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# Enhanced Diterpene Tanshinone Accumulation and Bioactivity of Transgenic *Salvia miltiorrhiza* Hairy Roots by Pathway Engineering

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Supporting Information

**ABSTRACT:** Tanshinones are health-promoting diterpenoids found in *Salvia miltiorrhiza* and 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 12.93 mg/g dry weight, which is the highest tanshinone content that has been achieved through genetic engineering. Furthermore, transgenic hairy root lines showed higher antioxidant and antitumor activities than control lines. In addition, contents of chlorophylls, carotenoids, indoleacetic 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 2-C-methyl-D-erythritol-4-phosphate (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, and 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 United States.[16] *S. 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 dime-

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thylallyl 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-xylulose-5-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 study 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 anticancer 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, indoleacetic 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, from plants in the future.

## MATERIALS AND METHODS

**Construction of Plant Expression Vectors.** pCAMBIA1304$^+$, pCAMBIA1304$^+$-*SmGGPPS*, and pCAMBIA1304$^+$-*SmDXSII* were constructed as stated in our previous paper.[5] *SmGGPPS* and *SmDXSII* were driven under the control of the constitutive CaMV35S promoter. *SmDXSII* was inserted into the pCAMBIA1304$^+$-*SmGGPPS* construct to express *SmGGPPS* and *SmDXSII*. The empty pCAMBIA1304$^+$ was used as the blank control. The constructs were introduced into *S. miltiorrhiza* via cells of a disarmed *Agrobacterium tumefaciens* strain C58C1, which harbored the Ri plasmid from *Agrobacterium rhizogenes A4*.[5]

**Plant Transformation and Hairy Root Cultivation.** Aseptic *S. miltiorrhiza* plants were grown in a greenhouse at 25 °C with 16 h light and 8 h dark periods in Murashige and Skoog (MS) basal medium with 3% sugar and 0.8% agar (pH 5.8).[5,28,29] Aseptic leaves of *S. miltiorrhiza* were cut into 0.5 cm$^2$ squares and cultured on half-strength MS medium (1/2 MS) in darkness for 2 days. Cells of *Agrobacterium tumefaciens* strain C58C1 carrying the transformation vectors were used to infect the leaves for about 30 min. After coculture for 2−3 days, the infected leaves were transferred to 1/2 MS supplemented with 300 mg/L carbencillin to kill the *Agrobacterium tumefaciens*. Two weeks later, the infected leaves were transferred to 1/2 MS with decreased concentration (500, 300, and 100 mg/L) of cefotaxime filtered with a sterile 0.2 μm filter (Pall Corp., USA) every 15 days. Rapidly growing root lines without bacterial contamination were used to establish hairy root lines. Fragments about 3−4 cm long were cut from PCR-positive hairy root colonies and cultured in 250 mL Erlenmeyer flasks containing 100 mL of 1/2 MS medium on an orbital shaker (100 rpm) at 25 °C in darkness. The hairy roots were subcultured 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 °C for 1.5 h and then inactivated at 70 °C 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 of denaturation at 95 °C; then 40 cycles of 15 s of denaturation at 95 °C, 30 s of annealing at 60 °C, and a 30 s extension at 72 °C.[7] Expression profiles of tanshinone biosynthetic genes, such as *GGPPS*, *DXSII*, *HMGR*, *DXR*, *IPPI*, *CPS*, and *KSL*, were investigated. All of the primer sequences are listed in Table S1.

**Determination of Tanshinone by HPLC.** The hairy roots were dried at 50 °C in an oven and then ground into powder; 100 mg of powder was placed in 16 mL of methanol/dichloromethane solvent (3:1, v/v) for 1 h of ultrasonic processing at a frequency of 40000 Hz at room temperature (KunshanHC-2002S, China). The extract was evaporated under a vacuum, and the residue was redissolved with 2 mL of methanol after being kept in the dark overnight. The filtered solution was used for HPLC on a Hitachi L2000 apparatus equipped with a Waters reversed-phase C18 symmetry column. Acetonitrile/water (65:35, v/v) worked as the mobile phase at a flow rate of 1 mL/min with the detection wavelength set at 270 nm.[5,7] Four components, dihydrotanshinone, cryptotanshinone, tanshinone I, and tanshinone IIA, were detected and quantified by comparison with authentic standards (Aladdin, China). The sum of dihydrotanshinone, cryptotanshinone, tanshinone I, and tanshinone IIA was calculated as the total tanshinone content.

