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Article

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Enhanced diterpene tanshinone accumulation and bioactivity of transgenic *Salvia miltiorrhiza* hairy roots by pathway engineering

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Abstract

Tanshinones are health-promoting diterpenoids found in *Salvia miltiorrhiza*, which have wide applications. Here, *SmGGPPS* (geranylgeranyl diphosphate synthase) and *SmDXSII* (1-deoxy-D-xylulose-5-phosphate synthase) were introduced into hairy roots of *S. miltiorrhiza*. Overexpression of *SmGGPPS* and *SmDXSII* in hairy roots produces higher levels of tanshinone than control and single-gene transformed lines, tanshinone production in the double-gene transformed line GDII10 reached to 12.93 mg/g dry weight, which is the highest tanshinone content that has been achieved through genetic engineering. Furthermore, transgenic hairy roots lines showed higher antioxidant and antitumor activity than control lines. In addition, contents of chlorophylls, carotenoids, indole acetic acid and gibberellins were significantly elevated in transgenic *Arabidopsis thaliana* plants. These results demonstrate a promising method to improve the production of diterpenoids including tanshinone as well as other natural plastid-derived isoprenoids in plants by genetic manipulation of the MEP pathway.

Keywords: hairy roots; isoprenoids; MEP pathway; *Salvia miltiorrhiza*; tanshinone

Introduction

Isoprenoids (terpenoids or terpenes) are a large group of biologically active compounds, numbering in the tens of thousands, which are found in almost all organisms.[1-3] Isoprenoids play major roles in a series of biological processes such as photosynthesis, respiration, growth and plant defense.[2] In higher plants, isoprenoids derive from a common five-carbon unit, isopentenyl pyrophosphate and its isomer dimethylallyl diphosphate, which are synthesized through two different routes. Plant isoprenoids are formed through two alternative pathways that operate in different subcellular compartments, the mevalonate (MVA) pathway localized in the cytosol and the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway localized in plastids.[4-7] The mevalonate pathway occurs in the cytoplasm, where sesquiterpenes and triterpenes such as sterols are synthesized, including such hormones as cytokinins, brassinosteroids and phytosterols.[1,8] The MEP pathway produces isopentenyl pyrophosphate, which is mainly used for the biosynthesis of isoprene, monoterpenes, diterpenes, carotenoids and phytol conjugates, such as chlorophylls, hormones such as gibberellins (GAs) and abscisic acid (ABA) (**Figure S1**). [1,2, 9,10] Moreover, isoprenoids produced by plants, for instance, vitamin E, are of importance for human health.[2,11]

Salvia miltiorrhiza Bunge (also named danshen), widely used for the clinical treatment of cardiovascular and cerebrovascular diseases, is a well-known traditional medicine in China.[12-15] Compound danshen dripping pills, which are listed in the official China pharmacopeia, have been widely used in many countries, this is also the first traditional Chinese medicine drug approved for clinical trials in the USA.[16]

Salvia miltiorrhiza consists of two types of bioactive components, the liposoluble tanshinone and phenolic compounds such as salvianolic acid A, salvianolic acid B, and rosmarinic acid.[7, 16-18] The abietane-type diterpenes (tanshinones), including tanshinone I, tanshinone IIA, dihydrotanshinone and cryptotanshinone, have a variety of biological activities including heart-protective, anti-ischemic, antioxidant, antibacterial and antitumor properties.[5, 8, 13, 19-23] Because of low contents of these compounds in cultivated *S. miltiorrhiza* plants, it is essential and urgent to improve the tanshinone production using biotechnological methods to meet the increasing clinical demand.

The biosynthesis of tanshinone is a complicated process that involves several catalytic steps (**Figure S1**). Tanshinones, which are diterpene compounds, are synthesized from common C5 precursors, isopentenyl pyrophosphate and its isomer dimethylallyl diphosphate, which are mainly derived through the MEP pathway.[4,5] Pyruvate and D-glyceraldehyde-3-phosphate are catalyzed by 1-deoxy-D-xylulose-5-phosphate (DXP) synthase (DXS) to form DXP. MEP is produced by 1-deoxy-D-xylulose5-phosphate reductoisomerase (DXR), and a subsequent series of condensations result in the formation of 20-carbon geranylgeranyl diphosphate (GGPP), catalyzed by GGPP synthase from 10-carbon geranyl diphosphate (GPP) and 15-carbon farnesyl diphosphate (FPP), providing a C20 parental structure for diterpenes including tanshinones (**Figure S1**).[13] Recently, the genes involved in tanshinone biosynthesis, such as *SmHMGR* (3-hydroxy-3-methylglutaryl CoA reductase), *SmHMGS* (3-hydroxy-3-methylglutaryl

CoA synthase), *SmDXR* (1-Deoxy-D-xylulose 5-phosphate reductoisomerase), *SmDXS* (1-deoxy-D-xylulose-5-phosphate synthase) and *SmGGPPS* (Geranylgeranyl diphosphate synthase) have been successfully isolated from *S. miltiorrhiza* by our team,[6, 8, 13, 24, 25] enabling us to elucidate the biosynthesis of tanshinone and to produce increased tanshinone yields in *S. miltiorrhiza* through genetic engineering. This is a feasible strategy for enhancing the production of natural products by manipulating a number of biosynthetic genes at regulatory points.[26, 27] However, there is, to our knowledge, no published report on the simultaneous introduction of key genes *DXS* and *GGPPS* into any isoprenoid-producing plants, including *S. miltiorrhiza* and *Arabidopsis thaliana.*

In this study, simultaneous introduction of *SmDXSII* (the first key gene upstream of the MEP pathway) and *SmGGPPS* (an important gene downstream of the tanshinone biosynthetic pathway) was achieved to increase tanshinone production in *S. miltiorrhiza* hairy roots. The correlation between content of tanshinone and other isoprenoids content and transcription levels of introduced transgenes was evaluated in the transgenic *S. miltiorrhiza* hairy roots. The antioxidant and anti-cancer activities of crude tanshinone extracts obtained from the transgenic hairy roots were also investigated by DPPH free radical assay and MTT assay, respectively. In addition, the contents of several plastid pathway-derived isoprenoids such as chlorophylls, carotenoids, indole acetic acid, gibberellins, and abscisic acid were examined in the transgenic *Arabidopsis* plants and wild-type controls. Taken together, this work provides useful information to improve the yield of isoprenoids, including tanshinone,

111 from plants in the future.

