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Molecular cloning and characterization of drimenol synthase from valerian plant (*Valeriana officinalis*)

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ABSTRACT

Drimenol, a sesquiterpene alcohol, and its derivatives display diverse bio-activities in nature. However, a drimenol synthase gene has yet to be identified. We identified a new sesquiterpene synthase cDNA (*VoTPS3*) in valerian plant (*Valeriana officinalis*). Purification and NMR analyses of the VoTPS3-produced terpene, and characterization of the VoTPS3 enzyme confirmed that VoTPS3 synthesizes (−)-drimenol. In feeding assays, possible reaction intermediates, farnesol and drimenyl diphosphate, could not be converted to drimenol, suggesting that the intermediate remains tightly bound to VoTPS3 during catalysis. A mechanistic consideration of (−)-drimenol synthesis suggests that drimenol synthase is likely to use a protonation-initiated cyclization, which is rare for sesquiterpene synthases. VoTPS3 can be used to produce (−)-drimenol, from which useful drimane-type terpenes can be synthesized.

1. Introduction

Terpenoids (or isoprenoids) are one of the major classes of natural products with 55 000 known structures [1]. Terpenoids in animals serve as primary physiological components of membrane constituents (e.g., cholesterol) and the electron transport chain (e.g., ubiquinone), while in plants and microbes, they occur as diverse secondary metabolites which have benefited human lives as aromas, flavors, and pharmaceutical substances [2,3]. Terpenoids of specific hydrocarbon backbones are synthesized by specific terpene synthases (TPSs), which can fold prenyl diphosphate precursors, such as geranyl (C_{10}), farnesyl (C_{15}), and geranyl geranyl (C_{20}) diphosphate, with subsequent carbocation formation to produce a large variety of different terpenoids [4–6]. Hence, at the entry point of the terpenoid metabolism, the TPS enzyme dictates terpene skeletal types. In nature, some terpene skeletons (e.g., farnesene, caryophyllene, and germacrene) are commonly found in plants, but others are associated with specific lineages or species of plants through evolutionary adaptations (e.g., amorphadiene from *Artemisia annua* and (+)-*epi*-α-bisabolol from *Lippia dulcis*) [7,8]. Therefore, it is important to characterize TPSs from diverse taxa.

Drimane is one of the unique C_{15} sesquiterpene backbones (Fig. 1A), of which the biosynthetic gene has not been identified [9]. Hundreds of drimane-type sesquiterpenoids have been isolated from plants (primarily Canellaceae and Polygonum families), liverworts, fungi, and sponges [9–16]. Intriguingly, many of them show strong biological activities, such as antifungal, plant growth regulatory and antifeedant effects [9,13,17–19]. Hence, it is apparent that drimane derivatives are widespread in nature and deliver important eco-physiological roles to the synthesizers. However, it should be noted that drimane is rarely isolated as a natural product and that the simplest and closest drimane-type sesquiterpene found in nature is drimenol (Fig. 1B), which implies that drimenol or perhaps drimenyl pyrophosphate is the biosynthetic precursor to the various drimane sesquiterpenoids. A native sesquiterpene synthase (sesqui-TPS), that can synthesize drimenol from (*E*,*E*)-farnesyl diphosphate (FPP) in a Mg^{2+}-dependent manner, has been purified from the *Polygonum hydropiper* [20]. This plant produces a drimane sesquiterpenoid, polygodial (Fig. 1C), which possesses potent antifeedant activity against insects [9,17,21]. These observations support that drimenol is the first skeletal backbone synthesized from FPP, and that drimenol serves as the central precursor of

Abbreviations: FPP, farnesyl diphosphate; TPS, terpene synthase; sesqui-TPS, sesquiterpene synthase

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naturally occurring drimane-type sesquiterpenoids. However, the respective gene for drimenol biosynthesis had not been identified until now.

2. Materials and methods

2.1. Plasmid construct

Total RNA was isolated from the roots of *Valeriana officinalis* with 1% of cetyl trimethyl ammonium bromide (0.5 M Tris HCl, pH. 8.0, 0.25 M ethylene diaminetetraacetic acid (EDTA), 4% NaCl, 0.5% polyvinylpyrrolidone). The cDNA synthesized from total RNA was used as template for PCR. The PCR products were amplified with primers (Table S1) and cloned into pESC-Leu2d for yeast expression, pBluescript II for D307A sub-cloning, and pH9GW or pDEST17 for *Escherichia coli* expression.

