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Identification and functional characterization of a sesquiterpene synthase gene from *Eleutherococcus trifoliatus*

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Abstract

In the present study, one sesquiterpene synthase gene in *Eleutherococcus trifoliatus* was identified and characterized. Full-length cDNA was obtained from stems. It contained an open reading frame of 1671 bp (*EtCop*) with a predicted molecular mass of 64.5 kDa. The amino acid sequence of *EtCop* contained the common terpene synthase family motifs $RR(x)_8W$, RxR and DDxxD. The recombinant protein from *Escherichia coli* was incubated with farnesyl diphosphate in order to identify the function of *EtCop*. The product of *EtCop* could be identified as an α-copaene by means of gas chromatography-mass spectrometry analysis and comparison with an authentic standard.

Keywords: Araliceae; *Eleutherococcus trifoliatus*; sesquiterpene synthase.

Introduction

The chemistry of wood and bark in the context of the utilization of terpenes and terpenoids has a long history, mostly in the context of utilization of pine terpenoids as by products of kraft pulping (Fengel and Wegener 1989). In recent years, terpene release from wood and wood composites as volatile organic components (VOCs) has been frequently discussed in the context of environmental protection (Banerjee 2001; Radhakrishnan and Banerjee 2004; Makowski and Ohlmeyer 2006a,b). The potential use of terpenes against wood decay fungi (Wu et al. 2005) has also been investigated. Terpenes have antioxidant activities (Wang et al. 2002); cloning and characterization of α-pinene synthase from *Chamaecyparis formosensis* has been described by Chu et al. (2009).

Over the past few decades, much attention has been paid to plant secondary metabolites, of which terpenoids are the most abundant and structurally most diverse group. A wide range of biological functions have been identified for terpenoids including the mediation of antagonistic and beneficial interactions among organisms (Gershenzon and Dudareva 2007), biosynthesis of hormones, protection against ultraviolet radiation and photo-oxidative stress, thermal protection, pollinator attraction, membrane stabilization, and resistance against insects and microorganisms (Steele et al. 1998; Trapp and Croteau 2001; Copolovici et al. 2005; Baldwin et al. 2006; Keeling and Bohlmann 2006; Dornelas and Mazzafera 2007). They often function as phytoalexins in plant direct defense or as signals in the indirect defense response (Cheng et al. 2007). Terpenoids have been extensively utilized in pharmaceuticals, nutraceuticals, flavorings, fragrances, cosmetics, colorants, the food industry and as agrochemicals (Harada and Misawa 2009), and, because of their diverse functions, they have become a group with a considerable commercial value (Yu and Utsumi 2009).

The terpenoids of the three-leaved eleuthero [*Eleutherococcus trifoliatus* (L.) S.Y. Hu var. *trifoliatus*] (Syn. *Acanthopanax trifoliatus*) are remarkable for their pharmaceutical effects. The plant belongs to the Araliaceae family (Ohashi 1993; Kim and Sun 2004; Hu 2005), the species of which are deciduous shrubs or climbers with prickles on branches and petioles; their leaves are generally three-foliolate, and the drupes become black when mature. They are widely distributed from the Himalayas to China, India, Taiwan and the Philippines (Ohashi 1993; Kuo and Gao 2000; Wiart 2006). The roots, bark and leaves of this plant are used by practitioners of traditional Chinese medicine for many complaints, including prevention or amelioration of tumors and aging, and for improving cardiovascular function. They have been suggested to have antioxidant properties (Wiart 2006; Hsü 2007). One study, based on an animal model, claimed that three-leaved eleuthero may be effective against tumors (Chen 2007). In Thailand, for example, it is a traditional mosquito repellent and its essential oil was confirmed to have insect repellant properties (Tawatsin et al. 2006).

Only a few studies have focused on the origin of the three-leaved eleuthero's efficacy. Because of its fragrance, more and more studies focused on its terpenoids (Phuong et al. 2006) and triterpenes (Ty et al. 1984; Lischewski et al. 1985; Ty et al. 1985; Yook et al. 1999; Kiem et al. 2003). In the present study, one sesquiterpene synthase gene in *E. trifoliatus* was identified and characterized. The aim was to understand the biosynthetic mechanisms of this plant and to investigate the possibility of controlling the levels of terpenoid production during different periods of plant growth.

