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Research article

Molecular cloning and functional analysis of a 10-*epi*-junenol synthase from *Inula hupehensis*

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ABSTRACT

Junenol based-eudesmanolides have been detected in many compositae plant species and were reported to exhibit various pharmacological activities. So far, the gene encoding junenol synthase has never been isolated. Here we report the molecular cloning and functional analysis of a 10-*epi*-junenol synthase from *Inula hupehensis* (designated IhsTPS1). IhsTPS1 converts the substrate farnesyl diphosphate into multiple sesquiterpenes with the product 10-*epi*-junenol being predominant. The transcript levels of IhsTPS1 correlate well with the accumulation pattern of 10-*epi*-junenol in *I. hupehensis* organs, supporting its biochemical roles in vivo.

1. Introduction

Inula hupehensis is a special *Inula* species seemingly only distributed in China (Editorial Committee of the Administration Bureau of Chinese Plant Medicine, 1979). This plant is used as traditional Chinese medicine, which is prescribed for the treatment of bronchitis, diabetes and intestinal ulcers (Zhao et al., 2006). Phytochemical studies of *I. hupehensis* above ground tissues have revealed the presence of sesquiterpene lactones (STLs) which can be classified based on their backbones as germacranolide, eudesmanolide, guaianolide, pseudo-guaianolide, xanthanolide, and bakkenolide (Qin, 2011). A number of junenol type eudesmanolides were found in the genus *Inula* plant, including *I. hupehensis* (Qin, 2011). The eudesmanolides have also been reported from the other *genus* plant species (Wu et al., 2006). The biological role of eudesmanolides in plants is not very clear, but they have potentially useful pharmacological activities, including anti-inflammatory, anti-tumor, anti-fungal, anti-microbial and cytotoxicity activities (Park and Kim, 1998; Wang et al., 2013, 2014; Lin et al., 2015; Jin et al., 2006; Qin et al., 2010; Maoz et al., 1999). In spite of many studies concerning their structures and medicinal activities, the enzymes and genes involved in the biosynthesis of plant STLs are largely unknown.

The first committed step of STLs biosynthesis is the formation of STL skeleton by the cyclization of the universal precursor, farnesyl diphosphate (FPP) under the action of sesquiterpene synthases (sTPSs). For the germacranolides, the backbone-forming enzymes, including germacrene A synthase, germacrene B synthase, germacrene C synthase, and germacrene D synthase, have been characterized in many plant species at molecular levels (De Kraker et al., 1999; Bouwmeester et al., 2002; Bertea et al., 2006; Van der Hoeven et al., 2000; Colby et al., 1998; Prosser et al., 2004; Deguerry et al., 2006). Relatively little is known about the formation of other STLs. Several groups proposed that the other STLs might be derived from germacranolides by a variety of modifications, e.g. cyclizations, ring fissions, and methyl migrations (De Kraker, 2002; Pickel et al., 2012), however, there are no direct evidences at biochemical and molecular levels to support this proposal. Recently, the cDNA encoding aneudesmane skeleton-forming enzyme (β-eudesmol synthase) has been cloned from *Zingiber zerumbet* (Yu et al., 2008), which implicated that the

Abbreviations: ORF, open reading frame; sTPS (s), sesquiterpene synthase (s); FPP, farnesyl pyrophosphate; IPTG, isopropyl β-D-1-thiogalactopyranoside; QRT-PCR, quantitative reverse transcription polymerase chain reaction; RACE, rapid amplification of cDNA ends.

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eudesmane skeleton is formed by a unique sTPS on its own pathway while not, at least in part, by the rearrangement of germacranolides.

As indicated in Fig. 1, there are two possibilities for the formation of 10-*epi*-junenol: (1) a sTPS converts FPP to an olefinic sesquiterpene intermediate, which is then oxidized at C-6 position by a P450 enzyme or dehydrogenase to form the 10-*epi*-junenol; (2) a sTPS directly catalyzes the formation of 10-*epi*-junenol from FPP. The first possibility represents a general activity feature of sTPSs, which is only toward the formation of terpenoid skeletons while not the formation of sesquiterpene alcohols. Recently, a number of sesquiterpene alcohol synthases, including *epi*-cedrol synthase (Mercke et al., 1999), kunzeaol synthase (Pickel et al., 2012), drimenol synthase (Kwon et al., 2014) and τ-cadinol synthase (Jullien et al., 2014), have been identified from plants, and they directly convert FPP to the corresponding sesquiterpene alcohols (*epi*-cedrol, kunzeaol, drimenol, and τ-cadinol). The identification of these sesquiterpene alcohol enzymes therefore raises the second possibility as shown in Fig. 1, in which 10-*epi*-junenol also can be directly formed by the conversion of FPP under a unique sesquiterpene alcohol synthase.

