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ORIGINAL RESEARCH PAPER

RNA interference-mediated repression of *SmCPS* (*copalyldiphosphate synthase*) expression in hairy roots of *Salvia miltiorrhiza* causes a decrease of tanshinones and sheds light on the functional role of SmCPS

Qiqing Cheng · Ping Su · Yating Hu · Yunfei He · Wei Gao · Luqi Huang

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Abstract Tanshinones are a group of bioactive abietane-type norditerpenoid quinone compounds in *Salvia miltiorrhiza*. Copalyldiphosphate synthase of *S. miltiorrhiza* (SmCPS) is the first key enzyme in tanshinone biosynthesis from the universal diterpene precursor geranylgeranyl diphosphate. Hairy roots of *S. miltiorrhiza* were transformed with *Agrobacterium rhizogenes* carrying an RNA interference (RNAi) construct designed to silence *SmCPS*, and we examined the resulting *SmCPS* expression and tanshinone accumulation. In *SmCPS*–RNAi hairy roots, the transcript level of *SmCPS* was reduced to 26 % while the dihydrotanshinone I and cryptotanshinone levels were decreased by 53 and 38 % compared to those of the vector control hairy roots; tanshinone IIA was not detected. Therefore, the decreased expression of *SmCPS* caused a decrease in tanshinone levels which verifies that SmCPS is a key enzyme for tanshinone biosynthesis in *S. miltiorrhiza*.

Keywords Copalyldiphosphate synthase · Hairy root · RNA interference · *Salvia miltiorrhiza* · Tanshinone

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Introduction

The roots and rhizome of *Salvia miltiorrhiza*, have been widely used for the clinical treatment of cardiovascular and cerebrovascular diseases in China, Japan, the United States, and some European countries (Zhou et al. 2005). Tanshinones are a group of lipophilic, abietanoid, diterpenoid natural-products that may potentially be important in human nutrition and medicine due to their antibacterial, antioxidant, anti-inflammatory and antineoplastic properties (Wang et al. 2007; Dong et al. 2011). As shown in Fig. 1, the biosynthesis of tanshinone is initiated by a sequential pair of cyclisation reactions by diterpene synthases. In these reactions, the universal diterpene precursor,

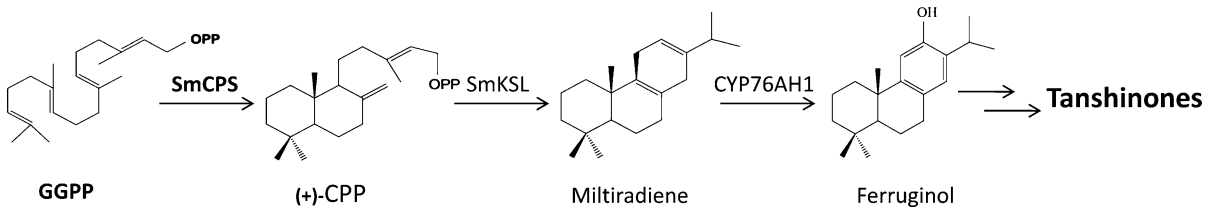

Fig. 1 The tanshinone biosynthesis pathway starting from GGPP in *S. miltiorrhiza*. Miltiradiene is synthesised from GGPP by SmCPS and SmKSL and is then converted into ferruginol by CYP76AH1. In the downstream pathway, ferruginol is converted into tanshinones by series of post-modification enzymes such as CYP450s

geranylgeranyl pyrophosphate (GGPP), is converted by SmCPS and kaurene synthase-like (SmKSL) to miltiradiene, which has recently been identified as a tanshinone precursor. The reaction in which SmCPS converts GGPP into (+)-Copalyldiphosphate [(+)-CPP] establishes the stereochemistry (Gao et al. 2009). In the next step, CYP76AH1 catalyses a unique four electron oxidation cascade on miltiradiene to produce ferruginol, both in vitro and in vivo (Guo et al. 2013). However, the downstream diterpenoid-modifying enzymes are still unknown, so further in-depth studies are needed to identify these enzymes, especially the cytochrome P450s, and reveal the whole tanshinone biosynthesis pathway.