2.2. Production and purification of drimenol in yeast

VoTPS3:pESC-Leu2d or the empty vector was transformed into the yeast strain, EPY300, and the terpene products from the transgenic yeast analyzed, according to published methods [22]. To purify the VoTPS3-product, the *VoTPS3*-expressing yeasts were cultured at 30 °C for 6 days in a 1-L cultivation scale. The yeast culture was extracted with diethyl ether (3 × 500 mL) and the organic extracts combined, concentrated and purified by flash column chromatography (silica, 45 g; 23 cm height; eluent = 95:5 hexanes:ethyl acetate; 180–250 mL fractions). Drimenol-containing fractions were pooled, concentrated, and fractionated again using flash column chromatography (silica, 15 g; 23 cm height; eluent = dichloromethane; 80∼180 mL fractions).

2.3. NMR spectroscopy

The purified product was dried with N_2 gas, dissolved in CD_3OD (600 μL) and analyzed by NMR. NMR spectra were recorded on a Varian Inova 600 MHz or 700 MHz spectrometer equipped with a $^{13}C/^{1}H$ dual cold probe. δ values were referenced to CD_3OD (3.31 ppm) or D_2O (4.90 ppm) for ^{1}H-NMR and to CD_3OD (49.0 ppm) for ^{13}C-NMR. Details of 1D and 2D NMR analyses are given in the Supplementary Materials.

2.4. Overexpression and purification of recombinant VoTPS3

An *E. coli* strain, Rosetta pLysS, harboring *VoTPS3*-pH9GW or *D307A*-pDEST17 was cultured in 500 mL of LB medium at 37 °C for 2 h and induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside at 22 °C overnight. The cells were pelleted at 6,000 rpm for 5 min at 4 °C and resuspended in lysis buffer (50 mM Na_3PO_4, pH. 7.5, 500 mM NaCl, 20 mM Imidazole). The cells were lysed by sonication and then centrifuged at 4000 rpm for 30 min at 4 °C. The soluble fraction was bound with Nickel–Nitrilotriacetic acid resin (Qiagen, Venlo, Netherlands) for 1 h. Proteins were eluted with 2 mL of elution buffer (50 mM Na_3PO_4, pH. 7.5, 500 mM NaCl, 50 mM Imidazole), followed by concentration and desalting using a membrane filter (Millipore, Massachusetts, USA).

2.5. Enzyme characterization

For the in vitro enzyme assay, 20 μg of purified enzyme was mixed with 50 mM Tris (pH 7.5), 10 mM $MgCl_2$ and 100 μM of (*E,E*)-FPP (Echelon Biosciences) or 1 mM (*E,E*)-farnesol (Sigma) or 100 μM of drimenyl diphosphate. The reaction mixture (500 μL) was overlaid with hexane (500 μL) and incubated at 30 °C for 2 h. The phases were separated and the aqueous layer extracted with hexane (2 × 500 μL). The combined hexane extracts were analyzed by GC–MS. To convert FPP to farnesol or drimenyl diphosphate to drimenol, the reaction mixture was incubated with 2 units of calf intestine phosphatase (CIP) (NEB). For the assay using heavy water, the reaction mixture was incubated with 79% of H_2O and 19% of $H_2^{18}O$ (Sigma). For kinetic analysis, cold FPP substrates were spiked with [1-^{3}H]-FPP (Perkin Elmer, 23 Ci mmol^{-1}) at 1% of the final concentration. A 100 μL mixture (3.2 μg of enzyme, 25 mM HEPES, pH. 7.4, 10 mM $MgCl_2$, 0.5–10 μM FPP) was incubated for 5 min at 30 °C. The reaction was terminated by adding 4 M NaOH and 1 M EDTA. The products were mixed with pentane (900 μL) and 400 μL of the upper layer was collected. The pentane extracts were mixed with 3.5 mL of liquid scintillation cocktail. The radioactivity of products was monitored by a liquid scintillation counter (Beckman LS6500). The kinetic parameters were calculated with the Enzyme Kinetics module in Sigmaplot 12.

