Functional characterization of ent-copalyl diphosphate synthase, kaurene synthase and kaurene oxidase in the *Salvia miltiorrhiza* gibberellin biosynthetic pathway

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*Salvia miltiorrhiza* Bunge is highly valued in traditional Chinese medicine for its roots and rhizomes. Its bioactive diterpenoid tanshinones have been reported to have many pharmaceutical activities, including antibacterial, anti-inflammatory, and anticancer properties. Previous studies found four different diterpenoid biosynthetic pathways from the universal diterpenoid precursor (\(E, E, E\)-geranylgeranyl diphosphate (GGPP)) in *S. miltiorrhiza*. Here, we describe the functional characterization of ent-copalyl diphosphate synthase (SmCPS\(_{ent}\)), kaurene synthase (SmKS) and kaurene oxidase (SmKO) in the gibberellin (GA) biosynthetic pathway. SmCPS\(_{ent}\) catalyzes the cyclization of GGPP to ent-copalyl diphosphate (ent-CPP), which is converted to ent-kaurene by SmKS. Then, SmKO catalyzes the three-step oxidation of ent-kaurene to ent-kaurenoic acid. Our results show that the fused enzyme SmKS-SmCPS\(_{ent}\) increases ent-kaurene production by several fold compared with separate expression of SmCPS\(_{ent}\) and SmKS in yeast strains. In this study, we clarify the GA biosynthetic pathway from GGPP to ent-kaurenoic acid and provide a foundation for further characterization of the subsequent enzymes involved in this pathway. These insights may allow for better growth and the improved accumulation of bioactive tanshinones in *S. miltiorrhiza* through the regulation of the expression of these genes during developmental processes.

*Salvia miltiorrhiza* Bunge has been widely used in China (and to a lesser extent in Japan, the United States, and European countries) for the treatment of cardiovascular and cerebrovascular diseases. This medicinal herb exhibits anti-inflammatory, antioxidant and radical scavenging effects\(^1,2\). Tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone I are the major diterpene quinones of the lipophilic constituents in Danshen and are responsible for much of its anti-inflammatory, antioxidant, antitumor and a variety of other activities\(^3-5\). Because these monomeric compounds have significant pharmacological activities, Danshen preparations are more frequently used in the clinic.

To accommodate the increasing need for clinical applications, researchers have deeply investigated the diterpenoid biosynthetic pathway to obtain the bioactive tanshinones directly using synthetic biology strategies in microbial cell factories. Previous works have indicated that at least four different diterpenoid biosynthetic pathways exist in *S. miltiorrhiza* (Fig. 1)\(^6\). Among them, the tanshinone biosynthetic pathway is uniquely initiated by a sequential pair of cyclization reactions catalyzed by SmCPS1 and SmKSL1 to produce abietane miltiradiene.

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which is a precursor of at least cryptotanshinone\(^7,8\). Then, SmCYP76AH1 catalyzes the turnover of miltiradiene to form ferruginol, thereby providing a solid foundation to elucidate the tanshinone biosynthetic pathway.

However, only two diterpene synthases (diTPSs) in the \(S. \text{miltiorrhiza}\) GA biosynthetic pathway have been reported to date, and the roles of GAs in \(S. \text{miltiorrhiza}\) root and rhizome development and the total yield of tanshinones per plant are less clear. GAs are formed from GGPP via a set of reactions catalyzed by different enzymes, including two consecutive diTPSs, cytochrome P450 (CYP) and 2-oxoglutarate-dependent dioxygenases (2ODDs) in plants\(^9\). As a group of plant-growth regulators, these GAs control different aspects of plant development, such as seed germination, stem elongation, flowering, fruit set and fruit development. Understanding GA biosynthesis will allow us to improve the tanshinone contents by regulating the expression of the genes involved in the \(S. \text{miltiorrhiza}\) GA biosynthetic pathway. Here, we cloned three genes (\(\text{SmCPS}_{\text{ent}}\), \(\text{SmKS}\) and \(\text{SmKO}\)) from \(S. \text{miltiorrhiza}\) hairy roots and then identified their functions by co-expressing them in \(\text{Saccharomyces cerevisiae}\).