112 **Materials and methods**

113 **Construction of plant expression vectors**

114 pCAMBIA1304^{+}, pCAMBIA1304^{+}-*SmGGPPS* and pCAMBIA1304^{+}-*SmDXSII* were
115 constructed as stated in our previous report.[5] *SmGGPPS* and *SmDXSII* were driven
116 under the control of the constitutive CaMV35S promoter. *SmDXSII* was inserted into
117 the pCAMBIA1304^{+}-*SmGGPPS* construct to express *SmGGPPS* and *SmDXSII*. The
118 empty pCAMBIA1304^{+} was used as the blank control. The constructs were
119 introduced into *S. miltiorrhiza* via cells of a disarmed *Agrobacterium tumefaciens*
120 strain C58C1 which harbored the Ri plasmid from *A.rhizogenes A4*[5].

121 **Plant transformation and hairy roots cultivation**

122 Aseptic *S. miltiorrhiza* plants were grown in a greenhouse at 25 °C with 16 h light and
123 8 h dark periods in Murashige and Skoog (MS) basal medium with 3% sugar and
124 0.8% agar (pH 5.8).[5, 28, 29] Aseptic leaves of *S. miltiorrhiza* were cut into 0.5 cm^2
125 squares and cultured on half-strength MS medium (1/2 MS) in darkness for 2 days.
126 Cells of *A. tumefacien* strain C58C1 carrying the transformation vectors were used to
127 infect the leaves for about 30 min. After co-culture for 2-3 days, the infected leaves
128 were transferred to 1/2 MS supplemented with 300 mg/L carbencillin to kill the *A.*
129 *tumefaciens*. Two weeks later, the infected leaves were transferred to 1/2 MS with
130 decreased concentration (500 mg/L, 300 mg/L and 100 mg/L) of cefotaxime filtered
131 with a sterile 0.2 μm filter (Pall Corporation, USA) every 15 days. Rapidly growing
132 root lines without bacterial contamination were used to establish hairy roots lines.

Fragments about 3-4 cm long were cut from PCR-positive hairy roots colonies and cultured in 250 mL Erlenmeyer flasks containing 100 mL 1/2 MS medium on an orbital shaker (100 rpm) at 25 ℃ in darkness. The hairy roots were sub-cultured every 30 days and harvested after 60 days for extraction of active compounds.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from hairy roots according to a previously described method.[7, 30] Reverse transcription was carried out using the RT-PCR system (TaKaRa, Japan) in a 20 μL volume consisting of 4 μL of 5×M-MLV buffer, 2 μL of 50 μM primer AP (5'-GGCCACGCGTCGACTAGTAC$(T)_{16}$-3') (Sangon, China), 1 μL of 10×dNTPs, 0.5 μL of 200 U/μL RNase M-MLV and 0.5 μL of 40 U/μL RNase inhibitor at 42 ℃ for 1.5 h, then inactivated at 70 ℃ for 15 min. A Super Real PreMix kit (Tiangen, China) was used to carry out the qRT-PCR reaction following the manufacturer's instructions. The qRT-PCR reaction was performed on the Applied Biosystem StepOne Real-Time PCR System (Applied Biosystems, USA) with an optional 48-well plate normalizing against ubiquitin[31] based on the relative quantitative analysis method ($2^{-\Delta\Delta Ct}$). Amplification parameters were as follows: 10 min denaturing at 95 °C; then 40 cycles of 15 s denaturing at 95 °C, 30 s annealing at 60 °C, and 30 s extension at 72 ℃.[7] Expression profiles of tanshinone biosynthetic genes, such *GGPPS*, *DXSII, HMGR*, *DXR*, *IPPI*, *CPS*, and *KSL*, were investigated. All the primers sequences are listed in Table S1.

Determination of tanshinone by HPLC

The hairy roots were dried at 50 ℃ in an oven and then ground into powder, 100 mg

155 powder was placed in 16 mL of methanol/dichloromethane solvent (3:1, v/v) for 1
156 hour's ultrasonic processing at a frequency of 40000 Hz at room temperature
157 (KunshanHC-2002S, China). The extract was evaporated under a vacuum, and the
158 residue was re-dissolved with 2 mL methanol after being kept in the dark overnight.
159 The filtered solution was used for HPLC on a HITACHI L2000 apparatus equipped
160 with a Waters reversed-phase C18 symmetry column. Acetonitrile-water (65:35, v/v)
161 worked as the mobile phase at a flow rate of 1 mL/min with the detection wavelength
162 set at 270 nm.[5,7] Four components-dihydrotanshinone, cryptotanshinone, tanshinone I
163 and tanshinone IIA-were detected and quantified by comparison with authentic
164 standards (Aladdin, China). The sum of dihydrotanshinone, cryptotanshinone,
165 tanshinone I and tanshinone IIA were calculated as the total tanshinone content.