RNA interference (RNAi) was first discovered as a response to double-stranded RNA, which resulted in gene-specific silencing (Fire et al. 1998). RNAi has since been shown to be a highly conserved mechanism in eukaryotic organisms, including animals, plants and fungi, and it functions as an innate defence system against alien nucleic acid molecules (Hannon. 2002; Pavli et al. 2010). To date, RNAi is considered as potent reverse genetic tool to identify gene function and regulate the biosynthesis of target compounds in plants such as *Ophiorrhiza pumila*, soybean, and *Nicotiana glauca* (Asano et al. 2013; Subramanian et al. 2005; DeBoer et al. 2009). In this paper, *Agrobacterium rhizogenes*-mediated RNAi allowed us to study the effect of decreased *SmCPS* expression on tanshinone biosynthesis. The hairy root transformation system was used to generate *SmCPS*–RNAi lines to explore the relationship between *SmCPS* expression level and tanshinone accumulation. This work showed that the gene silencing of *SmCPS* by RNAi strongly suppressed the expression of *SmCPS* and reduced the production of tanshinones in *S. miltiorrhiza* hairy roots.

Materials and methods

Plant materials and culture conditions

Mature seeds of *S. miltiorrhiza* were surface-sterilised with 0.1 % $HgCl_2$ and cultured on solid, hormone-free MS basal medium. The MS medium contained 30 g sucrose/l and 8 g agar/l without ammonium nitrate to promote germination. Cultures were grown in a growth chamber maintained at 25 °C with a 16 h light/8 h dark cycle.

Bioinformatic analysis

The nucleotide sequence, deduced amino acid sequence, and ORF were analysed using DNAMAN and ORF Finder. SmCPS and other CPSs retrieved from GenBank were aligned using Clustal W. A phylogenetic tree was constructed using the neighbour-joining method.

Construction of a *SmCPS*–RNAi vector and transformation of *A. rhizogenes*

The RNAi construct used to silence *SmCPS* (*SmCPS*–RNAi) was constructed as follows. Briefly, a 321 bp segment of *SmCPS* cDNA from nucleotides 901–1,221 was cloned into pDONR221 (Invitrogen, Carlsbad, CA, USA) using Gateway-adapted primers (forward, 5′-AAAAAGCAGGCTGGTTCCTTCCTCACCTCT CCCTC-3′; reverse, 5′-AGAAAGCTGGGTCATCCT CATAAGCCTCATTCCCA-3′) and the Gateway BP Clonase Enzyme Mix (Invitrogen). The segment was then cloned into the pK7GWIWG2D(II) binary transformation vector (Invitrogen) which contains the kanamycin resistance gene, using the Gateway LR Clonase Enzyme Mix. The resulting recombinant

construct, pK7GWIWG2D(II)-*SmCPS*, was then introduced into *A. rhizogenes* ACCC10060 via a freeze–thaw method. The appropriate vector without the RNAi sequence was used for control transformation.

Agrobacterium rhizogenes culture

Agrobacterium rhizogenes ATCC10060 was used. Empty vector control (VCK) and *SmCPS*–RNAi transformed strains were maintained by culturing them on YEB agar. All media contained 50 mg kanamycin/l and 100 mg spectinomycin/l. Stock cultures were grown in YEB broth and stored long-term in 25 % (v/v) glycerol at −70 °C. Cultures for plant inoculation were grown in 25 ml YEB broth containing an appropriate amount of kanamycin and spectinomycin at 28 °C for 2 days until the OD_{600} was ~0.8.

Hairy root transformation of *S. miltiorrhiza*

The pK7GWIWG2D(II)-*SmCPS* and pK7GWIWG2-D(II)-control vectors were transformed into *S. miltiorrhiza* using the *A. rhizogenes* strain as described previously (Cheng et al. 2013). The transformed hairy roots were identified using a fluorescence microscope to check for the green fluorescence protein (GFP) marker. After culturing on MS solid medium for 30 days, the successfully transformed hairy roots were transferred to 6,7-V liquid medium. The hairy roots were collected on 30 days after inoculation in the 6,7-V liquid medium.

