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ORIGINAL ARTICLE

Identification of a novel hedycaryol synthase gene isolated from *Camellia brevistyla* flowers and floral scent of *Camellia* cultivars

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Abstract

Main conclusion **A novel terpene synthase (*Tps*) gene isolated from *Camellia brevistyla* was identified as hedycaryol synthase, which was shown to be expressed specifically in flowers.**

Camellia plants are very popular because they bloom in winter when other plants seldom flower. Many ornamental cultivars of Camellia have been bred mainly in Japan, although the fragrance of their flowers has not been studied extensively. We analyzed floral scents of several Camellia cultivars by gas chromatography–mass spectrometry (GC–MS) and found that *Camellia brevistyla* produced various sesquiterpenes in addition to monoterpenes, whereas *Camellia japonica* and its cross-lines produced only monoterpenes, including linalool as the main product. From a flower of *C. brevistyla*, we isolated one cDNA encoding a terpene synthase (TPS) comprised of 554 amino acids, which was phylogenetically positioned to a sole gene clade. The cDNA, designated *CbTps1*, was expressed in mevalonate-pathway-engineered *Escherichia coli*, which carried the *Streptomyces* mevalonate-pathway gene cluster in addition to the acetoacetate-CoA ligase gene. A terpene product was purified from recombinant *E. coli* cultured with lithium acetoacetate, and analyzed by ^{1}H-nulcear magnetic resonance spectroscopy (^{1}H-NMR) and GC–MS. It was shown that a sesquiterpene hedycaryol was produced, because ^{1}H-NMR signals of the purified product were very broad, and elemol, a thermal rearrangement product from hedycaryol, was identified by GC–MS analysis. Spectroscopic data of elemol were also determined. These results indicated that the *CbTps1* gene encodes hedycaryol synthase. Expression analysis of *CbTps1* showed that it was expressed specifically in flowers, and hedycaryol is likely to be one of the terpenes that attract insects for pollination of *C. brevistyla*. A linalool synthase gene, which was isolated from a flower of *Camellia saluenensis*, is also described.

Keywords Hedycaryol · Elemol · Camellia · Sesquiterpene synthase · *Escherichia coli*

Abbreviations

Cm	Chloramphenicol
DMAPP	Dimethylallyl diphosphate
FPP	Farnesyl diphosphate
GC	Gas chromatography
GC-MS	Gas chromatography–mass spectrometry
GPP	Geranyl diphosphate
HPLC	High-performance liquid chromatography

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IPP	Isopentenyl diphosphate
IPTG	Isopropyl β-D-thiogalactopyranoside
Km	Kanamycin
LAA	Lithium acetoacetate
MEP	Methylerythritol phosphate
MVA	Mevalonate
NMR	Nuclear magnetic resonance spectroscopy
ORF	Open-reading frame
RACE	Rapid amplification of cDNA ends
RT	Reverse transcription
RT-PCR	Reverse transcription-polymerase chain reaction
SPME	Solid phase micro-extraction
TPS	Terpene synthase

Introduction

Higher plants possess species-specific floral scents. These are a mixture of various volatile compounds of low molecular weight (30–300 Da), such as terpenes (monoterpenes and sesquiterpenes), aliphatics, benzenoids, and phenylpropanoids (Knudsen and Gershenzon 2006; Knudsen et al. 2006). These compounds mediate plant–insect communications, and in insect–pollinated flowers, terpenes provide an invitation for butterflies, moths, and bees (Dobson 2006). Terpenes, of which there are more than 55,000 known, are a series of compounds composed of C_5 isoprene units, initially biosynthesized from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Jackson et al. 2014; Köksal et al. 2011; Thulasiram et al. 2007). IPP and DMAPP are supplied by two independent pathways, the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway. The MVA pathway exists in the cytosol of eukaryotes and in some of actinobacteria and archaea, whereas the MEP pathway is present in prokaryotes and the chloroplast of plants (Wu et al. 2006; Kirby and Keasling 2009). DMAPP is condensed with IPP to yield geranyl diphosphate (GPP; C_{10}), which are then converted to farnesyl diphosphate (FPP; C_{15}) with IPP, by prenyltransferases. Terpene synthases (TPSs; also called terpene cyclases) release diphosphate from GPP and FPP, and the remaining carbonated forms are intra-molecularly cyclized in almost all cases, yielding a huge variety of monoterpenes and sesquiterpenes, respectively (Misawa 2011). Genome sequence analyses have predicted numerous *Tps* genes in grape (*Vitis vinifera*), poplar (*Populus trichocarpa*), rice (*Oryza sativa*), and *Arabidopsis thaliana* amounting to 69, 49, 40, and 32 *Tps* sequences, respectively. The functions of these *Tps* genes have been characterized to some extent (Martin et al. 2010). Although *A. thaliana* has long been considered devoid of volatile terpenes (Ginglinger et al. 2013), 24 monoterpenes and 26 sesquiterpenes were found to be emitted from its inflorescences and other aerial parts (Rohloff and Bones 2005), and cytochrome P450 isoforms were revealed to metabolize volatile terpenes (Ginglinger et al. 2013).

Camellia plants grow spontaneously in Japan, China, Vietnam, and other Asian countries (Jullien et al. 2008; Li et al. 2013; Tanikawa et al. 2013). *Camellia japonica*, called 'Tsubaki' in Japan, is present from Okinawa to Aomori prefectures (Japan Camellia Soc 2010). *C. japonica* is not only a typical ornamental plant, but it is also a practical plant in traditional horticulture. Its breeding for ornamental purposes has long been performed (Shibata et al. 2004), and a large number of varieties exhibiting different flower shapes and colors are now available. It has long been planted as a hedge or shelter belt, or for firewood. Oil from the seeds of *C. japonica* has been used for hair dressing, cooking oil, and fuel. Recently, a new sort of tea was produced from *C. japonica* leaves from Gotoh island, Nagasaki prefecture (Tamaru et al. 2013), as a health product while the authentic tea plant is taxonomically *Camellia sinensis*. Most flowers of *Camellia*, such as those of *C. japonica*, are poorly fragrant (Jullien et al. 2008). Hence, some *Camellia* species that emit fragrance abundantly are known as 'Kaori Tsubaki' in Japanese, meaning scented Camellia. *C. lutchuensis* emits the strongest fragrance in the genus *Camellia*, and it has been crossed with other *Camellia* species to produce various fragrant *Camellia* plants (Oyama-Okubo et al. 2009). The floral scent of *Camellia* has been analyzed in a limited number of studies. Omata et al. (1989) investigated 63 *Camellia* species, from many of which they detected monoterpenes, linalool, and linalool oxide by gas chromatography–mass spectrometry (GC–MS) and gas chromatography–infrared spectrometry analysis. Jullien et al. (2008) analyzed the floral scents emitted from each floral organ of six fragrant *Camellia* species, and they detected the highest concentration of volatiles in the stamens.