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

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

**Anticancer Activity by MTT Assay.** The total tanshinone extracted from transgenic hairy root lines was used to measure its anticancer 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 re-equilibrate.[34] Then 3 and 5 μL of total tanshinone, respectively, were added to the wells and cultured for 12 or 24 h. Next, 25 μL of MTT solution was added and incubated for another 4 h. At the end of


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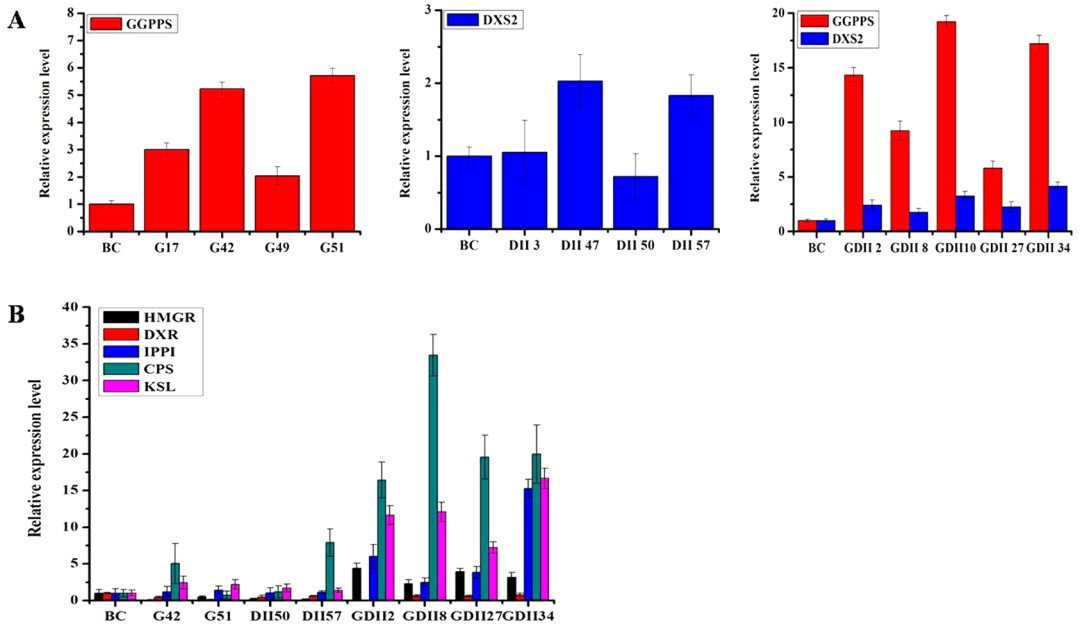


**Figure 1.** (A) Analysis of *SmGGPPS* and *SmDXSII* levels in *S. miltiorrhiza* hairy roots. (B) Transcripts of multiple genes (*HMGR, DXR, IPPI, CPS, KSL*) involved in tanshinone biosynthesis. Values are means ± standard deviation of triplicate analyses ($P < 0.05$).

drug exposure, the enzyme reaction was stopped by the addition of 100 μL of DMSO for 10 min to dissolve the formazan. Cell viability was measured by ELISA at 492 nm according to the following 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 of light and 8 h of darkness and 60% humidity. Vectors, including pCAMBIA1304$^+$-*SmDXSII*, pCAMBIA1304$^+$-*SmGGPPS*, and pCAMBIA1304$^+$-*SmGGPPS-SmDXSII*, were delivered into *Agrobacterium 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 *Arabidopsis thaliana*.** Leaves (0.2 g) were cut into pieces and added to 25 mL of 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 absorbance at wavelengths of 663, 646, 470, and 652 nm. A sample of solution mixture without samples was set as the control. The concentration of chlorophyll was measured according to the following formulas:

$$\text{chlorophyll } a = 12.21 \times A_{663} - 2.81 \times A_{646}$$

$$\text{chlorophyll } b = 20.13 \times A_{646} - 5.03 \times A_{663}$$

$$\text{total chlorophyll} = A_{652} \times \frac{\text{volume of extract}}{34.5 \times \text{weight of sample}}$$