Quantitative real-time PCR

Total RNA was extracted using Trizol and 1 μg total RNA was used to synthesize the first strand cDNA in the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Tokyo, Japan) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (Takara) on an ABI 7500 apparatus according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The primer pairs used for these assays were as follows: *SmCPS* (forward, 5′-GAGGG AGAGGTGAGGAAGGAA-3′; reverse, 5′-AGGGA ACAAAAGTTGAAAAGG-3′) and *β-actin* (forward, 5′- AGGAACCACCGATCCAGACA-3′; reverse, 5′- G GTGCCCTGAGGTCCTGTT-3′). Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method.

HPLC analysis

The analysis was conducted using a HPLC system equipped with an photodiode array detector. Chromatographic separation was conducted using a SB-C_{18} analytical column (Zorbax; μm, 4.6 × 250 mm; Agilent, USA) kept at 30 °C. The mobile phase was acetonitrile/water (75:25, v/v) containing 0.01 % phosphoric acid and was run at 1 ml/min. The injection volume was 10 μl for each sample, and the detector wavelength was 268 nm. The hairy roots were ground into small pieces, placed at −80 °C for 4 h, and freeze-dried for 8 h. Methanol was added in a ratio of 50 ml/g dry wt sample. Samples were incubated at room temperature overnight and tanshinones were extracted in an ultrasonic water bath for 30 min. All of the sample solutions were filtered through a 0.22 μm membrane before injection into the HPLC. Four tanshinone compounds (cryptotanshinone, dihydrotanshinone I, tanshinone I, and tanshinone IIA) were identified and quantified by comparing the retention times and UV spectral data with those of the reference compounds.

Results and discussion

Sequence and phylogenetic tree analyses suggest the function of SmCPS

The full-length cDNA of *SmCPS* (GenBank accession no. EU003997) is 2,688 bp long and has a 147 bp 5′-untranslated region (UTR), a 128 bp 3′-UTR, and a polyA tail. An ORF search showed that *SmCPS* contains a 2,382 bp ORF that encodes a protein with 793 amino acids. The calculated molecular weight of this protein is 90,362 Da, and the protein has a theoretical isoelectric point of 6.45.

A phylogenetic tree was constructed based on the deduced amino acid sequences of SmCPS and other CPSs or diterpene synthases from various plants (Fig. 2). The result revealed that SmCPS is located on its own sub-branch and is closely related to previously characterised CPSs from the dicotyledons *Arabidopsis thaliana*, *Solanum lycopersicum*, *Scoparia dulcis*, *Croton sublyratus*, *Cucurbita maxima*, and *Lactuca sativa*, which form a branch. The neighbouring branch was composed of diterpene synthases from gymnosperms and other dicotyledons. The distant