As part of our long-term project to investigate the STL biosynthesis in *I. hupehensis*, here we report the molecular cloning and biochemical property of a 10-*epi*-junenol synthase which stays in the early stage shown in Fig. 1 and is involved in the formation of the eudesmanolide biosynthesis. To the best of our knowledge, this is the first report concerning the cDNA cloning of a 10-*epi*-junenol synthase.

2. Materials and methods

2.1. Plant materials

I. hupehensis plants were collected from Enshi, Hubei Province, PR China. Seedlings were grown in open field conditions at Wuhan Botanical Garden, Chinese Academy of Sciences. When plants were at the full-bloom stage (about three month-old seedlings), plant materials, including roots, stems, young leaves, and flowers, were collected for the experiments of this study. The second leaf from the top of the plant was considered as the young leaf.

2.2. Isolation of full-length IhsTPS1 cDNA

Based on the highly conserved amino acid regions of several known sTPSs, which are (−)-limonene synthase from spearmint, 5-*epi*-aristolochene synthase from tobacco, and casbene synthase from castor bean, a pair of degenerate oligonucleotide primers 1a and 1b were synthesized (Wallaart et al., 2001). The PCRs were carried out for 40 cycles of 1 min for 94 °C, 2 min for 40 °C, 75 s for 72 °C, and a final elongation step of 72 °C for 10 min using cDNA from *I. hupehensis* young leaves as a template. The fragments with an expected size of 500–600 bp were separated by electrophoresis, gel-purified and ligated into a pMD19-T vector for sequencing. To obtain the entire sequence of IhsTPS1, the 5′ and 3′ regions were amplified separately using the SMART RACE cDNA Amplification kit (Invitrogen, Carlsbad, CA, USA) according to the provided protocols. The primers 2a, 2b, 3a, and 3b were used to amplify the 3′ region, and 4a, 4b, 5a, and 5b were used to amplify the 5′ region. The complete sequences were then amplified using specific primers 6a and 6b, and designated IhsTPS1. The sequences of all the nucleotide primers were listed in Supplemental Table S1.

2.3. Preparation of the purified recombinant IhsTPS1

The ORF of IhsTPS1 was cloned into an *E. coli* expression vector pET28b (Novagen, Germany) under the SalI/XhoI sites, giving the construct pET28b-IhsTPS1. The construct pET28b-IhsTPS1 was then transferred into the BL21(DE3) *E. coli* cells by heat shock at 42 °C for 90 s. Transgenic colonies were selected on LB plates with 50 μg mL^{-1} of kanamycin. A single positive colony was grow up at 37 °C overnight in 5 mL of LB liquid medium containing 50 μg mL^{-1} of kanamycin. The overnight culture was then diluted 100 folds into 300 mL of the fresh medium and grown until OD_{600} value of 0.5, at which point the expression of IhsTPS1 was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) in a final concentration of 1 mM and the *E. coli* cells were continually grown in the 180 rpm-shaker at 16 °C for 12 h.

After the induction, the cells were pelleted by centrifugation, resuspended in 10 mL of lysis buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 0.5 mg mL^{-1} lysozyme), and incubated 4 °C for 30 min. The lysozyme treated cells were then sonicated for the total protein extraction. After centrifugation at 12,000 × g at 4 °C for 20 min, the total protein supernatant was passed through a HisPur Ni-NTA resin-packed column (Thermo Scientific, U.S.A) and the HIS-tagged IhsTPS1 was finally eluted with 10 mL of the elution buffer (50 mM sodium phosphate, pH 7.5, containing 300 mM NaCl and 250 mM imidazole). The purified recombinant IhsTPS1 was desalted into the enzyme assay buffer (25 mM Tris-Cl, pH 7.5, 10% glycerol, 1 mM DTT, 1 mg mL^{-1} BSA, 10 mM $MgCl_2$, and 1 mM $MnCl_2$) and concentrated by centrifugation with 30-kDa cut-off Millipore concentrator. The purity of the recombinant IhsTPS1 was examined by an electrophoresis on SDS-PAGE gel followed by Coomassie Blue staining and its concentration was estimated using Bradford assays (Bio-Rad).