We have studied terpene synthases that synthesize fragrant terpene volatiles in edible plants belonging to the *Zingiberaceae* family (Hattan and Misawa 2015), e.g., several *Tps* genes were isolated from ginger (*Zingiber officinale*) and shampoo ginger (*Z. zerumbet*) (Fujisawa et al. 2010; Yu et al. 2008a, b), and their functions were characterized. Koo and Gang (2012) isolated other *Tps* genes from ginger and turmeric (*Curcuma longa*), and functionally characterized them by GC–MS. Here, we analyzed fragrant terpenes from several *Camellia* flowers, including *Camellia brevistyla*, which emits strong fragrance, and isolated *Tps* genes from the flowers of *C. brevistyla* and another scented Camellia *C. saluenensis*

Fig. 1 Flowers of scented Camellia, *Camellia brevistyla* (**a**) and *Camellia saluenensis* 'Inokuchi-Kaori' (**b**), photographed by Mr. Seizoh Matsui

(Fig. 1), and characterized the function of the TPS gene products with our original functional analysis system using an MVA-pathway-engineered *E. coli* strain that can utilize lithium acetoacetate as a substrate (Harada and Misawa 2009).

Materials and methods

Plant materials

The *Camellia* genus belongs to the Theaceae family. Two Camellia cultivars, 'Koto-no-kaori' (*Camellia japonica* × *C. lutchuensis*) and 'Momoiro-bokuhan' (*C. japonica*), were purchased at the Camellia Festival of Nonoichi-shi. Branches with several leaves and flowers of *C. brevistyla* and *C. saluenensis* 'Inokuchi-Kaori' (Fig. 1), and of two cultivars, 'Rozuki' (*C. japonica*) and 'Hai Duong' (*C. amplexicaulis*) × 'Kaga-hassaku' (*C. japonica*), were kindly provided by Seizou Matsui. Each organ was separated, frozen in liquid nitrogen, and stored at −80 °C until use.

Gas chromatography (GC)–mass spectrometry (MS) analysis of floral scent compounds

A 7890A gas chromatograph system coupled to a 5975C mass spectrometer detector (Agilent Technologies, CA, USA) was used for floral scent analyses. Sample constituents were separated using a DB-WAX column (60 m, 0.25 mm ID, 0.25 μm Df). Volatile constituents were extracted using a solid phase micro-extraction (SPME) fiber (50/30 μm divinylbenzene/carboxene/polydimethylsiloxane) attached to an auto-sampler, MPS2 (GERSTEL, Mülheim an der Ruhr, Germany), at 80 °C for 40 min (10 min preheating, 30 min extraction). Then, the SPME fiber was transferred into the GC injection room, and the sample was injected at 250 °C. The GC oven was programmed to start at 40 °C, hold for 10 min, then ramp at 5 °C/min to 220 °C, and hold for 12 min.

Isolation of terpene synthase cDNAs from *C. brevistyla* and *C. saluenensis*

Flowers from *C. brevistyla* and *C. saluenensis* were frozen in liquid nitrogen and ground in a mortar. Total RNA was extracted from the frozen powder using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany) with RNase-Free DNase set (Qiagen) to remove genomic DNA. A cDNA library was synthesized from 1.0 μg of the total RNA with reverse transcription (RT) reaction using a Clontech SMARTer RACE cDNA amplification kit (Takara Bio, Shiga, Japan) in accordance with the manufacturer's instructions. To amplify *Tps* gene fragments, PCR was performed using two degenerate primers (5′-TTYCGAY-TIYTIMGRMARCAIGG-3′ and 5′-TAIGHRTCAWAIR TRTCRTC-3′; Yu et al. 2008a). PCR conditions were the same as described by Yu et al. (2008a). PCR products were cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA), and sequenced using BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI3130 sequencer (Applied Biosystems).

Cloning of cDNAs for full-length terpene synthase genes

The 3′ end of a *C. brevistyla Tps* cDNA was isolated by rapid amplification of cDNA ends (RACE) using 2.5 μl of cDNA template. A primer (5′-TTTAGACAAACAGCTAT GGGACAA-3′) was designed from the cDNA fragment cloned above. PCR conditions were as follows: 94 °C for 2 min, five cycles of three steps (94 °C for 30 s, 63.6 °C for 30 s, and 72 °C for 90 s), and 30 cycles of three steps (94 °C for 30 s, 58.6 °C for 30 s, and 72 °C for 90 s). The PCR products were cloned into a pGEM-T Easy vector and sequenced. A cDNA fragment containing the entire *Tps*

open reading frame (ORF) [from initial codon (ATG) to terminal codon (TGA)] was then amplified by PCR using Clontech Advantage-2 polymerase (Takara Bio) with primers (5′-AGACAGAAGATCTCATGGCTTCATCTCA AGTTGGTGA-3′ and 5′-GAGGTACCTCGAGTCACA TGGGAATTGGATCTTCGA-3′) in accordance with the manufacturer's instructions. These primers included *Bgl*II and *Xho*I restriction endonuclease recognition sites (underlined) upstream of the initial codon and downstream of the terminal codon of the gene, respectively. PCR conditions were as follows: 94 °C for 2 min, five cycles of three steps (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min), and 30 cycles of three steps (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min).

The 5′ and 3′ ends of a *C. saluenensis Tps* were obtained using SMARTer RACE cDNA amplification kit and Clontech Advantage-2 polymerase with primers (5′-TCG AGCTTGATGATGAGAAAGATG-3′ and 5′-TCAC AAAACCCATCTCTTTCATCT-3′). Then, a cDNA fragment containing the entire *C. saluenensis Tps* ORF was isolated using the Advantage-2 polymerase and the cDNA template with primers (5′-AGACAGACATATGCAAAT CTTCCACTGTGCAT-3′ and 5′-GGTACCCTAAGGTA ATTTATCATAGAACATAGACTTCATG-3′). These primers included *Nde*I and *Kpn*I restriction endonuclease recognition sites (underlined). PCR conditions were as follows: 94 °C for 2 min, five cycles of three steps (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 4 min), and 25 cycles of three steps (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 4 min).

The PCR products were cloned into a pGEM-T Easy vector, and their sequences were confirmed. Other molecular experiments were carried out in accordance with to Sambrook and Russell (2001).