Biochemical studies suggested that CPS and KS might interact with one another\(^10\); therefore, we constructed a fused \(\text{SmCPS}_{\text{ent}}\) and \(\text{SmKS}\) protein and showed that the production of \(\text{ent}-\text{kaurene}\) was significantly improved.

**Results**

**Cloning and sequence analysis of \(\text{SmCPS}_{\text{ent}}, \text{SmKS}\) and \(\text{SmKO}\) from \(S. \text{miltiorrhiza}\) hairy roots.** The full-length \(\text{SmCPS}_{\text{ent}}\) and \(\text{SmKS}\) cDNAs were determined by 5’ RACE and 3’ RACE, and the corresponding cDNA sequences were submitted to the National Center for Biotechnology Information (Supplementary Fig. S1). The full-length \(\text{SmCPS}_{\text{ent}}\) cDNA (GenBank accession number KT934789) is 2413 nt and encodes a polypeptide of 793 amino acids. \(\text{SmCPS}_{\text{ent}}\) clusters most closely to \(\text{SmCPS}_5\) of \(\text{S. miltiorrhiza}\) f. alba and to \(\text{SdCPS}\) from \(\text{Scoparia dulcis}\) (Fig. 2). The first 21 N-terminal amino acids are rich in serine and threonine (19%), which is a common characteristic of transit peptides that target the diTPSs to plastids\(^11,12\). The amino acid sequence also contains a conserved DIDD motif (Fig. 3), which strongly suggests that \(\text{SmCPS}_{\text{ent}}\) can catalyze GGPP to CPP as a class II diTPS. The \(\text{SmKS}\) cDNA (GenBank accession number KT934790) is 2636 nt in length and encodes a predicted protein of 806 amino acid residues. At the protein level, the KS sequence from the hairy roots of \(\text{S. miltiorrhiza}\) exhibits 99% identity with the \(\text{SmKSL2}\) from \(\text{S. miltiorrhiza}\) f. alba (Fig. 2). The first 27 N-terminal amino acids are rich in serine and threonine (22%), suggesting that \(\text{SmKS}\) is also localized in plastids. Its amino acid sequence contains a DDFFD motif but lacks the...
DxDD motif (Fig. 3), indicating that SmKS is a plant KS protein with monofunctional class I diTPS activity. The SmKO cDNA (GenBank accession number KJ606394) is 1930 nt in length and has an open reading frame (ORF) encoding 519 amino acid residues, containing a cytochrome P450 conserved site (amino acids 451–460, Fig. 3). The deduced amino acid sequence shows 64% and 66% identity with AtKO (Arabidopsis thaliana, AAC39507) and PsKO (Pisum sativum, AAP69988) (Fig. 2). The gene was identified as a multifunctional kaurene oxidase catalyzing three sequential oxidations (ent-kaurene to ent-kaurenoic acid) in the GA biosynthetic pathway14,15.

Recombinant expression and functional characterization of SmCPS\textsubscript{ent} and SmKS. Previously reported evidence suggested that SmCPS\textsubscript{ent} and SmKS might be involved in the S. miltiorrhiza GA biosynthetic pathway. To confirm the biochemical functions of SmCPS\textsubscript{ent} and SmKS in vivo, the SmCPS\textsubscript{ent} ORF and SmKS ORFs were ligated individually or in combination in the yeast expression vector pESC-Trp (Fig. 4A) and expressed in the yeast strain BY-T20 (provided by Prof. Xueli Zhang's lab, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, China). As expected, CPP was detected as the SmCPS\textsubscript{ent} product.

Figure 2. Phylogenetic tree of CPS, KS and KO from different species. The neighbor-joining phylogenetic trees were constructed using the bootstrap method in MEGA 5.1. The number of bootstrap replications was 1000. Descriptions of the three different types of synthases used in the phylogeny are listed in Supplementary Table 2.
Figure 3. Alignment of plant CPS, KS and KO. The boxes represent the conserved regions. (A) AtCPS (Q38802), SmCPS5 (AHJ59324), SmCPS₆ent (ALX18648), PpCPS/KS (BAF61135), AgAS (Q38710), GbLPS (Q947C4), AtKS (AAC39443), SmKSL2 (AHJ59325), SmKS (ALX18649). (B) AtKO (AAC39507), OsKO (AAT81230), SmKO (AJF93403).