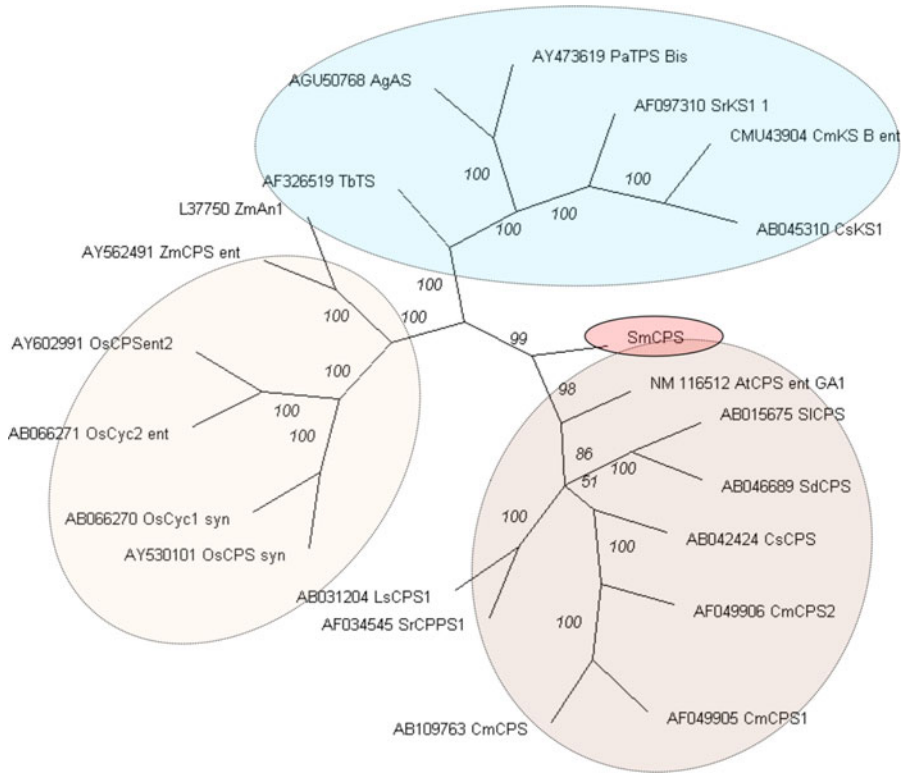

Fig. 2 Un-rooted phylogenetic tree of SmCPS sequences. Neighbour-joining tree bootstrap analysis was performed using 1,000 bootstraps (only values above 50 are shown). The accession numbers of the diterpene synthase sequences used were EU003997 (*S. miltiorrhiza*, SmCPS), AB015675 (*S. lycopersicum*, SlCPS), AF034545 (*Stevia rebaudiana*, SrCPPS1), AB031204 (*L. sativa*, LsCPS1), AF049906 (*C. maxima*, CmCPS2), AF049905 (*C. maxima*, CmCPS1), AB109763 (*C. maxima*, CmCPS), AB046689 (*S. dulcis*, SdCPS), NM_116512 (*A. thaliana*, AtCPS-ent GA1), AB042424 (*C. sublyratus*, CsCPS), AY530101 (*O. sativa*, OsCPS-syn), AB066271 (*O. sativa*, OsCyc2-ent), AY473619 (*Picea abies*, PaTPS-Bis), AB045310 (*Cucumis Sativus*, CsKS1), AB066270 (*O. sativa*, *OsCyc1*-syn), AY562491 (*Z. mays*, ZmCPS-ent), AY602991 (*O. sativa*, OsCPSent2), AF097310 (*S. rebaudiana*, SrKS1-1), AF326519 (*Taxus brevifolia*, TbTS), AGU50768 (*Abies grandis*, AgAS), L37750 (*Z. mays*, *ZmAn*1), and CMU43904 (*C. maxima*, CmKS-B-ent)

branch was made up of diterpene synthases from monocotyledons such as *Oryza sativa* syn-CPS, *O. sativa* ent-CPS, and *Zea mays* ent-CPS. Therefore, SmCPS is similar to CPSs from dicotyledons.

In gymnosperms and angiosperms, diterpene synthases convert GGPP into diterpenes through multistep cycloisomerization. Diterpene synthases can be classified into bifunctional synthases, monofunctional class II synthases and monofunctional class I synthases. The class II synthases contain a DxDD signature motif that is responsible for catalysing the protonation-initiated cyclisation of GGPP to a bicyclic diphosphate intermediate. SmCPS works as a monofunctional class II diterpene synthase by catalysing the protonation-initiated cyclisation of GGPP to (+)-CPP, a bicyclic Copalyldiphosphate, and it has so far been the only one reported from the angiosperm *S. miltiorrhiza* (Gao et al. 2009; Hall et al. 2013). SmKSL subsequently converts (+)-CPP to miltiradiene. Miltiradiene has been produced at 365 mg/l in *S. cerevisiae* using a modular pathway engineering (MOPE) strategy to construct a protein fusion of SmCPS and SmKSL (Zhou et al. 2012). Knowing the functions of these two diterpene synthases lays the basis for the further investigation of tanshinone biosynthesis, such as the identification of downstream CYP450 enzymes.

RNAi-mediated silencing of *SmCPS* in hairy roots using *A. rhizogenes*-mediated leaf transformation

The pK7GWIWG2D(II)-*SmCPS* vector (Fig. 3) and the pK7GWIWG2D(II)-control vector were separately introduced into *S. miltiorrhiza* leaf explants

using *A. rhizogenes* strain ACCC10060, and the generated hairy roots were screened using kanamycin. Pictures representing the different phases of hairy root growth following transformation are shown in Fig. 4. Within 2 days, the inoculated cut surface of the leaves turned brown, and the leaves curled (Fig. 4a). After just 10 days, a ridge of callus grew from the leaf stalk, and hairy roots began to form (Fig. 4b). By 30 days, abundant roots formed, and in some cases, more than 15 roots formed per leaf (Fig. 4c). The successfully transformed hairy roots were cultured in 6,7-V liquid medium for 30 days (Fig. 4d).