2.6. GC–MS analysis

GC–MS analysis was performed with an Agilent 6890N gas chromatography system, Agilent 5975B mass spectrometer and DB1-MS column (30 m × 250 μm × 0.25 μm). 2 μL of sample was injected at 250 °C, and the temperature was increased at a rate of 10 °C min^{-1} from 40 °C to 250 °C. Helium (1 mL min^{-1}) was used as a carrier gas. The National Institute of Standards and Technology (NIST) Standard Reference Database (version 5) and the Wiley Registry™ of Mass Spectral Data (version 10) were used to identify drimenol.

2.7. Quantitative read mapping

Illumina read mapping: for quantitative analysis, CLC Genomics Workbench software (version 5.5.1) was employed using a single

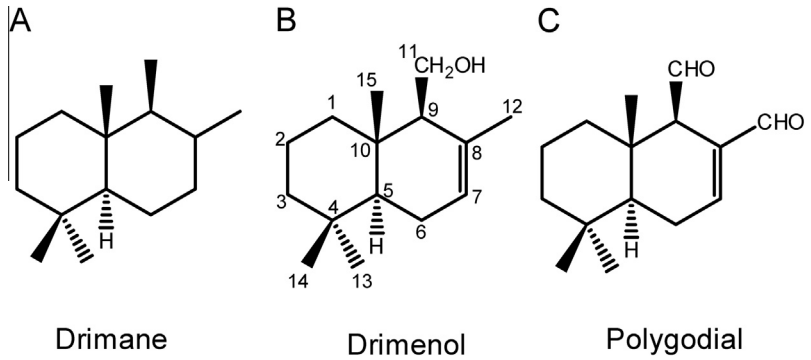

Fig. 1. Structures of drimane, drimenol, and polygodial.

read Illumina data. The parameters used were: mismatch cost, 2; insertion cost, 1; deletion cost, 1; length fraction, 0.8; similarity fraction, 0.98.

3. Results and discussion

3.1. Isolation of new sesqui-TPS from V. officinalis

Drimenol was reported in the valerian plant (*V. officinalis*) root as a minor constituent [23–26], and we searched for the valerian transcriptome data as a source of drimenol synthase gene isolation. From the valerian root transcriptome database, generated by 454 sequencing, valerenadiene synthase (*VoTPS1*) and germacrene C/D synthase (*VoTPS2*) were previously identified and characterized [27,28]. To acquire deeper transcript sequencing, Illumina sequencing was additionally performed, and the resulting reads were assembled by a Trinity algorithm [29]. The BLAST-searchable databases of *V. officinalis* 454 and Illumina transcriptomes are publicly accessible through the PhytoMetaSyn website (www.phytometasyn.ca) [30,31].

From this transcriptome database, the third full-length sesqui-TPS cDNA was identified, and its open reading frame (ORF) was PCR-amplified using primers designed at the start and stop codon of the assembled transcript. The cloned cDNA encodes a polypeptide of 556 amino acids with M_w of 64.8 kDa. The newly identified sesqui-TPS was named *VoTPS3* (GenBank number: JQ437841). *VoTPS3* was quite divergent from other sesqui-TPSs as it displayed only 41% and 39% sequence identity to *VoTPS1* and *VoTPS2*, respectively, and its closest homolog in the public database showed limited amino acid identity (49% identity with vetispiradiene synthase from *Solanum tuberosum*; GenBank number, BAA82108). To infer the relative expression level of *VoTPS3*, ~30 million raw Illumina reads were computationally mapped to *VoTPS1/2/3* ORF nucleotide sequences. In this *in silico* analysis, *VoTPS1* and *VoTPS2* showed a comparable level of expression to each other, but the read count of *VoTPS3* was only 2.4% of *VoTPS1* (Table 1). Therefore, *VoTPS3* appears to only marginally contribute to the terpene constituents of the valerian root, in comparison to the *VoTPS1* (valerenadiene synthase) that is responsible for a major sesquiterpenoid, valerenic acid [27].