Materials and methods

Plant materials, RNA isolation, and cDNA synthesis

Eight individual plants (5 years old) were collected from the Chiufenershan mountain of Nantou County, Taiwan, in July 2009 and identified by Professor Yen-Hsueh Tseng. The plants were incubated under greenhouse conditions at 25°C with natural light before investigation. The plant tissue was harvested and flash frozen in liquid nitrogen. The tissues were stored at -80°C prior to RNA isolation.

For total RNA isolation, fresh tissue was weighed and ground to a fine powder under liquid nitrogen, <100 mg per sample, then 450 μl of Plant RNA Lysis Solution B/2-Mercaptoethanol (Plant Total RNA Miniprep Purification Kit, Hopegen, Taichung, Taiwan) was added. The subsequent steps were carried out according to the manufacturer's manual. First, a Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) was used to synthesize cDNA with total RNA.

Construction and identification of full-length cDNA clones

Degenerate primers were designed based on sesquiterpene synthase sequences from *Magnolia grandiflora*, *Populus balsamifera* subsp. *trichocarpa* × *Populus deltoides* and *Actinidia deliciosa* available in GenBank (Accession numbers EU366429, AAR99061 and AY789791). The forward primer was 5′-CAACGCYTWGGYRTVKCYTACCAYTTTG-3′ and the reverse was 5′-GTACCRTAKACRTCRTAGRTGTCATC-3′. Rapid amplification of cDNA ends (3′-RACE and 5′-RACE) (Invitrogen) was then carried out to obtain the full-length cDNA sequence. There was only one kind of sequence obtained from the amplification by means of these degenerated primers. The complete open reading frame was amplified by Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) with specific primers 5′-ATGGCCACGTATCTTCAGGCTTCATC-3′ (forward) and 5′-TTATATTTGAACTGGATCTATGAGCACGC-3′ (reverse). The polymerase chain reaction (PCR) program was as follows: an initial denaturing step at 98°C for 30 s, followed by 35 cycles of 98°C for 15 s, 58°C for 20 s, 72°C for 1 min 30 s and then 72°C for 7 min. The PCR product was then cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) following standard procedure and transformed into *E. coli* strain DH5α (Lee et al. 2006). The full-length of *EtCop* was then sequenced.

The deduced amino acid sequence of *EtCop* was aligned with other sesquiterpene synthase 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). The 3D-structure of the protein was predicted by SWISS-MODEL (http://swissmodel.expasy.org; Arnold et al. 2006).

Detection of *EtCop* transcript in different tissues

The transcript of *EtCop* in different tissues was detected by reverse transcription (RT)-PCR. To amplify *EtCop*, the forward and reverse primers were applied: 5′-CAGGAAGACCATCATACCGTGTTCAC-3′ and 5′-CATCCTTAATTATATTTGAACTGGATCTATGAGCAC-3′. The forward primer was 581 bp upstream from the stop codon and the reverse primer included the stop codon and a 9-bp three prime untranslated region (3′UTR). cDNA from the roots, stems, drupes and young and old leaves was prepared as previously described. As 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 forward and reverse primers: 5′-CATGTATGTTGCTATCCAGGCTGTG-3′ and 5′-GTTGTTTCGTGAATTCCAGCAGATTC-3′.

Heterologous expression of *EtCop* in *E. coli*

The N-terminal of the full-length *EtCop* 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). For cloning into pTYB12, the 5′-specific primers were designed with the *Nde*I restriction endonuclease site immediately upstream of the start methionine codon of *EtCop*. The 3′-specific primer was designed with a *Xho*I restriction site immediately downstream of the stop codon. The specific primers were: 5′-GGAATTCCATATGATGGCCACGTATCTTCAGGCTTCATC-3′ for forward and 5′-CCGCTCGAGTTATATTTGAACTGGATCTATGAGCACCG-3′ for reverse. The PCR product was digested with the restriction enzyme described above and inserted into the vector pTYB12. After transformation, the DH5α cells were incubated overnight on a Luria-Bertani (LB) plate with ampicillin (100 μg ml^{-1}). To ensure that the insertion was successful, the plasmids were extracted and the insert was sequenced.