2.4. Enzyme assays

In vitro enzyme assays were performed in a final volume of

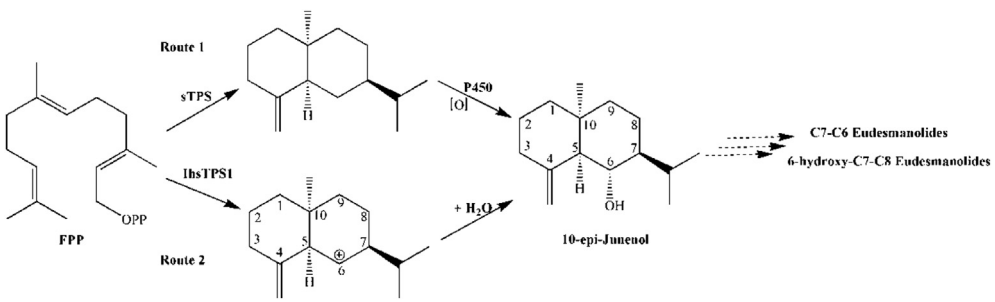

Fig. 1. Proposed two possibilities for the biosynthesis of 10-*epi*-junenol which is then forward for the production of eudesmanolides. Route 1, a sTPS converts the substrate farnesyl diphosphate (FPP) to an olefinic sesquiterpene intermediate, which is then oxidized at C-6 position by a unique P450 or dehydrogenase to form 10-*epi*-junenol; Route 2, a sTPS (e.g., IhsTPS1) directly catalyzes the formation of 10-*epi*-junenol from FPP.

500 μL reaction mixture containing enzyme assay buffer (25 mM Tris-Cl, pH 7.5, 10% glycerol, 1 mM DTT, 1 mg mL^{-1} BSA, 10 mM $MgCl_2$, 1 mM $MnCl_2$), 35 μg purified recombinant IhsTPS1, and 100 μM FPP (Sigma-Aldrich). The mixture was overlaid with 500 μL of pentane and incubated at 30 °C for 2 h. As a control, the purified IhsTPS1 was inactivated by being boiled at 100 °C for 10 min. After the reactions, the pentane layer was directly used for GC-MS analysis.

2.5. Measurement of 10-epi-junenol content in I. hupehensis organs

Fresh plant materials were ground to powder in liquid nitrogen and about 0.2 g of the powder was extracted with 500 μL of pentane containing 43.2 ng μL^{-1} nonyl acetate as an internal standard in a sonicator for 30 min. After centrifugation at 12, 000 rpm for 1 min, 1 μL of the supernatant was injected for GC/MS analysis. The peak areas of 10-*epi*-junenol between organs were normalized with the internal standard nonyl acetate and compared.

2.6. GC/MS analysis

GC/MS analysis were carried out on an Agilent 7890A GC instrument (Agilent Technologies, Waldbronn, USA) coupled to an Agilent 5975C mass selective detector. The injection temperature was 250 °C. One μL of sample was injected and analyzed on a HP-5MS column (0.25 mm ID9 30 m, 0.25 μm film thickness, Agilent). The carrier gas was helium with a flow rate of 1.2 mL min^{-1}. The temperature program was 45 °C for 1 min, followed by a 5 °C min^{-1} ramp to 250 °C, and held at 250 °C for 5 min. Full mass spectra were generated for metabolite identification by scanning within the *m*/*z* range of 40–300. Alkane standard mix (C_8–C_{20}) was used to determine retention indices. The identities of peaks were tentatively identified by searching their mass spectrums and retention indexes (RIs) against the MassFinder 4.0 terpenoids library. To further confirm the identity of 10-*epi*-junenol, samples were compared with the *Ferula galbaniflua* essential oils which contain 10-*epi*-junenol whose structure has been previously determined by NMR studies (Thomas et al., 1976). The *F. galbaniflua* oil extracts were commercially obtained from POLI Company (SH POLI TRADING CO., LTD).

