cDNA Cloning and Functional Analyses of Ashitaba (*Angelica keiskei*) Sesquiterpene Synthase Genes

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Abstract: *Angelica keiskei* (ashitaba) is an edible plant belonging to the Apiacea family. We focused on sesquiterpenes in the leaves eaten by humans (specifically, in the Japanese population), and confirmed the presence of several sesquiterpenes by GC-MS. Thus, total RNA was extracted from the ashitaba leaves, reverse transcribed, and the resultant cDNAs were used for degenerate PCR followed by rapid amplification of cDNA ends. Consequently, we were able to isolate two full-length *Tps* genes (designated *AkTps1* and *AkTps2*). Functional analysis of these two genes was carried out with *Escherichia coli* cells that expressed mevalonate pathway genes to increase the substrate (farnesyl diphosphate) amount of sesquiterpene synthase, revealing that *AkTps1* encodes germacrene D synthase, and *AkTps2* codes for an enzyme that catalyzes the generation of germacrene B and smaller amounts of germacrene D (a germacrene B and D synthase). We proposed biosynthetic routes of these two sesquiterpenes from farnesyl diphosphate (FPP) via farnesyl cation.

Key words: sesquiterpene, terpene synthase, ashitaba

1 Introduction

Sesquiterpenes, which are major components of floral scents, are C₁₅ members of the terpenoid family that are composed of three isoprene (C₅) units. More than 7000 sesquiterpenes have been identified from higher plants and other organisms^{1,2}. It is well known that sesquiterpenes possess various pharmaceutical functions, as well as flavoring effects, *e.g.*, artemisinin from *Artemisia annua* has anti-malarial activity³, zerumbone from *Zingiber zerumbet* has antitumor activity⁴, and nerolidol, farnesol, bisabolol, and apritone enhance bacterial permeability and susceptibility to exogenous antimicrobial compounds⁵. These findings suggest that sesquiterpenes are important compounds for human life and health.

Angelica keiskei (ashitaba) is an edible plant belonging to family Apiacea, which is native to the Pacific coast of Japan⁶. Humans ingest the boiled, baked, or fried leaves and stems of ashitaba, which possess a bitter taste and specific flavor. In addition, the extract of this plant has been reported to exhibit anti-inflammatory, antiobesity, antioxidant, anticoagulant, antitumor, antimutagenic, antidiabetic, antibacterial, and hepato-protective activities⁷. In addition, several bioactive compounds have been isolated

from ashitaba, *e.g.*, chalcones in the roots, which function as antibacterial, antidiabetic, and antiplatelet compounds^{8,9}, and ashitabaol A in the seeds, which functions as an antioxidant⁹. However, except for ashitabaol A, no studies have reported the formation of sesquiterpenes in ashitaba. On the other hand, other Apiacea family plants have been reported to produce many sesquiterpenes, including epikuzeanol, δ -cadinene, (*E*)- β -caryophyllene, α -humulene, germacrene D, farnesene, guaiol, and bulnesol¹⁰⁻¹³.

Sesquiterpenes are synthesized from farnesyl diphosphate (FPP) by terpene synthases (TPSs). Harada and Misawa (2009) created a similar process in *Escherichia coli*, establishing an efficient system for synthesis of sesquiterpenes using acetoacetate as the main substrate¹⁴. Using this system, the functions of TPSs from various species have been analyzed and many sesquiterpenes have been synthesized artificially¹⁵. Thus, we hypothesized that this system would be useful for studying ashitaba TPSs. In this study, we used gas chromatography-mass spectrometry (GC-MS) to analyze sesquiterpenes in ashitaba leaves, identified TPS genes from these leaves, and analyzed their enzymatic functions. This is the first study to report sesquiterpenes and TPSs in the edible ashitaba plant.

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2 Materials and Methods

2.1 Plant

Ashitaba was purchased from a commercial dealer (Ouchi Saien, Kanagawa, Japan). The leaves were sampled and immediately frozen in liquid nitrogen. The tissues were stored at -80°C until use.

2.2 GC-MS analyses of ashitaba extract

Five grams of ashitaba leaves were ground into powder in liquid nitrogen and the volatile constituents were extracted with 3 mL methanol. After adding 1.5 mL *n*-hexane, the mixture was shaken by vortex and the upper phase was collected for GC-MS analysis. GC-MS analysis was performed as described by Hattan *et al.*¹⁶⁾.

