Identification of a Novel (-)-5-Epieremophilene Synthase from *Salvia miltiorrhiza* via Transcriptome Mining

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**INTRODUCTION**

*Salvia miltiorrhiza*, a perennial herb in the family of Lamiaceae (Menthaceae), is one of the most commonly used medicinal plants in China. Its dry root, known as Danshen in traditional Chinese medicine (TCM), is used in the treatment of coronary heart diseases, particularly angina pectoris and myocardial infarction (Wang et al., 2007). Danshen has been reported to contain two types of active ingredients: the diterpenoid tanshinones and phenolic salvianolic acids (Zhou et al., 2005; Yang et al., 2016).

Lamiaceae is the sixth largest angiosperm family which comprises about 240 genera and more than 7000 species widely distributed in the world (Li et al., 2016). The chemistry of the Lamiaceae plants is dominated by the great diversity and considerable amounts of terpenoids, especially monoterpenes and sesquiterpenes. Within the family, *Salvia* is the largest genus, and plants of this genus are used for medicinal purposes not only in China but also in many other areas. For example, *S. officinalis* is one of the most important medicinal and aromatic plants in Europe, which has antioxidant, antimicrobial, astringent, antidiuretic and specific sensorial properties (Cuvelier et al., 1994; Ghorbanpour et al., 2016; Guneser et al., 2016).
and produces rich amounts of monoterpenes and sesquiterpenes (Bouajaj et al., 2013). Root of S. miltiorrhiza accumulates a specific group of diterpenoids, known as tanshionines, which are the major active constituents against cerebral infarction (Lin and Hsieh, 2010) and inflammation (Chen and Xu, 2014). In recent years, extensive investigations of biosynthesis of tanshionines have resulted in important progresses. (Gao et al., 2009; Guo et al., 2013, 2016; Cui et al., 2015; Ma et al., 2016). Based on a review of chemical constituents of Salvia plants, 46 sesquiterpenoids have been identified from 15 Salvia species (Wu et al., 2012). However, until now sesquiterpenes have not been reported from S. miltiorrhiza, though more than 40 diterpenoids have been identified.

Plants produce a rich array of terpenoids with diverse structures, which play important roles in both basic biological processes and interactions with environmental factors. Terpene synthases (TPSs), including mono-, sesqui-, and di-terpene synthases, function as key enzymes in biosynthesis of terpenoids, and TPS genes often have different expression patterns in different plant tissues. Most of TPSs produce one single dominant product restricted in limited genera or even a single species. For example, (+)-Valencene is a high added-value commercial flavoring responsible for the overall flavor of citrus fruit oils and the corresponding enzyme has been characterized in the genus Citrus (Sharon-Asa et al., 2003). Thus, functional characterizations of TPS gene in more species will allow us access to unusual secondary metabolisms previously not encountered. Furthermore, plant secondary metabolisms could be biosynthesized in a tight regulated fashion (Yu et al., 2015) in which the intermediates may be transformed immediately and thus could not be found by traditional phytochemistry way. Genome and transcriptome mining are alternative methodologies developed recently to reveal these inaccessible natural products, such as thalianol (Fazio et al., 2004) and its derives (Castillo et al., 2013) from Arabidopsis thaliana. Our previous analysis of S. miltiorrhiza transcriptomes has annotated 24 unigenes as TPSs (Yang et al., 2013), most of them have not been functionally characterized. Here, we report three of them, which encode the sesquiterpene synthase for a single product, (-)-5-epieremophilene. To the best of our knowledge, this is the first time (-)-5-epieremophilene and its synthase being reported.

**MATERIALS AND METHODS**

**Plant Materials and Chemicals**

Seeds of S. miltiorrhiza were collected from Anhui Province and the plants were grown in nursery of Shanghai Chenshan Botanical Garden. For experimental analysis, the plants were grown in greenhouse at 25°C under a light intensity of 150 µmol of photons·m⁻²·s⁻¹ with 14-h-light/10-h-dark cycle. Chemicals were purchased from Sigma-Aldrich, TCI and Fluka.