in the products of the yeast strain SGH1 (BY-T20/pESC-Trp::SmCPS₆ent) compared with the product of the A. thaliana AtCPS using GGPP as the substrate. No CPP was found in the yeast strain carrying the empty pESC-Trp
vector. Using the product of the *A. thaliana* AtKS as the authentic standard, ent-kaurene was detected as the SmKS product in the products of the yeast strain SGH3 (BY-T20/pESC-Trp::SmCPS<sub>ent</sub>/SmKS) but not SGH2 (BY-T20/pESC-Trp::SmKS), confirming that SmKS possessed monofunctional class I dITPS activity and catalyzed the formation of ent-kaurene using the SmCPS<sub>ent</sub> product CPP as the substrate. Therefore, the absolute configuration of the SmCPS<sub>ent</sub> product CPP was identified as an enantiomer (i.e., ent-CPP) (Fig. 4B).

Protein complexes have been reported to improve the efficiency of specific pathways by protecting substrates and intermediates from diffusion and degradation<sup>16</sup>. Zhou et al. reported that a recombinant strain containing the fused enzyme SmKSL1-SmCPS<sub>1</sub> produced 2.8-fold more miltiradiene compared with another recombinant strain in which SmCPS<sub>1</sub> and SmKSL1 were expressed separately<sup>17</sup>. Hence, we constructed the fused enzyme SmKS-SmCPS<sub>ent</sub> in the yeast strain SGH4 (BY-T20/pESC-Trp::SmKS-SmCPS<sub>ent</sub>) using the RF cloning method, and the results showed that SGH4 produced approximately 4.25-fold more ent-kaurene than SGH3 (Fig. 4D).

Recombinant expression and functional characterization of SmKO in vivo. As a strategy to characterize the biochemical function of SmKO in vivo, first we constructed the fused enzyme SmKS-SmCPS<sub>ent</sub>, which improved the ent-kaurene precursor supply as expected. Then, SmKO was coexpressed with the fused enzyme SmKS-SmCPS<sub>ent</sub> and a NADPH-cytochrome P450 reductase (SmCPR1) in the yeast strain SGH5 (BY-T20/pESC-Trp::SmCPS<sub>ent</sub>/SmKS-SmKO<sub>+</sub>pESC-Leu::SmCPR1). After extraction and methylation, the ent-kaurenolic acid methyl ester was detected by a comparison with the methylated authentic standard (Sigma, USA) (Fig. 5). This result confirmed that SmKO encoded a functional ent-kaurene oxidase that was involved in the three-stage oxidation of ent-kaurene to ent-kaurenolic acid in the *S. miltiorrhiza* GA biosynthetic pathway.

Discussion

We identified three consecutive enzymes (SmCPS<sub>ent</sub>, SmKS and SmKO) involved in the *S. miltiorrhiza* GA biosynthetic pathway. SmCPS<sub>ent</sub> catalyzes the formation of ent-CPP from GGPP; then, SmKS converts ent-CPP to ent-kaurene. Subsequently, SmKO converts ent-kaurene to ent-kaurenolic acid via a three-stage oxidation reaction. ent-Kaurene biosynthesis was reported to be catalyzed by a one-to-one CPS/KS complex in which CPP could be channeled from CPS to the KS catalytic site<sup>10</sup>. Therefore, we fused SmCPS<sub>ent</sub> and SmKS to obtain a close proximity between the active sites of the two consecutive enzymes. As expected, the fused enzyme SmKS-SmCPS<sub>ent</sub> produced 4.25-fold more ent-kaurene than the separate expression of SmCPS<sub>ent</sub> and SmKS in the yeast strain, suggesting that the protein fusion treatment was an efficient approach to improve the catalytic activity and enlarge the heterologous production of ent-kaurene. With an increased supply of the ent-kaurene precursor, SmKO catalyzed the formation of ent-kaurenolic acid. However, the intermediates ent-kaurenol and ent-kaurenal were not detected. One possible explanation is that the intermediates were unstable and were changed into other
intermediates during the extraction process. The enzymes involved in the early steps of the GA biosynthetic pathway (i.e., CPS, KS, KO, and KAO) are primarily encoded by single genes, whereas those involved in the later steps (i.e., GA20ox, GA3ox, and GA2ox) are encoded by gene families. The SmCPS\textsubscript{ent}, SmKS, and SmKO genes are likely single copy genes responsible for GA biosynthesis in \textit{S. miltiorrhiza}.