166 **DPPH free radical scavenging potential**

167 Total tanshinone from individual transgenic hairy roots lines (BC, DII50, DII57, G28,
168 G53, GDII2, GDII8, GDII10, GDII27) was used to measure the DPPH free radical
169 scavenging activities according to the DPPH method.[5, 32] Briefly, 1, 4, 6, 8, and 10 μL
170 of total tanshinone from these lines were diluted with methanol to 1 mL and added to
171 0.2 mM DPPH solution. The reaction mixture was incubated at room temperature for
172 30 min, and then analyzed at 517 nm on a spectrophotometer with 0.2 mM DPPH
173 solution in methanol as a control. The radical scavenging activity of total tanshinone
174 was calculated based on the formula: [5, 7, 33]

175 $$\text{DPPH radical scavenging activity (\%)} = \left[1 - \frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}}\right] \times 100$$

176 **Anti-cancer activity by MTT assay**

The total tanshinone extracted from transgenic hairy roots lines was used to measure its anti-cancer activity. NCI-H460 lung cancer cells were plated in 96-well microtiter plates with a volume of 100 μL in each well. The plates were incubated with 5% CO_2 at 37 °C for 12 h to allow cells to reattach and reequilibrate.[34] Then 3 μL and 5 μL total tanshinone, respectively, were added to the wells and cultured for 12 h or 24 h. Next, 25μL MTT solution was added and incubated for another 4 h. At the end of drug exposure, the enzyme reaction was stopped by the addition of 100 μL DMSO for 10 min to dissolve the formazan. Cell viability was measured by ELISA at 492 nm according to the formula:

$$\text{Cell viability} = \left(\frac{\text{Optical density of test group}}{\text{Optical density of control group}} \right) \times 100\%$$

The two concentrations were tested in three replicate wells on each plate, and each experiment was repeated three times.

Genetic transformation of *Arabidopsis thaliana*

Arabidopsis thaliana plants were grown from seeds in a greenhouse at 22 °C with 16 h light and 8h darkness and 60% humidity. Vectors, including pCAMBIA1304^{+}-*SmDXSII*, pCAMBIA1304^{+}-*SmGGPPS* and pCAMBIA1304^{+}-*SmGGPPS*-*SmDXSII* were delivered into *A.tumefaciens* strain GV3101 and used to transform *Arabidopsis* via the floral dip method [35]. T_0 seeds were sterilized and spread on MS medium with hygromycin (30 mg/L) for selection. Green and rooted seedlings were transferred to soil to harvest T_1 seeds. Then T_1 seeds were sown to produce T_2 populations.

Determination of chlorophyll and carotenoid in transgenic *A. thaliana*

Leaves (0.2 g) were cut into pieces and added to 25 mL mixture (acetone: ethanol: H_2O, 4:5:1) in a tube. The tube was put in the dark for 12 h, and shaken every 3 h until the fragments became white. Spectrophotometry was used to detect the content of chlorophyll and carotenoid, by analyzing obsorbance at wavelengths of 663 nm, 646 nm, 470 nm and 652 nm. A sample of solution mixture without samples was set as the control. The concentration of chlorophyll was measured according to the formulas:

$$\text{Chlorophyll a} = 12.21 \times A663 - 2.81 \times A646$$

$$\text{Chlorophyll b} = 20.13 \times A646 - 5.03 \times A663$$

$$\text{Total chlorophyll} = A_{652} \times \frac{\text{Volume of extract}}{34.5 \times \text{Weight of sample}}$$

The concentration of carotenoid[36] was:

$$\text{Carotenoid} = \frac{(1000A_{470} - 3.27[\text{Chlorophyll a}] - [\text{Chlorophyll b}])}{229}$$

Determination of phytohormones in transgenic *A. thaliana* by HPLC

Leaves of transgenic *A. thaliana* (10 g) were cut into fragments, ground to a homogenate in 60 mL 80% methanol using a mortar and pestle, and kept at 4 °C in the dark for 24 h. The homogenate was filtered through four layers of gauze and the residue dissolved in 20 mL methanol. The extract was blended and evaporated to half volume under a vacuum, and 10 mL petroleum ether was used to re-dissolve the pigment. The solution was evaporated under a vacuum until only the water phase remained. The pH was adjusted to 2.0-2.5 with 1M HCl. An equal volume of ethyl acetate was added to extract gibberellins, abscisic acid and indole acetic acid using

separatory funnel three times. An equal volume of ethyl acetate with abscisic acid, gibberellins, and indole acetic acid was evaporated under vacuum, after which 2 mL methanol was used to re-dissolve the residue. The solution was filtered and used for HPLC analysis, which was performed on a HITACHI L2000 apparatus equipped with a Waters reversed-phase C18 symmetry column (5 μm, 250 mm×4.6 mm). The separation conditions were as follows: The temperature of the column was 35 °C, the detection wavelength was 269 nm, the mobile phase consisted of methanol and 1% acetic acid (40:60, v/v) with a flow rate of 1 mL/min. Abscisic acid, gibberellin, and indole acetic acid were detected and quantified by comparison with authentic standards (Aladdin, China) and their retention time.

Statistical analysis

All experiments including the culture of hairy roots clones, PCR identification, qRT-PCR, HPLC analysis, measurement of antioxidant activity and MTT assay were repeated three times. Results for tanshinone and isoprenoid content are presented as mean values ± standard deviation. Error bars were obtained biological triplicates. The statistical significant difference was analyzed by a one-sample *t* test, and the errors of different hairy roots lines and transgenic plants were used in the one-way analysis of variance (ANOVA) using SPSS 11.5 software (SPSS Inc.).

Results

Generation of transgenic hairy roots lines of *S. miltiorrhiza*

Three plasmids containing *SmDXSII* or *SmGGPPS* full-length cDNA, or both, were

242 transformed into modified *A. tumefacien* C58C1 strain separately and subsequently
243 transferred into *S. miltiorrhiza* to generate transgenic hairy roots lines. A total of 50
244 lines transformed with *SmGGPPS* (G lines), 58 lines transformed with *SmDXSII* (DII
245 lines) and 60 lines transformed with both *SmGGPPS* and *SmDXSII* (GDII lines) were
246 generated. Three lines transformed with blank vector pCAMBIA1304^{+} were used as a
247 control. Hygromycin-resistant hairy roots lines with normal growth and phenotypes
248 were randomly chosen for further research.