**Phytohormone Treatment**

For hormone treatments, plants of 3-month-old, when biosynthesis of secondary metabolites was active and the transcription levels of the corresponding enzymes were high, were used. The plants were sprayed with the following solutions: 5 mM salicylic acid (SA), 100 µM ethylene (ET), 100 µM abscisic acid (ABA), 50 µM methyl jasmonate (MeJA), and 50 µM gibberelrin A₃ (GA₃), in addition of a dimethyl sulfoxide (DMSO) solution (5%) as control. Leaf were then collected for isolation of total RNAs 2 h post-spray. Each experiment was performed with at least three biological replicates.

**Plant Terpenoids Extraction**

The following materials from the 6-month-old plants in bloom were harvested for extraction of chemicals: primary root (6 cm length from the apical); stem (the second to the fourth internodes from the top); the third pair of true leaves; and the inflorescence. The 6-month-old plants contained a high level of terpenoids and suitable for extraction.

Fresh materials (0.5 g) were ground in liquid nitrogen, followed by adding 2.5 mL hexane to extract the terpenoids in a shaker at 30°C for 1 h. A short column of aluminum oxide overlaid with anhydrous MgSO₄ was used to purify the extracts, and the obtained hexane phase was analyzed by Gas chromatography–mass spectrometry (GC-MS) by the total ion chromatogram mode.

**cDNA Isolation and Analysis**

Total RNAs from the plant tissues were extracted using CTAB solution (2% CTAB, 2% PVP, 100 mM Tris-HCl, 25 mM EDTA, 2M NaCl, 2% β-mercaptoethanol) and treated with DNase I (Takara) according to the manufacturer’s protocol. Total RNA of 1 µg was reverse-transcribed using the ReverTra Ace qPCR RT Kit (TOYOBO, FSQ-101). Rapid amplification of cDNA end (RACE) was performed by the 5′- and the 3′-Full RACE Core Set Ver.2.0 (Takara) according to manufacturer’s instructions. PCR products were cloned into the pMD18-T Vector (Takara) for sequencing. Full-length cDNAs were obtained by assembling fragments obtained by RACE in combination with annotated unigene sequences.

For gene expression analysis, real-time PCR was performed with gene-specific primer pairs on the reverse transcripts derived from total RNAs, using the SYBR® Premix Ex Taq™ II (Perfect Real Time) kit (Takara) on a Mastercycler system (Eppendorf, Germany). ACTIN (HM231319) was used as internal reference (Yang et al., 2010). The relative expression value was calculated via the 2⁻ΔΔCt method. All PCR primers used in this investigation are listed in Supporting Table S1.

Terpene synthase sequences from other plant species were obtained through searching the GenBank database at NCBI. Alignment of protein sequences was performed with CLUSTAL W program of MEGA 6 software with default parameters. Phylogenetic tree was built with the neighbor-joining method with MEGA 6 program. Chloroplast signal peptide prediction was performed by SignalP software.

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1http://www.cbs.dtu.dk/services/SignalP/
Prokaryotic Expression and Protein Purification
The open reading frames of cDNAs were amplified with fast-Pfu DNA polymerase, the PCR products were then digested by EcoRI and HindIII, followed by ligation into pET-32a expression vector. After confirmation by DNA sequencing, the plasmids were transferred into Escherichia coli Rosetta (DE3) for protein production. Recombinant proteins were purified with Ni-NTA resin (Thermo) according to manufacturer’s manual. Protein concentrations were determined with the Bradford method using BSA as standard.

Enzyme Assay
The TPS activity was assayed as described (Ruan et al., 2016). The reaction was conducted in a volume of 500 µL buffer containing 20 µg purified recombinant protein, 25 mM hydroxyethyl piperazineethanesulfonic acid (HEPES, pH 7.0), 5 mM MgCl2, 5 mM dithiothreitol (DTT) and 40 µM FPP, and incubated at 30°C for 1 h unless otherwise indicated. The reaction mixture was extracted with 800 µL hexane and subjected to analysis by GC-MS. To determine the quantities of the products, nonyl acetate was added to the hexane as internal standard.