In addition to the identification and characterization of SmCPS\textsubscript{ent}, SmKS and SmKO, we provided insights into the genes encoding the enzymes involved in all steps of the GA biosynthetic pathway from GGPP to ent-kaurenoic acid. Our results provide a foundation for further characterization of the subsequent enzymes (i.e., SmKAO and the CYP88A subfamily) involved in the GA biosynthetic pathway using this yeast expression system. In plants, GA levels vary at different sites and during different development processes. It is possible to control the GA levels by regulating the expression of these genes to acquire better growth of the \textit{S. miltiorrhiza} roots and rhizomes, thereby improving the total yield of tanshinones per plant.

In conclusion, we functionally characterized three consecutive enzymes (SmCPS\textsubscript{ent}, SmKS and SmKO) involved in the GA biosynthetic pathway from GGPP to ent-kaurenoic acid, thereby laying the foundation for further characterization of GA biosynthesis. Based on these results, we could regulate the expression of all genes involved in the GA biosynthetic pathway to acquire better growth and an increased accumulation of the bioactive tanshinones involved in the \textit{S. miltiorrhiza} developmental processes. Protein fusion is an applicable and efficient approach that can be used to direct metabolic flux to the bioactive diterpenoid tanshinones pathway for the heterologous production of isoprenoids in microbial cell factories.

**Methods**

**RNA isolation and cDNA cloning.** Hairy roots were induced from the \textit{S. miltiorrhiza} leaf explants under the mediation of \textit{Agrobacterium rhizogenes} strain ACCC10060 as described previously and maintained in 6,7-V liquid medium at 25°C on a gyratory shaker (80 rpm) in the dark. Total RNA was extracted using the TRIZol reagent (Invitrogen, Carlsbad, CA, USA). The 5' and 3' ends of the targeted SmCPS\textsubscript{ent} and SmKS genes were cloned by RACE (Invitrogen) according to the manufacturer's directions using the corresponding \textit{S. miltiorrhiza} genome sequences released by the National Center for Biotechnology Information (NCBI). The primer sequences are shown in Supplementary Table 1. An aliquot (1 μg) of the total RNA was used to synthesize the first strand cDNA according to the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio, Dalian, China) manufacturer's protocol. The full-length cDNA for each ORF was cloned using the PrimeSTAR DNA polymerase (Takara Bio). The PCR products were purified and cloned into the pEASY-T3 cloning vector (TransGen Biotech, Beijing, China), transformed into \textit{Escherichia coli} Trans5α cells (TransGen Biotech), and then cultured in Luria-Bertani (LB) medium at 37°C in the dark. Positive clones were sequenced. The full-length cDNA of SmKO was cloned previously.

**Bioinformatics analysis.** The SmCPS\textsubscript{ent}, SmKS and SmKO sequences were confirmed at NCBI (http://www.ncbi.nlm.nih.gov/). The open reading frames (ORFs) and deduced amino acid sequences were analyzed using the online tool ORF Finder (http://www.ncbi.nlm.nih.gov/orffinder.html) and the ExPASy online tool (http://web.expasy.org/translate/), respectively. The ChloroP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/) was used to predict chloroplast transit peptides. The sequences of the SmCPS\textsubscript{ent}, SmKS and SmKO as well as other corresponding sequences downloaded from GenBank were aligned using the DNA MAN program, and the phylogenetic trees for SmCPS\textsubscript{ent}, SmKS and SmKO were constructed using sequences from other plants (Supplementary Table 2) using the neighbor-joining method in MEGA5.1.