2.7. Measurement of IhsTPS1 transcript abundance in I. hupehensis organs

Total RNA was isolated from the *I. hupehensis* organs, including its roots, stems, leaves and flowers, using the EASYspin Plant RNA isolation kit (Aidlab, Beijing, China). The first-strand cDNA was synthesized from 1.5 μg of the total RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with an oligo $(dT)_{17}$ primer. The primers 7a/7b were used to amplify a 180-bp fragment of the IhsTPS1 transcript and the primers 8a/8b were used to amplify a 214-bp fragment of *I. hupehensis* actin cDNA (GenBank accession no. KT218679). QPCRs were performed on Step One Plus Real-Time PCR System (ABI, U.S.A.) using Fast Start Universal SYBR Master (Rox) Mix (Roche, Switzerland). The thermal cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Relative gene expression was calculated using the $2^{\Delta\Delta CT}$ method described by Schmittgen (Schmittgen, 2006). The sequences of the primers used for QPCRs were shown in the Supplemental Table S1.

2.8. Phylogenetic tree analysis

The phylogenetic tree was constructed with MEGA 5.1 software using a neighbor-joining method. A bootstrap resampling analysis with 1000 replicates was performed to evaluate the tree topology. The scale bar represents 0.2 amino acid substitutions per site. The known terpene synthases from different sub-families were selected in reference to the TPS phylogenetic tree previously constructed by Li et al. (Li et al., 2016).

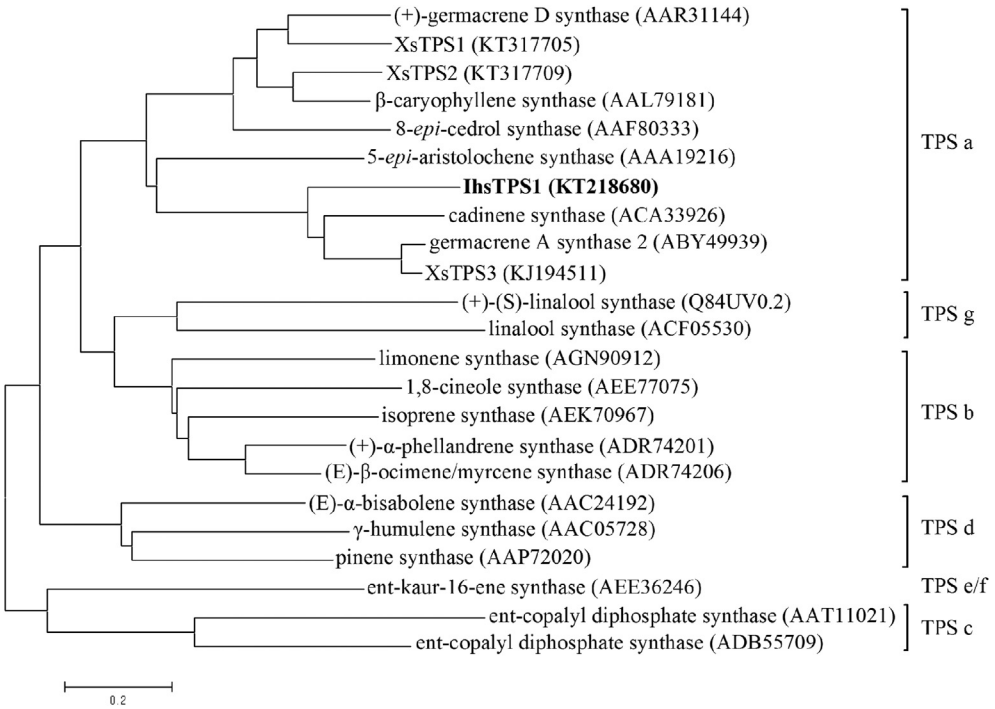

Fig. 2. Phylogenetic analyses of IhsTPS1 with other known TPSs from different sub-families. The tree was constructed by the neighbor-joining method using MEGA 5.1. The TPS sequences selected for the tree construction are in reference to the previous report by Li et al. (Li et al., 2016).

3. Results and discussions

3.1. Full-length cDNA cloning of IhsTPS1 from I. hupehensis

Degenerate oligonucleotide primers were used in RT-PCR experiments with the *I. hupehensis* leaves cDNA as the template, which resulted in a 550 bp-amplified product. Its full-length cDNA was then discovered by RACE strategy and designated *IhsTPS1* (the GenBank accession no. KT218680). The open reading frame (ORF) of IhsTPS1 encodes a 529 amino acid-polypeptide which shows the most similarity (61% amino acid identity) to the germacrene A synthase of *Cichorium intybus* (CiGAS) (Bouwmeester et al., 2002). As shown in the Supplemental Fig. S1, the deduced amino acid sequences of IhsTPS1 possess the DDxxD and NSE/DTE motifs

