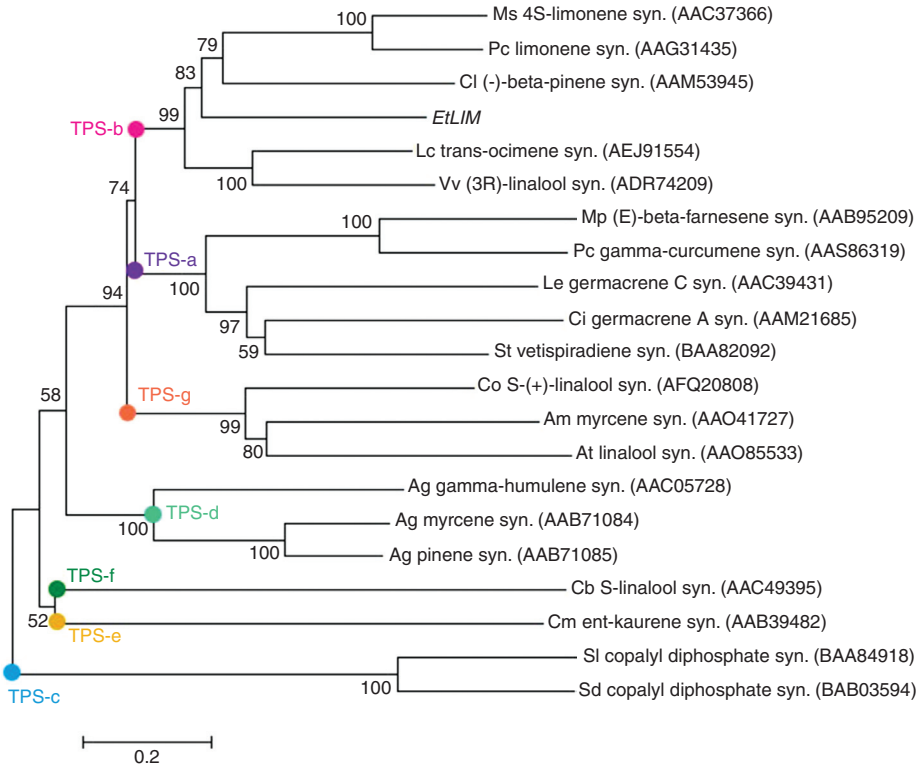

Figure 4 Phylogenetic tree of *EtLIM* with other known TPS amino acid sequences analyzed by the neighbor-joining method. TPS subfamilies are classified TPS-a to TPS-g based on Bohlmann et al. (1998). The GenBank accession numbers used in this analysis are shown in Appendix Table 1.

with the gymnosperm limonene synthases. According to the features of the amino acid sequences, the gymnosperm monoterpene synthases are classified to the TPS d-1 subfamily, not the TPS-b subfamily. Based on Table 2, we also found that the *EtLIM* sequence did not closely resemble any currently characterized angiosperm limonene. The composition of the *EtLIM* sequence seems to be positioned between known angiosperm and gymnosperm limonene synthases.

Expression of *EtLIM* in different tissues

RT-PCR was performed to examine the expression of *EtLIM* in different tissues of *E. trifoliatus*, including the stems, drupes, young leaves, and old leaves. The stem of *E. trifoliatus* showed a high level of expression of *EtLIM*. The expression in drupes and young leaves was lower than in stem, and the old leaves showed a low level of expression

Table 2 Pairwise percentage identity of deduced amino acid sequences of *EtLIM* with limonene synthases from other species.

	EtLIM	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**	**(7)**	**(8)**	**(9)**	**(10)**	**(11)**
Ca limonene sy. (1)	46%											
Cs limonene sy. (2)	45%	49%										
Cl limonene sy. (3)	43%	46%	46%									
Pt limonene sy. (4)	43%	47%	46%	85%								
Pf limonene sy. (5)	43%	49%	46%	44%	42%							
Ml limonene sy. (6)	43%	48%	44%	45%	44%	68%						
Ms limonene sy. (7)	42%	47%	42%	43%	42%	65%	98%					
Ro limonene sy. (8)	42%	51%	45%	47%	46%	53%	51%	51%				
Ts limonene sy. (9)	42%	49%	45%	44%	45%	42%	41%	40%	43%			
La limonene sy. (10)	40%	46%	43%	45%	43%	50%	51%	51%	51%	42%		
Pa limonene sy. (11)	33%	32%	31%	33%	34%	32%	31%	31%	31%	31%	30%	
Ps limonene sy. (12)	33%	32%	31%	33%	34%	31%	31%	31%	31%	31%	30%	98%

The GenBank accession numbers used in this analysis are listed in Appendix Table 1.

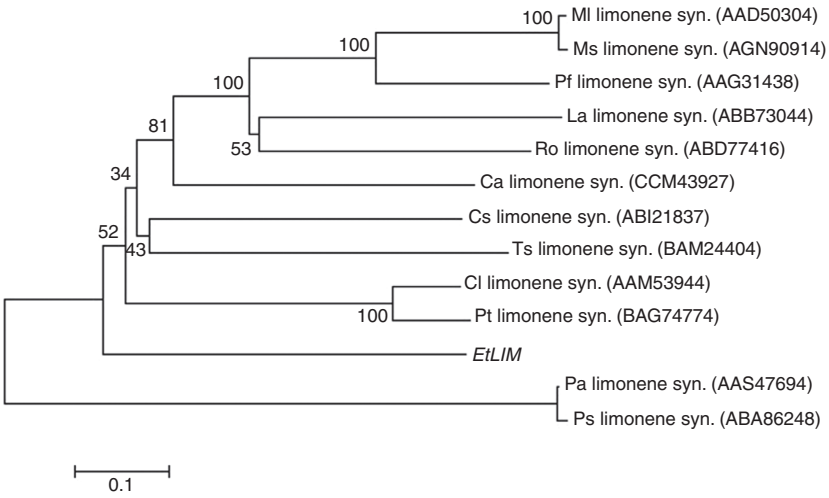

Figure 5 Phylogenetic tree of *EtLIM* with other known limonene synthases amino acid sequences.
The GenBank accession numbers used in this analysis are listed in Appendix Table 2.