249 **PCR analysis of genetically engineered hairy roots**

250 Genomic DNA was isolated from all the hairy roots lines individually and used for
251 PCR analysis with primers specially designed for the amplification of CaMV35S
252 promoter and the N-terminal part of the *SmGGPPS* or *SmDXSII* genes. Hairy roots
253 containing the *rolB* gene in *A. tumefacien* strain C58C1 were also detected by PCR
254 using the primers of *rolB*. Part of the PCR analysis results of genetically engineered
255 hairy roots are shown in **Figure S2**. As a positive control, a fragment with the
256 expected size was amplified from plasmids containing the delivered genes. Samples
257 NC1 (wild-type plant), NC2 (water) and blank-pCAMBIA1304^{+} were set as negative
258 controls and showed no amplified fragment. Amplicons of the same size as the
259 positive control were detected in some of the hairy roots lines. The PCR-positive rates
260 of hairy roots lines for G, DII and GDII lines were 14/50 (28.0%),11/58 (19.0%) and
261 25/60 (41.7%), respectively. These results suggested that these PCR-positive lines
262 contained introduced *SmGGPPS* or *SmDXSII* transgenes. We randomly selected five G,
263 five DII and eight GDII lines from the PCR-positive lines with normal phenotypes for

264 further experiments.

265 **Gene expression levels in transgenic hairy roots**

266 As shown in **Figure 1**, the expression of *SmGGPPS* in the transgenic G line and
267 *SmDXSII* in the transgenic DII line was higher than in control, respectively, while in
268 double-gene transformed GDII lines, the variation range of *SmGGPPS* expression
269 level was much higher than that for *SmDXSII*. Expression levels of gene *HMGR* (in
270 the MVA pathway), *DXR* (from the MEP pathway), *IPPI*, *CPS* and *KSL* (involved in
271 the downstream pathway) in the transgenic hairy roots lines (G42, G51, DII50, DII57,
272 GDII2, GDII8, GDII27, GDII34) were also detected. The expression profiles of these
273 tanshinone biosynthetic genes could be grouped into three categories. First, *HMGR*
274 was slightly downregulated in transgenic *GGPPS* or *DXS* lines compared with the
275 control, whereas in double-gene transformed GD lines, the expression level of *HMGR*
276 was elevated by two fold to five fold compared with the control. Second, the
277 expression of *DXR* did not show an apparent change in any of the transgenic hairy
278 roots lines and *IPPI* only showed obvious changes in single-gene transgenic hairy
279 roots lines, while *IPPI* obviously increased in all the double-gene transformed GD
280 lines. Third, expression of *CPS* and *KSL* was a little higher in G lines or DII lines than
281 in the control, but were dramatically enhanced by 16-33 fold and 7-16 fold in the
282 GDII line, respectively. These results indicated that all the cDNAs were introduced
283 into *S. miltiorrhiza* and expressed in corresponding transgenic lines, but with different
284 expression levels.

285 **Tanshinone content in transgenic hairy roots**

To examine the effect of the introduction of transgenes *SmDXSII* and *SmGGPPS* in the transgenic hairy roots, the contents of dihydrotanshinone, cryptotanshinone, tanshinone I and tanshinone IIA in *S. miltiorrhiza* hairy roots lines were determined by HPLC (**Figure 2)**. Transgenic lines (DII) harboring the *SmDXSII* gene produced tanshinone with the content varying from 2.02 to 4.58 mg/g, which is higher than the control (0.61 mg/g), reflecting that *SmDXS* is a key regulatory enzyme in the biosynthesis of tanshinone for genetic engineering. Hairy roots lines overexpressing *SmGGPPS* (G line) led to enhanced tanshinone production up to 4.95 mg/g, this confirmed that *SmGGPPS* is an important regulatory target for tanshinone biosynthesis. Furthermore, the highest tanshinone production was detected in double-gene transformed line GDII10 with the amount of 12.93 mg/g which was about 21 times that of the control, and nearly 3 times that of the highest single-gene transformed lines, G42 and DII47. Our results testified that co-operation of *SmDXSII* and *SmGGPPS* has an accumulative effect on tanshinone accumulation, leading to an almost tribling of tanshinone accumulation compared with single-gene-transformed lines (G or DII lines).

Antioxidant activities of crude tanshinone extract

In this study, DPPH radical scavenging experiments were carried out to evaluate the antioxidant activities of the crude extraction of tanshinone from transgenic hairy roots. As shown in **Figure 3**, all the tested samples possess strong radical scavenging capacity. The transgenic hairy roots lines DII2, DII57, G28, G53, GDII2, GDII8, GDII10 and GDII27 line exhibited higher total antioxidant potential than the

blank-vector control (BC). All the samples tested possess a strong radical scavenging capacity, which implied that the transgenic hairy roots lines had a higher total antioxidant activity, owing to the higher total tanshinone content.

Antitumor activity of crude tanshinone extract

To examine the antitumor activity of crude tanshinone extract from *S. miltiorrhiza* transgenic hairy roots, dihydrotanshinone was set as a positive control and pure methanol without samples as a blank control. Our results showed that the addition of 3 μL or 5 μL methanol had little effect on cell viability, while the addition of tanshinone from transgenic hairy roots decreased cell viability (**Figure 4**). Increasing the volume of tanshinone extract from 3 μL to 5 μL caused a deeper reduction in cell viability, extending the incubation time from 12 h to 24 h also further decreased cell viability, indicating that transgenic lines with higher tanshinone production resulted in lower cell viability.