The expression of *EtCop* was examined in *E. coli* strain BL21 (DE3). Recombinant *E. coli* cells were grown in 5 ml LB with carbenicillin (50 μg ml^{-1}) at 37°C overnight and refreshed in 500 ml LB with carbenicillin (50 μg ml^{-1}) for 2.5 h to an OD_{600} of 0.6. Then, 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and incubated at 20°C for 20 h. Following that, the cells were centrifuged at 4°C, 8000 rpm. The cells were resuspended in 25 ml column buffer (with TritonX) (IMPACT™-CN, New England Biolabs) with proteinase inhibitor and disrupted by sonication. To collect the soluble protein, the cells were centrifuged (4°C, 8500 rpm) to clear the cell lysate; the remaining steps were carried out as described in the instruction manual (IMPACT™-CN, New England Biolabs). The soluble protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme characterization

For determination of the function of the protein, an assay was done in volumes of 1 ml with 34 μl protein sample (0.106 μg μl^{-1}) in sesqui-TPS buffer (25 mM HEPES, pH 7.3, 10 mM $MgCl_2$, 10% glycerol, 10 mM dithiothreitol; Martin et al. 2004) and 50 μm farnesyl diphosphate (FPP; Sigma, St Louis, MO, USA), overlaid with 1 ml pentane at 30°C for 1 h. The product was extracted with 1 ml pentane (Steele et al. 1998) three times and passed through silica columns overlaid with ethyl acetate (EA). Pentane extract was concentrated under nitrogen and stored at -20°C and then analyzed by gas chromatography-mass spectrometry (GC-MS).

Identification of the product by solid-phase microextraction and GC-MS

The pentane extract obtained, as explained above, was analyzed by a Trace GC PoLaris Q mass system (Thermo Finnigan, San Jose, CA, USA) with a TR-5MS SQC column (30 m×0.25 mm×0.25 μm, Agilent Technologies, Santa Clara, CA, USA). Before analysis, the pentane extract was incubated at 60°C for 30 min after which solid-phase microextraction (SPME) was performed. Conditions: Carboxen™/polydimethylsiloxane, CAR/PMDS, 75 μm; (Supelco, Bellefonte, PA, USA); adsorption for 30 min, followed by desorption at GC-MS injector port for 1 min. The sample was then injected at an injector port temperature of

280°C; helium (constant flow rate of 1 ml min^{-1}) was used as the carrier gas. The temperature program was started at 60°C, followed by an increase of 5°C min^{-1} to 200°C, then 15°C min^{-1} to 300°C and held at 300°C for 10 min.

Results

Cloning of *EtCop*

EtCop (GenBank accession GU987104) has an open reading frame containing 1671 bp and codes for 556 amino acids; before the start codon there is a 5′-UTR of 94 bp and after the stop codon there is a 3′-UTR of 183 bp. *EtCop* has a predicted molecular weight of 64.5 kDa and its theoretical isoelectric point is 5.76. To predict the function of *EtCop*, the sequence was blasted by blastx (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). It was predicted to be a sesquiterpene synthase gene. The amino-acid sequence of *EtCop* shows a high degree of sequence similarity with a grapevine (*Vitis vinfera* L.) (-)-germacrene D synthase (*VvGerD*) (GenBank accession AAS66357) and a (+)-δ-cadinene synthase (*cdn*1-C4) (GenBank accession AAX44034) from upland cotton (*Gossypium hirsutum* L.) (63% and 51%, respectively) (Figure 1).

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                *        20         *        40         *        60         *        80
EtCop   : -MATYLQASSGPCSTIVPEITRRSANYHPNIWGDQFLKYNSFDLSKTHANTKEHFRQLKEEVK-KMLVDAAPN--QQLNLIDD :  79
VvGerD  : MSVQSSGVLLAPSKNLSPEVGRRCANFHPSIWGDHFLSYAS-EFTNTDDHLKQHVQQLKEEVR-KMLMAADDDSAQKLLLIDA :  81
cdn1-C4 : MAKQVSQLLSSSPLTSNKDEMRLKADFQPSIWGDLFLTCLD---NDIDAETEQRHQQLEGGVR-KMIVAPMANSTQKLTFIDS :  79
DCS     : MASQVSQMPSSSPLSSNKDEMRPKADFQPSIWGDLFLNCPD---KNIDAETEKRHQQLKEEVR-KMIVAPMANSTQKLAFIDS :  79
VvVal   : ---MSTQVSASSLAQIPQPKNRPVANFHPNIWGDQFITYTP-EDKVTRACKEEQIEDLKKEVKRKLTAAAVANPSQLLNFIDA :  79
                                  RR(x)8W

