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Purification and structural analysis of volatile sesquiterpenes produced by *Escherichia coli* carrying unidentified terpene synthase genes from edible plants of the family *Araliaceae*

Kazutoshi Shindo[a], Jun-ichiro Hattan[b], Mariko Kato[a], Miho Sato[a], Tomoko Ito[a], Yurika Shibuya[a], Arisa Watanabe[a], Maki Sugiyama[a], Yuri Nakamura[a] and Norihiko Misawa[b]

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ABSTRACT

A simple method to purify volatile sesquiterpenes from recombinant *Escherichia coli* was developed using the cells that carried known sesquiterpene synthase (*Tps*) genes *ZzZss*2 (*ZSS*2) and *ZoTps*1. This method was applied for the purification and structural analyses of volatile sesquiterpenes produced by *E. coli* cells that carried unidentified *Tps* genes, which were isolated from the *Aralia*-genus edible plants belonging to the family *Araliaceae*. Recombinant cells carrying each Tps gene were cultured in the two-layer medium (*n*-octane/TB medium), and volatile sesquiterpenes trapped in *n*-octane were purified through two-phase partition, silica gel column chromatography, and reversed-phase preparative high-performance liquid chromatography, if necessary. Further, their structures were confirmed by nuclear magnetic resonance, $[\alpha]_D$, and gas chromatography–mass spectrometry analyses. Herein, the products of *E. coli* cells that carried two *Tps* gene (named *AcTps*1 and *AcTps*2) in *Araria cordata* "Udo" and a *Tps* gene (named *AeTps*1) in *Aralia elata* "Taranoki" were studied resulting in identifying functionalities of these cryptic *Tps* genes.

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Functional identification of cryptic terpene synthase (*Tps*) genes have often been performed using recombinant micro-organisms containing *Escherichia coli* that can functionally express the corresponding *Tps* genes and generate the sesquiterpene synthase (TPS) products [1–4]. In many cases, the generated sesquiterpenes (the TPS products) have been analyzed by gas chromatography–mass spectrometry (GC–MS) using the MS library or co-chromatography with the authentic samples [2,3,5–8]. However, such methods are sometimes insufficient for the unambiguous determination of their chemical structures and consequent identification of the *Tps* genes.

To recover the generated volatile sesquiterpenes without losses, recombinant micro-organisms that express the *Tps* genes, such as *E. coli* and *Saccharomyces cerevisiae*, have often been cultured with two phases [*n*-dodecane (C12 alkane)/H_2O (medium), e.g. 20% (v/v)] to recover the products in *n*-dodecane [5,6,8,9]. The two-phase partition is adequate when the researchers identify the products using the GC–MS, whereas, because of its high boiling point, *n*-dodecane is an inconvenient solvent when the researchers purify the products. The present report shows a simple method to purify volatile sesquiterpenes from recombinant *E. coli* followed by functional identification of *Aralia*-derived cryptic *Tps* genes and structural identification of the generated products.

Material and methods

Tps genes and mevalonate pathway-engineered E. coli

The *ZzZss*2 (*ZSS*2) and *ZoTps*1 genes were described previously [5,8]. Entire *Tps* gene sequences were isolated by similar methods described [2]. Firstly, total RNA was extracted from young leaves of *Araria cordata* "Udo" or *Aralia elata* "Taranoki" using RNeasy Plant Mini kit (Qiagen). Next, the cDNA pool was synthesized from the total RNA using Clontech SMARTer RACE cDNA amplification kit (Takara Bio) and used as the template of following polymerase chain reactions (PCRs). Full length sequence of *Tps* gene was cloned using degenerate PCR and subsequent RACE method, isolating two *Tps* gene (named *AcTps*1 and *AcTps*2) in *A. cordata* and a *Tps* gene (named *AeTps*1) in *A. elata*. The cloned *Tps* gene was transferred into a mevalonate (MVA) pathway-engineered *E. coli* [10] using the *E. coli* expression vector pETDuet-1 (Merck). The MVA pathway-engineered

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E. coli was prepared by introducing plasmid pAC-MeV/Scidi/Aacl into *E. coli* Bl21 (DE3). The plasmid contains the MVA-pathway gene cluster from *Streptomyces* sp. strain CL190, the *Saccharomyces cerevisiae* TPP isomerase gene (*Scidi*), and acetoacetate-CoA ligase gene (*Aacl*) for the utilization of lithium acetoacetate (LAA) as the substrate [2,10].

Preparation of LAA

Ethylacetoacetate (Tokyo Chemical Industry) (5.2 g), H_2O (10 mL), and 4 M LiOH (10 mL) were added into capped 200 mL glass container and the container was kept at 40 °C in water bath for 4 h for saponification reaction. The solution was concentrated to dryness *in vacuo*, and a quantity of 45 mL anhydrous MeOH was then added into the flask and sonicated for 5 min. The solution was filtered, and the filtrate was recovered in a 500 mL Erlenmeyer flask. Furthermore, 255 mL of diethylether was added in the recovery flask; it was shaken well and stored overnight at 5 °C. The precipitation was then recovered by filtration to obtain pure LAA (2.23 g).

Production of volatile sesquiterpenes by fermentation of recombinant E. coli

The recombinant *E. coli* was inoculated into the 100 mL TB medium in a 500 mL Erlenmeyer flask containing 1 mM ampicillin (Ap) and chloramphenicol (Cm) and was cultured at 25 °C with 150 rpm until the OD (600 nm) of the culture reached 1.0. Furthermore, 2 mL of the culture was inoculated into a 500 mL Sakaguchi flask containing 100 mL TB medium that included 1 mM Ap, 1 mM Cm, 1 mM isopropylthiogalactoside, and 100 mM LAA. In addition, 20 mL of *n*-octane was added to each flask, and two-layer-cultured at 20 °C with 150 rpm for 48 h. The culture without the *n*-octane addition was also carried out, as needed.