Generation of transgenic *Arabidopsis* plants

Genomic DNA was isolated from random chosen *A. thaliana* individual line and used for PCR analysis with primers (listed in **Table S1**) specially designed to amplify the fragment overlapping the N-terminal of the *SmGGPPS* or the *SmDXSII* gene and the C-terminal of CaMV35S promoter. Plasmids containing the delivered genes were used as a positive control and showed amplicons at the expected size, while the negative controls, NC1 (wild-type plant), NC2 (water) and blank-pCAMBIA1304^{+}, did not show any amplicon. Amplicons of the same size as the positive control were detected in some of the transgenic lines. The PCR-positive rates of transgenic T_1 *Arabidopsis*

thaliana plant lines from G, DII and GDII lines were 15/54 (27.8%), 14/54 (25.9%) and 18/60 (30.0%), respectively. These results suggested that these PCR-positive lines contain the *SmGGPPS* or the *SmDXSII* gene. No obvious visual phenotype difference was observed between transgenic and untransformed control plants.

Chlorophyll and carotenoid content in transgenic *Arabidopsis* ***thaliana***

The contents of chlorophyll and carotenoids in transgenic *A. thaliana* were determined by HPLC (**Figure 5**). Transgenic *A.thaliana* plants overexpressing *SmGGPPS* (G line) produced higher levels of chlorophylls than the control line (1.18 mg/g), the average content was 2.12 mg/g. Transgenic line DII harboring only the *SmDXSII* gene produced up to 1.82 mg/g chlorophyll. The highest chlorophyll production was detected in line GDII 2.36 mg/g. However, the content of chlorophyll b did not obviously vary in transgenic lines and controls; thus, the increase in total chlorophyll content was attributed to chlorophyll a. The total carotenoid content in the transgenic plants was also analyzed. The results indicated that overexpression of *SmGGPPS* alone increased the total carotenoids content (0.32 mg/g for G lines) whereas *SmDXS* did not (0.26 mg/g for DII lines), implying that *SmGGPPS* plays a more important role in the biosynthesis of carotenoids than *SmDXS*. Transgenic T2 lines overexpressing *SmGGPPS* and *SmDXSII* revealed expression of up to 0.36 mg/g carotenoids, 1.4 times higher than the control.

Phytohormone content in transgenic *Arabidopsis* ***thaliana***

The content of gibberellins, abscisic acid and indole acetic acid in transgenic *A. thaliana* were analyzed by HPLC (**Figure 5**). The average content of gibberellins was

352 1.75 mg/g in transgenic DII lines and 3.18 mg/g in G lines, while a higher gibberellin
353 content was found in double-gene transformed GDII lines at 5.94 mg/g (5.8-fold of
354 the control), suggesting that geranylgeranyl diphosphate synthase (GGPPS) was
355 more effective than DXS in catalyzing the formation of gibberellins. Levels of indole
356 acetic acid in DII lines were slightly increased from 0.31mg/g (control line) to 0.36
357 mg/g, but were clearly boosted to 2.75 mg/g in G lines overexpressing GGPPS.
358 Interestingly, indole acetic acid content in double-gene transformed GDII lines was
359 0.72 mg/g, which was higher than control but lower than G lines. In contrast to the
360 change of gibberellin and indole acetic acid, abscisic acid production altered very
361 little between transgenic lines and controls.
362
363 **Discussion**
364 Tanshinones, a type of diterpene, are derived from two common C5 precursors,
365 isopentenyl pyrophosphate and dimethylallyl diphosphate.[2-3, 5] These two C5 units are
366 synthesized via two different pathways in separate cellular compartments, the MVA
367 pathway in the cytosol and the MEP pathway in the plastids. The MEP pathway
368 consists of seven enzymatic reactions starting from pyruvate and glyceraldehyde
369 3-phosphate;[37] it is considered as the main resource of C5 precursors for the
370 biosynthesis of tanshinones.[5,13] DXS, which catalyzes the first step of the MEP
371 pathway for isoprenoid biosynthesis, has been intensively studied and identified as a
372 limiting enzyme in the MEP pathway.[1, 2, 37, 38] The overexpression of *DXS* in *A.*
373 *thaliana* and tomato (*Lycopersicon esculentum*) has resulted in higher levels of

carotenoids and other terpenoids.[2] Furthermore, constitutive expression of *DXS* in spike lavender could improve essential oil yield,[38] suggesting that DXS plays an important role in isoprenoid biosynthesis in plants. Our previous studies demonstrated that DXS is rate-limiting for tanshinone biosynthesis.[5] In this study, we found that overexpression of single DXS gene in *S.miltiorrhiza* hairy roots resulted in large increases in tanshinone, this finding is in good agreement with other reports and demonstrated that DXS is a key regulatory point in modulating isoprenoid metabolism.[38] Introduction of *SmDXS* into *A. thaliana* resulted in increased total chlorophylls and gibberellins, but produced no obvious difference for carotenoids, indole acetic acid or abscisic acid, this finding was slightly different from the results of Estévez J.M [2] and implied the complexity of metabolic regulation of single DXS.[2,39] Our results indicated that the MEP pathway played an important role in the yield of tanshinone as well as other plastid-derived isoprenoids. This suggests that genetic manipulation of the MEP pathway is an effective strategy for providing sufficient precursors for the production of related isoprenoids.[40]