              *       100        *       120        *       140        *       160
EtCop   : IQRLGVAYQFEAEIDAALQRMN-VIFQGNDDD--LHTISLRFRLLRQHGYNVSSDVFRKFMDNNGKFKECLISDLRGVLSLYE : 159
VvGerD  : IQRLGVAYHFESEIDEVLKHMFDGSVVSAEED--VYTASLRFRLLRQQGYHVSCDLFNNFKDNEGNFKESLSSDVRGMLSLYE : 162
cdn1-C4 : VQRLGVSYRFTKEIEDELENIY---HNNNDAENDLYTTSLRFRLLREHGFNVSCEVFNKFKDEQGDFKSSLTSDVRGLLELYE : 159
DCS     : VQRLGVSYHFTKEIEDELENIY---HNNNDAENDLYTTSIRFRLLREHGYNVSCDVFNKFKDEQGNFKSSVTSDVRGLLELYQ : 159
VvVal   : VQRLGVAYHFEQEIEEALQHICNSFHDCNDMDGDLYNIALGFRLLRQQGYTISCDIFNKFTDERGRFKEALISDVRGMLGLYE : 162

                 *       180        *       200        *       220        *       240
EtCop   : ATHFRVHGEDILEGALEFTTSHLERLKSHLKNPLAAQVIRALKCPIHKGLNRLEAKHYISIYQQEDDSHNKVLLNFAKLDFNL : 242
VvGerD  : ATHFRVHGEDILDEALAFTTTHLQSATKHSSNPLAEQVVHALKQPIRKGLPRLEARHYFSVY-QADDSHNKALLKLAKLDFNL : 244
cdn1-C4 : ASYLRVHGEDILDEAISFTTDHLTLAVATLEYPLSEHVSHALKKSIRRGLPRIEARHYLSVY-QDIESHNTALLEFAKIDFNM : 241
DCS     : ASYLRVHGEDILDEAISFTTHHLSLAVASLDHPLSEEVSHALKQSIRRGLPRVEARHYLSVY-QDIESHNKALLEFAKIDFNM : 241
VvVal   : AAHLRVHGEDILAKALAFTTTHLKAMVESLGYHLAEQVAHALNRPIRKGLERLEARWYISVY-QDEAFHDKTLLELAKLDFNL : 244

              *       260        *       280        *       300        *       320        *
EtCop   : LQKMHQGELSHITRWWKELNFAKKLPFARDRVVECYFWILGVYFEPQYLIARRFLTKIIAMASVADDIYDVYGTLEELVILTD : 325
VvGerD  : LQKLHQKELSDISAWWKDLDFAHKLPFARDRVVECYFWILGVYFEPQFFFARRILTKVIAMTSIIDDIYDVYGTLEELELFTE : 327
cdn1-C4 : LQLLHRKELSEICRWWKDLDFKRKLPYVRDRVVECFFWILGVYFEPQYSLGRKILTKVIAMTSVIDDTYDSYATYDELIPYTN : 324
DCS     : LQFLHRKELSEICRWWKDLDFQRKLPYARDRVVEGYFWISGVYFEPQYSLGRKMLTKVIAMASIVDDTYDSYATYEELIPYTN : 324
VvVal   : VQSLHKEELSNLARWWKELDFATKLPFARDRLVEGYFWMHGVYFEPQYLRGRRILTKVIAMTSILDDIHDAYGTPEELKLFIE : 327
                                    RxR                                  DDxxD

                   340        *       360        *       380        *       400        *
EtCop   : AIERWDMDALDQIPECMRVYHRALLDVYTEMEEEMAKTGRPSYRVHYAKEAYKELVRQYLAEAKWFQEDYDPTLEEYLRVALI : 408
VvGerD  : AVERWDISAIDQLPEYMRVCYQALLYVYSEIEEEMAKEGR-SYRLYYAKEAMKNQVRAYYEEAKWLQVQQIPTMEEYMPVALV : 409
cdn1-C4 : AIERWDIKCIDQLPEYMKLSYKALLDVYEEMEQLMAEDGR-QYRVEYAKNIMIQLAQAFLMEAKWTLQNHKPSFEEFKATALQ : 406
DCS     : AIERWDIKCIDEIPEYMKPSYKALLDVYEEMVQLVAEHGR-QYRVEYAKNAMIRLAQSYLVEAKWTLQNYKPSFEEFKANALP : 406
VvVal   : AIERWDINSINQLPEYMKLCYVALLDVYKEIEEEMEKEGN-QYRVHYAKEVMKNQVRAYFAEAKWLHEEHVPAFEEYMRVALA : 409