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 *Arabidopsis thaliana* by HPLC.** Leaves of transgenic *Arabidopsis thaliana* (10 g) were cut into fragments, ground to a homogenate in 60 mL of 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 of methanol. The extract was blended and evaporated to half volume under a vacuum, and 10 mL of petroleum ether was used to redissolve 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 1 M HCl. An equal volume of ethyl acetate was added to extract gibberellins, abscisic acid, and indoleacetic acid using a separatory funnel three times. An equal volume of ethyl acetate with abscisic acid, gibberellins, and indoleacetic acid was evaporated under vacuum, after which 2 mL of methanol was used to redissolve 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 indoleacetic 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 root clones, PCR identification, qRT-PCR, HPLC analysis, measurement of antioxidant activity, and MTT assay were repeated three times. Results for tanshinone and isoprenoid contents are presented as mean values ± standard deviation. Error bars were obtained for biological triplicates. The statistically significant difference was analyzed by a one-sample *t* test, and the errors of different hairy root 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 transformed into modified *Agrobacterium. tumefaciens* C58C1 strain separately and subsequently transferred into *S. miltiorrhiza* to generate transgenic hairy roots lines. A total of 50 lines transformed with *SmGGPPS* (G lines), 58 lines transformed with *SmDXSII* (DII lines), and 60 lines transformed with both *SmGGPPS* and *SmDXSII* (GDII lines) were generated. Three lines transformed


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with blank vector pCAMBIA1304$^+$ were used as a control. Hygromycin-resistant hairy root lines with normal growth and phenotypes were randomly chosen for further research.

**PCR Analysis of Genetically Engineered Hairy Roots.** Genomic DNA was isolated from all of the hairy root lines individually and used for PCR analysis with primers especially designed for the amplification of CaMV35S promoter and the N-terminal part of the *SmGGPPS* or *SmDXSII* genes. Hairy roots containing the *rolB* gene in *Agrobacterium tumefaciens* strain C58C1 were also detected by PCR using the primers of *rolB*. Part of the PCR analysis results of genetically engineered hairy roots are shown in Figure S2. As a positive control, a fragment with the expected size was amplified from plasmids containing the delivered genes. Samples NC1 (wild-type plant), NC2 (water), and blank-pCAMBIA1304$^+$ were set as negative controls and showed no amplified fragment. Amplicons of the same size as the positive control were detected in some of the hairy root lines. The PCR-positive rates of hairy root lines for G, DII, and GDII lines were 14/50 (28.0%), 11/58 (19.0%), and 25/60 (41.7%), respectively. These results suggested that these PCR-positive lines contained introduced *SmGGPPS* or *SmDXSII* transgenes. We randomly selected five G, five DII, and eight GDII lines from the PCR-positive lines with normal phenotypes for further experiments.

**Gene Expression Levels in Transgenic Hairy Roots.** As shown in Figure 1, the expression of *SmGGPPS* in the transgenic G line and *SmDXSII* in the transgenic DII line was higher than in control, respectively, whereas in double-gene transformed GDII lines, the variation range of *SmGGPPS* expression level was much higher than that for *SmDXSII*. Expression levels of gene *HMGR* (in the MVA pathway), *DXR* (from the MEP pathway), *IPPI*, *CPS*, and *KSL* (involved in the downstream pathway) in the transgenic hairy root lines (G42, G51, DII50, DII57, GDII2, GDII8, GDII27, GDII34) were also detected. The expression profiles of these tanshinone biosynthetic genes could be grouped into three categories. First, *HMGR* was slightly down-regulated in transgenic *GGPPS* or *DXS* lines compared with the control, whereas in double-gene transformed GD lines, the expression level of *HMGR* was elevated by 2−5-fold compared with the control. Second, the expression of *DXR* did not show an apparent change in any of the transgenic hairy root lines, and *IPPI* only showed slight changes in single-gene transgenic hairy roots lines, whereas *IPPI* obviously increased in all of the double-gene transformed GD lines. Third, expressions of *CPS* and *KSL* was a little higher in G lines or DII lines than in the control, but were dramatically enhanced by 16−33- and 7−16-fold in the GDII line, respectively. These results indicated that all of the cDNAs were introduced into *S. miltiorrhiza* and expressed in corresponding transgenic lines, but with different expression levels.