A Shimadzu GCMS-QP2010 (Shimadzu, Kyoto, Japan) equipped with a DB-5ms capillary column (0.25-mm internal diameter $\times$ 0.25 μm $\times$ 30 m, Agilent Technologies) was used for analysis. Split injections (1 μL) were made at a ratio of 10:1 with an injection room temperature of 260°C . The heating program of the column oven was heating to 40°C (held for 1 min), increasing at $4^{\circ}\text{C}/\text{min}$ up to 120°C , then increasing by $15^{\circ}\text{C}/\text{min}$ up to 260°C (held for 4 min). Mass spectra were monitored in the mass range of m/z 45–250 with an electron voltage at 1.15 kV and an interface temperature at 280°C .

2.3 RNA extraction and cDNA synthesis

Frozen leaves were ground and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Madison, USA). After the concentration of total RNA was determined using a Nano Drop 2000 (Thermo Fisher Scientific, Tokyo, Japan), first strand cDNA was synthesized from 1.0 μg of total RNA using the SMARTer RACE cDNA amplification kit (Takara Bio, Shiga, Japan).

2.4 cDNA cloning of *AkTps1*

In order to amplify *Tps* gene fragments, PCR was performed using *Taq* DNA polymerase (+ dNTPs) Robust buffer (Bioacademia, Osaka, Japan) with two degenerate primers (5'-TT(C/T)CGA(C/T)TI(C/T)TI(A/C)G(A/G)(A/C)A(A/G)CAIGG-3' and 5'-TAIG(A/C/T)(A/G)TCA(A/T)AI(A/G)T(A/G)TC(A/G)TC-3'), which were successfully used for cloning *Tps* gene fragments from *Zingiber zerumbet* Smith¹⁷⁾. The PCR condition were as follows: 94°C for 3 min, 5 cycles of three steps (94°C for 30 s, 35°C for 60 s and 72°C for 60 s) and 30 cycles of three steps (94°C for 30 s, 40°C for 60 s and 72°C for 60 s). Unless otherwise stated, PCR mixture contained 1 μL cDNA template, 5 μM of each primer, 125 μM each of dNTP and 1U of *Taq* DNA polymerase in 20 μL .

Using PCR product as a template, nested PCR was performed with two primers (5'-TTGCTAGCGTGTTGGAA-3' and 5'-TAIG(A/C/T)(A/G)TCA(A/T)AI(A/G)T(A/G)TC(A/G)TC-3'). The PCR condition were as follows: 94°C for 3

min and 30 cycles of three steps (94°C for 30 s, 40°C for 60 s, and 72°C for 60 s).

The PCR products were ligated into pGEM-T Easy vector (Promega) and transformed into XL-1 Blue. Sequences of PCR products were analyzed using BigDye Terminator version 3.1 (Applied Biosystems, CA, USA) and ABI3130xl genetic analyzer (Applied Biosystems). Unless otherwise stated, sequences of PCR products were analyzed in the same manner.

To amplify the *Tps* 3' region, primers were designed from the cDNA fragment cloned in above and 3' RACE was performed. PCR was performed with 5'-GGTGTACTTTGAGCCACGTT-3' and 5'-AACTGGAAGAATTCGCGGCC-3', and the PCR conditions were as follows: 94°C for 3 min, 30 cycles of three steps (94°C for 30 s, 58°C for 30 s, and 72°C for 70 s). Using the PCR product as a template, nested PCR was performed with primers (5'-CAGCCC-GAATATTTCTTACCAAAG-3' and 5'-TCGCGGCCGAG-GAA-3'). The PCR conditions were as follows: 94°C for 3 min, 35 cycles of three steps (94°C for 30 s, 58°C for 30 s, and 72°C for 70 s).

BLASTX searching showed that the cloned cDNA fragment was similar to germacrene D synthase of *Thapsia villosa* var. *laciniata* (KR822706). Thus, for getting the longer fragments of ashitaba *Tps*, the forward primer (5'-ATCATCCTAGCATCTGGGGAGAC-3') was designed from the germacrene D synthase gene of *Thapsia villosa* var. *laciniata* and the reverse primer 5'-GTACACCCCCGT-TATCCAAAAGTAGC-3' was designed from cDNA fragment cloned above, respectively. PCR with these primers was performed under the following conditions: 94°C for 3 min, 35 cycles of three steps (94°C for 30 s, 60°C for 30 s, and 72°C for 45 s).