Kinetic parameters of the enzymes were determined as described (Ruan et al., 2016). Briefly, the purified recombinant enzyme (5 µg) was added to each assay mixture containing FPP range from 2.3 to 184.6 µM. The reaction was allowed to proceed for 5 min at 30°C, stopped by the addition of 0.5 M EDTA (pH 8.0) and the reaction products were extracted with 800 µL hexane containing 10 ng/µL nonyl acetate (10 ng/µL) was added to the hexane as internal standard.

Gas Chromatography–Mass Spectrometry Analysis
Gas chromatography–mass spectrometry analysis was carried out on Agilent 6890 Series GC System coupled to an Agilent 5973 Network Mass Selective Detector, with the carrier gas helium at 1 mL/min, splitless injection, a agilent HP-5MS column (5% phenyl methyl siloxane, length 30.0 m, diameter: 250.00 µm, film thickness: 0.25 µm) for non-chiral analysis. For volatile terpenes detection of S. miltiorrhiza, a temperature program from 60°C (5 min hold) at 5°C/min to 260°C was used; For enzymatic assay, the following temperature program was used: initial temperature of 60°C (5 min hold), increase to 250°C by 10°C/min, and ramp to 300°C by 50°C/min (5 min hold). Compounds were identified by comparison with NIST data base (National Institute of Standards and Technology) library and retention indices (Garms et al., 2010).

RESULTS
Volatile Terpenes of S. miltiorrhiza Determined by GC-MS
To analyze volatile terpenes in root, stem, leaf and inflorescence of S. miltiorrhiza, fresh tissues were extracted with hexane and subjected to GC-MS. In total 20 terpenes were detected, including 8 monoterpenes, 11 sesquiterpenes, and 1 diterpenes (Figure 1). Of the four organs sampled, leaf contained the most diversified terpenes, with at least 18 compounds (8 monoterpenes, 10 sesquiterpenes) being identified by searching NIST database and retention indices (Garms et al., 2010). By contrast, root had the lowest diversity, as GC-MS identified only 1 diterpene (ferruginol). Notably, ferruginol was not present at a detectable level in the aerial tissues examined.

Isolation of Terpene Synthase Genes and Phylogenetic Analysis
We searched S. miltiorrhiza transcriptomes (Yang et al., 2013) for TPSs, and amplified cDNAs by 5′- and 3′-RACE. Three cDNAs, namely SmSTPS1, SmSTPS2, and SmSTPS3 (GenBank accession numbers are KY432512, KY432513, and KY432514), were isolated. They all encode proteins of 546 amino acids, which are highly identical to each other with the sequence identity of 97~99%. Based on S. miltiorrhiza genome sequence database (Xu et al., 2016), SmSTPS1, SmSTPS2, and SmSTPS3 are duplicates arranged as a cluster in the genome. Searching of National Center for Biotechnology Information (NCBI) non-redundant protein database showed that the three proteins...
FIGURE 1 | Volatile terpenes detected from S. miltiorrhiza plant. Samples of root, stem, leaf, and inflorescence, 0.5 g (fresh weight) each, were extracted with 2.5 mL hexane for 1 h at room temperature and analyzed by GC-MS. Peaks are: 1, α-pinene; 2, Camphene; 3, sabinene; 4, β-pinene; 5, β-myrcene; 6, β-thujene; 7, trans-β-ocimene; 8, cis-β-ocimene; 9, α-copaene; 10, β-elemene; 11, β-caryophyllene; 12, β-copaene; 13, cadina-3,5-diene; 14, α-humulene; 15, trans-cadina-1(6),4-diene; 16, germacrene D; 17, β-cadinene; 18, germacrene D-4-ol; 19, germacrene B; 20, ferruginol.

share a high (62%) sequence identity to germacrene A synthase PatTpsCF2 (AY508728) from Pogostemon cablin (Deguerry et al., 2006), another Lamiaceae species which is particularly rich in sesquiterpenes (Swamy and Sinniah, 2015). The three proteins of S. miltiorrhiza contain the conserved domain DDxxD involved in the coordination of divalent ions and water molecules, and a modified NSE/DTE domain [ND][D][LIV][x][ST]x3E at the C-terminal involved in metal cofactor binding (Whittington et al., 2002). In addition, a modified RRx8W motif exists at the N-terminal (Figure 2), which is related to cyclic products formation (Martin et al., 2010; Liu et al., 2014).