3.2. In vivo functional assessment of sesqui-TPS

To assess the catalytic function of *VoTPS3*, its ORF was expressed under a galactose-inducible promoter in the EPY300 strain, a yeast strain engineered to accumulate an elevated level of FPP [32,33]. After galactose-induction, volatile terpenes were sequestered by dodecane (2 mL) overlaid on the yeast culture (20 mL) during the 3-day cultivation. The dodecane layer was then analyzed by GC–MS. In the GC–MS profile, a new peak (Fig. 2, peak 2) was identified from the yeast expressing *VoTPS3*, whereas this peak was absent in the control yeast harboring an empty plasmid. On the other hand, (*E*,*E*)-farnesol (Fig. 2, peak 1) derived from the dephosphorylation of FPP was the most abundant compound in the control yeast, but it was markedly reduced in the yeast expressing *VoTPS3*. This observation implied that an efficient conversion of FPP to the new terpene product was occurring in the *VoTPS3*-expressing yeast. In the MS spectral database search, the electron-impact (EI) fragmentation pattern of the new terpene showed a strong spectral match (98%) to that of drimenol with a parental *m*/*z* of 222. This result indicated that VoTPS3 might catalyze the synthesis of the sesquiterpene alcohol, drimenol, from FPP.

Table 1
Quantitative mapping of three valerian sesqui-TPSs.

TPS name	Reference length (base pairs)	Unique read count[a]	Relative abundance
VoTPS1	1689	24137	100%
VoTPS2	1692	7674	31.8%
VoTPS3	1671	575	2.4%

[a] 30362677 reads generated by Illumina sequencings were mapped to the three reference sequences by the parameters described in the method.

3.3. Structural elucidation of (−)-drimenol

To unambiguously assign the chemical identity of the VoTPS3-product as drimenol, the cultivation of the yeast expressing *VoTPS3* was scaled up to 1-L, and the whole yeast culture was extracted with diethyl ether (3 × 500 mL). The diethyl ether fraction was fractionated using flash column chromatography (see Section 2.2) to yield 6 mg of the VoTPS3-product with >95% purity. The purified terpene was fully characterized by one- and two-dimensional NMR analyses. ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, HMBC, and TOCSY were used to validate the drimenol skeleton. 1D-NOESY experiments were additionally used to assign the relative stereo-configuration of the stereo-centers at C5, C9 and C10 (details of structural determination are given in the Supporting Information). To determine if the isolated drimenol was the *RRR* or *SSS* enantiomer, a specific rotation value was measured, and its $[\alpha]_D$ value compared to a previously reported synthetic *SSS* standard [34]. Both the isolated drimenol sample and the synthetic literature compound had comparable negative values (see Supplementary Materials), which allowed us to assign the absolute configuration as the *SSS* enantiomer, (−)-drimenol. From these results, we concluded that *VoTPS3* encodes the *Valeriana officinalis* (−)-drimenol synthase (*VoDMS*).

3.4. Characterization of recombinant drimenol synthase

In order to evaluate the kinetic properties of VoDMS, VoDMS was N-terminally tagged with nine histidines (9×His-VoDMS) and was expressed in *E. coli*. Approximately, 0.5 mg of the recombinant enzyme with 99% purity, as assessed by SDS–PAGE, was purified by a Nickel-NTA affinity column (Supplementary Fig. 1A). Incubation of purified VoDMS with FPP and the cofactor Mg^{2+} resulted in the in vitro synthesis of drimenol (Supplementary Fig. 1B/C). Furthermore, kinetic properties of VoDMS were obtained using ^{3}H-labeled FPP. The K_m and k_{cat} values of VoDMS were determined to be 9.6 ± 2.7 μM and 0.025 ± 0.004 s^{-1} (mean ± S.D; n = 3). This result assured that *VoDMS* encodes a catalytically active sesqui-TPS enzyme.

3.5. Mechanism of drimenol synthesis by VoDMS

Terpene synthases are known to utilize two different mechanisms: (1) Mg^{2+}-dependent "ionization-initiated" reaction (class I) by cleaving an allylic diphosphate bond or (2) "protonation-initiated" reaction (class II) by protonating a double-bond at the terminal isoprene unit (Fig. 3) [35–37]. The ionization-initiated reaction is mediated by the conserved DDxxD motif, which binds a divalent metal ion for substrate ionization. On the other hand, the "protonation-initiated" reaction involves an acidic residue, often conserved in DxDD motif, which donates a proton to the double-bond at a terminal isoprene unit. Both reactions produce reactive carbocation intermediates which undergo carbocation cascade reactions to yield various isoprene products. A large majority of sesqui-TPSs uses the class I "ionization-initiated" mechanism, whereas the class II "protonation-initiated" reaction is a typical terpene-forming reaction for triterpene synthases.

