Metabolic Engineering 13 (2011) 319–327

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Metabolic engineering tanshinone biosynthetic pathway in *Salvia miltiorrhiza* hairy root cultures

Guoyin Kai [a,*], Hui Xu [a], Congcong Zhou [a], Pan Liao [a], Jianbo Xiao [a], Xiuqin Luo [a], Lijia You [a], Lin Zhang [b]

[a] Laboratory of Plant Biotechnology, College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, PR China
[b] Department of Pharmacy, Shaoxing People's Hospital, Shaoxing 312000, PR China

ARTICLE INFO

Article history:
Received 7 September 2010
Received in revised form
27 December 2010
Accepted 8 February 2011
Available online 16 February 2011

Keywords:
Salvia miltiorrhiza bunge
Diterpenoid
Tanshinone
Transformation
Metabolic engineering

ABSTRACT

Tanshinone is a group of active diterpenes widely used in treatment of cardiovascular diseases. Here, we report the introduction of genes encoding 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and geranylgeranyl diphosphate synthase (GGPPS) involved in tanshinone biosynthesis into *Salvia miltiorrhiza* hairy roots by *Agrobacterium*-mediated gene transfer technology. Overexpression of *SmGGPPS* and/or *SmHMGR* as well as *SmDXS* in transgenic hairy root lines can significantly enhance the production of tanshinone to levels higher than that of the control ($P<0.05$). *SmDXS* showed much more powerful pushing effect than *SmHMGR* in tanshinone production, while *SmGGPPS* plays a more important role in stimulating tanshinone accumulation than the upstream enzyme *SmHMGR* or *SmDXS* in *S. miltiorrhiza*. Co-expression of *SmHMGR* and *SmGGPPS* resulted in highest production of tanshinone (about 2.727 mg/g dw) in line HG9, which was about 4.74-fold higher than that of the control (0.475 mg/g dw). All the tested transgenic hairy root lines showed higher antioxidant activity than the control. To our knowledge, this is the first report on enhancement of tanshinone content and antioxidant activity achieved through metabolic engineering of hairy roots by push–pull strategy in *S. miltiorrhiza*.

1. Introduction

Salvia miltiorrhiza Bunge (Dan shen in Chinese) is a well-known traditional Chinese medicine and has been used widely for clinical purposes for the treatment of many cardiovascular diseases such as menstrual disorder, blood circulation disturbance, inflammation and angina pectoris (Zhou et al., 2005; Wu and Shi, 2008; Xu et al., 2010).

The bioactive components of *S. miltiorrhiza* consisted of two main groups, namely the water-soluble phenolic acids derived from caffeic acid and the lipid-soluble tanshinone belonging to diterpene quinines (Li, 1998; Chen et al., 2001; Zhou et al., 2005). The diterpenoid tanshinone including tanshinone I, tanshinone IIA, tanshinone IIB, dihydrotanshnone I and cryptotanshinone have also roused extensive attention, as well as the polyphenols (Wu et al., 2009). Tanshinones share a variety of biological activities. For example, tanshinone I could enhance learning and memory (Kim et al., 2009), while tanshinone IIA has anti-oxidative (Wang et al., 2003; Yang et al., 2008), anti-inflammatory (Jang et al., 2003, 2006), anti-proliferative (Liu et al., 2006) and anti-tumor properties (Dong et al., 2007). Dihydrotanshnone I, tanshinone IIB and cryptotanshinone have antibacterial activity against a broad range of gram positive bacteria (Honda et al., 1988; Lee et al., 1999). Therefore, *S. miltiorrhiza* has been widely used to produce a number of traditional Chinese medicine preparations (Hu et al., 2005). However, because of low content, long growth cycle and serious degradation of the quality, the supplement of tanshinone for the increasing needs of clinical applications has become a research spotlight (Zhou et al., 2007).