**Recombinant expression and functional characterization of SmCPS\textsubscript{ent} and SmKS.** The SmCPS\textsubscript{ent} and SmKS ORFs (alone or in combination) were subcloned into the yeast epitope-tagging vector pESC-Trp under the control of the GAL1 or GAL10 inducible promoter (Agilent Technologies, USA) via digestion by the
corresponding restriction endonucleases. The resulting constructs were verified by complete gene sequencing
and then transformed into the yeast strain BY-T20 (BY4742, ΔTrp1, Trp1::HIS3-PGK1-BTS1/ERG20-ADH
1-PTEF/Thr1::GGPS::Trp1::PTEF-HTMG1-TCYC), provided by Prof. Xueli Zhang’s lab, Tianjin Institute of Industrial
Biotechnology, Chinese Academy of Sciences, China. Then, the recombinant strains SGH1 (containing the
plasmid pESC-Trp::SmKS-SmCPS), SGH2 (containing the plasmid pESC-Trp::SmKS), and SGH3 (containing the
plasmid pESC-Trp::SmCPS/SmKS) were selected on synthetic drop-in medium -Trp-His (SD-Trp-His) con-
taining 20 g/L glucose and grown at 30 °C for 2–3 d. Single transformed yeast colonies were grown in SD-Trp-His
liquid medium supplemented with 20 g/L glucose at 30 °C for approximately 2 d. The yeast cells were pelleted
and resuspended in 100 mL of SD-Trp-His liquid induction medium supplemented with 20 g/L galactose and
grown at 30 °C for 3 d. Finally, the induced yeast cells were extracted three times with an equal volume of hex-
ane. The organic fractions were pooled and dried using a nitrogen evaporator (Baojingkeji, Henan, China).
The dried samples were dissolved in 100 μL of hexane for GC-MS analysis as described previously28. To confirm
the products of these strains, the identified products ent-CPP and ent-kaurene of A. thaliana AtCPS and AtKS
were used as the authentic standards29,30. The detailed protocols for the constructions of the recombinant plas-
mids and strains and the recombinant expression and enzymatic assay for AtCPS and AtKS are described in
the Supplementary Methods.

Construction of the module producing the fused protein SmKS-SmCPS<sub>ent</sub> and the functional
classification of SmKO. To prepare the module producing the fused protein SmKS-SmCPS<sub>ent</sub>, a
restriction-free (RF) cloning method was used31. The genes encoding the fusion enzyme were constructed by
inserting a widely used GGGS linker encoded by a “GGT GGT GTT TCT” sequence between the two corre-
ponding genes<sup>2,3,33</sup>. The recombinant plasmid pESC-Trp::SmKS-SmCPS<sub>ent</sub> was transformed into the yeast strain
BY-T20 to generate SGH4 and induced with D-galactose as described above. Then, the products of SGH4 were
analyzed by GC-MS. The detailed protocols for the RF cloning are described in the Supplementary Methods.
The ORF region ligated into the recombinant plasmid pESC-Trp::SmKS-SmCPS<sub>ent</sub> was described above and then transformed into the yeast strain BY-T20 with another recombinant plasmid pESC-Leu::SmCPR1
(SmCPR1, S. miltiorrhiza cytochrome P450 reductase)<sup>8</sup>. The recombinant strain SGH5 (containing the plasmids
pESC-Trp::SmKS-SmCPS<sub>ent</sub>/SmKO and pESC-Leu::SmCPR1) was induced with D-galactose and extracted once
with an equal volume of hexane and twice with an equal volume of ethyl acetate. The organic fractions were
pooled and dried and then dissolved in 50 μL of methanol and methylated with approximately 200 μL of (tri-
methylsilyl)diazomethane (Aladdin Industrial Inc., Shanghai, China). The methylated samples were redried and
then dissolved in 100 μL of ethyl acetate for GC-MS using a Thermo TRACE 1310/TSQ 8000 gas chromatograph
(splitsless; injector temperature 250 °C) with a DB-5 ms (30 m × 0.25 mm × 0.25 μm) capillary column. The GC
conditions were the same as those described previously34.

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Author Contributions

L.H. and W.G. conceived and designed the study. P.S. and Y.T. performed the experiments and wrote the manuscript. Q.C., Y.H., M.Z., J.Y. and Z.T. participated in the research and analyzed the data. All authors read and approved the final manuscript.

Additional Information

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