To amplify a *Tps* 5' end of the ashitaba *Tps* gene, primers were designed from the cDNA fragment cloned above and 5' RACE was performed. First, PCR was performed with UPM (mixture of 5'-CTAATACGACTCATATAGGGCAAGCAGTGTTATCAACGCAGAGT-3' and 5'-CTAATACGACTCACTATAGGGC-3') and another primer 5'-GCCGCCGTCTGATGGCTTCCGGTCT-3' under the following conditions: 94°C for 3 min, 25 cycles of three steps (94°C for 30 s, 68°C for 30 s, and 72°C for 180 s). Then, using PCR product as a template, nested PCR was performed with 5'-CTAATACGACTCACTATAGGGC-3' and 5'-CAGCTTCAAAGTGGTACGACAACCCAGCC-3' under the same condition as above.

2.5 cDNA cloning of *AkTps2*

In order to amplify another *Tps* fragments, PCR was performed using *Taq* DNA polymerase (+ dNTPs) Robust buffer (Bioacademia) with two degenerate primers (5'-GATGTTGC(A/T/C)CT(A/T)(A/T/C)G(C/T)TT(C/T)(A/C)G-3' and 5'-TC(C/T)T(A/C/G)ATT(A/T/C)AT(A/G)TC(C/T)TTCCA-3'), which were designed using relatively well-

conserved regions in Apiaceae *Tps* sequences. The PCR condition were as follows: 94°C for 3 min, 35 cycles of three steps (94°C for 30 s, 40°C for 30 s, and 72°C for 50 s).

To amplify the 3' region, primers were designed from the cDNA fragment cloned above and 3' RACE was performed. PCR was performed with 5'-TACTACCTCTCACCTTCATCTCT-3' and 5'-AACTGGAAGAATTCGCGGCC-3', and the PCR conditions were as follows: 94°C for 3 min, 30 cycles of three steps (94°C for 30 s, 58°C for 30 s, and 72°C for 50 s). Using PCR product as a template, nested PCR was performed with primers (5'-GGGGCTT-TACTTTGAGCCCCAA-3' and 5'-TCGCGGCCGCGAGGAA-3'). The PCR conditions were as follows: 94°C for 3 min, 35 cycles of three steps (94°C for 30 s, 58°C for 30 s, and 72°C for 60 s).

To amplify the 5' end of *ashitaba Tps*, primers were designed from the cDNA fragment cloned above and 5' RACE was performed. First, PCR was performed with UPM (mixture of 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' and 5'-CTAATACGACTCACTATAGGGC-3') and 5'-CCATGAA-CACTCAACGGCTGACGGTATA-3' under the following conditions: 94°C for 3 min, 25 cycles of three steps (94°C for 30 s, 68°C for 30 s, and 72°C for 180 s). Then, using PCR product as a template, nested PCR was performed with 5'-CTAATACGACTCACTATAGGGC-3' and 5'-ATA-ATCGAAGCCGTGTGTGCGGCCCTGCTTC-3' under the same conditions as above.

The generated PCR products were analyzed as described just before in the Materials and methods section of cDNA cloning of *AkTps1*.

2.6 Phylogenetic analysis

BLASTX searches were carried out against the GeneBank non-redundant protein database on NCBI. Alignment of multiple protein sequences was carried out using BioEdit or EMBOSS Needle, and the phylogenetic tree was drawn using Mega-X.

2.7 Functional analysis of *AkTps1*

PCR was performed using KOD-Plus-Neo (Toyobo, Fukui, Japan) with primers 5'-GGGCATATGGCTCTGTTTGTGCTCTC-3' and 5'-CCCCTCGAGCTACCATGATACTGGAATAGGATC-3'. These primers contained *NdeI* and *XhoI* restriction endonuclease recognition sites, respectively. PCR mixture contained 1 µL cDNA template, 0.3 µM of each primer, 400 µM each of dNTP and 1U of KOD-Plus-Neo in 50 µL. PCR conditions were as follows: 94°C 2 min, 25 cycles of three steps (98°C 10 s, 55°C 30 s, and 68°C 60 s). After these cycles, 5 U of *Taq* DNA polymerase was added to the mixture, and reacted for 600 s at 72°C. The PCR products were cloned into pGEM-T Easy vector and their sequences were analyzed. The entire *Tps* open reading frame (ORF) from *ashitaba* was separated from the

plasmid by *NdeI* and *XhoI* digestion and inserted into the *NdeI-XhoI* site of pETDuet-1 (Merck, Darmstadt, Germany) to construct the plasmid named pET-AkTps1. The obtained plasmid was introduced into *E. coli* BL21 (DE3) together with plasmid pAC-Mev/Scidi/AacI¹⁴.