                 420        *       440        *       460        *       480        *       5
EtCop   : SGGYKMLATHSFVGMGDLATKEAFDWVSNNPLIVKASSVICRLSDDMVGHEVEHERGDVASAVECYMKQYGVTKQEVYIEFQK : 491
VvGerD  : TSAYSMLATTSFVGMGDAVTKESFDWIFSKPKIVRASAIVCRLMDDMVFHKFEQKRGHVASAVECYMKQHGASEQETPNEFPQ : 492
cdn1-C4 : TTGYAMLAITALVDMGDIVTPETFTWAANNPKIIQASTIICRFMDDVAEHKFKQRREDDFSGIECYMEEYGVMVQEAYNVFYK : 489
DCS     : TCGYAMLAITSFVGMGDIVTPETFKWAASDPKIIQASTIICRFMDDVAEHKFKHRREDDCSAIECYMEEYGVTAQEAYDVFNK : 489
VvVal   : SSGYCLLATTSFVGMGEIATKEAFDWVTSDPKIMSSSNFITRLMDDIKSHKFEQKRGHVTSAVECYMKQYGVSEEQVYSEFQK : 492

                 00         *       520        *       540        *       560
EtCop   : QISNAWKDMNQECLHPTTVTMPLLTVIFNMTRVINLLYDEEDGYTNSNTRTKDFITSVLIDPVQI : 556
VvGerD  : PVREAWKDINEECLIPTAVPMPILMRVLNLARVIDVIYKNEDGYTHFGAVLKDFVTSMLIDPVPI : 557
cdn1-C4 : HIESAWKDVNKGFLKPTEMPIEVLNRILNLARVMNVLYNEGDGYTYVGKATKGIISILLIEPVTL : 554
DCS     : HVESAWKDLNQEFLKPTEMPTEVLNRSLNLARVMDVLYREGDGYTYVGKAAKGGITSLLIEPIAL : 554
VvVal   : QIENAWLDINQECLKPTAVSMPLLARLLNFTRTMDVIYKEQDSYTHVGKVMRDNIASVFINAVI- : 556
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Figure 1 Alignment of the deduced amino acid sequence of selected sesquiterpene synthase genes. *EtCop,* a α-copaene synthase gene, was isolated from three-leaved Eleuthero. *VvGerD*: (-)-germacrene D synthase from grapevine (*Vitis vinfera* L.; accession number AAS66357), *cdn*1-C4: (+)-δ-cadinene synthase from upland cotton (*Gossypium hirsutum* L.; accession number AAX44034), DCS: (+)-δ-cadinene synthase from tree cotton (*Gossypium arboretum* L.; SWISS-PROT accession code Q39761) and *VvVal*: (+)-valencene synthase from grapevine (*Vitis vinfera* L.; accession number AAS66358). The amino acids marked are the conserved motifs: $RR(x)_8W$, RxR and DDxxD.

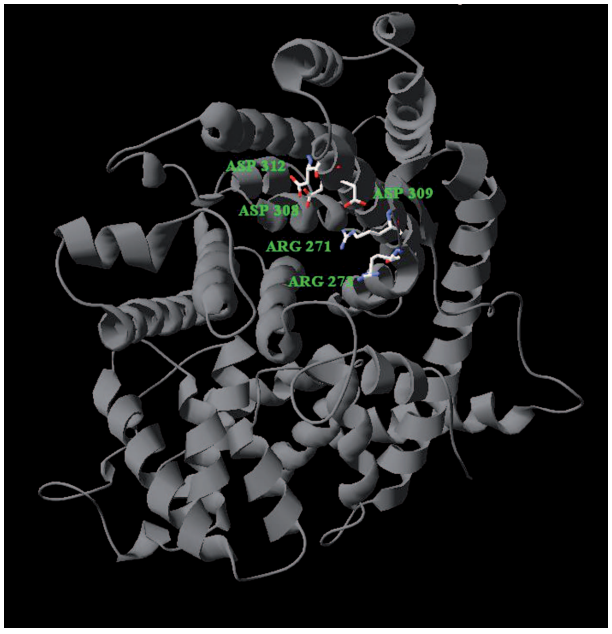

Figure 2 Predicted 3-dimensional structure of *EtCop*. The side chains illustrated are motifs $R^{271}x\ R^{273}$ and $D^{308}D^{309}xxD^{312}$.