Terpene synthases of plants are divided into eight clades or subfamilies designated TPS-a through TPS-h, with a minimum identity of 40% in each clade, and most angiosperm sesquiterpene
FIGURE 2 | Alignment of deduced amino acid sequences of SmSTPS1, SmSTPS2, SmSTPS3 with Pogostemon cablin germacrene A synthase, PatTpsCF2 (AY508728). The modified RRxW and DDXXD motifs and the metal binding motif NSE/DTE domain are framed. The sequences were aligned by Clustal W.

FIGURE 3 | Phylogenetic analysis of three sesquiterpene synthases from Salvia miltiorrhiza with other plant terpene synthases of different clades. In TPS-a the three S. miltiorrhiza (-)-5-epieremophilene synthases share high identities with Pogostemon cablin germacrene A synthase. The phylogenetic tree was constructed by Dayhoff's distances between proteins using the neighbor-joining method. Scale bar indicates 0.2 amino acid substitutions per site. Numbers are the actual bootstrap values of branches. The Protein ID in NCBI protein database are given in parentheses.
Fang et al. New Sesquiterpene Synthases from Salvia miltiorrhiza

Enzyme Activities of the Three TPSs

The three putative terpene synthases were then expressed in E. coli cells (Supplementary Figure S1). The purified fusion proteins were incubated with farnesyl diphosphate (FPP) as the substrate and the products were analyzed by GC-MS. The total ion chromatograms of hexane extracts of SmSTPS1, SmSTPS2, and SmSTPS3 after incubation with FPP as substrate are shown in Figure 4A. The protein tag produced by pET32a was used as control.

The chemical structure of the identified compound (-)-5-epieremophilene and structural related compounds are shown in Figure 4B. The mass spectrum of the identified (-)-5-epieremophilene is shown in Figure 4C.

FIGURE 4 | Identification of the products catalyzed by recombinant SmSTPS1, SmSTPS2, and SmSTPS3 in vitro. (A) Total ion chromatograms from GC-MS analysis of hexane extracts of SmSTPS1, SmSTPS2, and SmSTPS3 after incubation with FPP as substrate. The protein tag produced by pET32a was used as control. (B) Chemical structure of the identified compound (-)-5-epieremophilene and structural related compounds. (C) Mass spectrum of the identified (-)-5-epieremophilene.
and 1.53 s

14 20.23 0.94 s, 3H

J

13 108.33 4.74 d,

12 21.53 1.75 br s, 3H

11 150.11 –

10 144.08 –

9 120.51 5.36 m

8 30.11 1.69 m, 2H

7 38.51 2.06 m

6 38.11 –

5 32.89 –

4 37.05 1.53 m

3 27.09 1.47 m, 2 H

2 25.41 2.07 m; 1.97 m

1 120.51 5.36 m

-|$

\text{Position}$ | $^{13}{C}$ [s in ppm] | $^{1}{H}$ [s in ppm, m]

| 1 | 120.51 | 5.36 m |
| 2a/b | 25.41 | 2.07 m; 1.97, m |
| 3 | 27.09 | 1.47 m, 2 H |
| 4 | 37.05 | 1.53 m |
| 5 | 38.11 | – |
| 6α/β | 39.76 | 2.00 m; 1.55, m |
| 7 | 38.51 | 2.06 m |
| 8 | 30.11 | 1.69 m, 2H |
| 9α/α | 28.40 | 2.41 m; 1.97 m |
| 10 | 144.08 | – |
| 11 | 150.11 | – |
| 12 | 21.53 | 1.75 br s, 3H |
| 13 | 108.33 | 4.74 d, $J = 15$ Hz, 2H |
| 14 | 20.23 | 0.94 s, 3H |
| 15 | 16.02 | 0.89 d, $J = 10$ Hz, 3H |

of (+)-eremophilene and (+)-valencene mix, peak of (+)-valencene eluted before (+)-eremophilene (Schifrin et al., 2015), whilst in our GC-MS analysis of (+)-valencene and this sesquiterpene product mix, (+)-valencene eluted after this compound (Supplementary Figure S2). All of these suggest that these compounds are structurally similar. The detailed 2D NMR analysis allowed the construction of the structure of the sesquiterpene (Supplementary Figures S3–S8). The $^{1}H$-$^{1}H$ chemical shift correlation spectroscopy (COSY) correlation of an olefinic proton ($\delta_H = 5.36$) to H-2, and its heteronuclear multiple bond correlation (HMBC) correlations to C-3, and C-9 indicate the presence of C1-C10 double bound. The other olefinic protons