Crude extracts from the recombinant *E. coli* cells cultured without the *n*-octane addition [2], which included all produced sesquiterpnes, were subjected to a 7890A gas chromatography system coupled to a 5975C mass spectrometer detector (GC–MS; Agilent Technologies, CA, USA) to determine the production of volatile sesquiterpenes. A DB-WAX column (60 m, 0.25 mm ID, 0.25 μm Df) and N_2 (carrier gas 1.12 mL/min) was utilized. The GC oven was programmed to start at 40 °C for 10 min, then ramped at 5 °C/min to 230 °C, and held for 12 min.

Purification of volatile sesquiterpenes from two-layer cultures with recombinant E. coli cells

The 10 or 20 flasks of each culture were mixed with 200 or 400 mL of *n*-hexane, and poured into a separatory funnel and shaken well. The alkane layer (*n*-octane + *n*-hexane) was collected and washed with a equal amount of alkaline 50% MeOH (0.5 M KOH in 50% MeOH) twice. The washed alkane layer was concentrated to a small volume of 5 mL *in vacuo*. This was applied on silica gel (Silica Gel 60, Kanto Chemical) column chromatography (20 i.d. × 200 mm) filled with *n*-pentane and developed with *n*-pentane (200 mL) (fraction (fr.) 1–30) → *n*-pentane-EtOAc (5:1) (200 mL) (fr. 31 -60), stepwisely. Eluates including sesquiterpenes in subsequent fractions were detected as blue spots on silica gel TLC (fr.s 1–30 were developed by *n*-pentane and fr.s 31–60 were developed by *n*-pentane–EtOAc (5:1)) by heating TLC plates after spraying molybdophophoric acid solution (12 g disodium molybdate (VI) dihydrate, 1.5 mL H_3PO_4, 25 mL H_2SO_4 in 500 mL distilled water).

Purification from E coli (pET-ZSS2 + pAC-MeV/Scidi/Aacl) (ZzZss2 (ZSS2) in Zingiber zerumbet) [5] (10 flasks)

Fraction 38–42, which contained one silica gel TLC (Merck) spot (Rf 0.40, solvent: *n*-pentane-EtOAc (5:1)), was combined and concentrated to a small volume of 3 mL *in vacuo* and further purified through preparative high-performance liquid chromatography (HPLC) (column: C30-UG-5 (10 i.d. × 250 mm, Developsil), solvent: 80% CH_3CN, flow rate: 3.0 mL/min), detection: DAD (200–300 nm)); the peak that eluted at 9.6 min was collected.

The eluate (30 mL) were mixed with a equal amount of water, and partitioned with *n*-pentane (60 mL) in a separatory funnel. The *n*-pentane layer was dried over Na_2SO_4 for 30 min and concentrated to dryness *in vacuo* (100 mm Hg) to procure pure β-eudesmol (**1**) (6.2 mg).

Purification from E coli (pET-ZoTps1 + pAC-MeV/Scidi/Aacl) (ZoTps1 in Zingiber officinale) [8] (10 flasks)

Fraction 8–10, which contained one silica gel TLC spot (Rf 0.61, solvent: *n*-pentane), was combined and concentrated to a small volume of 3 mL *in vacuo* and further purified through preparative HPLC (column: C30-UG-5 (10 i.d. × 250 mm), solvent: 95% CH_3CN, flow rate: 3.0 mL/min, detection: DAD (200–300 nm)), and the peak that eluted at 18.0 min was collected. The eluate (40 mL) was further treated as described above to procure β-bisabolene [11] (**2**) (20.1 mg).

Purification from E. coli (pET-AcTps1 + pAC-MeV/Scidi/Aacl) (AcTps1 in Araria cordata) (20 flasks)

Fraction 10–14, which contained one silica gel TLC spot (Rf 0.61, solvent: *n*-pentane), was combined and concentrated to dryness *in vacuo* (100 mm Hg) to procure pure germacrene A [11] (**3**) (10.1 mg).

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Purification from E. coli (pET-AcTps2 + pAC-MeV/Scidi/Aacl) (AcTps2 in Araria cordata) (20 flasks)

Fraction 8–12 (Rf 0.81, solvent: *n*-pentane) and fr. 13–17 (Rf 0.75, solvent: *n*-pentane), containing one silica gel TLC spot, was combined and concentrated to dryness *in vacuo* (100 mm Hg) to procure pure β-panasinsene [12] (**4**) (fr. 8–12 5.6 mg) and β-caryophyllene [12] (**5**) (fr. 13–17 4.9 mg), respectively. Fr. 22–24, which contained two silica gel spots (Rf 0.58 and 0.52, solvent: *n*-pentane), was further purified through preparative HPLC (column: ADME (10 i.d. × 250 mm, Shiseido), solvent: 80% CH_3CN, flow rate: 3.0 mL/min, detection: DAD (200–300 nm)), and the peaks that eluted at 21.5 and 22.5 min were collected separately. Subsequent eluates (20 mL (*t*R 21.5 min) and 25 mL (*t*R 22.5 min)) were further treated as described above to procure pure germacrene A (**3**) (*t*R 21.5 min, 0.8 mg) and α-humulene (**6**) (*t*R 22.5 min, 0.6 mg), respectively.

Purification from E. coli (pET-AeTps1 + pAC-MeV/Scidi/Aacl) (AeTps1 in A. elata) (20 flasks)

The fr.s that contained one silica gel TLC spot (fr. 8–9 (Rf 0.75, solvent: *n*-pentane) and fr. 17–25 (Rf 0.51, solvent: *n*-pentane) were combined and concentrated to dryness *in vacuo* (100 mm Hg), respectively, to procure pure α-copaene [13] (**7**) (fr. 8–9, 5.0 mg) and δ-cadinene [14] (**8**) (fr. 17–25, 4.2 mg).