As expected, VoTPS sequences align seamlessly with those of other sesqui-TPSs and possess the conserved DDxxD motif for

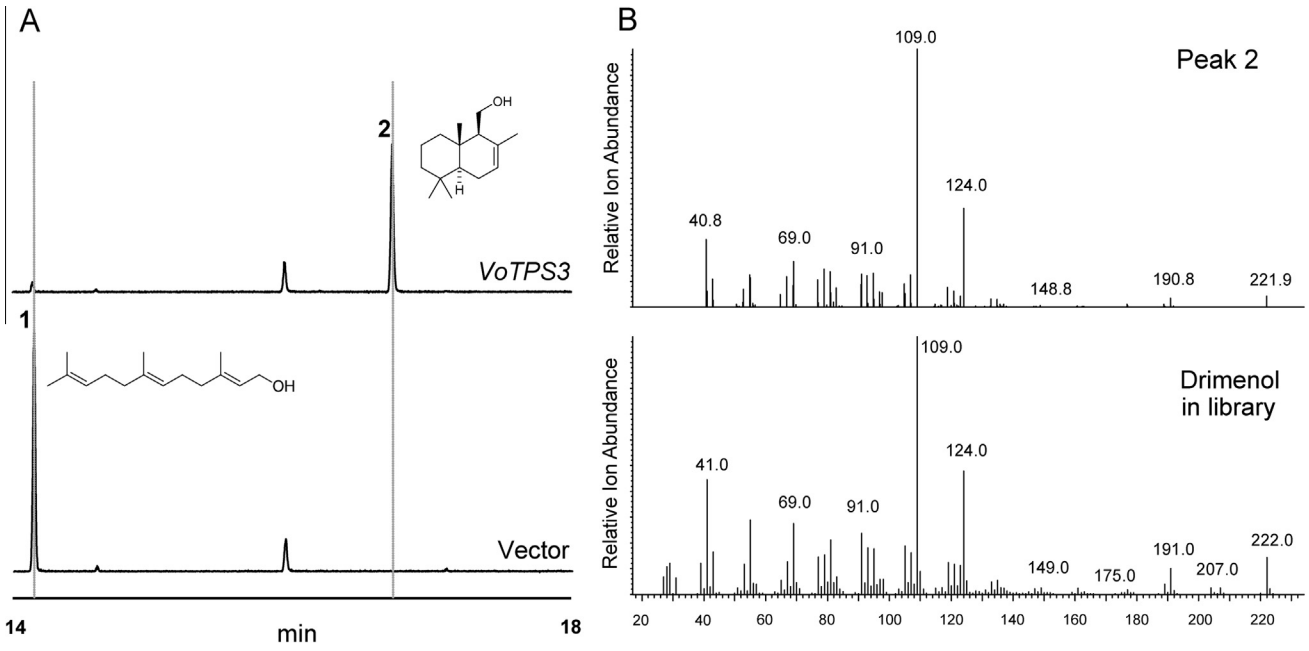

Fig. 2. GC–MS analyses of volatiles from *VoTPS3*-expressing yeast. (A) Gas chromatographic separation of volatile products from empty plasmid and *VoTPS3*-expressing yeast. Peaks 1 and 2 are farnesol and VoTPS3-product (drimenol), respectively, and their structures are shown beside the peaks. (B) Mass spectra of VoTPS3-product and its closest spectral match in the GC–MS library.