### Table 1 | Kinetic Parameters of SmSTPS1, SmSTPS2, and SmSTPS3.

| Enzyme       | $K_m$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$) |
|--------------|------------|---------------------|----------------------------------|
| SmSTPS1      | 25.35 ± 1.88 | 2.09 ± 0.05         | 8.27 ${\times}$ 10$^4$ ± 4.06 ${\times}$ 10$^4$ |
| SmSTPS2      | 24.20 ± 0.84 | 2.07 ± 0.13         | 8.56 ${\times}$ 10$^4$ ± 3.82 ${\times}$ 10$^4$ |
| SmSTPS3      | 10.44 ± 1.07 | 1.53 ± 0.02         | 1.48 ${\times}$ 10$^5$ ± 1.32 ${\times}$ 10$^5$ |

SmSTPS1, SmSTPS2, and SmSTPS3 were expressed in E. coli as recombinant proteins, and the enzyme activity was assayed at 30°C for 5 min with FPP as substrate.

### Table 2 | $^{1}H$ (500 MHz) and $^{13}C$ (125 MHz) NMR spectroscopic data for (+)-5-epieremophilene in CDCl$_3$.

| Position | $^{13}C$ [s in ppm] | $^{1}H$ [s in ppm, m] |
|----------|---------------------|----------------------|
| 1        | 120.51              | 5.36 m               |
| 2a/b     | 25.41               | 2.07 m; 1.97, m      |
| 3        | 27.09               | 1.47 m, 2 H          |
| 4        | 37.05               | 1.53 m               |
| 5        | 38.11               | –                    |
| 6α/β     | 39.76               | 2.00 m; 1.55, m      |
| 7        | 38.51               | 2.06 m               |
| 8        | 30.11               | 1.69 m, 2H           |
| 9α/α     | 28.40               | 2.41 m; 1.97 m       |
| 10       | 144.08              | –                    |
| 11       | 150.11              | –                    |
| 12       | 21.53               | 1.75 br s, 3H        |
| 13       | 108.33              | 4.74 d, $J = 15$ Hz, 2H |
| 14       | 20.23               | 0.94 s, 3H           |
| 15       | 16.02               | 0.89 d, $J = 10$ Hz, 3H |

substrate, and Mg$^{2+}$ as a metal cofactor. SmSTPS1, SmSTPS2 and SmSTPS3 all converted FPP into a single and same 204 Da sesquiterpene product, corresponding to the chemical formula C$_{15}$H$_{24}$ (Figure 4). Its MS spectrum is closely resemble those of valencene by searching NIST database, however, the retention time of this enzymatic product in GC-MS analysis is different to that of authentic (+)-valencene (Supplementary Figure S2), suggesting that it may share the same skeleton with valencene.

Kinetic analysis with FPP in the presence of Mg$^{2+}$ showed that the $K_m$ values of SmSTPS1, SmSTPS2 and SmSTPS3 are 25.35, 24.20, and 10.44 µM, the estimated $k_{cat}$ are 2.09 s$^{-1}$, 2.07 s$^{-1}$ and 1.53 s$^{-1}$, and the $k_{cat}/K_m$ values are 8.27 $\times$ 10$^4$ s$^{-1}$·M$^{-1}$, 8.56 $\times$ 10$^4$ s$^{-1}$·M$^{-1}$, and 1.48 $\times$ 10$^5$ s$^{-1}$·M$^{-1}$, respectively (Table 1). Thus, these three synthases have similar catalytic activities, though SmSTPS1 and SmSTPS2 are slightly more efficient. These parameters are in the normal range of plant TPSs.