Fraction 11–12 that contained two silica gel TLC spots (Rf 0.65 and 0.61, solvent:*n*-pentane) were combined and concentrated to dryness *in vacuo* (100 mm Hg), and further purified through preparative HPLC (column: C30-UG-5 (10 i.d. × 250 mm), solvent: 90% CH_3CN, flow rate: 3.0 mL/min, detection: DAD (200–300 nm)), and the peaks that eluted at 27.0 min and 33.0 min were collected separately. Each eluate (30 mL (*t*R 27.0 min) and 40 mL (*t*R 33.0 min)) was further treated as described above to procure pure β-cubebene [15] (**9**) (*t*R 27.0 min, 6.3 mg) and germacrene D [16] (**10**) (*t*R 33.0 min, 1.8 mg), respectively.

Fraction 13–16, which contained several spots (Rf 0.58 (main spot), solvent: *n*-pentane), was combined and concentrated to small volume (3 mL) *in vacuo* and further purified through preparative HPLC (column: C30-UG-5 (10 i.d. × 250 mm), solvent: 90% CH_3CN, flow rate: 3.0 mL/min, detection: DAD (200–300 nm)), and the main peak eluted at 27.0 min was collected. The eluate (30 mL) was further treated as described above to procure pure δ-cadinene (**8**) (4.1 mg).

Structural analyses of the isolated volatile sesquiterpenes

Each isolated sesquiterpene was analyzed by NMR (Bruker, AMX400), $[\alpha]_D$ (AUTOPOL IV, Rudolph Research Analytical), and GC–MS as described above. NMR spectra were measured in $CDCl_3$ using the residual solvent peak as an internal standard (δ_C 77.0, δ_H 7.26 ppm); $[\alpha]_D$ was measured in $CHCl_3$.

Physico-chemical properties of β-eudesmol (1)

GC–MS *t*R 48.1 min, *m/z* 222 (M^+). $[\alpha]_D^{20}$ 32.9 (*c* 0.52). ^1H NMR (Figure S1) δ: 0.69 (s, 3H, H-15), 1.20 (s, 6H, H-13 and H-14), 1.20–1.22 (2H, H-1b and H-9b), 1.35(m, 1H, H-7), 1.45–1.52 (2H, H-1a and H-9a), 1.55–1.65 (6H, H-2a, H-2b, H-6a, H-6b, H-8a, and H-8b), 1.76 (m, 1H, H-5), 1.95 (m, 1H, H-3b), 2.30 (m, 1H, H-3a), 4.44 (s, 1H, H-11b), and 4.70 (s, 1H, H-11a). ^{13}C NMR (Figure S2) δ: 16.3 (C-15), 22.4 (C-6), 23.5 (C-2), 25.0 (C-8), 27.1 (C-13), 27.2 (C-14), 35.9 (C-10), 36.9 (C-3), 41.1 (C-9), 41.8 (C-1), 49.4 (C-7), 49.8 (C-5), 72.9 (C-12), 105.3 (C-11), and 151.1 (C-4).

Physico-chemical properties of β -bisabolene (2)

GC–MS *t*R 38.4 min, *m/z* 204 (M^+). $[\alpha]_D^{24}$-69.3 (*c* 2.0).^1H NMR (Figure S3) δ: 1.45 (m, 1H, H-4b), 1.61 (s, 3H, H-15), 1.65 (s, 3H, H-13), 1.69 (s, 3H, H-12), 1.81 (m, 1H, H-4a), 1.90 (m, 1H, H-3b), 2.05–2.15 (6H, H-6b, H-6a, H-8b, H-8a, H-9b, and H-9a), 2.09 (m, 1H, H-5), 2.10 (m, 1H, H-3a), 4.74 (s, 1H, H-14b), 4.76 (s, 1H, H-14a), 5.12 (m, 1H, H-10), 5.41 (m, 1H, H-1). ^{13}C NMR (Figure S4) δ: 17.7 (C-15), 23.5 (C-13), 25.7 (C-12), 26.8 (C-9), 28.3 (C-4), 30.8 (C-3), 31.4 (C-6), 34.9 (C-8), 39.8 (C-5), 107.1 (C-14), 120.8 (C-1), 124.3 (C-10), 131.5 (C-11), 133.8 (C-2), 154.3 (C-7).

Physico-chemical properties of germacrene A (3)

GC–MS *t*R 34.4 min (β-elemene), *m/z* 204 (M^+). $[\alpha]_D^{24}$ −1.90 (*c* 0.10). The signals in ^1H NMR spectrum of gemacrene A (**3**) were very broad because of its conformational equilibrium in the solution, and we could not assign them. The ^1H spectrum of **3** was shown in Figure S5 as supporting material.

Physico-chemical properties of β-panasinsene (4)

GC–MS *t*R 33.4 min, *m/z* 204 (M^+). $[\alpha]_D^{24}$-37.3 (*c* 0.29).^1H NMR (Figure S6) δ: 0.75 (s, 3H, H-14), 0.86 (s, 3H, H-12), 1.07 (s, 3H, H-13), 1.09 (m, 1H, H-5b), 1.24 (m, 1H, H-5a), 1.38 (2H, H-3b and H-6b), 1.60 (m, 1H, H-6a), 1.69 (m, 1H, H-3a), 1.70 (m, 1H, H-2b), 1.95 (m, 1H, H-2a), 1.97 (m, 1H, H-7b), 2.10 (m, 1H, H-1), 2.20 (m, 1H, H-7a), 4.80 (s, 1H, H-15b), 4.90 (s, 1H, H-15a). ^{13}C NMR (Figure S7) δ: 18.2 (C-14), 23.1 (C-6), 24.7 (C-2), 24.8 (C-12), 30.5 (C-10), 30.6 (C-13), 33.8 (C-7), 35.7 (C-11), 36.0 (C-5), 41.1 (C-3), 45.6 (C-4), 52.6 (C-1), 52.8 (C-9), 108.3 (C-15), 152.3 (C-8).