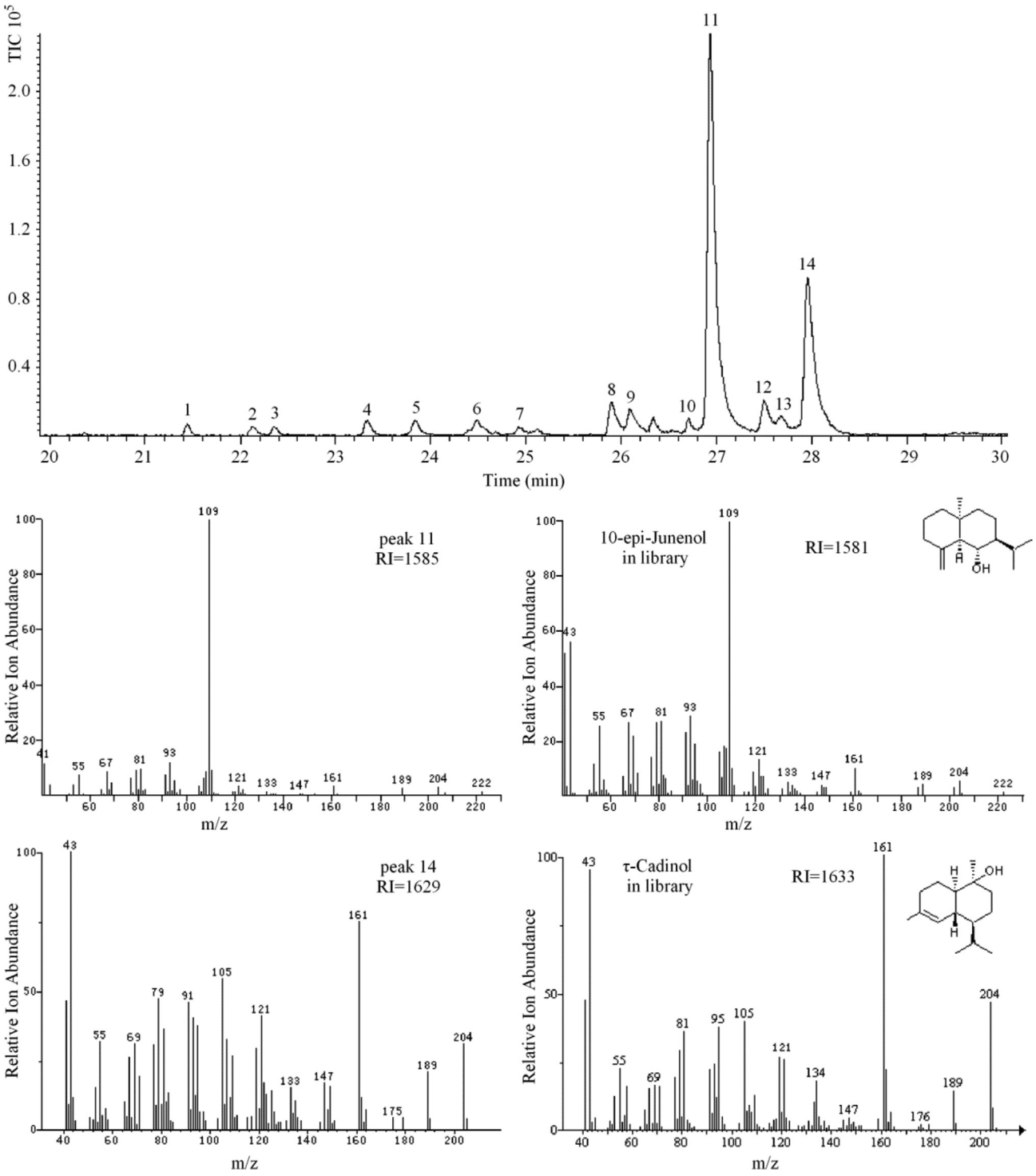

Fig. 3. GC-MS analyses of the products by IhsTPS1 in the *in vitro* reactions with FPP. Total ion chromatograms (TICs) were shown for the products (peaks 1–14) from the reactions with the peak 11 and peak 14 being the major products. By searching their retention indexes and mass spectrums against the Mass Finder 4.0 terpenoids library, the peak 11 and peak 14 were tentatively identified as 10-*epi*-junenol and τ-cadinol, respectively.

characteristic of the sTPS superfamily, which are thought to form the divalent metal ion binding sites in the interaction with diphosphate group (Degenhardt, 2009; Harris et al., 2015; Miller and Allemann, 2012). In addition, IhTPS1 contains the RxR motif that plays an important role in the complexation of the diphosphate group after substrate ionization (Christianson, 2006). Thus, the predicated amino acid sequences indicated IhsTPS1 as a sTPS. Phylogenetic tree analysis revealed that IhsTPS1 falls into the TPS-a subfamily (Fig. 2).