Hairy root cultures induced by *Agrobacterium rhizogenes* have the advantages of high genetic stability, rapid and hormone-free growth (Wu and Shi, 2008) and have been considered as a promising system to produce secondary bioactive components from the medicinal plants (Guillon et al., 2006). Elicitation with elicitors such as methyl jasmonate (MJ), yeast elicitor (YE) and jasmonic acid (JA) is one of the attractive approaches to improve secondary metabolism compounds in cell lines or hairy roots (Zhao et al., 2006; Nims et al., 2006; Peebles et al., 2009). *In vitro* cultures of *S. miltiorrhiza* hairy roots to improve tanshinone yield were investigated by various kinds of elicitors including YE, MJ, Ag^{+}, Co^{2+}, α-amino isobutyric acid and β-aminobutyric acid (BABA), which have been extensively studied and well documented recently (Zhang et al., 2004a; Ge and Wu, 2005a, 2005b; Yan et al., 2005). Plant metabolic engineering provides an alternative way to improve the accumulation of tanshinone by transferring key genes involved in the tanshinone biosynthetic pathway into

* Corresponding author. Fax: +86 21 643212 91.
E-mail addresses: gykai@shnu.edu.cn, gykai@yahoo.com.cn (G. Kai).

1096-7176/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2011.02.003

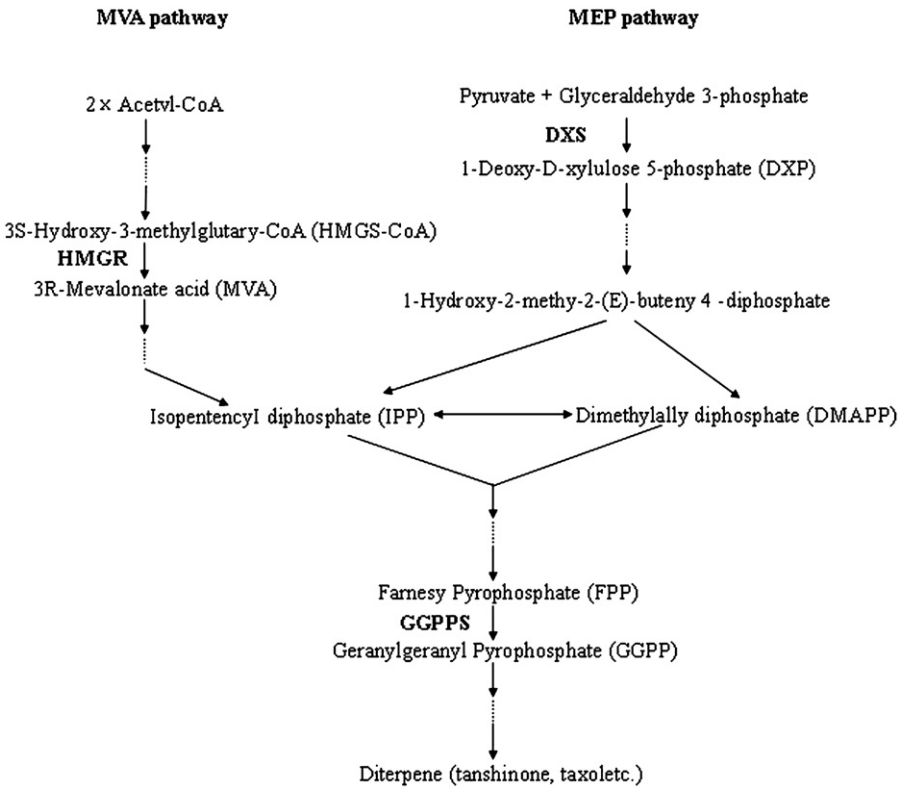

Fig. 1. The metabolic pathway for tanshinone. *SmHMGR*, 3-hydroxy-3-methylglutaryl-CoA reductase; *SmDXS*, 1-deoxy-D-xylulose-5-phosphate synthase; *SmGGPPS*, Geranylgeranyl diphosphate synthase; all the three genes were used to study in this work.