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Physico-chemical properties of β-caryophyllene (5)

GC–MS *t*R 37.2 min, *m/z* 204 (M^+). $[\alpha]_D^{24}$ -16 (*c* 0.16).^{1}H NMR (Figure S8) δ: 0.90 (s, 3H, H-12), 1.19 (s, 3H, H-13), 1.35–1.55 (2H, H-2a and H-2b), 1.53 (s, 3H, H-14), 1.55 (m, 1H, H-11b), 1.72 (m, 1H, H-11a), 1.74 (m, 1H, H-7b), 1.88 (ddd *J* = 2.5, 12.0, 12.0 Hz, 1H, H-3b), 2.00 (m, 1H, H-6b), 2.08 (m, 1H, H-3a), 2.12 (m, 1H, H-1), 2.33 (m, 1H, H-6a), 2.48 (dd J = 7.0, 12.5 Hz, 1H, H-7a), 2.90 (ddd *J* = 8.3, 8.3, 11.8 Hz, 1H, H-9), 4.79 (s, 1H, H-15b), 4.86 (s, 1H, H-15a), 5.11 (dd *J* = 3.2, 11.6 Hz, 1H, H-5). ^{13}C NMR (Figure S9) δ: 18.6 (C-14), 25.1 (C-2), 25.3 (C-12), 27.3 (C-6), 29.8 (C-13), 32.9 (C-10), 38.1 (C-11), 39.5 (C-7), 41.6 (C-3), 42.9 (C-9), 53.7 (C-1), 112.7 (C-15), 121.8 (C-5), 136.5 (C-4), 149.7 (C-8).

Physico-chemical properties of α-humulene (6)

GC–MS *t*R 37.0 min, *m/z* 204 (M^+). ^{1}H NMR (Figure S10) δ: 1.06 (s, 6H, H-14 and H-15), 1.43 (s, 3H, H-13), 1.64 (s, 3H, H-12), 1.91 (d *J* = 7.4 Hz, 2H, H-7), 2.05–2.15 (4H, H-3b, H-3a, H-4b, and H-4a), 2.51 (d *J* = 7.3 Hz, 2H, H-11), 4.87 (t *J* = 7.4 Hz, 1H, H-6), 4.93 (m, 1H, H-2), 5.16 (d *J* = 15.4 Hz, 1H, H-9), 5.59 (dt *J* = 7.3, 15.9 Hz, 1H, H-10). ^{13}C NMR (Figure S11) δ: 15.1 (C-13), 17.9 (C-12), 23.3 (C-3), 27.1 (C-14 and C-15), 37.4 (C-8), 39.7 (C-4), 40.4 (C-11), 42.0 (C-7), 125.0 (C-6), 125.8 (C-2), 127.7 (C-10), 133.2 (C-5), 139.2 (C-1), 141.0 (C-9).

Physico-chemical properties of α-copaene (7)

GC–MS *t*R 31.9 min, *m/z* 204 (M^+). The assignment of α-copaene was performed by co-GC–MS analysis with the authentic sample procured from Taiyo Corporation. The physico-chemical properties of **7** will be described in another report (Higuchi et al. in preparation).

Physico-chemical properties of δ-cadinene (8)

GC–MS *t*R 39.1 min, *m/z* 204 (M^+). $[\alpha]_D^{24}$ -57.6 (*c* 0.12, $CHCl_3$). Electron ionization-MS *m/z* 204 (M^+). ^{1}H NMR (Figure S12) δ: 0.78 (d *J* = 7.0 Hz, 3H, H-14), 0.96 (d *J* = 7.0 Hz, 3H, H-15), 1.05 (m, 1H, H-9), 1.58 - 1.64 (2H, H-8b and H-8a), 1.65 (s, 3H, H-12), 1.67 (s, 3H, H-11), 1.90 - 2.00 (4H, H-3b, H-3a, H-7b, and H-7a), 2.05 (m, 1H, H-13), 2.06 (m, 1H, H-4b), 2.50 (m, 1H, H-10), 2.71 (m, 1H, H-4a), 5.44 (m, 1H, H-1). ^{13}C NMR (Figure S13) δ: 15.6 (C-14), 18.5 (C-12), 21.2 (C-8), 21.7 (C-15), 23.6 (C-11), 26.6 (C-4), 26.7 (C-13), 32.0 (C-3), 32.3 (C-7), 39.4 (C-10), 45.4 (C-9), 124.5 (C-6), 124.7 (C-1), 129.9 (C-5), 134.2 (C-2).