Identification of *EtCop*

The amino acid sequence of *EtCop* contained the common terpene synthase family motifs $RR(x)_8W$ (R^{21}RSANYH-PNIW^{31}), RxR ($R^{271}DR^{273}$) and DDxxD ($D^{308}DIYD^{312}$) (Figure 1). DDxxD is found in almost all terpene synthases from bacteria and plants and is a putative substrate binding site (Back and Chappell 1995; Bohlmann et al. 1998; Cheng et al. 2007). The RxR has also been reported to be a conserved terpene synthase deduced amino-acid sequence which is involved in terpenoid catalysis and is conserved in *Arabidopsis thaliana* (Aubourg et al. 2002).

The 3D-structure of the protein was predicted by SWISS-MODEL with a (+)-δ-Cadinene synthase (DCS, isoenzyme XC1; SWISS-PROT accession code Q39761; Gennadios et al. 2009) as template (Figure 2) as it had the highest sequence similarity (50.841%) with *EtCop* in the SWISS-MODEL database. DCS is from tree cotton (*Gossypium arboretum* L.) and has the motif $D^{307}DTYD^{311}$ instead of DDIYD.

Phylogenetic analysis The phylogenetic tree constructed with amino-acid sequence data from the NCBI is shown in Figure 3. Based on a *Cycas* sesquiterpene synthase sequence as the root, we found that the gymnosperms form a distinct group. On the other hand, the angiosperms formed a large

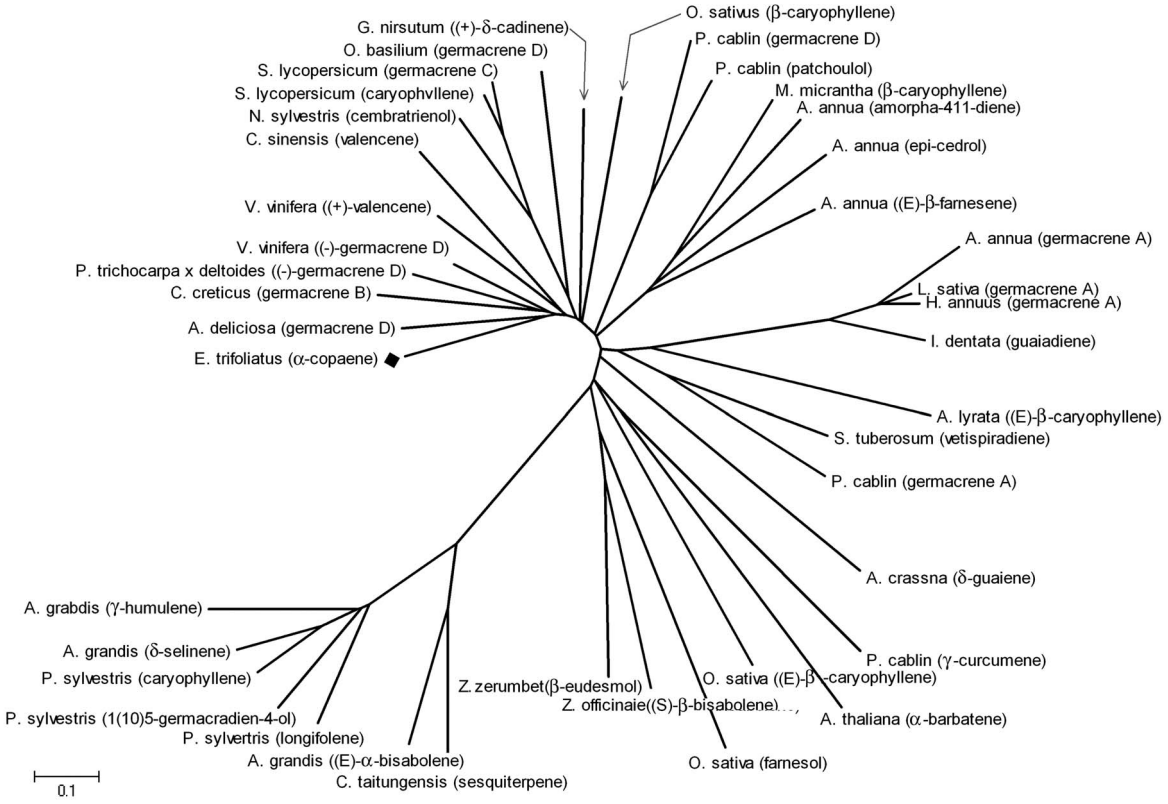

Figure 3 Phylogenetic tree of deduced amino acid sequences of different vascular plant sesquiterpene synthase genes from GenBank. The GenBank accession numbers are shown in Supplementary Data Table S1. The phylogenetic tree was constructed using MEGA 4.1 software Tamura et al. 2007) using the neighbor-joining method.