The plastidic pathway produces isopentenyl pyrophosphate, which is used in the biosynthesis of isoprenoids including monoterpenes, diterpenes, carotenoids, and phytol conjugates, such as chlorophylls and tocopherols. Geranylgeranyl diphosphate synthase catalyzes the sequential condensation of the dimethylallyl diphosphate with three molecules of isopentenyl pyrophosphate to produce GGPP, which is a key precursor for diterpenes including tanshinone.[5] Being a rate-limiting enzyme in the MEP pathway, the role of DXS has been well documented in several plants as

mentioned already, whereas comparatively limited information concerning GGPPS gene is available. GGPPS was found to be a key enzyme in the biosynthesis of such diterpenes as forskolin, taxol and tanshinone in *Coleus forskohlii*, *Taxus canadensis* and *S. miltiorrhiza*, respectively.[5,41] Here, the higher yield of tanshinone and other plastid pathway-derived isoprenoids, compared with control pCAMBIA1304^{+}-transformed lines, was observed in *GGPPS*-transformed *S. miltiorrhiza* lines, while *SmGGPPS* had a greater effect than *SmDXS* in the production of tanshinone and other isoprenoids including total chlorophylls, carotenoids, gibberellins, and indole acetic acid, it may be interpreted from this finding that *GGPPS* is downstream of the diterpene biosynthetic pathway, and may regulate diterperne biosynthesis more tightly than *DXS* in plants.

The "push-pull" strategy in genetic manipulation has been utilized successfully to significantly increase valuable metabolites, including terpenoid indole alkaloid, tropane alkaloid and camptothecin accumulation in *Catharanthus roseus*, *Anisodus acutangulus*, and *Ophiorrhiza pumila*, respectively.[5, 39, 42] Although the MEP pathway exhibited a critical role in diterpene biosynthesis, however, until now there has been no report on the enhancement of diterpene content by co-overexpression of DXS and GGPPS in any isoprenoid-producing plants, including the medicinal plant *S. miltiorrhiza* and even the model plant *A. thaliana*. In this study, overexpression of *GGPPS* or *DXS*, or both, had a difference on the genes involved in the tanshinone biosynthesis pathway. *HMGR* was elevated several-fold in double-gene transformed lines compared with the control, although it was slightly downregulated in single-gene

418 *GGPPS* or *DXS* transgenic lines, while there was no obvious change in *DXR*
419 expression in any of the transgenic lines. Interestingly, transcripts of *CPS* and *KSL* as
420 well as *IPPI* were distinctly increased in double-gene transformed GDII lines,
421 implying that many more intermediates, such as GGPP, were generated and led to an
422 increase transcripts of consecutive genes including *CPS* and *KSL*, involved in the
423 tanshinone production chain. Our results demonstrated that co-overexpression of
424 *SmGGPPS* and *SmDXSII* can dramatically affect the expression of other tanshinone
425 biosynthetic genes besides the single gene, which may be responsible for high levels
426 of tanshinone accumulation in double-gene transformed GDII lines.

427 Transgenic hairy roots lines expressing both *SmGGPPS* and *SmDXSII* produced
428 significantly higher ($P < 0.05$) levels of tanshinone and other isoprenoids compared
429 with the control and single-gene (*GGPPS* or *DXSII*) transformed line. The best line
430 (GDII10) produced 12.93 mg/g of tanshinone, to our knowledge, this is the highest
431 level of tanshinone accumulation achieved through genetic engineering in *S.*
432 *miltiorrhiza*, and is even slightly higher than that obtained in field-cultivated *S.*
433 *miltiorrhiza* plants.[43] In the current study, the transgenic hairy roots lines exhibited
434 higher total antioxidant potential than the control, as revealed by DPPH radical
435 scavenging activity analysis. Furthermore, an MTT assay showed that total tanshinone
436 from different lines exhibited antitumor activity on NCI-H460 lung cancer cells in a
437 time-and concentration-dependent manner, and transgenic hairy roots lines showed
438 higher antitumor activity than the control. Transgenic *A.thaliana* harboring *GGPPS*
439 and *DXSII* showed elevated levels of chlorophyll, carotenoids, gibberellins, and indole

acetic acid which were 2.0, 1.4, 5.8, 2.3 times the levels obtained with non-transgenic lines, respectively.

Our work showed that the co-overexpression of *SmGGPPS* and *SmDXSII* can produce a positive co-operative effect in stimulating accumulation of diterpene tanshinone and plastid pathway-derived isoprenoids. The genetically engineered *S. miltiorrhiza* hairy roots can be used further for mass-production of tanshinones in bioreactors by optimizing the bioreactor design and bioprocess control. Further, a combination of transgenic technology with elicitor treatments as well as bioreactor optimization may be a feasible strategy for large-scale production of tanshinone in *S. miltiorrhiza* hairy roots.

Abbreviations Used

Sm, *Salvia miltiorrhiza*; MVA, Mevalonate; MEP, Methylerythritol phosphate; IPP, Isopentenyl pyrophosphate; DMAPP, Dimethylallyl diphosphate; *SmGGPPS*, geranylgeranyl diphosphate synthase; *SmDXS*, 1-deoxy-D-xylulose-5-phosphate synthase; CaMV, cauliflower mosaic virus; HPLC, High-performance liquid chromatography; TT, total tanshinone; HT, Dihydrotanshinone; CT, Cryptotanshinone; T1, Tanshinone I; T2A, Tanshinone IIA; GAs, Gibberellins; ABA, Abscisic acid; IAA, Indole acetic acid; Ca, Chlorophyll a; Cb, Chlorophyll b.

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638 **Figure captions**

639 **Figure.1** (A) Analysis of *SmGGPPS* and *SmDXSII* levels in *S. miltiorrhiza* hairy roots.
640 (B)Transcripts of multiple genes (*HMG*R, *DXR*, *IPPI*, *CPS*, *KSL*) involved in
641 tanshinone biosynthesis. Values are means±standard deviation of triplicate analyses
642 (P<0.05).