Physico-chemical properties of β-cubebene (9)

GC–MS *t*R 33.7 min, *m/z* 204 (M^+). ^{1}H NMR (Figure S14) δ: 0.57 (m, 1H, H-7b), 0.85 (m, 1H, H-10), 0.88 (m, 1H, H-8b), 0.89 (d *J* = 6.8 Hz, 3H, H-14), 0.92 (d *J* = 6.8 Hz, 3H, H-15), 0.98 (d *J* = 6.8 Hz, 3H, H-12), 1.10 (m, 1H, H-9), 1.38 (m, 1H, H-1), 1.40 (m, 1H, H-8a), 1.54 (m, 1H, H-4b), 1.60 (2H, H-7a and H-13), 1.78 (m, 1H, H-6), 1.97 (m, 1H, H-4a), 2.02 (m, 1H, H-3b), 2.11 (m, 1H, H-3a), 4.57 (s, 1H, H-11b), 4.76 (s, 1H, H-11a). ^{13}C NMR (Figure S15) δ: 18.8 (C-12), 19.4 (C-14), 20.1 (C-15), 26.3 (C-8), 28.5 (C-10), 29.0 (C-3), 30.8 (C-4), 31.6 (C-7), 33.4 (C-13), 35.8 (C-1), 38.1 (C-5), 44.1 (C-9), 100.8 (C-11), 155.3 (C-2).

Physico-chemical properties of germacrene D (10)

GC–MS *t*R 38.0 min, *m/z* 204 (M^+). $[\alpha]_D^{24}$ -209 (*c* 0.17). ^{1}H NMR (Figure S16) δ: 0.80 (d *J* = 6.8 Hz, 3H, H-13), 0.86 (d *J* = 6.8 Hz, 3H, H-14), 1.40 - 1.52 (2H, H-8b, and H-8a), 1.42 (m, 1H, H-12), 1.52 (s, 3H, H-15), 1.95 (m, 1H, H-2b), 2.02 (m, 1H, H-7), 2.08 (m, 1H, H-3b), 2.10 - 2.30 (2H, H-9b, and H-9a), 2.40 (2H, H-2a, and H-3a), 4.74 (s, 1H, H-11b), 4.79 (s, 1H, H-11a), 5.13 (m, 1H, H-1), 5.25 (dd *J* = 10.0, 15.8 Hz, 1H, H-6), 5.80 (d *J* = 15.8 Hz, 1H, H-5). ^{13}C NMR (Figure S17) δ: 15.9 (C-15), 19.3 (C-13), 20.7 (C-14), 26.5 (C-8), 29.2 (C-2), 32.7 (C-12), 34.5 (C-3), 40.7 (C-9), 52.9 (C-7), 109.0 (C-11), 129.6 (C-1), 133.5 (C-6), 134.0 (C-10), 135.5 (C-5), 148.9 (C-4).

Accession numbers

Accession numbers of the new genes deposited are LC307165 (AcTps1), LC307166 (AcTps2), and LC307167 (AeTps1).

Results

In the present study, we isolated three novel *Tps* genes (*AcTps*1 and *AcTps*2 from *A. cordata*, and *AeTps*1 from *A. elata*). Amino acid sequences deduced from these genes were phylogenetically analyzed (Figure 1). The *AcTps*1 and *AcTps*2 gene products were closely related. Although they fall into the same clade, the *AeTps*1 gene product was found to be categorized into another single clade.

To recover volatile sesquiterpenes – TPS protein products that are produced by *E. coli* carrying the corresponding *Tps* genes—the recombinant *E. coli* had often been cultured with two phases (*n*-dodecane (C12 alkane)/H_2O (medium); 20% (v/v)) to recover the products in *n*-dodecane [5,6,8]. The use of *n*-dodecane was adequate when the researchers would identify the products (volatile sesquiterpenes) using GC–MS, whereas *n*-dodecane was an inconvenient solvent when the researchers would purify the products because of its high boiling point (*n*-dodecane is not removable using diaphragm pump). Thus, we screened more suitable alkane for two-phase culture to purify the products using *E. coli* that harbored plasmid pET-ZSS2 carrying the *Z. zerumbet Tps* gene

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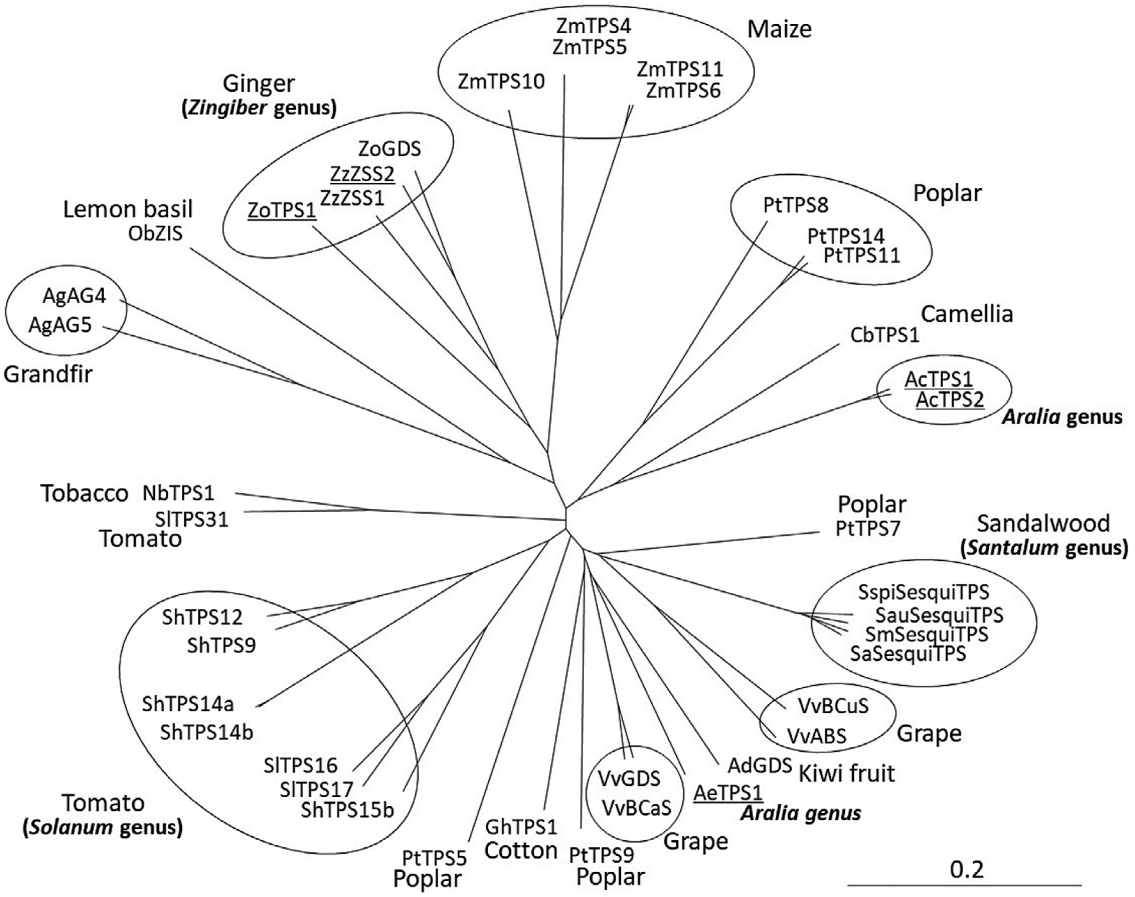