### Structural Analysis of the Enzymatic Product

To elucidate the structure of the enzymatic product of three putative terpene synthases, we collected 4.5 mg this compound through large scale incubation experiment. The $^{1}H$ and $^{13}C$ NMR spectroscopic data of this sesquiterpene product (Table 2) were analogous to those of (+)-eremophilene (Schifrin et al., 2015), a stereoisomer of (+)-valencene, but their MS data are different; furthermore, in GC-MS separation

![Figure 4](image-url)
(δ_H = 4.74, J = 15 Hz) have HMBC cross peaks with C-7 and C-13 methyl group, locating the exo-isopropylidene group at C-7. The HMBC correlations of C-15 methyl group to C-3, C-4, and C-5; and C-14 methyl group to C-4, C-5, C-6, and C-10 attached these two methyls at C-4 and C-5, respectively. Thus, this compound has the same planar structure as valencene and eremophilene, which harbors three chiral centers of C-4, C-5, and C-7, indicating the existence of eight stereoisomers.

The GC-MS and NMR data of this compound are different to those of (+)-valencene, (-)-4-epieremophilene (Zhao et al., 2004) and (+)-eremophilene (Schifrin et al., 2015), and thus their enantiomers (-)-valencene, (+)-4-epieremophilene, and (-)-eremophilene, only leaving (+)-5-eremophilene and (-)-5-eremophilene as two possible candidates. The rotating frame overhauser effect spectroscopy (ROESY) data are in agreement with this assignment. The optical rotation analysis gave a [α]_24 value of -29.4 (ethanol). Taken together, this compound was unambiguously determined as (-)-5-epieremophilene, which is neither isolated nor synthesized previously. And these three sesquiterpene synthases are _S. miltiorrhiza_ (-)-5-epieremophilene synthases.

**Transient Expression of SmSTPS1 in _Nicotiana benthamiana_**

Because (-)-5-epieremophilene was not detected from the extracts of _S. miltiorrhiza_ plants, we adopted a transient expression system to produce recombinant proteins in leaves of _N. benthamiana_ (Peyret and Lomonossoff, 2013; Sainsbury and Lomonossoff, 2014), in order to further assay the activity of _S. miltiorrhiza_ (-)-5-epieremophilene synthase in planta. After 4 d of the infiltration of Agrobacterium harboring pEAQ-SmSTPS1, the leaves were gathered and extracted. We found that, compared to the extracts from control _N. benthamiana_ leaves, an extra peak with the retention time of approximately 16 min appeared when SmSTPS1 was expressed, which was determined to be (-)-5-epieremophilene by GC-MS (Figure 5).

**Spatial Expression Patterns and Hormone Induction**

Relative expression levels SmSTPS1, SmSTPS2, and SmSTPS3 in root and aerial organs were analyzed by quantitative real-time PCR (qRT-PCR). The three (-)-5-epieremophilene synthase genes were mainly expressed in leaf and inflorescence, and their transcript levels were low or undetectable in stem and root. In general, SmSTPS1 was expressed at a higher level than the other two, and its transcripts were most abundant in leaf. SmSTPS2 was weakly expressed and exhibited a clear expression only in leaf, whereas SmSTPS3 was expressed predominantly in inflorescence (Figures 6A–C). Thus, the three STPS genes were differentially regulated in _S. miltiorrhiza_ plants.

Next, we examined responses of the three sesquiterpene synthase genes to phytohormone treatments. Since SmSTPS1,
SmSTPS2, and SmSTPS3 were all expressed in S. miltiorrhiza leaves, changes in their expression levels in leaves were analyzed after hormone treatments. We found that, although the three sesquiterpene synthases share high similarities in sequences and catalytic activities, they responded differentially to different phytohormones at transcription level. SmSTPS1 was induced strongly by ethylene (ET) and moderately by MeJA, abscisic acid (ABA) and gibberellin A3 (GA3) (Figure 6D), SmSTPS2 responded not only to ET but also to ABA (Figure 6E), and SmSTPS3 was up-regulated slightly only by ABA (Figure 6F).