Phylogenetic analysis

Sequence similarity of the *Tps* genes was analyzed as described previously (Fujisawa et al. 2010). Multiple alignment of plant TPSs was carried out using BioEdit free software (Hall 1999). Phylogenetic analysis of these TPSs was carried out using DNASIS Pro (Hitachi Solutions, Ltd. Tokyo, Japan). Deduced amino acid sequences of known TPSs were obtained from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Known plant sesquiterpene synthases and their accession numbers were as follows: grape (*V. vinifera*) (*E*)-α-bergamotene synthase (VvABS), accession no. NP_001267972; (*E*)-β-caryophyllene synthase (VvBCaS), ADR74193; β-curcumene synthase (VvBCuS), ADR74200; (−)-germacrene D synthase (VvGDS), Q6Q3H3; kiwi fruit (*Actinidia deliciosa*) germacrene D synthase (AdGDS), AAX16121; shampoo ginger (*Z. zerumbet*) α-humulene synthase (ZzZSS1), BAG12020; β-eudesmol synthase (ZzZSS2), BAG12021; ginger (*Z. officinale*) germacrene D synthase (ZoGDS), AAX40665; (*S*)-β-bisabolene synthase (ZoTPS1), BAI67934; maize (*Zea mays*) terpene synthases (ZmTPS4, AAS88571; ZmTPS5, AAS88574; ZmTPS6, AAS88576; ZmTPS10, AAX99146; ZmTPS11, ACF58240); lemon basil (*Ocimum basilicum*) α-zingiberene synthase (ObZIS), AAV63788; grand fir (*Abies grandis*) δ-selinene synthase (AgAG4), AAC05727; γ-humulene synthase (AgAG5), AAC05728; western balsam poplar (*P. trichocarpa*) terpene synthases (PtTPS5, KF776503; PtTPS7, KF776505; PtTPS8, KF776506; PtTPS9, KF776507; PtTPS11, KF776509; PtTPS14, KF776512); sandalwood (*Santalum album*) sesquiterpene synthase (SaSesquiTPS), ACF24768; (*S. austrocaledonicum*) sesquiterpene synthase (SauSesquiTPS), HQ343281; (*S. murrayanum*) sesquiterpene synthase (SmSesquiTPS), JF746810; (*S. spicatum*) sesquiterpene synthase (SspiSesquiTPS), HQ343282.

GC–MS analysis of terpene products with mevalonate-pathway-engineered *E. coli*

The entire *Tps* ORFs from *C. brevistyla* and *C. saluenensis* were inserted into the *Bgl*II–*Xho*I and *Nde*I–*Kpn*I sites of the *E. coli* expression vector pRSFDuet-1 [Merck (formerly Novagen), Darmstadt, Germany], yielding the desired plasmids, named pRSF-CbTps1 and pRSF-CsTps1, respectively. Each plasmid was introduced into *E. coli* BL21-CodonPlus (DE3) cells (Stratagene, La Jolla, CA, USA) together with plasmid pAC-Mev/Scidi/Aacl (Harada et al. 2009), which contains the MVA-pathway gene cluster from *Streptomyces* sp. strain CL190, the *Saccharomyces cerevisiae* IPP isomerase gene (*Scidi*), and the acetoacetate-CoA ligase gene (*Aacl*) for the utilization of Li acetoacetate (LAA) as the substrate. Recombinant *E. coli* cells were cultured in TB medium (Sambrook and Russell 2001) containing kanamycin (Km; 50 μg/ml) and chloramphenicol (Cm; 30 μg/ml) at 18 °C with the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 1 mg/ml LAA (Tokyo Kasei, Tokyo, Japan). The culture medium was centrifuged briefly, and the *E. coli* cell pellet was extracted with ethyl acetate. A terpene product in the extract was concentrated about fivefold by decompression, and analyzed using a GC–MS (Shimadzu, GCMS-QP5050) as described previously (Harada et al. 2009). An authentic sample of elemol was provided by Takasago International Corporation.

Cultivation of recombinant *E. coli*, purification and spectroscopic analyses of a CbTPS1 product

The *E. coli* cells transformed with plasmids pRSF-CbTps1 and pAC-Mev/Scidi/Aacl were grown in LB medium (Sambrook and Russell 2001) containing Km and Cm at 25 °C with reciprocal shaking for 7–8 h until the absorbance at OD 600 nm had reached approximately 1.0. One milliliter of this culture was inoculated into 100 ml of TB medium (Sambrook and Russell 2001) in a Sakaguchi flask containing 50 μg/ml Km, 30 μg/ml Cm, and 1 mg/ml LAA, and cultured at 37 °C with rotary shaking (180 rpm) for 3–4 h until the absorbance at OD 600 nm reached approximately 1.0. After cooling the flask to 20 °C, 25 μM IPTG (final concentration) and 20 ml *n*-octane were added to each flask, and cultured at 20 °C with rotary shaking (180 rpm) for 2 days.

The contents of 10 flasks [1 L culture and *n*-octane layer (200 ml)] was added to *n*-hexane (200 ml), and partitioned between alkane (*n*-octane + *n*-hexane) and H_2O. The alkane layer was washed with 400 ml of 50 % alkaline MeOH [MeOH (150 ml) + 0.1 N KOH (150 ml)] twice, and concentrated to a small volume (1.0 ml). The concentrated alkane layer (each 0.1 ml) was subjected to preparative ODS high-performance liquid chromatography (HPLC) developed using the following conditions (column: C30-UG-5 (Developsil) 20 mm × 250 mm, solvent: 90 % CH_3CN, flow rate: 8.0 ml/min, detect 212 nm). A peak that eluted at 15.0 min was collected (100 ml) and an equal volume of water added to achieve 45 % CH_3CN solution (200 ml), which was partitioned between *n*-pentane (200 ml) and 45 % CH_3CN. The *n*-pentane layer was dried over Na_2SO_4 for 30 min and concentrated in vacuo to afford a pure terpene product (hedycaryol 13.2 mg). This product was subjected to ^{1}H-nuclear magnetic resonance spectrometry (^{1}H NMR; 400 MHz, Bruker AMX400) and GC–MS analyses.

Spectroscopic data of elemol

GC–MS retention time 36.9 min, *m/z* 222 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 0.98 (s, 3H, H-13), 1.20 (s, 6H, H-8 and H-9), 1.38 (m, 1H, H-1), 1.40–1.50 (2H, H-6), 1.44 (m, 1H, H-2), 1.59 (m, 1H, H-2), 1.60–1.70 (2H, H-5), 1.71 (s, 3H, H-12), 1.96 (dd, J = 3.3. 12.4 Hz, H-3), 4.59 (s, 1H, H-11), 4.82 (s, 1H, H-11), 4.89 (d, J = 11.8 Hz, 1H, H-15), 4.90 (d, J = 16.5 Hz, 1H, H-15), 5.80 (dd, J = 11.8, 16.5 Hz, 1H, H-14). ^{13}C-NMR ($CDCl_3$) δ: 16.5 (C-13), 22.5 (C-6), 24.8 (C-12), 27.1 (C-8 and C-9), 28.4 (C-2), 39.7 (C-4), 39.8 (C-5), 49.3 (C-1), 52.6 (C-3), 72.4 (C-7), 109.9 (C-15), 112.0 (C-11), 147.9 (C-10), 150.2 (C-14).