Figure 1. Phylogenetic tree of plant TPSs.

Notes: ZzZSS2, ZoTPS1, AcTPS1, AcTPS2, AeTPS1 (underlined) and known TPSs were phylogenetically analyzed. TPS accession numbers are as follows: ZmTPS4 (AAS88571), ZmTPS5 (AAS88574), ZmTPS6 (AAS88576), ZmTPS10 (AAX99146), ZmTPS11 (ACF58240), ObZIS (AAV63788), AgAG4 (AAC05727), AgAG5 (AAC05728), VvABS (NP_001267972), VvBCaS (ADR74193), VvBCuS (ADR74200), VvGDS (Q6Q3H3), AdGDS (AAX16121), ZoTPS1 (BAI67934), ZoGDS (AAX40665), ZzZSS1 (BAG12020), ZzZSS2 (BAG12021), PtTPS5 (KF776503), PtTPS7 (KF776505), PtTPS8 (KF776506), PtTPS9 (KF776507), PtTPS11 (KF776509), PtTPS14 (KF776512), SaSesquiTPS (ACF24768), SauSesquiTPS (HQ343281), SmSesquiTPS (JF746810), SspiSesquiTPS (HQ343282). *Circles* indicate TPSs in the same genus or the same species.

(*ZzZss*2; *ZSS*2) [5] and *E. coli* harbored plasmid pET-ZoTps1 carrying the *Z. officinale Tps* gene (*ZoTps*1) [8]. We tested *n*-hexane (C-6), *n*-octane (C-8), and *n*-decane (C-10), and found that *n*-hexane was toxic to recombinant *E. coli*; it did not grow because of high solubility of *n*-hexane in water, and *n*-octane and *n*-decane could be used as alternative phases, which do not have any effect on the growth of *E. coli*. Because *n*-octane was removable using diaphram pump, we selected *n*-octane as the new material for the two-phase culture. Consequently, pure β-eudesmol (**1**) (6.2 mg) and β-bisabolene (**2**) (20.1 mg) were obtained from total 1 L culture of *E. coli* carrying plasmid pET-ZSS2 and *E. coli* carrying plasmid pET-ZoTps1, respectively.

Using the purification method described in the Material and Methods section, we successfully isolated the volatile sesquiterpenes that were produced not only by the above-mentioned recombinant *E. coli* but also by the recombinant *E. coli* carrying the unidentified *Araliaceae Tps* genes. The extracts from the recombinant *E. coli* cells were analyzed by GC–MS to investigate the product property of the enzyme. The results are shown in Supplementary Figures S18 and Table S1–S4 (the result of ZzZSS2 (ZSS2) is not shown, since it was described [5]) . Each purified volatile sesquiterpene was subjected to spectroscopic analysis along with GC–MS analysis to identify its chemical structure.

The molecular formula for **1** was determined as $C_{15}H_{26}O$ by the observation of M^+ ion peaks in GC–MS analyses (*m/z* 222). The analyses of ^{1}H NMR (Figure S-1), ^{13}C NMR (Figure S-2), and HSQC spectrum of **1** proved the presence of three singlet methyl carbons [δ_C 17.0 (C-15), δ_C 27.8 (C-13), and δ_C 27.9 (C-14)], six sp^2 methylene carbons [δ_C 23.1 (C-6), δ_C 24.4 (C-2), δ_C 25.8 (C-8), δ_C 37.7 (C-3), δ_C 42.0 (C-9), and δ_C 42.7 (C-1)], two sp^3 methine carbons [δ_C 50.1 (C-7) and δ_C 50.5 (C-5)], two sp^3 quaternary carbons [δ_C 36.5 (C-10) and δ_C 72.4 (C-12)], one sp^2 (exo methylene) carbon [δ_C 106.3 (C-11)], and one quaternary sp^2 carbon [δ_C 151.6 (C-4)] in **1**. The attachment of oxygen at C-12 was also confirmed according to its ^{13}C chemical shift.