Recombinant *E. coli* were culture in LB medium containing 50 µg/mL ampicillin (Am) and 30 µg/mL chloramphenicol (Cm) at 37°C overnight. One milliliter of this culture was inoculated into 100 mL of TB medium in a 500-mL Erlenmeyer flask containing 50 µg/mL Am, 30 µg/mL Cm, and 1 mg/mL lithium acetoacetate (LAA) and cultured at 37°C with rotary shaking (150 rpm) for 3–4 h. After cooling the flask to 20°C, 25 µM isopropyl β-D-thiogalactopyranoside (IPTG) and 20 mL *n*-octane were added to the flask and cultured at 20°C with rotary shaking for 2 days.

The contents of 12 flasks were added to *n*-hexane (400 mL) and poured into a separatory funnel and shaken well. The alkane layer (*n*-octane + *n*-hexane) was washed with an equal amount of alkaline 90% MeOH (0.5 M KOH in 90% MeOH) twice and concentrated to a small volume (3 mL) *in vacuo*. The concentrated alkane was applied on a silica gel (Silica Gel 60, Kanto Chemical) chromatography column (20 i.d. × 200 mm) filled with *n*-pentane, and developed with *n*-pentane.

The eluates were fractionated every 5 g, and the sesquiterpenes included in each fraction were analyzed by silica gel TLC developed with *n*-hexane. By spraying molybdophosphoric acid solution (12 g disodium molybdate (VI) dihydrate, 1.5 mL H₃PO₄, 25 mL H₂SO₄ in 500 mL distilled water) on the developed TLC plate and heating to 200°C, the produced sesquiterpenes were detected as blue spots (R_f value 0.7).

The fractions containing a sesquiterpene (fr. 8–10, 9.8 mg) were concentrated to dryness *in vacuo* and analyzed by ¹H NMR (Bruker, AMX400) using TMS as an internal standard (δ 0.00) and GC-MS.

2.8 Functional analysis of *AkTps2*

PCR was performed using KOD-Plus-Neo with primers 5'-CGCAGAGTACATGGGGTGATAA-3' and 5'-GCAAGTG-GCAAACACCAACTCA-3'. PCR condition was as follows: 94°C 2 min, 25 cycles of three steps (98°C 10 s, 55°C 30 s, 68°C 60 s). PCR mixture contained 1 µL cDNA template, 0.3 µM of each primer, 400 µM each of dNTP and 1U of KOD-Plus-Neo in 50 µL. With PCR product as a template, nested PCR was performed with 5'-CATATGGCGGCTCTTGTTCA-3' and 5'-AGATCTTTACTGGAATAGGATCAATT-3' under the same condition above. These primers contained *NdeI* and *BglII* restriction endonuclease recognition sites, respectively. After these cycles, 5 U of *Taq* DNA polymerase was added to the mixture, and reacted for 600 s at 72°C. The PCR products were cloned into pGEM-T Easy vector and their sequences were analyzed.

The entire *Tps* ORF from ashitaba was separated from the plasmid by *Nde*I and *Bgl*III digestion and inserted into the *Nde*I-*Bgl*III site of the pRSFDuet-1 (Merck) and constructed the plasmid was named pRSFD-AkTps2. The obtained plasmid was introduced into *E. coli* BL21 (DE3) together with plasmid pAC-Mev/Scidi/AacI¹⁴.

Isolation of the produced sesquiterpenes was performed according to the method described in the Materials and methods section of functional analysis of *AkTps1*. The silica column fractions that contained one sesquiterpene (Rf 0.7) (fr. 8–11, 1.8 mg) or other one (Rf 0.5) (fr. 23–31, 2.5 mg) were collected separately, and analyzed by ¹H NMR and GC-MS.

2.9 ¹H NMR analyses of the produced sesquiterpenes

Each isolated sesquiterpene was analyzed by ¹H NMR (Bruker, AMX400). ¹H NMR spectra were measured in CDCl₃ using the residual solvent peak as an internal standard (δ_{H} 7.26 ppm).

Germacrene D 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).