S. miltiorrhiza with *A. rhizogenes* infection (Liao et al., 2009; Kai et al., 2010).

In higher plants, there are two distinct isoprenoid biosynthesis pathways in separate cellular compartments, the mevalonate (MVA) pathway in the cytosol and the methylerythritol phosphate (MEP) pathway in the plastids (Liao et al., 2009; Yan et al., 2009; Wu et al., 2009). Isopentenyl pyrophosphate (IPP), the universal precursor of all isoprenoids, can be synthesized by both MVA and MEP pathways (Laule et al., 2003). Being a kind of diterpene, tanshinone are generally considered to be mainly synthesized via the MEP pathway, but MVA pathway also played a certain role for the crosstalk between the two pathways (Fig. 1) (Ge and Wu, 2005a; Yan et al., 2009). 3-Hydroxy-3-methylglutaryl CoA reductase (HMGR), which catalyzes the formation of MVA from 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), has been identified as a key enzyme in the MVA pathway (Chapell, 1995; Aquil et al., 2009). 1-Deoxy-D-xylulose-5-phosphate synthase (DXS) catalyzes the first committed step of the MEP pathway (Lois et al., 2000; Estévez et al., 2001; Enfissi et al., 2005; Munoz-Bertomeu et al., 2006). Geranylgeranyl diphosphate synthase (GGPPS) catalyzes the consecutive condensation of the dimethylallyl diphosphate (DMAPP) with three molecules of IPP to produce geranylgeranyl diphosphate (GGPP), the universal precursor for biosynthesis of diterpenes such as tanshinone (Wang and Ohnuma, 1999; Engprasert et al., 2004). GGPPS has been considered as an important regulatory target in the tanshinone biosynthetic pathway (Kai et al., 2010).

Manipulating multiple biosynthetic genes at multiple regulatory points may be a feasible strategy to achieve considerable metabolic alterations (Leech et al., 1998). A successful example for this strategy is the engineering of the scopolamine biosynthetic pathway in *Hyoscyamus niger* by co-overexpressing the two key enzymes putrescine N-methyltransferase (PMT) and hyoscyamine 6 β-hydroxylase (H6H), and the final scopolamine production in the best transgenic line was increased nine times than the control (Zhang et al., 2004b). Recently, the cDNAs of *SmHMGR*, *SmDXR*, *SmHMGS*, *SmDXS* and *SmGGPPS* from *S. miltiorrhiza* have been successfully cloned by our laboratory (Liao et al., 2009; Yan et al., 2009; Zhang et al., 2010; Kai et al., 2010), which provided the possibility to improve the yield of tanshinone by metabolic engineering of *S. miltiorrhiza* hairy root cultures. In this present work, we report the introduction of gene constructs containing cDNAs of *SmHMGR* and/or *SmGGPPS* as well as *SmDXS* under the control of the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter into *S. miltiorrhiza* cells.

2. Materials and methods

2.1. Construction of plant expression vectors

The complete *SmHMGR* and *SmGGPPS* cDNAs were cloned from the sterile seedlings of *S. miltiorrhiza* in our laboratory as reported before (Liao et al., 2009; Kai et al., 2010), and complete *SmDXS* cDNA was also isolated recently. The vectors pBI121 (Clontech) and pCAMBIA1304 (CAMBIA) were double-digested with HindIII and EcoRI. The purified smaller DNA fragment containing a GUS expression cassette from pBI121 was cloned into the large pCAMBIA1304 fragment to generate the recombinant plasmid pCAMBIA1304$^+$ (Kai et al., 2009). The full-length *SmGGPPS* cDNA was inserted into pCAMBIA1304$^+$ in place of the *GUS* gene of the GUS gene expression cassette from pBI121 to generate the expression plasmid pCAMBIA1304$^+$–*SmGGPPS* containing *SmGGPPS* gene (see Fig. S1A), while full-length *SmHMGR* cDNA was inserted into the pCAMBIA1304$^+$ in place of the *mGFP5* and *GUSA* genes originally from pCAMBIA1304 to generate the expression plasmid pCAMBIA1304$^+$-*SmHMGR* containing *SmHMGR* gene (see Fig. S1B). On the basis of pCAMBIA1304$^+$-*SmHMGR*, the