The analyses of ^{1}H-^{1}H DQF COSY and HSQC-TOCSY spectra of **1** proved the presence of ^{1}H-^{1}H vicinal spin networks C-1 ~ C-3 and C-5 ~ C-9 in **1** (Figure 2). The linkages of C-1, C-5, and C-9 through C-10 were proved by the observation of ^{1}H-^{13}C long-range coupling

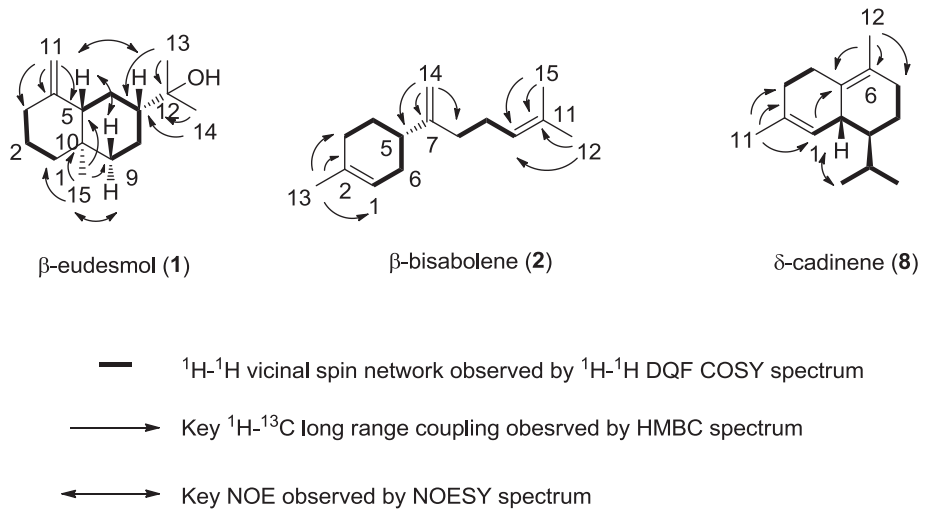

Figure 2. NMR spectral analyses for β-eudesmol (**1**), β-bisabolene (**2**), and δ-cadinene (**8**).

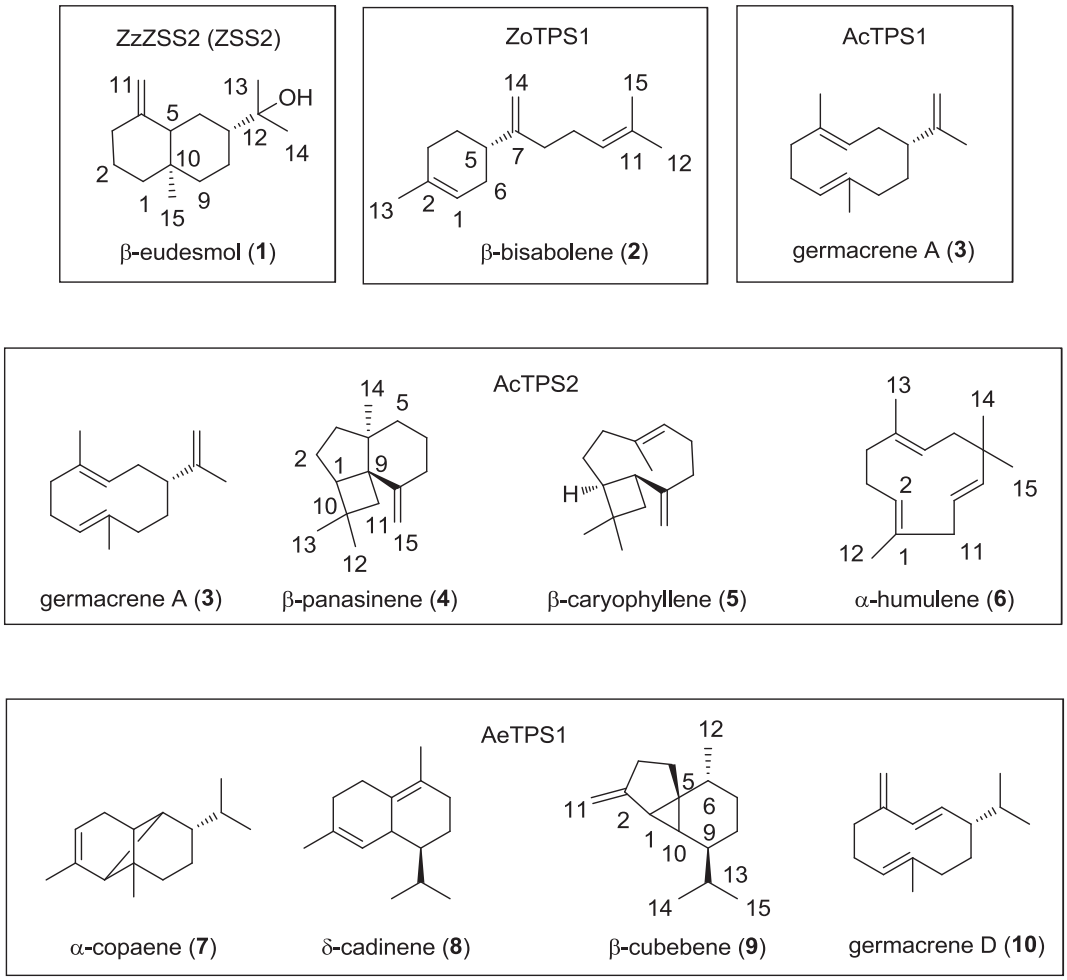

Figure 3. The structures of purified volatile sesquiterpenes.

from H-15 (δ_H 0.78) to C-1, C-5, and C-9 in the HMBC spectrum of **1**, and the linkages of C-3 - C-4 - C-5 and C-7 – C-12 – C-13 (C-14) were also proved by the ^{1}H-^{13}C long-range coupling from H-11 (δ_H 4.65 and δ_H 4.89) to C-3, C-4, and C-5 and from H-13 and H-14 (δ_H 1.07) to C-7 and C-12, respectively (Figure 2). From these observations, the planar structure of **1** was established. The key NOEs observed among H-5, H-7, and H-9β (1,3-diaxials) and between H-9α and H-15) (Figure 3) in NOESY spectrum of **1** clarified the relative configurations of **1**, and the absolute configuration of **1** was proved as shown in Figure 2 by the $[\alpha]_D$ value (32.9 (*c* 0.52) [16]. From these observations, **1** was proved to identical to β-eudesmol. This ZzZSS2 product was already identified

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by Yu et al. [5] while its NMR and $[\alpha]_D$ analysis was first shown here.