Expression analysis of *CbTps1*

Total RNA extraction from *C. brevistyla* leaves and branches and subsequent cDNA synthesis were performed as described above. PCR conditions were as follows: 94 °C for 2 min, 35 cycles of three steps (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 24 s). The primers used for *CbTps1* expression analysis were as follows: 5′-TTGCTAAGTTT GAAGACAATGAGG-3′ and 5′-AGAGTTTCATTCCTA GCAGCATCT-3′. A partial fragment of a *C. brevistyla* actin-like gene was also amplified by RT-PCR as a control. The primers used were as follows: 5′-GCCAAAAAGAT GCATATGTTGGAGAC-3′ and 5′-TGAAGGAGTAGCC ACGTTCC-3′. These actin-like gene-specific primers were designed based on the sequence of the cloned fragment of the actin-like gene, which was amplified using degenerate RT-PCR. Primers used were as follows: 5′-AATGGWA CTGGAATGGTBAAGGCTGG-3′ and 5′-TTAGAAGCA YTTYCTGTGVACAATGSM-3′. These degenerate primers were designed based on the conserved amino acid and mRNA sequences observed in an alignment of several plant actin sequences. The accession numbers of actin mRNAs used for the alignment were as follows: *A. thaliana* ACT8 (NM_103814), *Brassica napus* actin2.1 (FJ529167), *Brachypodium distachyon* actin-1-like (XM_003560541), *Cymbidium sinense* actin-like (GU181353), *Glycyrrhiza uralensis* actin 2 (GQ404511), *Gossypium hirsutum* actin 15 (AY305736), *Malus* × *domestica* actin (AB638619), *Pisum sativum* actin 14 (U76193), *Sorghum bicolor* actin-11-like (XM_002441128).

Accession numbers of *CbTps1* and *CsTps1*

The nucleotide sequences reported in this paper were submitted to DDBJ under the accession numbers LC070683 (*CbTps1*) and LC096084 (*CsTps1*).

Results

GC–MS analysis of Camellia floral scent

Omata et al. (1989) demonstrated the existence of linalool and linalool oxide in the floral scents of various *C. japonica* plants. Jullien et al. (2008) examined six fragrant *Camellia* species and revealed that several sesquiterpenes and monoterpenes other than linalool were synthesized in specific *Camellia* species. Here, we performed qualitative analysis of the floral scents of five *Camellia* cultivars by GC–MS (Table 1). *Camellia brevistyla* (Fig. 1a) and

Table 1 Terpenes and their ratio in the volatile compounds trapped by solid phase micro-extraction from the flowers of six *Camellia* cultivars

Terpene	*C. brevistyla*	'Inokuchi-Kaori' *C. saluenensis*	'Rozuki' *C. japonica*	'Momoiro-bokuhan' *C. japonica*	'Koto-no-kaori' *C. lutchuensis* × *C. japonica*	'Hai Duong' × 'Kaga-hassaku' *C. amplexicaulis* × *C. japonica*
Trans-Linalool oxide		23.89	0.57		2.54	0.48
Linalool	0.48	21.94	11.3	21.86	16.91	25.9
β-Elemene	0.43					
Neral						
Terpineol		1.91	0.22	3.04	1.41	1.08
Borneol		2.01	0.18		1.04	
E-Citral			0.15			
α-Bisabolene	25.06					
Epoxylinalool		9.89	0.91		1.22	1.35
Myrtenol		1.01			0.98	
Geraniol	0.84	0.35	3.14	11.22	1.31	1.73
(1*S*)-*cis*-Calamenene						0.26
Nerolidol		0.31				
Elemol	0.48					
γ-Eudesmol	1.56					
Agaruspirol	0.11					
α-Eudesmol	1.54					
β-Eudesmol	1.92					
Farnesol	0.34	0.12				
Total	32.76	61.43	16.61	36.12	25.41	30.8