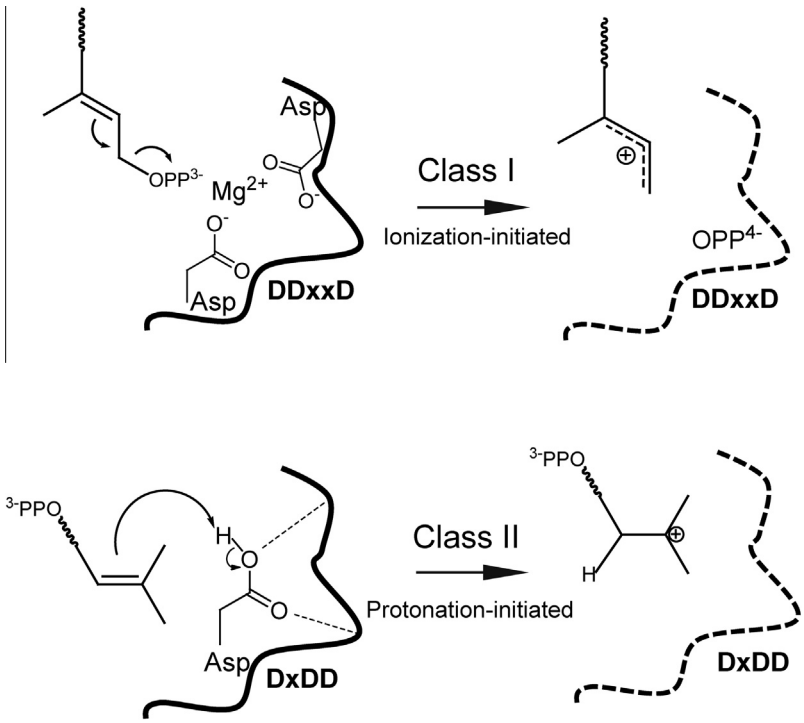

Fig. 3. Schematic description of class I and class II reaction mechanisms by terpene synthases.

FPP ionization (Supplementary Fig. 2). Therefore, we considered the biosynthesis of drimenol using an "ionization-initiated" carbocation formation at C11. However, a simple mechanism for drimenol formation by this typical ionization (class I) mechanism is not readily apparent. Rather, the drimenol formation from FPP can be postulated without difficulty by employing the class II reaction by protonating at C3 (Fig. 4A). In this mechanism, the reaction proceeds with a protonation at C3, followed by two sequential double-bond rearrangements and a deprotonation. Then the cleavage of diphosphate by DDxxD and Mg^{2+} could generate a primary carbocation which can be quenched by a water molecule. In this model, both class I and II mechanisms contribute to the formation of drimenol.

We recognized, however, the occurrence of an unstable primary carbocation in this mechanism and hence reasoned that drimenol could be synthesized by an enzymatic hydrolysis of the diphosphate. To probe the biosynthetic nature of the alcohol moiety at C11, VoDMS enzyme was incubated with $H_2^{18}O$. If C11 is ionized by the DDxxD motif and Mg^{2+}, ^{18}O will be incorporated into drimenol. On the other hand, if a hydrolysis of diphosphate is

responsible for the reaction, ^{18}O from the heavy water will not be incorporated into the drimenol. With $H_2^{18}O$ as 19% of total H_2O in the enzyme assay, comparable amount (20.3%) of drimenol parental ions with two mass increase ($m/z = 224$) was detected by GC–MS analysis (Fig. 5). This result proved that the oxygen atom from water is incorporated into drimenol, supporting the class I ionization reaction by VoDMS. The proposed homoallylic cation could be stabilized by its resonance structure, cyclopropylcarbinyl and cyclobutyl cation (Fig. 4A). In this proposed catalysis, drimenyl diphosphate is a key intermediate, and thus VoDMS could utilize drimenol diphosphate as a substrate. To test this possibility, drimenyl diphosphate was synthesized from the purified drimenol and incubated with VoDMS, but no evidence of drimenol formation from drimenyl diphosphate was obtained (Supplementary Fig. 3). From this result, we can deduce two possible explanations: (1) drimenyl diphosphate is not an intermediate in VoDMS reaction or (2) drimenyl diphosphate remains tightly bound to the active site of VoDMS during catalysis. Even though the drimenyl diphosphate is an intermediate in VoDMS reaction, it is apparent that VoDMS does not have a separate active site for the intermediate (i.e., drimenyl diphosphate) as shown in the bifunctional abietadiene synthase that encodes two distinct active sites in a single polypeptide [38].