Germacrene B δ : 1.50 (s, 3H, H-15), 1.54 (s, 3H, H-11), 1.69 (s, 3H, H-13), 1.71 (s, 3H, H-14), 1.80 (m, 1H, H-8b), 1.95 (m, 1H, H-3b), 2.04–2.07 (2H, H-9b and H-2b), 2.17 (m, 1H, H-3a), 2.21 (m, 1H, H-9a), 2.29 (m, 1H, H-2a), 2.52–2.58 (2H, H-8a and H-6b), 2.92 (m, 1H, H-6a), 4.50 (m, 1H, H-5), 4.72 (m, 1H, H-1).

3 Results

3.1 GC-MS analysis of ashitaba leaf extract

GC-MS analysis of the ashitaba leaf extract detected several sesquiterpenes, including β -caryophyllene, β -himachalene, germacrene D, and germacrene B, which were deduced from the MS database (Fig. 1).

3.2 cDNA cloning of ashitaba *Tps1* and ashitaba *Tps2*

Two full-length *Tps* genes, designated *AkTps1* and *AkTps2*, were isolated (accession No. LC506578 and LC506579, respectively). The deduced amino acid sequences of AkTps1 and AkTps2 consisted of 569 and 567 amino acids, respectively. BLAST searching showed that AkTps1 has 81% identity to germacrene D synthase of *Trachyspermum ammi* (AUZ98407) and AkTps2 has 72%

Fig. 1 Gas chromatography-mass spectrometry (GC-MS) analysis of the volatile compounds in ashitaba leaf extract. TIC of the extract (a), MS of peak 1 (b), MS of β -caryophyllene in the database (c), MS of peak 2 (d), MS of germacrene B in the database (e), MS of peak 3 (f), MS of β -Himachalene in the database (g), MS of peak 4 (h), MS of germacrene D in the database (i), and structures of each sesquiterpene (j).

identity to germacrene D synthase of *Thapsia villosa* var. *laciniatta* (AMU19318). The similarity between AkTPS1 and AkTPS2 was 84.8%. In general, TPSs have aspartate rich motifs, such as the RDR, DDxxD, and NSE/DTE motifs¹⁶⁾. These motifs were all conserved in AkTPS1 and AkTPS2 (Fig. 2). Phylogenetic analyses revealed that AkTPS1 and AkTPS2 are more similar to germacrene D synthases of species belonging to the Apiaceae family than ones of other families (Fig. 3).

3.3 Functional analyses of AkTPS1 and AkTPS2

3.3.1 AkTPS1

Silica gel column fractions 8–10, which contained a sesquiterpene, were concentrated to dryness (9.8 mg) and analyzed by ¹H NMR. The sesquiterpene was identified as germacrene D by comparison the observed ¹H NMR spectrum (Fig. S-1) with the previously reported ¹H NMR data¹⁵⁾. GC-MS analysis using the conditions described in material and method section also proved the sesquiterpene as germacrene D (retention time 24.6 min, MS data match rate

98%).

3.3.2 AkTPS2

The silica gel column fractions 8–12 and 23–28, containing different sesquiterpenes, were concentrated to dryness (1.8 mg and 2.5 mg, respectively) and analyzed by ¹H NMR. These sesquiterpenes were identified as germacrene D (fractions 8–12) and germacrene B (fraction 23–28), respectively, by comparison the observed ¹H NMR spectra (germacrene D (Fig. S-1) and germacrene B (Fig. S-2)) with the previously reported ¹H NMR data^{15, 18)}. GC-MS analysis using the conditions described in material and method section also proved them as germacrene D (retention time 24.6 min, MS data match rate 98%) and germacrene B (retention time 23.8 min, MS data match rate 99%).

4 Discussion

Angelica keiskei (ashitaba) is an edible plant belonging

Fig. 2 Sequence alignment of AkTPS1 and AkTPS2.

Amino acid sequences deduced from *Tps* genes isolated from ashitaba were aligned. The TPS conserved motifs RDR, DDxxD, and NSE/DTE are indicated by gray lines. Amino acids conserved between AkTPS1 and AkTPS2 are indicated in black boxes.

Fig. 3 Phylogenetic tree of AkTPS1, AkTPS2, and known germacrene D synthases.