3.2. Biochemical characterization of IhsTPS1

To investigate the biochemical activity of IhsTPS1, the HIS-tagged recombinant IhsTPS1 was purified by an expression in *E. coli* cells (Supplementary Fig. S2). Through the *in vitro* enzyme assays, the purified IhsTPS1 was able to convert the substrate FPP into a number of sesquiterpenes (Fig. 3). Among its enzymatic products, the peak 11 at the retention time of 26.94 min and peak 14 at the retention time of 27.97 min are major products, accounting for 57.69% and 26.99% of the total products, respectively (Fig. 3). By comparing the mass spectrums and retention time index (RI) of the products against the Mass Finder 4.0 terpenoid library, the peak 11 was tentatively identified as 10-*epi*-junenol while the peak 14 as τ-cadinol (Fig. 3).

To further confirm the identity of the peak 11, the *F. Galbanurn* essential oils containing the compound 10-*epi*-junenol (Thomas et al., 1976) were used as the reference. As shown in Fig. 4, the peak 11 had identical retention index and mass fragmented products with those of 10-*epi*-junenol from the *F. Galbanurn* essential oils. It should be noted that the RI of 10-*epi*-junenol is apparently different from that of its isomer, junenol. For instance, the RI of 10-*epi*-junenol is 1581 while the RI of junenol is 1617 in the Mass Finder 4.0 terpenoids library. Thus, the data here suggested that the compound corresponding to the peak 11 is 10-*epi*-junenol. By searching the Mass Finder 4.0 terpenoid library, the minor products including the peaks 2, 4, 5, 8 and 9 were tentatively identified as β-elemene, pacifigorgia-2(10),11-diene, melanene, 10-*epi*-cis-dracunculifoliol and elemol, respectively (Fig. S3), while the identities of the rest minor products were not determined and their mass spectrums were available in the Supplemental Fig. S3. The second major product by IhsTPS1 is τ-cadinol. A cDNA encoding the τ-cadinol-forming sTPS (LaCADS: accession number is JX401282) has been cloned from *Lavandula angustifolia* (Jullien et al., 2014). In *I. hupehensis* young leaves extracts, 10-*epi*-junenol was detectable but at very low levels (Fig. 4C), while other products of IhsTPS1, including the product τ-cadinol, were not found in the plant extracts under the conditions of this study (data not shown), which could be due to the low amount of these products accumulated in