**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 root lines overexpressing *SmGGPPS* (G line) led to enhanced tanshinone production up to 4.95 mg/g;

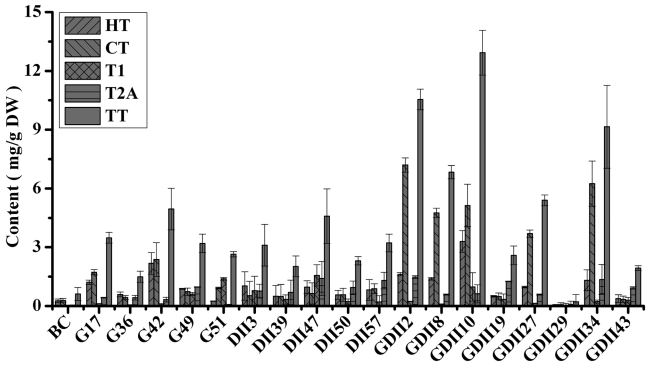


**Figure 2.** HPLC analysis of tanshinone production in transgenic hairy roots lines. BC, control hairy roots cultures generated from blank-vector transformation; DII, *SmDXSII* transgenic hairy roots lines; G, *SmGGPPS* transgenic hairy roots lines; GDII, *SmGGPPS* and *SmDXSII* transgenic hairy roots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

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 trebling 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 of the tested samples possess strong radical scavenging capacity. The transgenic hairy root lines DII50, DII57, G28, G53, GDII2, GDII8, GDII10, and GDII27 line exhibited higher total antioxidant potential than the blank-vector control (BC).

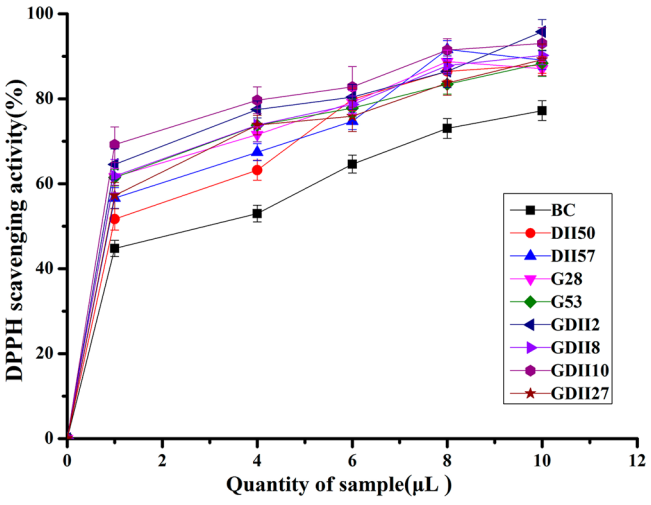


**Figure 3.** DPPH scavenging activities in transgenic hairy roots lines. Nine different hairy roots lines (BC, DII50, DII57, G28, G53, GDII2, GDII8, GDII10, and GDII27) were tested. The values are means ± standard deviation of triplicate analyses ($P < 0.05$).



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All of 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 or 5 μL of methanol had little effect on cell viability, whereas the addition of tanshinone from transgenic hairy roots decreased cell viability (Figure 4). Increasing the volume of tanshinone extract from 3 to 5 μL caused a deeper reduction in cell viability, and extending the incubation time from 12 to 24 h also further decreased cell viability, indicating that transgenic lines with higher tanshinone production resulted in lower cell viability.

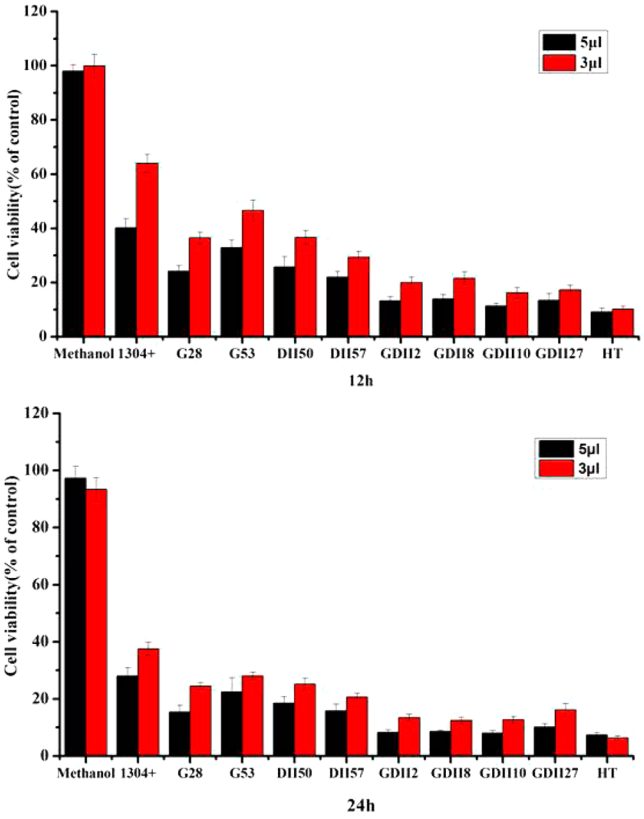


**Figure 4.** Effect of tanshinone on cell viability of NCI-H460 lung cancer cells. Total tanshinone extracts from DII50, DII57, G28, G53, GDII2, GDII8, GDII10, GDII27, and BC lines (3 and 5 μL) were used to test the effect on cell viability. Values are means ± standard deviation of triplicate analyses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