AkTPS1, AkTPS2, and known germacrene D synthases were phylogenetically analyzed. Accession numbers are as follows: *Chengiopanax sciadophylloides* germacrene D synthase (BBE32331), *Ocimum basilicum* germacrene D synthase (AAV63786), *Pogostemon cablin* germacrene D synthase (AAS86320), *Solidago canadensis* germacrene D synthase (CAE47440), *Thapsia villosa* var. *laciniata* germacrene D synthase (AMU19318), *Vitis vinifera* germacrene D synthase (AAS66357), *Xanthium strumarium* germacrene D synthase (AMP42987), and *Zingiber officinale* germacrene D synthase (AAX40665).

to the Apiacea family. In the present study, we focused on sesquiterpenes present in the leaves eaten by humans (specifically, in the Japanese population), from which only one sesquiterpene has been reported previously. GC-MS analysis of the leaf extract resulted in the detection of β -caryophyllene, β -himachalene, germacrene D, and germacrene B. We identified two sesquiterpene synthase genes (designated *AkTps1* and *AkTps2*), which were germacrene D synthase and germacrenes B and D synthase, respectively.

Phylogenetic analyses revealed that AkTPS1 and AkTPS2 were more similar to germacrene D synthases from species belonging to the same Apiaceae family than ones from other families (Fig. 3). For example, similarity between AkTPSs and germacrene D synthase from *Thapsia villosa* var. *laciniata* (AMU19318) of the same family was over 80%, while only approximately 50% similarity was observed between AkTPSs and germacrene D synthase from *Zingiber officinale* (DAY860846). AkTPSs also showed over 80% similarity to kuzeeol synthase of *Thapsia garganica* (K4LMW2). Structural diversity of germacrene D synthase enzymes is likely to be the result of convergent evolution. Thus, it is difficult to estimate function of each unknown TPS sequences by sequence-based dry analysis. Therefore, functional analysis experiments of individual TPSs should be needed, e.g., by using a mevalonate-pathway-engineered *E. coli*.

As germacrene D is reported to have antioxidant and antibacterial activities^{19, 20}, this compound may contribute to

the relevant activities of ashitaba leaves. In addition, sesquiterpenes, including germacrene D, are known to be emitted in response to biotic environmental stress^{21, 22}. As insect pests have become problematic in the culture of ashitaba²³, sesquiterpenes should be a focus of research directed toward this problem.

Many TPSs contribute to multi-sesquiterpene synthesis^{13, 24, 25}, and these multi-sesquiterpene synthases contribute to the diversity of sesquiterpenes in plants²⁶. AkTPS2 found in this study also belongs to this multi-sesquiterpene synthase due to the production of two sesquiterpenes, germacrene D and germacrene B. *Tps* genes encoding enzymes that catalyze the formation of germacrene D and germacrene B, like AkTPS2, have been identified in *Valeriana officinalis*²⁴. Here, we propose biosynthetic routes from FPP to these two sesquiterpenes by way of a farnesyl cation, as shown in Fig. 4. Cation A (Fig. 4), generated from farnesyl cation, is likely to be a starting point for germacrene D and germacrene B synthesis. Considering the products of AkTPS1 (germacrene D) and AkTPS2 (germacrene B and germacrene D), the selective dehydrogenation of H-7 from cation A in AkTPS1 may be caused by a strict steric interaction between the binding pocket of AkTPS1 and the substrate.

In this study, we were not able to identify *Tps* genes for β -caryophyllene synthase or β -himachalene synthase, although these sesquiterpenes were detected by GC-MS. Nine sesquiterpenes were detected in the case of carrot (*Daucus carota*), a plant belonging to family Apiacea, and

Fig. 4 Proposed biosynthetic routes of germacrene B and germacrene D.

22 sesquiterpene synthases were suggested to exist in that species²⁷⁾. It is thus likely that many unidentified sesquiterpene synthases exist in *ashitaba*, which can contribute to its diverse biological functions.

5 Conclusions

In the present study, we confirmed the presence of several sesquiterpenes in *Angelica keiskei* (*ashitaba*) for the first time. We cloned two *Tps* genes from the edible leaves and expressed them in MVA-pathway-engineered *E. coli*. Products of the TPSs were analyzed by ¹H NMR and GC-MS. Consequently, a germacrene D synthase gene, named *AkTps1*, and a germacrenes B and D synthase gene, named *AkTps2*, were identified. This is the first study reporting TPSs of the *ashitaba* plant.

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Supporting Information

This material is available free of charge via the Internet at <http://dx.doi.org/jos.69.10.5650/jos.ess19275>

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