A triterpene synthase-class enzyme, squalene-hopene cyclase, has been shown to catalyze an enzymatic conversion of (*E*,*E*)-farnesol to drimenol by its promiscuous protonation activity on the C3 of farnesol [35,39,40]. Based on this observation, we investigated if farnesol could be an intermediate of the VoDMS reaction as shown in Fig. 4B. In this model, ionization occurs at C11, and the C11 carbocation is immediately quenched by a water molecule, yielding a farnesol intermediate. Farnesol can undergo C3 protonation, double-bond rearrangements, and deprotonation to produce drimenol. To test this possible mechanism, we incubated VoDMS with up to 10 mM of the farnesol substrate. However, no evidence of drimenol formation was obtained from this assay (Supplementary Fig. 4), suggesting that farnesol is not likely to be an intermediate in the VoDMS reaction. Despite this result, we cannot exclude the possibility that the enzyme initially binds only FPP, which may induce a conformational change so that exogenous farnesol cannot access the active site despite the possible transient existence of that species during the reaction.

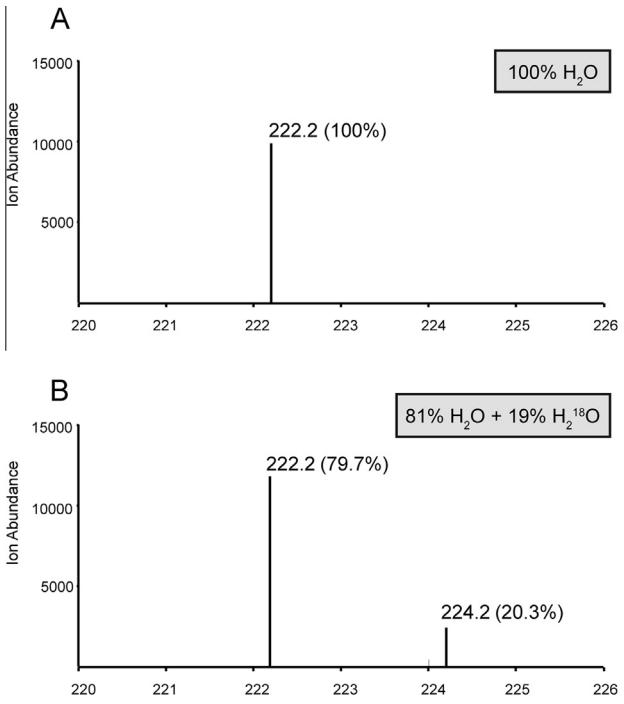

Fig. 5. Mass spectra of the drimenol synthesized by VoDMS in the presence of $H_2^{18}O$. Mass spectrum of drimenol synthesized from VoDMS assay with 100% H_2O (A) and with 81% H_2O and 19% $H_2^{18}O$ (B). The m/z value of 222 is measured for drimenol parental ions with ^{16}O.