The transformation of hairy roots with the vector control or the RNAi construct was monitored by root fluorescence due the GFP marker. The roots, shown in Fig. 4e and f, were transformed with the binary vector containing the *SmCPS*–RNAi construct. On average, we observed more than 60 roots each for the vector control and the *SmCPS*–RNAi constructs. In all, 86 % of vector control lines and 88 % of *SmCPS*–RNAi lines were successfully transformed. Therefore, the rapid growth and high transformation rate make the hairy root culture of *S. miltiorrhiza* a suitable model transformation system for exploring tanshinone biosynthesis and identifying the functions of key diterpene synthases in the pathway, especially the downstream enzymes SmKSL and CYP450.

Analysis of transcript levels in hairy roots of *SmCPS*–RNAi

qRT-PCR analysis showed that the expression of *SmCPS* was successfully regulated through genetic manipulation. Hairy roots that expressed the empty

Fig. 3 Construction of the *SmCPS* RNAi vector pK7GWIWG2-D(II)-*SmCPS*. The T-DNA construct in the plasmid pK7 GWIWG2D-*SmCPS* that contains the *SmCPS* gene used for genetic transformation of *S. miltiorrhiza* is shown. LB and RB indicate the *left* and *right borders*, respectively

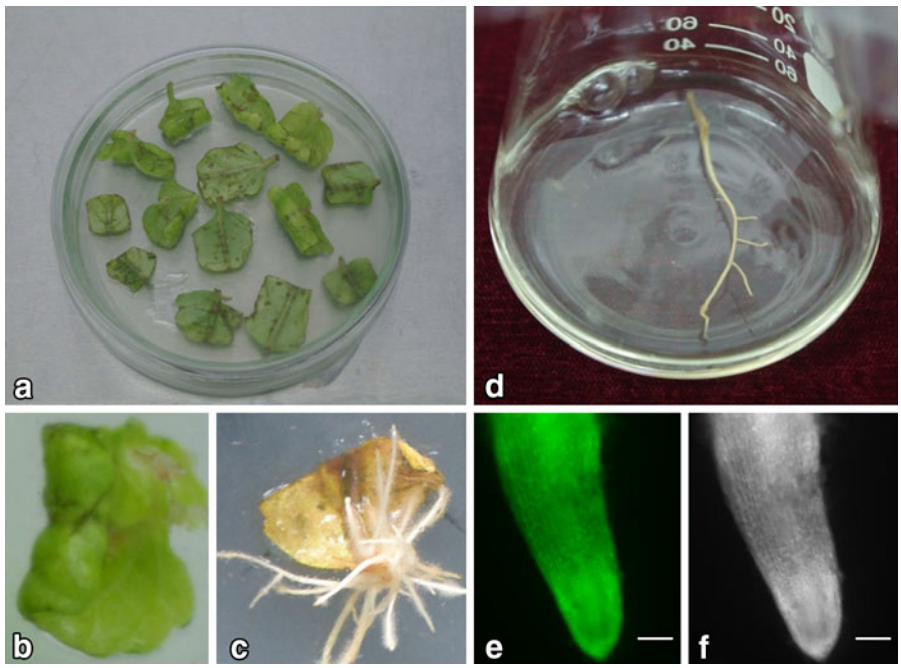

Fig. 4 Stages of transformation and hairy root formation on *S. miltiorrhiza* leaves. Leaves are shown at 2 days (**a**), 10 days (**b**), and 30 days (**c**) after infection with ACCC10060. A root cultured in liquid medium for 30 d after transferring from agar culture medium is also shown (**d**). GFP-positive hairy roots under fluorescence microscopy with *blue light* (**e**) and *white light* (**f**) are also shown. *Scale marker bars* 100 μm