**Generation of Transgenic *Arabidopsis* Plants.** Genomic DNA was isolated from random chosen *Arabidopsis thaliana* individual line and used for PCR analysis with primers (listed in Table S1) especially 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, whereas 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 Contents in Transgenic *Arabidopsis thaliana*.** The contents of chlorophyll and carotenoids in transgenic *Arabidopsis thaliana* were determined by HPLC (Figure 5). Transgenic *Arabidopsis 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 contents of gibberellins, abscisic acid, and indole acetic acid in transgenic *Arabidopsis thaliana* were analyzed by HPLC (Figure 5). The average content of gibberellins was 1.75 mg/g in transgenic DII lines and 3.18 mg/g in G lines, whereas a higher gibberellin content was found in double-gene-transformed GDII lines at 5.94 mg/g (5.8-fold of the control), suggesting that geranylgeranyl diphosphate synthase (GGPPS) was more effective than DXS in catalyzing the formation of gibberellins. Levels of indoleacetic acid in DII lines were slightly increased from 0.31 mg/g (control line) to 0.36 mg/g, but were clearly boosted to 2.75 mg/g in G lines overexpressing GGPPS. Interestingly, indoleacetic acid content in double-gene-transformed GDII lines was 0.72 mg/g, which was higher than control but lower than G lines. In contrast to the change of gibberellin and indoleacetic acid, abscisic acid production altered very little between transgenic lines and controls.

## DISCUSSION

Tanshinones, a type of diterpene, are derived from two common C5 precursors, isopentenyl pyrophosphate and dimethylallyl diphosphate.[2,3,5] These two C5 units are synthesized via two different pathways in separate cellular compartments, the MVA pathway in the cytosol and the MEP pathway in the plastids. The MEP pathway consists of seven enzymatic reactions starting from pyruvate and glyceraldehyde 3-phosphate;[37] it is considered as the main resource of C5 precursors for the biosynthesis of tanshinones.[5,13] DXS, which catalyzes the first step of the MEP pathway for isoprenoid biosynthesis, has been intensively studied and identified as a limiting enzyme in the MEP pathway.[1,2,37,38] The overexpression of *DXS* in *Arabidopsis 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


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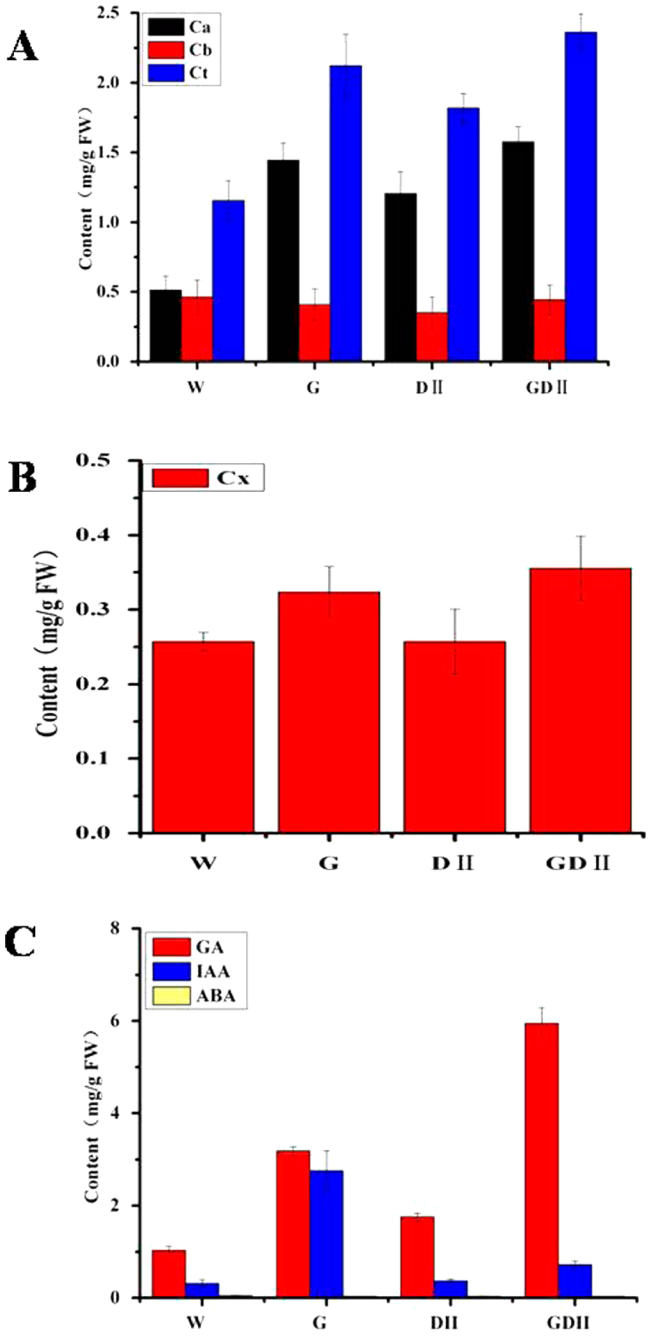