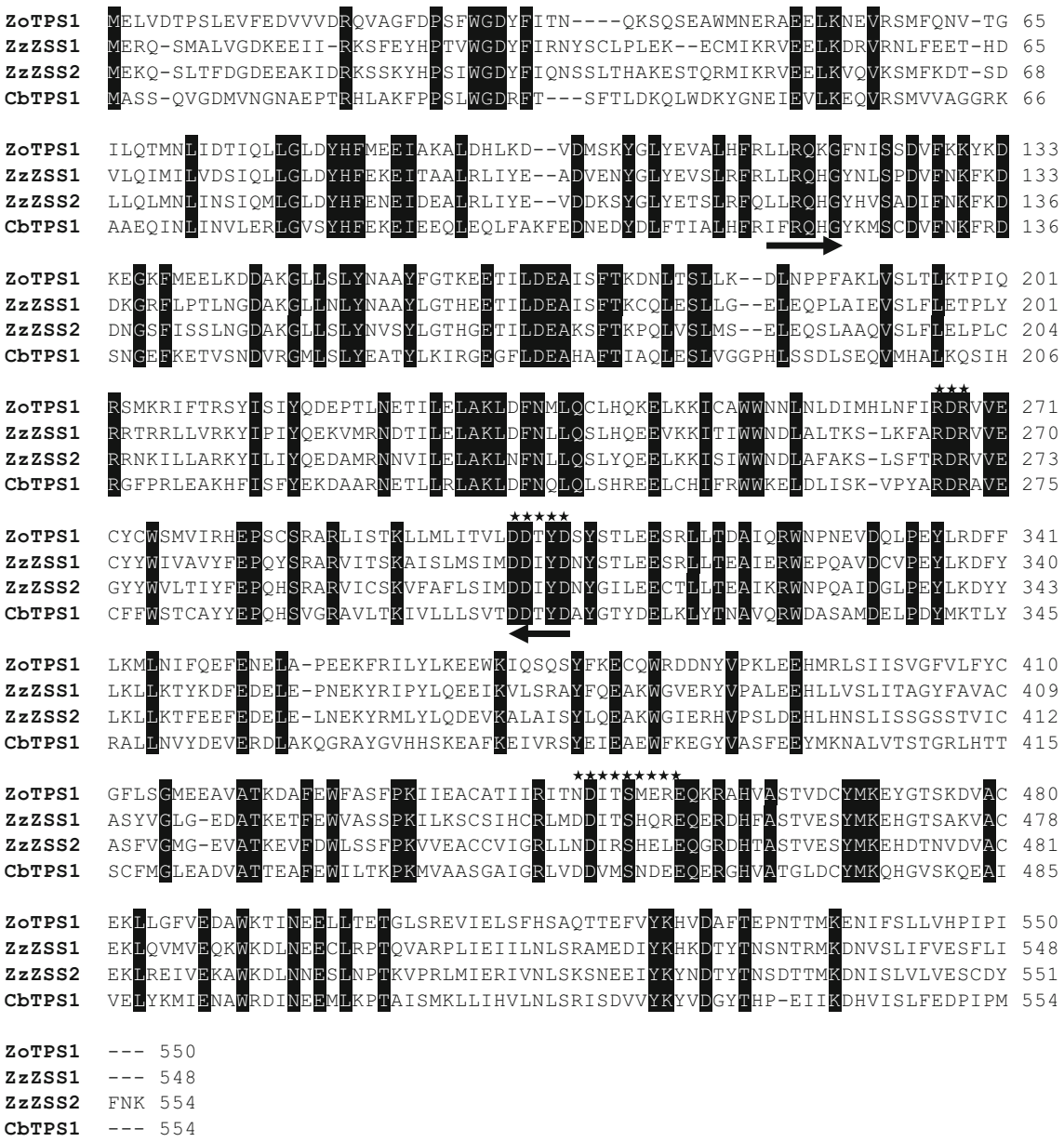


Fig. 2 Amino acid alignment of sesquiterpene synthases derived from *Zingiber* plants and *Camellia brevistyla* terpene synthase 1 (CbTPS1). The deduced amino acid sequences of the *Zingiber* terpene synthases (TPSs), isolated by our group, together with CbTPS1 were aligned. The TPS conserved motifs RDR, DDxxD, and NSE/DTE [(N/D)Dxx(S/T)xxxE] are indicated by *asterisks*. Amino acids conserved in all TPSs are shown in *black boxes*. *Arrows* indicate the sequences corresponding to the degenerate primers. ZoTPS1, *Zingiber officinale* β-bisabolene synthase (Fujisawa et al. 2010); ZzZSS1, *Z. zerumbet* α-humulene synthase (Yu et al. 2008b); ZzZSS2, *Z. zerumbet* β-eudesmol synthase (Yu et al. 2008a)

'Koto-no-kaori' were selected as representatives of fragrant *Camellia* plants. 'Rozuki', 'Momoiro-bokuhan', and 'Hai Duong' × 'Kaga-hassaku' were selected as representatives of poorly fragrant *Camellia* plants. As expected, monoterpenes and sesquiterpenes were detected from the floral scents of the two fragrant *Camellia*s, and these volatile terpenes accounted for more than 30 % of the total floral scent compounds. Monoterpenes, such as linalool and geraniol, were also detected from the three poorly fragrant *Camellia*s. The floral scents of *C. japonica* cultivars contained linalool as the main terpene, which agreed with the results of Omata et al. (1989). The floral scent of *C. brevistyla* contained α-bisabolene and other sesquiterpenes, including elemol.

Isolation of *CbTps1* and *CsTps1* from *Camellia* flowers

First of all, we selected *C. brevistyla* as the source for *Tps* isolation, because the above GC–MS analysis showed that *C. brevistyla* flowers included additional sesquiterpene compounds different from those of *C. japonica* and its cross-lines. Degenerate primers were designed based on the nucleotide sequences deduced from the highly conserved amino acid motifs in known plant TPSs. For example, aspartic acid (D)-rich motifs, which interact with Mg^{2+} required for the TPS enzymatic reaction, are conserved across many plant species (Martin et al. 2010; Gennadios et al. 2009). Such motifs were used for the design of the degenerate primers (Yu et al. 2008a). A partial *Tps* cDNA fragment including its 5′-end was obtained by degenerate RT-PCR, using cDNAs prepared from a *C. brevistyla* flower as the template. Subsequently, a full-length *Tps* sequence was acquired by performing 3′-RACE. As a result, a full-length *Tps* gene, designated *CbTps1*, was isolated, and the protein encoded by the cDNA was called CbTPS1. Figure 2 shows the alignment of CbTPS1 and other TPS amino acid sequences isolated by our group. CbTPS1 consisted of 554 amino acids (aa) with a calculated molecular mass of 64.0 kDa. D-rich motifs mentioned above, such as the RDR, DDxxD, and NSE/DTE motifs, are almost conserved in CbTPS1. In general, sesquiterpene synthases are smaller than monoterpene synthases by 50–70 aa. This difference is due to the lack of the N-terminal transit peptide, which is required for plastidial targeting of monoterpene synthases (Bohlmann et al. 1998). Actually, iPSORT (http://ipsort.hgc.jp/) predicted that no transit peptide exists at the N terminus of the CbTPS1 protein.

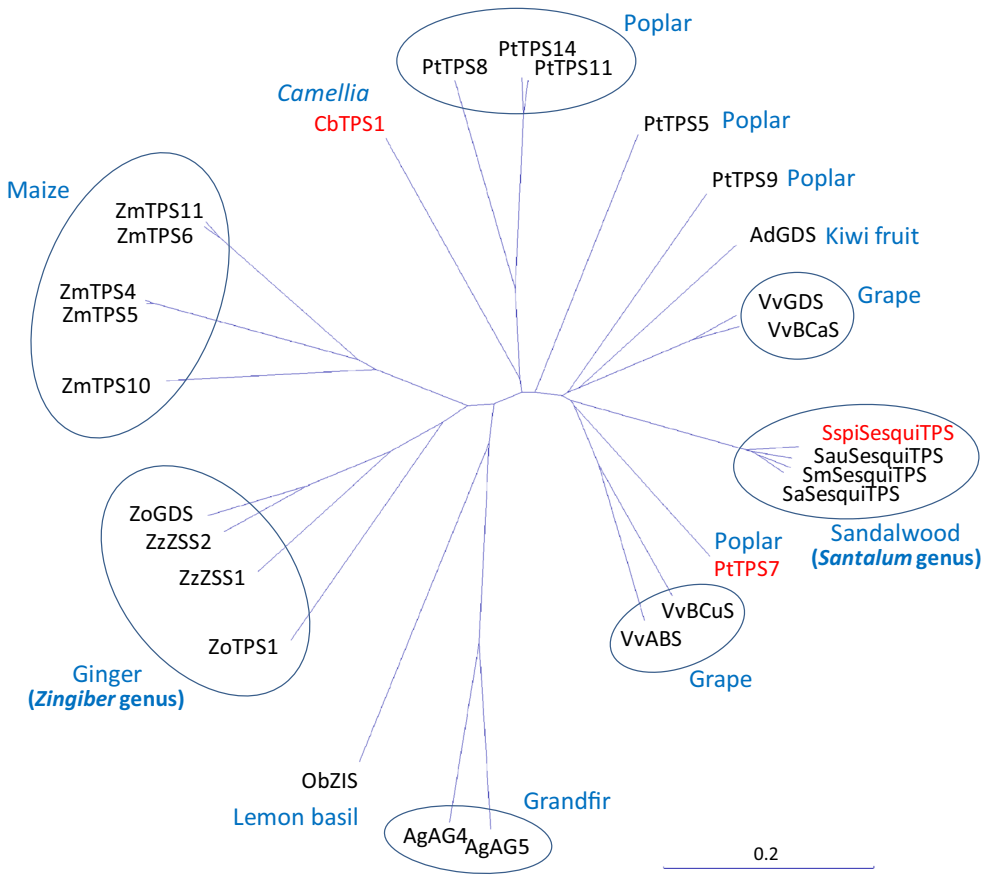


Fig. 3 Phylogenetic tree of plant sesquiterpene synthases. CbTPS1 and known sesquiterpene synthases (TPSs) were analyzed phylogenetically. TPS accession numbers are as follows: ZmTPS4 (AAS88571), ZmTPS5 (AAS88574), ZmTPS6 (AAS88576), ZmTPS10 (AAX99146), ZmTPS11 (ACF58240), ObZIS (AAV63788), AgAG4 (AAC05727), 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; *red texts* indicate TPSs that can synthesize hedycaryol. TPSs from ginger and turmeric, which were reported by Koo and Gang (2012), are not shown in this figure, because their accession numbers were not available