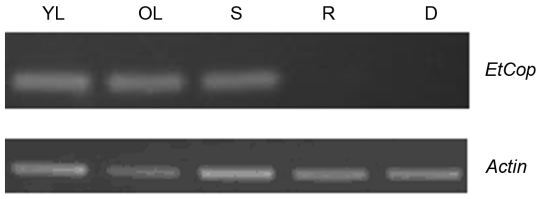

Figure 4 Expression of *EtCop* in different tissues. RT-PCR analysis of *EtCop* expression using the constitutive gene actin as the internal control. Total RNA was extracted from young leaves (YL), old leaves (OL), stems (S), roots (R) and drupes (D).

group and displayed a pattern that seemed to be influenced by the product, but not the species. From the phylogenetic tree, it can be seen that *EtCop* is more homologous to germacrene B, germacrene D and valencene synthase. The minor group formed within *EtCop* contained *Vitis* sp. and *Actinidia* sp.; both were woody plants with odor from their fruits.

Expression of *EtCop* in different tissues

The old leaves and stem of three-leaved eleuthero showed high levels of expression of *EtCop*. The root had only low level of expression (Figure 4). This result contrasts with several other terpene synthases in other plants species that showed high levels of expression in roots (Hiltpold and Turlings 2008; Göpfert et al. 2009). In addition, the drupes showed no detectable expression of *EtCop*.

Functional characterization of *EtCop*

For functional identification, the *EtCop* gene was heterologously expressed in *E. coli* to produce soluble protein and then assayed for in vitro enzyme activity. The pentane extract was analyzed by GC-MS to detect the product, which was predicted to be a sesquiterpene. The enzyme was active with FPP but not with geranyl diphosphate. The major product of *EtCop* was identified as α-copaene (Figure 5), based on comparison of the retention time, KI, and mass spectra from a study that investigated the composition of the essential oil extract from three-leaved eleuthero (Muselli et al. 1999). In order to double confirm the correction, the product was co-injected with a candidate authentic compound, namely α-copaene.

Discussion

Chemists have attempted to synthesize terpenoids using chemical approaches, but this has often proven difficult or costly because of the structural complexity of this class of substance (Harada and Misawa 2009). Biosynthesis of terpenoids by means of genetically modified heterologous hosts provides an alternative, and promising, approach (Harada and Misawa 2009). Terpene synthases (TPSs) play a key role