DISCUSSION

To date, more than 700 secondary metabolites have been reported from plants of Salvia, most of which (>80%) are terpenes (Wu et al., 2012). In S. miltiorrhiza and closely related species distributed in China diterpenoids are dominant in root (Li et al., 2008). Other natural products, such as flavonoids, were also reported from a number of species of the genus (Cuvelier et al., 1994). In this study, we analyzed volatile terpenes in different parts of S. miltiorrhiza, and found that root is distinct from aerial organs in accumulating much less volatile terpenes. In Arabidopsis, 20 of the 32 terpene synthase genes annotated were found to be expressed in flowers, which are responsible for the emission of both monoterpenes and sesquiterpenes (Chen et al., 2003). Microarray expression profiling also showed the distinct patterns of AtTPS genes in different organs (Ro et al., 2006). The diterpene ferruginal detected in S. miltiorrhiza root is an intermediate in the biosynthetic pathway to tanshinones (Guo et al., 2013; Ma et al., 2016), which are mainly synthesized in root (Yang et al., 1996). Our results demonstrate that S. miltiorrhiza synthesizes and accumulates distinct terpene compositions in different organs, suggesting a strategy of enhancing adaptation to various environmental factors through using diverse secondary metabolites, consistent with the report of Eucalyptus, which has a highly diverse family of TPS genes differentially regulated to generate unique terpene profiles in different parts of the plant (Kulheim et al., 2015).

Aroma is a characteristic of Lamiaceae family, and the volatiles with biological activities are often the major interests of the resources. Terpenes are often the important constituents of plant essential oils and have a variety of biological functions, among which sesquiterpenes form the largest group and are greatly valuable for their aromatic and pharmaceutical properties. S. miltiorrhiza is an important medicinal plant in China; differing from the European species S. officinalis, it has no particular scent even from the flowers. The three S. miltiorrhiza sesquiterpene synthases characterized here, SmSTPS1, SmSTPS2, and SmSTPS3, share a high sequence identity to PatTpsCF2 (AY508728) that catalyzes the biosynthesis of (+)-germacrene A as the dominant product with germacrene D, 4,5-di-epi-aristolochene, α-selinene and eremophilene as side products (Deguerry et al., 2006). The SmSTPS1, SmSTPS2, and SmSTPS3 reported here function as (-)-5-epieremophilene synthase in vitro, and the SmSTPS1 activity of synthesizing (-)-5-epieremophilene in planta was confirmed by transient expression. However, (-)-5-epieremophilene was not detected from S. miltiorrhiza in our experiments, neither was it reported from species of Salvia. One possibility is that there is a biosynthesis pathway that transforms (-)-5-epieremophilene into other non-volatile product(s), which awaits identification. Importantly, the transcriptome mining approach has enabled us to identify this previously unknown metabolite, suggesting a great potential of this method in further exploring the treasure of plant metabolites.

Sesquiterpenes bearing valencene backbone have been isolated from different plant species and attracted intensive attention due to their broad activity. (+)-Valencene was reported to significantly inhibit the activity of transient receptor potential ankyrin-1 (TRPV1) and calcium release-activated calcium modulator 1 (ORAI1), which are critical in mediating UV-induced skin aging, thus this natural product is suggested to be a potent candidate for the development of therapeutic agents for the prevention and treatment of UV-induced photaging (Nam et al., 2016). Its oxidative product, (+)-nootkatone, has also received interests in pharmaceutical industry for its anti-platelet aggregation effects (Choi et al., 2014) and anti-proliferative activity toward cancer cells (Gliszczynska et al., 2011), as well as its activity in repelling insects (Zhu et al., 2001). As (-)-5-epieremophilene is identified and can be produced by the three (-)-5-epieremophilene synthases from S. miltiorrhiza efficiently, it would be interesting to compare the activities of these structurally related sesquiterpenes.

AUTHOR CONTRIBUTIONS

LY and XF designed the study and wrote the manuscript. LY, YY, C-YL, and M-YC performed the experiment. X-YC helped in data analysis and manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00627/full#supplementary-material

FIGURE S1 | Analysis of recombinant proteins. (A–C) SDS-PAGE of recombinant SmSTPS1, SmSTPS2, SmSTPS3 (1: 0 mM IPTG; 2: 0.2 mM IPTG; 3: 0.5 mM IPTG; 4: 1 mM IPTG).
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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