of *EtLIM* (Figure 6). Traditionally, the stem of *E. trifoliatus* is used to cure stomach pain and limonene capsules are available commercially for many gastrointestinal diseases (Ghosh and Playford 2003). Based on our current study, we suggest that such activity may be associated with the limonene content in the stem of *E. trifoliatus*.

Conclusion

In this study, we isolated a monoterpene synthase from *E. trifoliatus*, a traditional medicinal herb with ginseng-like activity. To obtain the monoterpene synthase gene, pairs of degenerate primers were designed and then used in PCR; fragments of TPS were obtained including the conserved region. To obtain the full-length cDNA, the 5′- and 3′-end was obtained by means of 5′- and 3′-RACE. Finally, a full-length monoterpene synthase sequence was obtained from the leaves of *E. trifoliatus*. To identify the function of this monoterpene synthase, recombinant proteins from *E. coli* were reacted with GPP and FPP, the substrates of monoterpenes and sesquiterpenes, respectively. GC/MS analysis revealed that the major product of this TPS is limonene, so we designated this TPS *EtLIM* (GenBank accession no. KJ126717).

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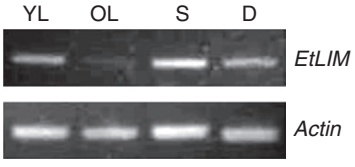
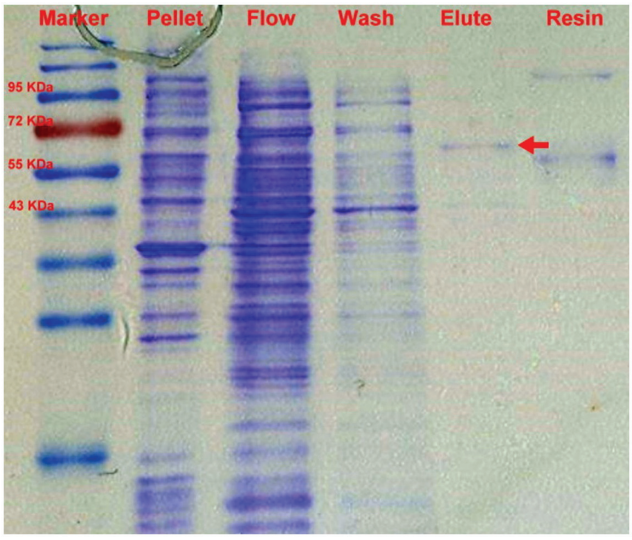


Figure 6 Expression of *EtLIM* in different tissues. RT-PCR analysis of *EtLIM* expression using the constitutive gene actin as an internal control.
Total RNA was extracted from young leaves (YL), old leaves (OL), stems (S), and drupes (D).

Appendix



Appendix Figure 1 The SDS-PAGE analyz of *EtTPS* recombination protein, the red arrow point to the band represent the *EtTPS* recombination protein product.

Appendix Table 1

Abbreviation	Species	Assension No.
Am myrcene syn.	*Antirrhinum majus*	AAO41727
Ag gamma-humulene syn.	*Abies grandis*	AAC05728
Ag myrcene syn.	*Abies grandis*	AAB71084
Ag pinene syn.	*Abies grandis*	AAB71085
At linalool syn.	*Arabidopsis thaliana*	AAO85533
Cb S-linalool syn.	*Clarkia breweri*	AAC49395
Cl (-)-beta-pinene syn.	*Citrus limon*	AAM53945
Ci germacrene A syn.	*Cichorium intybus*	AAM21658
Cm ent-kaurene syn.	*Cucurbita maxima*	AAB39482
Co S-(+)-linalool syn.	*Cinnamomum osmophloeum*	AFQ20808
Lc *trans*-ocimene syn.	*Litsea cubeba*	AEJ91554
Le germacrene C	*Lycopersicon esculentum*	AAC39431
Mp (E)-beta-farnesene syn	*Mentha* x *piperita*	AAB95209
Ms 4S-limonene syn.	*Mentha spicata*	AAC37366
Pc gamma-curcumene syn.	*Pogostemon cablin*	AAS86319
Pc limonene syn.	*Perilla citriodora*	AAG31435
Sd copalyl diphosphate syn.	*Scoparia dulcis*	BAB03594
Sl copalyl diphosphate syn.	*Solanum lycopersicum*	BAA84918
St vetispiradiene syn.	*Solanum tuberosum*	BAA82092
Vv (3R)-linalool syn.	*Vitis vinifera*	ADR74209

Appendix Table 2

Species	Accession no.
Coffea arabica	CCM43927
Cannabis sativa	ABI21837
Citrus limon	AAM53944
Poncirus trifoliate	BAG74774
Perilla frutescens	AAG31438
Mentha longifolia	AAD50304
Mentha spicata	AGN90914
Rosmarinus officinalis	ABD77416
Toona sinensis	BAM24404
Lavandula angustifolia	ABB73044
Picea abies	AAS47694
Picea sitchensis	ABA86248

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