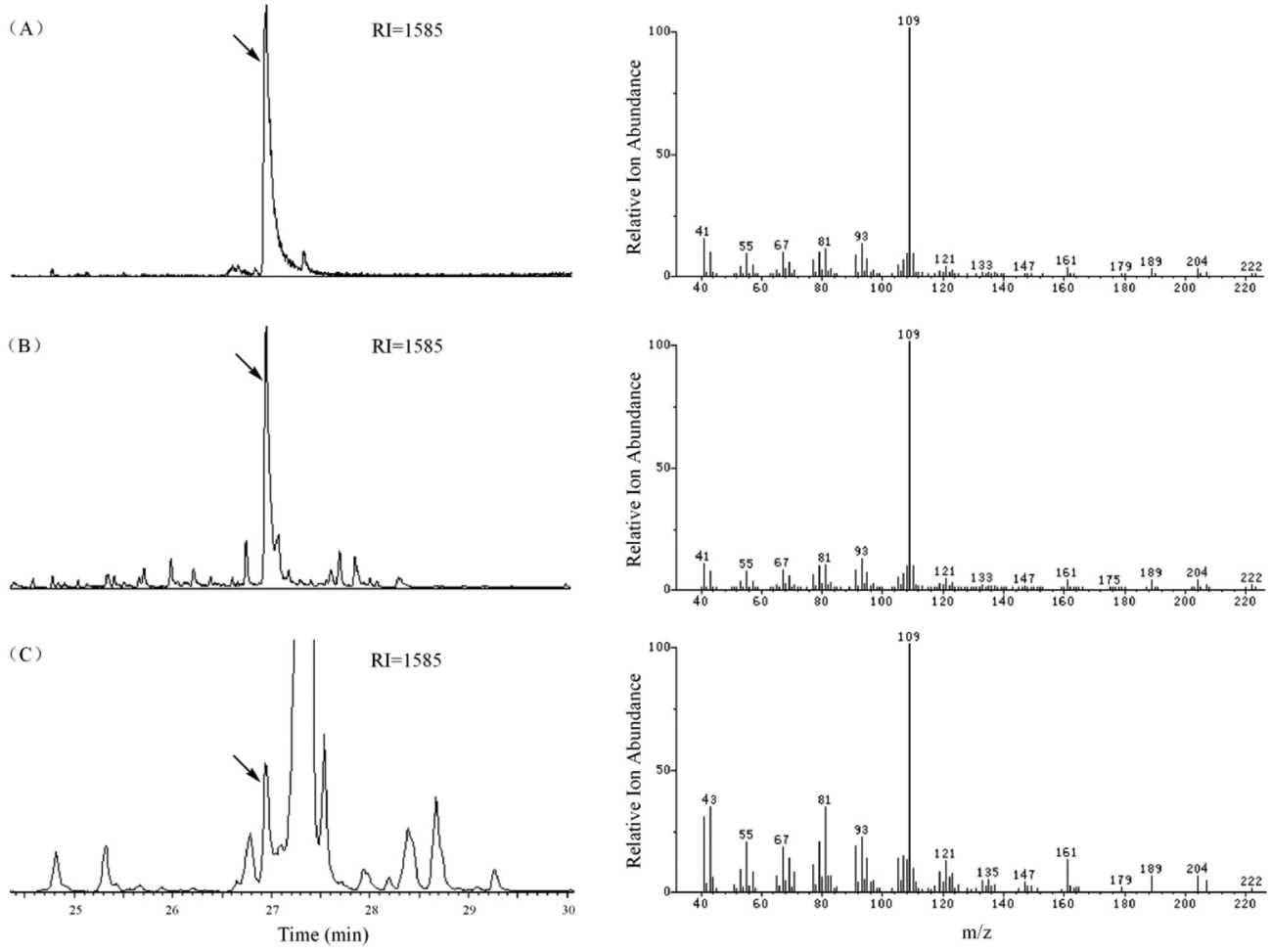

Fig. 4. Aligning of the chromatograms at a single-ion of *m/z* 109 for the products by IhsTPS1 *in vitro* (A), the *Ferula Galbanurn* essential oils (B), and the *I. hupehensis* young leaves extracts (C). The arrow pointed peaks correspond to the product 10-*epi*-junenol. The right panel showed the mass spectrums of the 10-*epi*-junenol product from each extract. The retention indexes and mass spectrums of the 10-*epi*-junenol product from each extract are identical. The structure of the 10-*epi*-junenol from the *F. Galbanurn* essential oils was previously confirmed by NMR studies (Thomas et al., 1976).

the plant or the much efficiently conversion of them to the downstream end eudesmanolides in vivo.

Considering that 10-*epi*-junenol was the predominant product by the *in vitro* enzyme assays, IhsTPS1 was thus judged to be a 10-*epi*-junenol synthase. To the best of our knowledge, this is the first report concerning the isolation of the gene encoding 10-*epi*-junenol synthase from the plant kingdom. A number of by-products formed by IhsTPS1 indicated that IhsTPS1 catalyzes a complex reaction in which a series of highly reactive carbon cationic intermediates are produced during the reaction. The reaction mechanism of IhsTPS1 with FPP is not clear, but the attachment of the alcohol group to sesquiterpene backbone may be the consequence of water solvent quenching the terminal carbocation of the reactive intermediates (Pickel et al., 2012; Kwon et al., 2014; Harris et al., 2015; Miller and Allemann, 2012; Salmon et al., 2015). IhsTPS1 shows the most sequence identity to CiGAS (Fig. S1) but did not exhibit an apparent germacrene A synthase activity (Fig. 2). On the other hand, although it shows only 32.9% amino acid identity with LaCADS (the first identified τ-cadinol synthase from *L. angustifolia*), τ-cadinol was found as the second major product in the reaction of IhsTPS1 with FPP (Fig. 2). Thus, the identification of IhsTPS1 provided another example of the fact that the biochemical role of sTPSs could not be simply inferred by their primary amino acid sequences alone.