**Figure 5.** Total chlorophyll and carotenoids contents in transgenic *Arabidopsis thaliana* lines and nontransgenic lines grown under the same conditions: (A) total chlorophyll carotenoids content; (B) total carotenoid content; (C) phytohormone contents (gibberellins, abscisic acid, indoleacetic acid) in transgenic and nontransgenic *Arabidopsis thaliana*. Each column represents the mean of three independent experiments. GAs, gibberellins; ABA, abscisic acid; IAA, indoleacetic acid; Ca, chlorophyll *a*; Cb, chlorophyll *b*; Cx, total carotenoid; W, wild-type.

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 a 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 *Arabidopsis thaliana* resulted in increased total chlorophylls and gibberellins, but produced no obvious difference for carotenoids, indoleacetic acid, or abscisic acid; this finding was slightly different from the results of Estévez[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, whereas *SmGGPPS* had a greater effect than *SmDXS* in the production of tanshinone and other isoprenoids including total chlorophylls, carotenoids, gibberellins, and indoleacetic 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, 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 *Arabidopsis thaliana*. In this study, overexpression of *GGPPS* or *DXS*, or both, produced a difference in 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 down-regulated in single-gene *GGPPS* or *DXS* transgenic lines, whereas there was no obvious change in *DXR* expression in any of the transgenic lines. Interestingly, transcripts of *CPS* and *KSL* as well as *IPPI* were distinctly increased in double-gene-transformed GDII lines, implying that many more intermediates, such as GGPP, were generated and led to an increase of transcripts of consecutive genes, including *CPS* and *KSL*, involved in the tanshinone production chain. Our results demonstrated that co-overexpression of *SmGGPPS* and *SmDXSII* can dramatically affect the expression of other tanshinone biosynthetic genes besides the single gene, which may be responsible for high levels of tanshinone accumulation in double-gene-transformed GDII lines.


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Transgenic hairy root lines expressing both *SmGGPPS* and *SmDXSII* produced significantly higher ($P < 0.05$) levels of tanshinone and other isoprenoids compared with the control and single-gene-transformed (*GGPPS* or *DXSII*) lines. The best line (GDII10) produced 12.93 mg/g of tanshinone; to our knowledge, this is the highest level of tanshinone accumulation achieved through genetic engineering in *S. miltiorrhiza* and is even slightly higher than that obtained in field-cultivated *S. miltiorrhiza* plants.[43] In the current study, the transgenic hairy root lines exhibited higher total antioxidant potential than the control, as revealed by DPPH radical scavenging activity analysis. Furthermore, an MTT assay showed that total tanshinone from different lines exhibited antitumor activity on NCI-H460 lung cancer cells in a time- and concentration-dependent manner, and transgenic hairy root lines showed higher antitumor activity than the control. Transgenic *Arabidopsis thaliana* harboring *GGPPS* and *DXSII* showed elevated levels of chlorophyll, carotenoids, gibberellins, and indoleacetic acid, which were 2.0, 1.4, 5.8, and 2.3 times the levels obtained with nontransgenic 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. Furthermore, 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.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b04697

Figures S1−S3 and Table S1 (PDF)

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### Notes

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## ■ 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, indoleacetic acid; Ca, chlorophyll *a*; Cb, chlorophyll *b*

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