The molecular formula for **2** was determined as $C_{15}H_{24}$ by the observation of M^+ ion peaks in GC–MS analyses (*m/z* 204). The analyses of ^{1}H NMR (Figure S-3), ^{13}C NMR (Figure S-4), and HSQC spectrum of **2** proved the presence of three singlet methyl carbons [δ_C 17.7 (C-15), δ_C 23.5 (C-13), and δ_C 25.7 (C-12)], five sp^2 methylene carbons [δ_C 26.8 (C-9), δ_C 28.3 (C-4), δ_C 30.8 (C-3), δ_C 31.4 (C-6), δ_C 34.9 (C-8)], one sp^3 methine carbons [δ_C 39.8 (C-5)], one sp^2 (exo methylene) carbon [δ_C 107.1 (C-14)], two sp^2 methine carbons [δ_C 120.8 (C-1) and δ_C 124.3 (C-10)], and three sp^2 quaternary sp^2 carbons [δ_C 131.5 (C-11), δ_C 133.8 (C-2), and δ_C 154.3 (C-7)] in **2**.

The analyses of ^{1}H-^{1}H DQF COSY and HSQC-TOCSY spectra of **2** proved the presences of ^{1}H-^{1}H vicinal spin networks C-3 ~ C-6 – C-1, and C-8 ~ C-10 in **2** (Figure 2). The linkages of C-1–C-2–C-3 was proved by the observation of ^{1}H-^{13}C long-range coupling from H-13 (δ_H 1.65) to C-1, C-2, and C-3 in the HMBC spectrum of **2**, and the linkages of C-5 - C-7 - C-8 and the linkage of C-10, C-12, and C-15 through C-11 were also proved by the ^{1}H-^{13}C long-range coupling from H-14 (δ_H 4.74 and δ_H 4.76) to C-5, C-7, and C-8 and from H-12 (δ_H 1.69) and H-15 (δ_H 1.61) to C-11 and C-10, respectively (Figure 2). From these observations, **2** was proved to be identical to β-bisabolene. The absolute configuration of **2** was shown to be 5*S* by chiral GC–MS analysis in the previous study [8], and it was also proved by the $[\alpha]_D$ value (−69.3 (*c* 2.0)) [17] (Figure 2). This ZoTPS1 product was already identified by Fujisawa et al. [8] while its NMR and $[\alpha]_D$ analysis was first shown here.

The planar structures of **4**–**10** produced by *E. coli* carrying the *Araliaceae Tps* genes were also determined by NMR analyses, whereas germacrene A (**3**) was identified by the observation of β-elemene in the GC–MS analysis because of its broadened (unassignable) ^{1}H signals (Figure S-5). The key NOEs observed in NOESY spectra to clarify the relative configurations of **8** are shown in Figure 2, whereas the relative stereochemistry of **4**–**7**, **9**, and **10** were not confirmed by NOESY analyses. The absolute configurations of **3**–**10** were certified by matching with the GC–MS library (the presence of all the compounds (**1**–**10**) was certified by the GC–MS library (Supplementary Figure S18 and Table S1-S4) and $[\alpha]_D$ values. The structures of the isolated volatile sesquiterpenes from each TPS are listed in Figure 3.

Discussion

Since 2008, Misawa et al. have identified several novel *Tps* genes for the production of volatile sesquiterpenes using recombinant *E. coli* cells carrying the corresponding unidentified *Tps* genes derived from edible or flavor plants [2,3,5,6,8,18]. They identified volatile sesquiterpenes produced by *E. coli* cells that carried the *ZzZss*2 gene and the *ZoTps*1 gene by GC–MS analyses (identification using GC–MS library and the authentic standards) to be β-eudesmol [5] (**1**) and (*S*)- β-bisabolene [8] (**2**), respectively. Recently, we were further required to purify **1** and **2** to analyze their structures in detail. For these purposes, we established an isolation method to purify the volatile sesquiterpenes using the *E. coli* cultures of pET-ZSS2 (carrying *ZzZss*2) and pET-ZoTps1 (carrying *ZoTps*1). *n*-Octane was first added to the culture to trap generated terpenes, followed by two-phase partition, silica gel column chromatography, and preparative ODS-HPLC, which is generally available in the modern chemical laboratories. We also successfully clarified the structures of **1** and **2** by NMR, $[\alpha]_D$ analyses, and GC–MS analyses.

Furthermore, we applied the above isolation method to the volatile sesquiterpenes, produced by *Araria* TPSs [pET-AcTps1 and pET-AcTps2 (*AcTps*1 and *AcTps*2 in *A. cordata*), and pET-AeTps1 (*AeTps*1 in *A. elata*)] that were not reported previously; we successfully identified the volatile sesquiterpenes. This is the first study to report on the volatile sesquiterpenes of these *Tsp* genes and their physico-chemical data (^{1}H and ^{13}C NMR, and $[\alpha]_D$), resulting in achievement of the functional identification of these cryptic *Tps* genes.

Author contributions

Kazutoshi Shindo (KS), Jun-ichiro Hattan, Mariko Kato, Miho Sato, Tomoko Ito, Yurika Shibuya, Arisa Watanabe, Maki Sugiyama, Yuri Nakamura performed experiments. Norihiko Misawa supervised the present study, and wrote the manuscript along with KS.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Acknowledgments

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Disclosure statement

No potential conflict of interest was reported by the authors.

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