3.3. *IhsTPS1 transcripts correlate well with the accumulation of 10-epi-junenol in vivo*

QPCRs analysis showed that the highest level of IhsTPS1 transcript was observed in *I. hupehensis* young leaves while relatively lower levels in its roots and flowers and no expressions in stems (Fig. 5A). On the other hand, by calculating GC peak areas which were normalized with the internal standard nonyl acetate, the highest concentration of 10-*epi*-junenol was observed in the young leaves followed by the roots and flowers whereas absent in stems (Fig. 5B), which generally paralleled with the gene expression of IhsTPS1. These data demonstrated that IhsTPS1 is involved in the production of 10-*epi*-junenol in *I. hupehensis* in vivo.

Taken together, our data here demonstrated that IhsTPS1 is the enzyme to synthesize 10-*epi*-junenol in *I. hupehensis*. Generally speaking, the biological role of 10-*epi*-junenol in plant is not very clear, but as an essential oil it is implicated to have several beneficial effects to human health (Sahebkar and Iranshahi, 2010). The identification of IhsTPS1 provides a potential to increase the production of 10-*epi*-junenol in plants by overexpressing IhsTPS1 or facilitate its production in microbial hosts by synthetic biology.

4. Conclusion

The biosynthetic mechanism for the formation of 10-*epi*-junenol was elucidated in this study, for the first time, by the molecular cloning and functional characterization of a sesquiterpene alcohol synthase, IhsTPS1, from the Chinese native composite plant, *I. hupehensis*. IhsTPS directly converts the substrate FPP to form the 10-*epi*-junenol. The transcript abundance of *IhsTPS1* correlates well with the accumulation pattern of 10-*epi*-junenol in various tissues of *I. hupehensis*, supporting the involvement of *IhsTPS1* in the biosynthesis of 10-*epi*-junenol in this plant.

Author contributions

JBG designed this study; JBG performed the gene cloning and biochemical reactions; ZQL, CFL, FFC, and SYL provided the assistance in determining the identities of the products discovered in this study; YSZ and JBG wrote the manuscript.

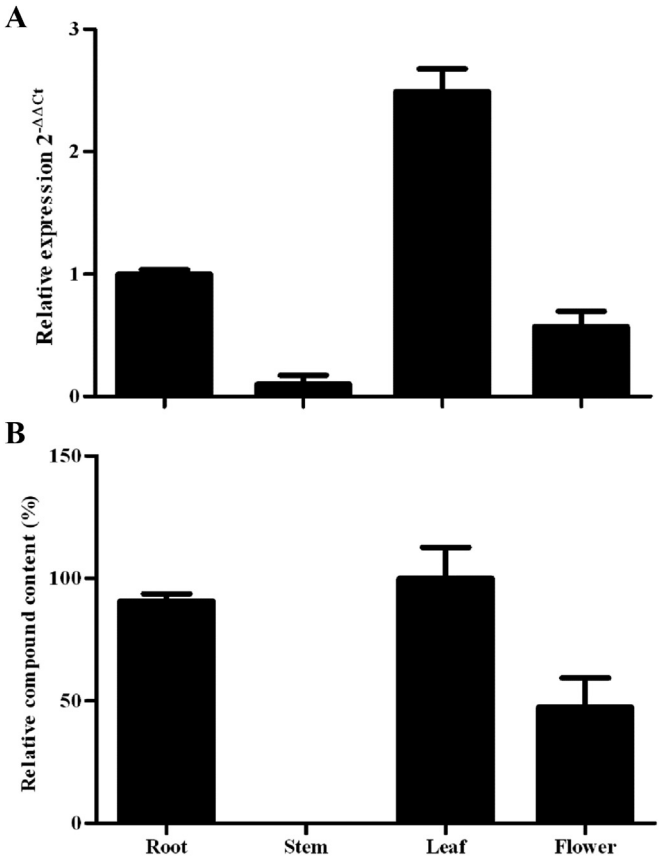

Fig. 5. IhsTPS1 transcript levels correlate well with the accumulation of 10-*epi*-junenol in *I. hupehensis* organs. IhsTPS1 transcripts were measured by QPCRs (A) and the relative amount of 10-*epi*-junenol between different organs was compared by calculating their GC peak areas which were normalized with the internal standard nonyl acetate (B). Error bars represent standard errors from three biological replicates.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.05.023.

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