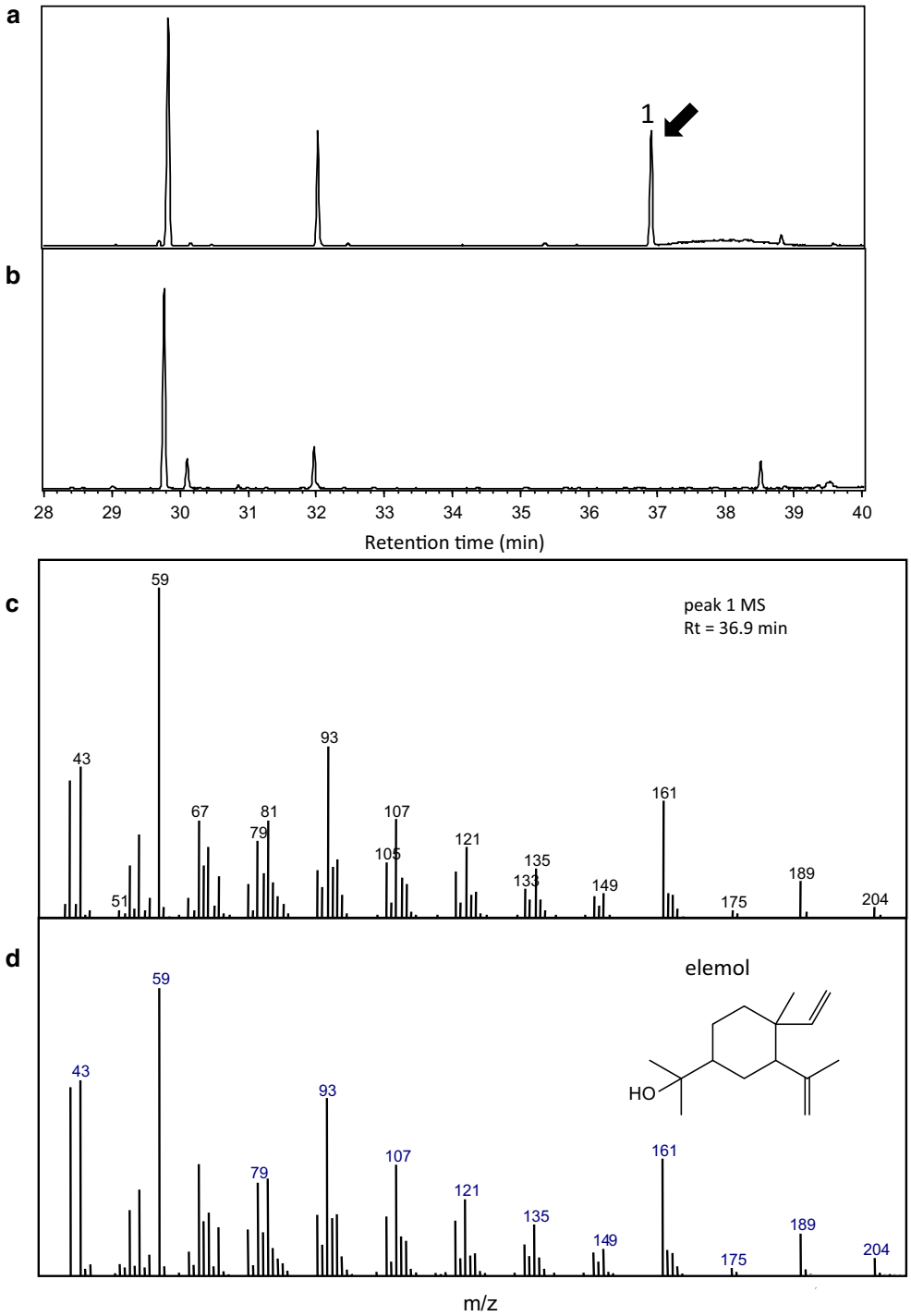
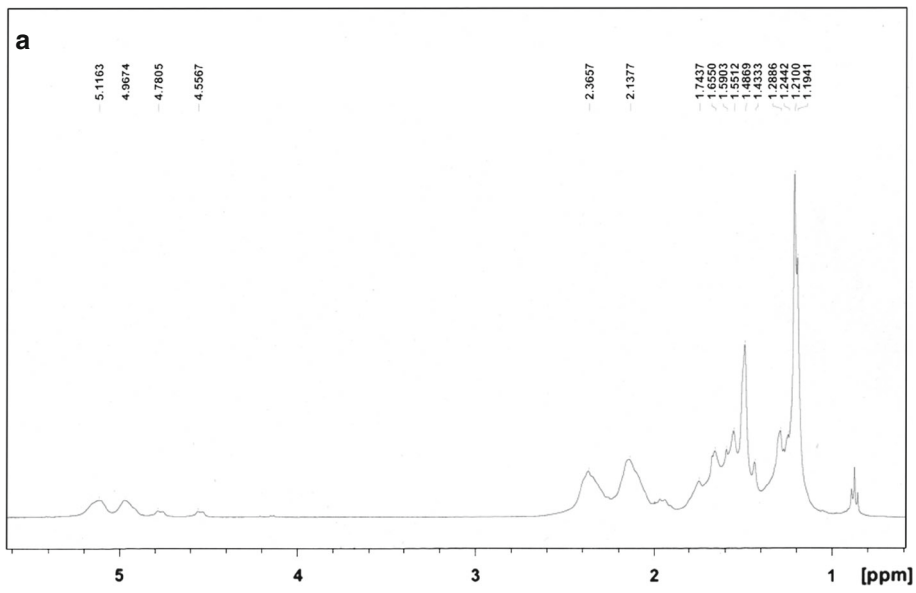


Fig. 4 Gas chromatography–mass spectrometry (GC–MS) analysis of the CbTPS1 product. The CbTPS1 product extracted from *E. coli* (pRSF-CbTps1 and pAC-Mev/Scidi/Aacl) was subjected to GC–MS. Total ion chromatograms of the CbTPS1 product (**a**) and of empty vector product (control) (**b**), MS of peak 1 in a (**c**), and MS of elemol in the database (**d**)

Next, we isolated a Tps gene, named *CsTps1*, from another scented Camellia *C. saluenensis* (Fig. 1b). CsTPS1 consisted of 575 aa with a calculated molecular mass of 66.1 kDa and pI 6.03, and was found to belong to the monoterpene synthase group, TPS-g subfamily, which lacks RRx8W motif and possesses a transit peptide sequence for chloroplasts at the N terminus (Supplementary Fig. 1) (Dudareva et al. 2003).