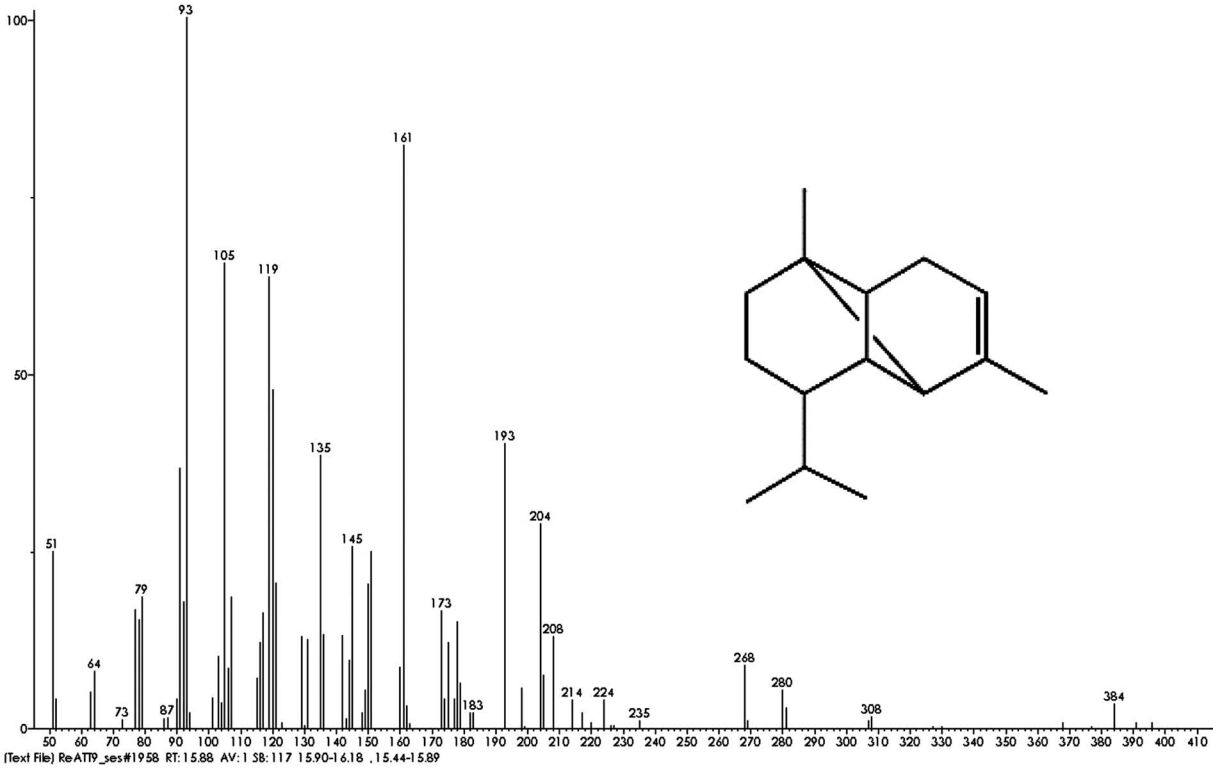

Figure 5 Mass spectra of peak at retention time 15.9 min (KI: 1366), the major sesquiterpene product generated by *EtCop*. The product was identified as α-copaene; the structure of the molecule is shown on the right.

in the biosynthesis of terpenoids. Cloning and functional characterization of TPSs has been an active area of plant secondary metabolism research and many genes have been isolated from various gymnosperm and angiosperm species. Advances have been made in metabolic engineering to increase terpenoid production by expression of TPS genes (Cheng et al. 2007). Recently, some terpenoids have been confirmed to have a potential use in targeting signaling to tumor cells (Ishibashi and Ohtsuki 2008). In this study, a sesquiterpene synthase gene was characterized from a traditional medical herb, the three-leaved eleuthero. To our knowledge, this is the first sesquiterpene synthase gene to be identified from a woody plant that has α-copaene as its main product.

α-Copaene is known to attract the Mediterranean fruit fly (*Ceratitis capitata* Wiedemann; Nishida et al. 2000), a costly agricultural pest. The bioactivity and ecological function of α-copaene in three-leaved eleuthero has not yet been identified, but in maize (*Zea mays* L.) it has been reported to have the ability to attract predators of beetles (Hiltpold and Turlings 2008). Hiltpold and Turlings (2008) found that, in maize, more turpene (E)-β-caryophyllen than α-copaene was emitted from damaged plants, but α-copaene diffused more readily in the soil. A sesquiterpene synthase gene that produces α-copaene as its sole reaction product has also been reported. It was highly expressed in potato and corresponded to the difference in tuber flavor between two cultivars (*Solanum tuberosum* group Phureja and *Solanum tuberosum* group Tuberosum; Ducreux et al. 2008). In three-leaved eleuthero, however, the α-copaene synthase *EtCop* did not show detectable expression in roots and no other minor products were detected; therefore, it may have other functions that need to be identified.

Traditional medicines and medical plants represent an abundant source of novel leads for drug discovery (Verpoorte et al. 2006) and, because they are a mixture of many compounds, their use may not result in drug resistance. However, the bioactivities and ecological role of the discovered terpene synthase gene still need to be further investigated.

Conclusions

A sesquiterpene synthease gene *EtCop* was cloned from the stems of the traditional medicine, three-leaved eleuthero. The full length was 1671 bp and it was functionally expressed in *E. coli*; the product of the protein was α-copaene. This is the first sesquiterpene synthase gene to be identified from a woody plant that has α-copaene as its main product.

Three-leaved eleuthero was previously confirmed to have anti-tumor activities. Because its medical efficacy has not been understood at the molecular level, the activities and functions of α-copaene still require more research.

Acknowledgements

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