643 **Figure.2** HPLC analysis of tanshinone production in transgenic hairy roots lines. BC,
644 control hairy roots cultures generated from blank-vector transformation; DII,
645 *SmDXSII* transgenic hairy roots lines; G, *SmGGPPS* transgenic hairy roots lines; GDII,
646 *SmGGPPS* and *SmDXSII* transgenic hairy roots lines. Values are means±standard
647 deviation of triplicate analyses (P<0.05). HT, Dihydrotanshinone; CT,
648 Cryptotanshinone; T1, Tanshinone I; T2A, Tanshinone IIA; TT, total tanshinone.

649 **Figure.3** DPPH scavenging activities in transgenic hairy roots lines. Nine different
650 hairy roots lines (BC, DII50, DII57, G28, G53, GDII2, GDII8, GDII10 and GDII27)
651 were tested. The values are means±standard deviation of triplicate analyses (P<0.05).

652 **Figure.4** Effect of tanshinone on cell viability of NCI-H460 lung cancer cells. 3 μL
653 and 5 μL of total tanshinone extract from DII50, DII57, G28, G53, GDII2, GDII8,
654 GDII10, GDII27 and BC lines were used to test the effect on cell viability. Values are
655 means±standard deviation of triplicate analyses (P<0.05).

656 **Figure.5** Total chlorophyll and carotenoids contents in transgenic *Arabidopsis*
657 *thaliana* lines and non-transgenic lines grown under the same conditions. (A) Total
658 chlorophyll carotenoids content; (B) Total carotenoid content; (C) Phytohormone
659 contents (gibberellins, abscisic acid, indole acetic acid) in transgenic and
660 non-transgenic *A.thaliana*. Each column represents the mean of three independent
661 experiments. GAs, Gibberellins; ABA, Abscisic acid; IAA, Indole acetic acid; Ca,
662 Chlorophyll a; Cb, Chlorophyll b; Cx, total carotenoid; W, wild-type.

Figure 1

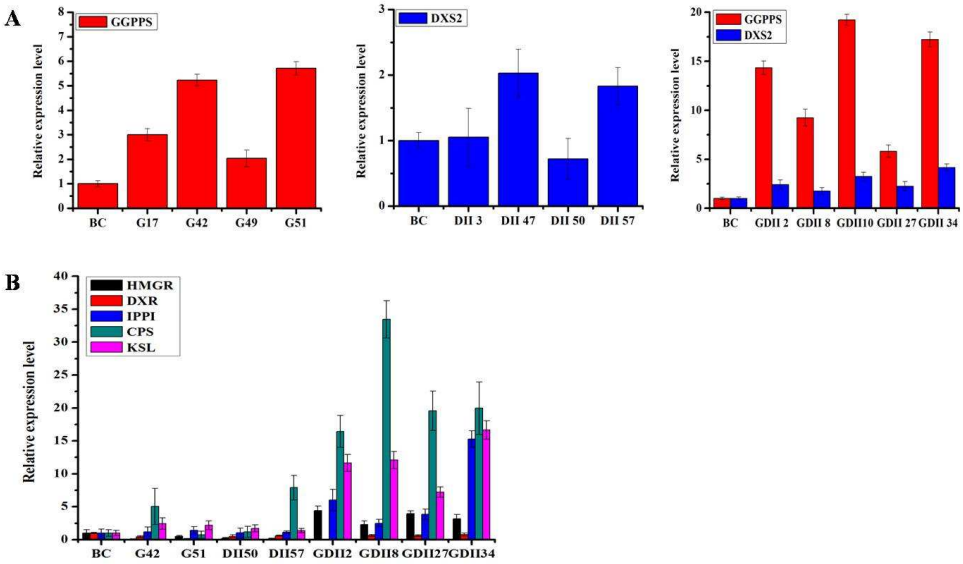

A
GGPPS
Relative expression level
BC
G17
G42
G49
G51
DXS2
DII 3
DII 47
DII 50
DII 57
GGPPS
DXS2
GDII 2
GDII 8
GDII10
GDII 27
GDII 34
B
HMGR
DXR
IPPI
CPS
KSL
DII50
DII57
GDII2
GDII8
GDII27
GDII34

Figure 2

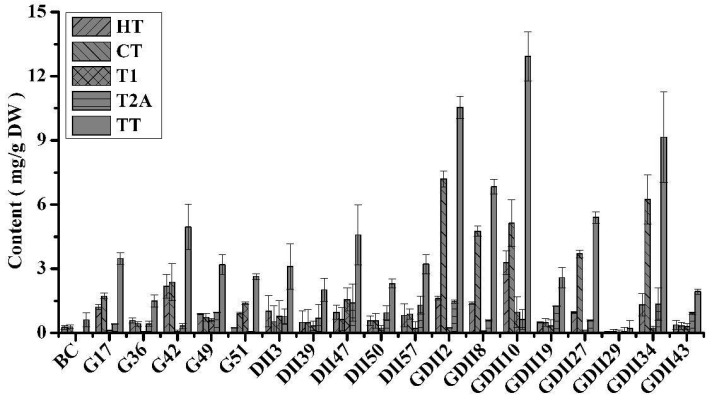

HT
CT
T1
T2A
TT
Content (mg/g DW)
0
3
6
9
12
15
BC
G17
G36
G42
G49
G51
DII3
DII39
DII47
DII50
DII57
GDII2
GDII8
GDII10
GDII19
GDII27
GDII29
GDII34
GDII43