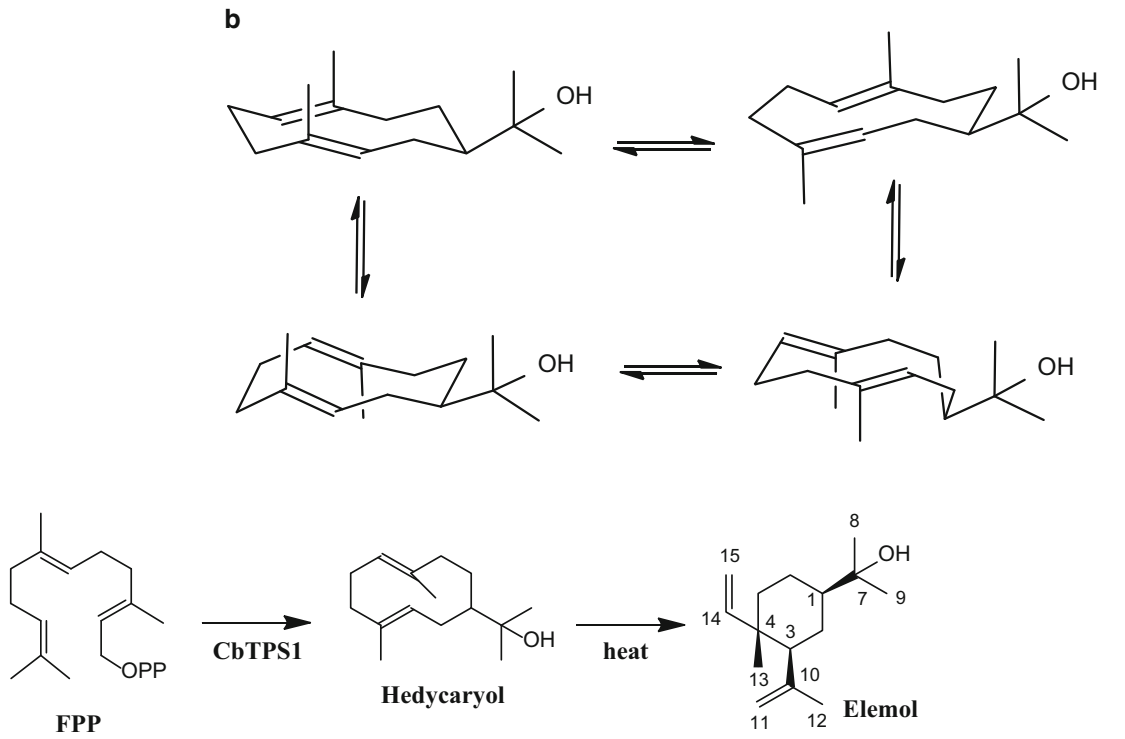


Fig. 5 ^{1}H-nuclear magnetic resonance spectroscopy (^{1}H NMR) spectrum of hedycaryol (**a**) and plausible conformations of hedycaryol (**b**)

Fig. 6 Structures of synthesized sesquiterpenes and the catalytic function of CbTPS1. Hedycaryol was the direct product with CbTPS1, which was transformed to elemol by heat during GC–MS analysis. *FPP* farnesyl diphosphate

Phylogenetic analysis of CbTPS1 and CsTPS1

The CbTPS1 protein was analyzed phylogenetically by comparing with known plant TPSs registered in the NCBI database (Fig. 3). As a result, CbTPS1 was phylogenetically positioned as a sole-gene clade, indicating that *CbTps* was a novel gene. This analysis also showed that TPS proteins derived from the same plant species or family tended to fall into one clade.

The phylogenetic tree of CsTPS1 is shown in Supplementary Fig. 2. This protein exhibited closer identity (71 %) to two known linalool synthases (ApLS and AaLS) from silver vine (*Actinidia polygama*) and hardy kiwi (*Actinidia arguta*), respectively.

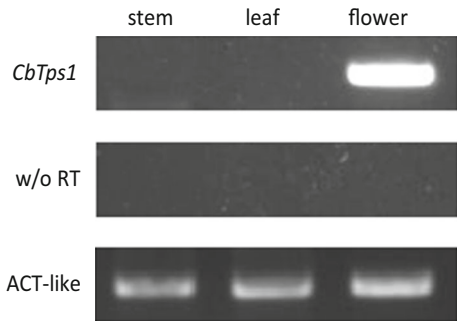


Fig. 7 Expression analysis of the *Camellia brevistyla CbTps1* gene by reverse transcription (RT)-polymerase chain reaction (PCR). The *CbTps1* transcript was amplified in each tissue of *C. brevistyla*. Total RNA treated without reverse transcriptase was used as the template for the 'w/o RT' reaction. An *actin*-like (ACT-like) gene was also amplified as an internal control

Functional characterization of CbTPS1 and CsTPS1 in the mevalonate-pathway-engineered *E. coli*

Figure 4 shows the result of GC–MS analysis of the ethyl acetate extract from *E. coli* co-transformed with plasmids pRSF-CbTps1 and pAC-Mev/Scidi/Aacl. MS (Fig. 4c) of the main peak (Fig. 4a) of this CbTPS1 extract, which was not observed in the control extract from *E. coli* harboring the empty vector pRSFDuet-1 (Fig. 4b), had the highest similarity to elemol in the database of the GC–MS system (Fig. 4d). When the CbTPS1 extract was mixed with an authentic sample of elemol, and analyzed by GC–MS, these peaks were overlapped with the same retention time. This result clearly showed the generation of elemol. Spectral data of elemol are described in the "Materials and methods" section. ^{1}H-NMR signals of the product purified from *E. coli* (pRSF-CbTps1 and pAC-Mev/Scidi/Aacl) were very broad in $CDCl_3$ and not suitable for direct structural analyses. Elemol was reported to be a thermal rearrangement product from hedycaryol (Jones and Sutherland 1968). The ^{1}H-NMR spectrum of the product (Fig. 5a) supported the conformational equilibrium in solution, which was reported for hedycaryol (Osawa et al. 1979). Thus, we identified the compound converted from FPP in recombinant *E. coli* as hedycaryol (Fig. 6). The plausible conformations of hedycaryol (Faraldos et al. 2007) are shown in Fig. 5b. These results indicated that CbTPS1 synthesizes hedycaryol, and *CbTps1* is a hedycaryol synthase gene (Fig. 6). Elemol that was detected in the floral scents of *C. brevistyla* by GC–MS (Table 1) was very likely to have been converted from hedycaryol during the heating-up process of GC–MS.