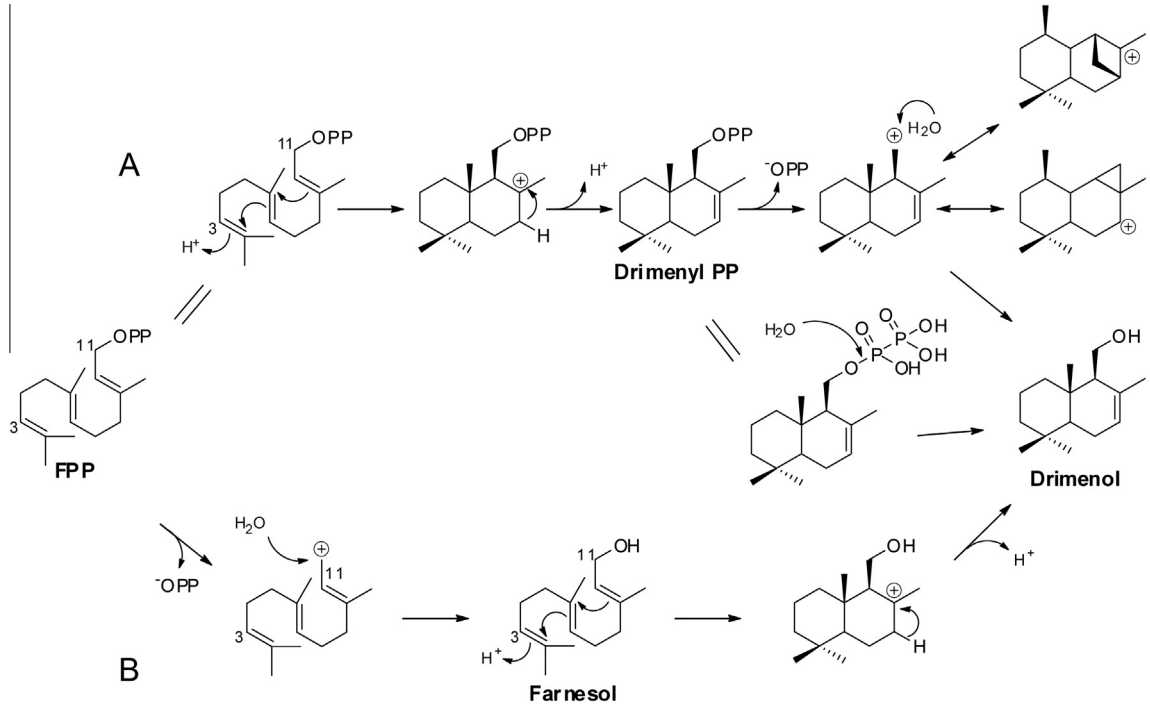

Fig. 4. A proposed biosynthetic mechanism of drimenol catalyzed by drimenol synthase. See Fig. 1 for carbon numbers. (A) Protonation at C3 of drimenol occurs first, followed by double-bond rearrangement and a quench by H_2O. (B) Ionization at C11 occurs first, followed by a quench by a H_2O and farnesol intermediate formation. Farnesol is further cyclized to drimenol by a protonation at C3. FPP can be converted to farnesol by a phosphatase enzyme reaction, in which phosphorus in FPP is attacked by a hydroxyl group of serine residue in phosphatase.

It is unprecedented that a sesqui-TPS possesses both class I and class II reactivity in a single polypeptide. We attempted to decouple the class I (ionization) and class II (protonation) reaction by mutating the DDxxD motif in VoDMS. The first Asp residue of the DDxxD motif, known to ionize the substrate in concert with Mg^{2+}, was mutated to Ala to generate an ADxxD motif (D307A). This mutant enzyme, possessing the weakened metal-binding motif, was incubated with FPP, and the product was dephosphorylated by phosphatase and analyzed by GC–MS. From this experiment, we aimed to examine the possibility that the substrate FPP binds, although weakly, to the active site, but is not ionized due to the D307A point-mutation in the conserved DDxxD motif. While FPP is being bound to the mutant enzyme, C3-protonation could still occur to produce drimane-diphosphate, which can be converted to drimenol by phosphatase treatment. However, in assays using the mutant VoDMS, drimenol was not detected by GC–MS analysis (Supplementary Fig. 5).

Taken together, a specific reaction mechanism could not be inferred either by feeding farnesol or drimenyl diphosphate to the enzyme or by using the weakened metal-biding motif (ADxxD; D307A). However, these results need to be cautiously interpreted. During the reaction, the reaction intermediates (farnesol or drimenyl diphosphate) could be deeply buried in the substrate-binding site of VoDMS by the conformational change of the enzyme, and hence exogenous intermediates may not be accessible to the active site. Also, FPP may not be able to bind strongly to the active site of the mutant VoDMS (D309A). Therefore, the lack of evidence for C3-protonation from these experiments cannot exclude the class II reaction in VoDMS. We predict that an unrevealed acidic residue in VoDMS may play a critical role in protonation.

4. Conclusion

In summary, a new sesqui-TPS, identified from the valerian root transcriptomic analysis, was comprehensively characterized and demonstrated to encode a (−)-drimenol synthase (VoDMS). Drimenol can serve as a useful synthon for the semi-synthetic preparations of various bioactive drimane-type sesquiterpenes. Hence, the *VoDMS* can be used to biologically synthesize drimenol, as shown in our yeast system. VoDMS appears to utilize both class I (ionization) and class II (protonation) mechanisms in a single active site that are unprecedented among the known sesqui-TPSs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.10.031.

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