Figure 3

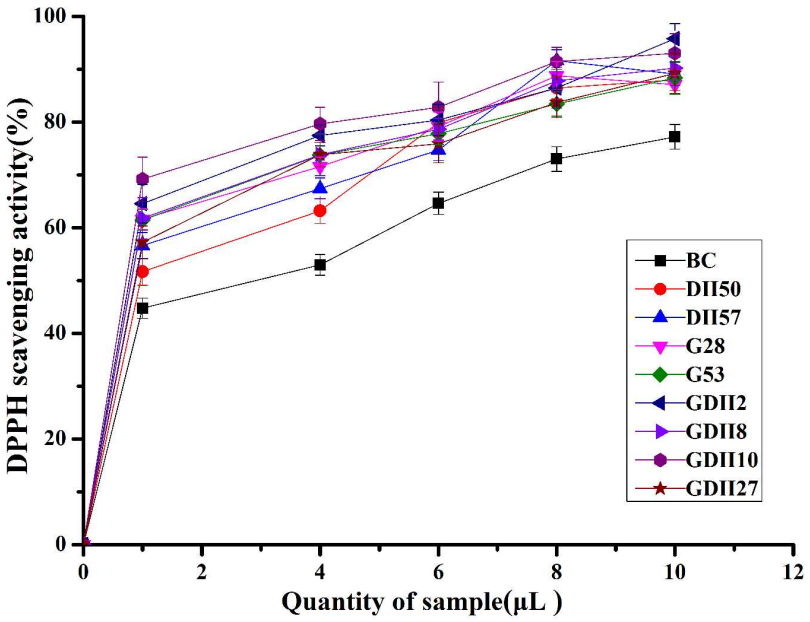

DPPH scavenging activity(%)
Quantity of sample(μL)
0
20
40
60
80
100
0
2
4
6
8
10
12
BC
DII50
DII57
G28
G53
GDII2
GDII8
GDII10
GDII27

Figure 4

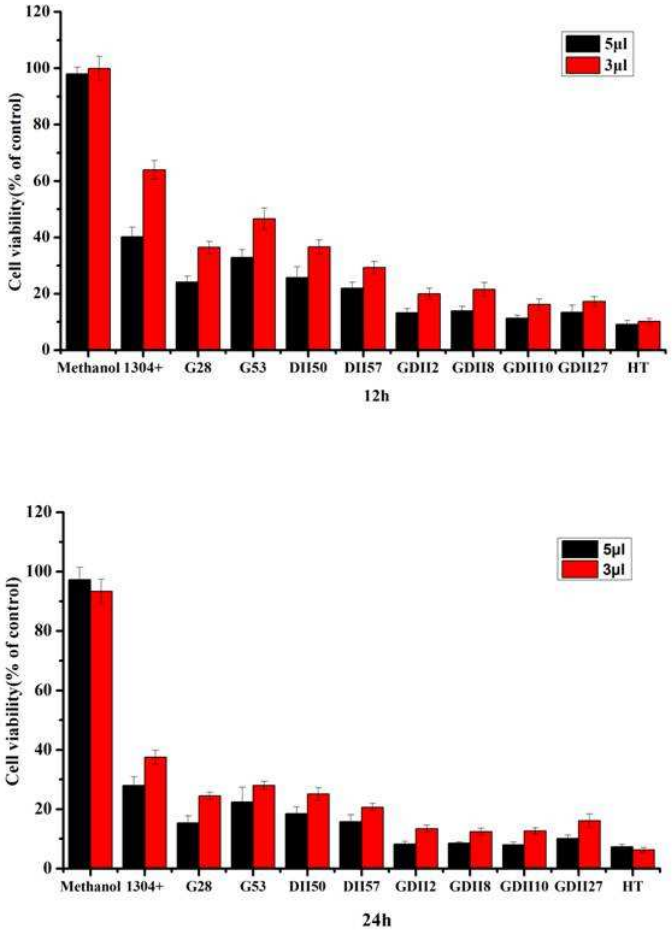
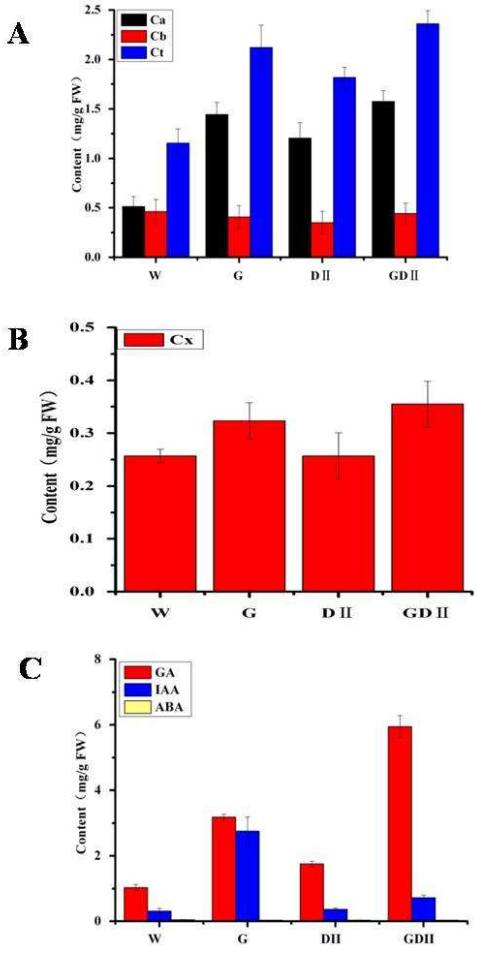

Cell viability(% of control)
120
100
80
60
40
20
0
5μl
3μl
Methanol
1304+
G28
G53
DII50
DII57
GDII2
GDII8
GDII10
GDII27
HT
12h
Cell viability(% of control)
120
100
80
60
40
20
0
5μl
3μl
Methanol
1304+
G28
G53
DII50
DII57
GDII2
GDII8
GDII10
GDII27
HT
24h

Figure 5


A
Ca
Cb
Ct
Content (mg/g FW)
2.5
2.0
1.5
1.0
0.5
0.0
W
G
D II
GD II
B
Cx
Content (mg/g FW)
0.5
0.4
0.3
0.2
0.1
0.0
W
G
D II
GD II
C
GA
IAA
ABA
Content (mg/g FW)
8
6
4
2
0
W
G
DII
GDII

Graphic for table of contents