Escherichia coli (pRSF-CsTps1 and pAC-Mev/Scidi/Aacl) was similarly cultured, extracted, and analyzed by GC–MS. As a result, only one peak generated, which was determined to be linalool by the MS database and by comparison with the authentic sample (Sigma-Aldolich Co.) (Supplementary Fig. 3), indicating that *CsTps1* is a linalool synthase gene.

Expression analysis of *CbTps1*

Figure 7 shows the result of the expression analysis of *CbTps1* in several tissues of *C. brevistyla*. The amount of *CbTps1* transcript in each tissue was compared by RT-PCR using total RNA extracted from a branch, leaf, or flower of *C. brevistyla*. A DNA fragment amplified using 35 PCR cycles, which indicated the expression of *CbTps1*, was observed only in the floral cDNA sample. This result showed that *CbTps1* was expressed specifically in flowers and not expressed in the branches or leaves. Thus, the *CbTps1* gene is likely to be regulated under a flower-specific promoter.

Discussion

Previous reports (Omata et al. 1989; Jullien et al. 2008) and this study (Table 1) showed that only linalool is a common floral scent of the *Camellia* genus. *C. japonica*, which usually emits little floral scent, is distributed at the northern limits of the natural habitats of the *Camellia* genus (Japan Camellia Soc 2010), and it blooms in the cold winter season when few insects are available for pollination. The reason why *C. japonica* flowers scarcely smell could be attributed to lack of benefit of insect pollination. *C. japonica* has long been bred for ornamental purposes in Japan, with its horticultural varieties first established in the 17th–19th centuries during the Edo era (Shibata et al. 2004). Human selection in those days, which appreciated the shape, size, and color of the flowers, and did not show interest in the fragrance, may have diminished the abundance of scent-emitting flowering *Camellia* lines. *C. japonica* is classical flowering plant in winter for Japanese tea ceremonies, where people enjoy the taste and scent of tea. If the flowers emitted a strong fragrance, it could mask the tea scent. Thus, fragrance-less *C. japonica* is likely to have been selected to accompany locations used for the Japanese tea ceremony. It typically takes 5 years for *Camellia* plants to flower after sowing (Shibata et al. 2004). Such a long generation time would make genetic analysis more difficult compared with model plants, such as *A. thaliana* that has a short generation time of 1.5 months. However, *Camellia* plants have several thousand cultivars with various flower shapes and colors (Japan Camellia Soc 2010). Recently, new varieties of transgenic ornamental plants have been produced (Chandler and Sanchez 2012), some of which emitted modified scents (Saxena et al. 2007). Although *C. sinensis* is the sole species for genetic modification in the *Camellia* genus

(Chen et al. 2006), transgenic research on *Camellia* plants, in which the fragrance or other floral properties are modified, could be a highly interesting subject for basic molecular or physiological research.

It has been reported that a single TPS often generated multiple products, and occasionally dozens of products (Ginglinger et al. 2013; Koo and Gang 2012; Yoshikuni et al. 2006). These secondary metabolites, promiscuous products of TPSs, are not indispensable for growth and development (Hartmann 1996; Bohlmann et al. 1998). As a result, TPSs may not show precise enzymatic specificity compared with enzymes involved in primary metabolism. Conversely, some TPSs could be still variable and undergoing evolutionary changes, and as a result they may synthesize multiple fragrant compounds. If such TPS duplicates and increases in number, and each one accumulates mutations in response to environmental changes, it could be beneficial for various interactions between plants and their environment, such as repelling herbivores, protecting from pathogenic microorganisms, and inviting pollinators (Hartmann 1996; Bohlmann et al. 1998). In this context, CbTPS1 and CsTPS1 obtained from *Camellia*, which generated one predominant terpene, appear to be unusual TPS enzymes. In fact, almost all linalool synthases that were previously analyzed indicated that they have neloridol synthase activity simultaneously (Koo and Gang 2012).

Terpene synthases that seem to synthesize hedycaryol were found in ginger (Koo and Gang 2012), sandalwood (*S. spicatum*) (Jones et al. 2011), and poplar (*P. trichocarpa*) (Irmisch et al. 2014), because elemol was detected by GC–MS analysis of their enzymatic-reaction products. Among them, only poplar TPS (PtTPS7) was able to produce hedycaryol predominantly, which is the same with CbTPS1. CbTPS1 and PtTPS7, although they are phylogenetically distant (Fig. 3), possess the same catalytic function. It is, therefore, considered that these two TPSs were generated as a result of convergent evolution. It is also interesting that TPSs derived from the same genus or the same species tended to fall into the same clade, as observed in those from the *Zingiber* genus, the *Santalum* genus, maize, and grand fir.

CbTps1 expression analysis showed that the *C. japonica CbTps1* gene was specifically expressed in flowers (Fig. 7). This result suggested that the floral scent of *C. brevistyla* could invite pollinators to the flowers. The southeast area of China, where the air temperature is relatively high and insects are still alive in winter, is the natural habitat of *C. brevistyla* (http://www.gene.affrc.go.jp/databases-plant_images.php#flower). Hence, the floral scent of *C. brevistyla* could have a selective advantage for insect-mediated pollination. Hedycaryol is likely to be one of the terpenes that attract insects for pollination to *Camellia* species.

The physiological functions of hedycaryol for human health have not been reported. On the other hand, elemol, which was converted from hedycaryol, was reported to have anti-ulcer activity (Matsunaga et al. 2000), and to be one of the components of a plant essential oils effective for dementia care (Miyazawa et al. 1998). *E. coli* is generally employed as a microbial host for the production of valuable industrial compounds (Martin et al. 2003; Kirby and Keasling 2009; Melillo et al. 2013; George et al. 2015). Currently, we are planning a scheme for the efficient production of elemol as well as hedycaryol using MVA-pathway-engineered *E. coli* to produce sufficient quantities for studies for their physiological activities. Finding novel or unknown activities may lead to the development of new medicines, functional foods, or food ingredients.

Fragrances emitted from flowers attract insects and fascinate many people together with floral shapes and colors. Despite these important characteristics, until now approximately only 0.25 % of angiosperms have been studied with regard to the chemical characteristics of their floral scents (Knudsen and Gershenzon 2006). Hence, research on the fragrance of various plant flowers could identify many novel scent compounds, and these compounds may have physiological activities useful for maintaining human health and to cure diseases (Tsuneki et al. 2005; Gertsch et al. 2008; Yao et al. 2008). Studies on plant fragrance continue to intrigue.

Author contribution statement NM designed research. JI, AK and NM supervised research. JH, KS, TI, YS, AW, CT, FO and TS performed experiments. JH and KS analyzed chromatographic data. JH, KS and NM wrote the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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