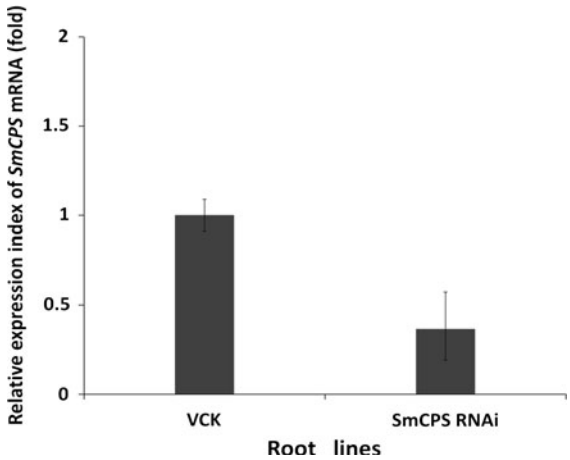

Fig. 5 Relative expression of *SmCPS* in VCK and *SmCPS*–RNAi hairy root lines. *Vertical bars* indicate the mean ± SD from three independent lines of the same type of hairy roots

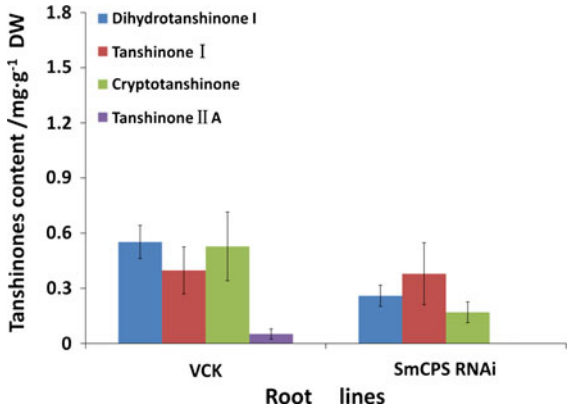

Fig. 6 Metabolites production involved in tanshinone biosynthesis pathway in VCK and *SmCPS* RNAi hairy root lines. *Vertical bars* indicate the mean ± SD from three independent lines of same type of hairy roots

vector served as a control. As shown in Fig. 5, in comparison with levels in the VCK lines, *SmCPS* transcript levels were reduced in the RNAi lines. The transcript level of *SmCPS* in *SmCPS*–RNAi hairy roots was decreased by 26 % compared with that of the VCK hairy root lines.

GGPP is the universal precursor of gibberellins in angiosperms and gymnosperms and of labdane-type specialised diterpenes in angiosperms, such as tanshinones in *S. miltiorrhiza* (Keeling et al. 2010; Peters 2010). Therefore, as tanshinone biosynthesis is repressed, the universal precursor GGPP may be shuttled into other pathways, such as general metabolism.

Tanshinone accumulation in *SmCPS*–RNAi transgenic hairy roots

HPLC revealed an alteration in tanshinone accumulation in engineered hairy root cultures that reflected the modulation in transcript profiles (Fig. 6). Except for tanshinone I, the tanshinone levels in *SmCPS*–RNAi hairy roots were reduced. The levels of dihydrotanshinone I and cryptotanshinone were decreased by 53 and 38 % compared with those of the VCK lines, and tanshinone IIA was below the detection limit.

The results indicate that the expression level of *SmCPS* correlated with the tanshinone levels. To explore the influence of SmCPS on specific metabolites, we evaluated the levels of four major tanshinones. As the expression level of *SmCPS* decreased, we expected the tanshinone levels to decrease. Consistent with this assumption, the levels of dihydrotanshinone I, crypotanshinon, and tanshinone IIA were reduced. Therefore, we hypothesise that the formation of these tanshinones was more sensitive to *SmCPS* RNAi than the formation of tanshinone I.

Conclusion

We have further elucidated the molecular biology of tanshinone biosynthesis from GGPP and the function of SmCPS by silencing the corresponding gene in *S. miltiorrhiza*. Transforming leaves with *A. rhizogenes* carrying an RNAi construct for *SmCPS* resulted in a suppression of *SmCPS* and a large decrease of tanshinones in transgenic hairy roots. This suggests that SmCPS is the key enzyme controlling diterpenoid metabolic flux. Silencing *SmCPS* provided strong evidence of the association between *SmCPS* expression and tanshinone accumulation and identified the function of SmCPS in *S. miltiorrhiza* culture for the first time. Additionally, hairy root culture of *S. miltiorrhiza* is a stable and effective model transformation system for exploring tanshinone biosynthesis and functionally identifying key enzymes in the pathway.

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