full-length *SmGGPPS* cDNA was used to replace the GUS gene of the GUS gene expression cassette from pBI121 to generate the expression vector pCAMBIA1304$^+$-*SmHMGR*–*SmGGPPS* containing both *SmHMGR* and *SmGGPPS* genes (see Fig. S1C). On the basis of pCAMBIA1304$^+$–*SmGGPPS*, the full-length *SmDXS* cDNA was used to replace the *SmGGPPS* gene to generate the expression vector pCAMBIA1304$^+$-*SmDXS* containing *SmDXS* gene (see Fig. S1D). The genes *SmHMGR* and/or *SmGGPPS* as well as *SmDXS* gene were under the control of the strong cauliflower mosaic virus (CaMV) 35S promoter. The blank vector pCAMBIA1304$^+$ without exogenous genes (such as *SmHMGR*, *SmGGPPS* or *SmDXS*) was used as the control. The disarmed *A. tumefaciens* strain C58C1 harboring both the *A. rhizogenes* Ri plasmid pRiA4 (Kai et al., 2009) and each of the five plasmids constructed above was used for plant genetic transformation.

2.2. Plant transformation and hairy root culture

The sterile *S. miltiorrhiza* plants were grown and kept in our lab (Zhou et al., 2007; Liao et al., 2009; Kai et al., 2010). In order to induce hairy roots with disarmed *A. tumefaciens* strain C58C1 (pRi A4), the sterile leaf sections were submerged in the bacterial suspension for 30 min, blot-dried on sterile filter paper and then placed in MS medium (Murashige and Skoog, 1962) supplemented with 30% sucrose, 0.8% agar (pH 5.8), at 22 °C in darkness. After co-cultivation for 3 days, the cultures were washed with 60 ml sterilized water three times, and blot-dried on sterile filter paper then transferred to 1/2 MS medium supplemented with 30% sucrose, 0.8% agar (pH 5.8), and 500 mg/l of cefotaxime. After 2–3 weeks, hairy roots were derived from sterile leaves and rapidly growing hairy roots (exceeded 2 cm) were excised and cultivated individually on solid 1/2 MS medium supplemented with 30% sucrose, 0.8% agar (pH 5.8) and different concentrations of cefotaxime (each subsequent subculture had lesser cefotaxine than the previous one during this subcultured period) for 3–4 weeks, at 25 °C in darkness. Rapidly growing root lines that showed hygromycin resistance with no bacterial contamination were used to establish hairy root lines. Approximately 200 mg of normally growing hairy roots were inoculated in 250-mL Erlenmeyer flasks each containing 200 mL 1/2 MS liquid medium on an orbital shaker controlled at 100 rpm in darkness at 25 °C. The hairy roots clones were routinely subcultured every 30 days and harvested after 60 days.

2.3. DNA isolation and PCR analysis

Genomic DNA was isolated from harvested hairy root samples using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). The DNA was then used as the template in PCR analysis for detecting the presence of *SmHMGR*, *SmDXS* and *SmGGPPS* genes in transgenic hairy roots. The sequences of 35SF (forward primer) located at the 35S promoter of pBI121 vector and GGPPS161R (reverse primer) in the interior of *SmGGPPS* gene for the *SmGGPPS* detection were as follows: 5′-GAGGACCTAACAGAACTCGCC-3′ and 5′-ATGAATCCGACCAGGGATTTGGAGGGGCAT-3′, respectively, and those of primers 35SF at the 35S promoter of pCAMBIA1304 plasmid and HMGR171R (reverse primer) in the interior of *SmHMGR* gene used for the amplification of the *SmHMGR* gene were as follows: 5′-GAGGACCTAACAGAACTCGCC-3′ and 5′-CAGAGGCTTTAGGGGAGGAGGGTTGGTGAT-3′, respectively. *SmDXS* gene in transgenic hairy roots was also detected by the same method as *SmHMGR* and *SmGGPPS* genes. The primers were as follows: 35SF (5′-GAGGACCTAACAGAACTCGCC-3′); DXS197R (5′-GGAAGAGTAGCAGTAGTGCGA-3′). PCR was carried out in total volume of 25 μL reaction mixtures, containing 1 μL of each primer, 0.5 μL of 10 mmol/L dNTPs, 2.5 μL of 10 × PCR buffer (Mg^{2+} plus), 1 μL genomic DNA as template and 1.25 units of Ex Taq DNA polymerase (TaKaRa), following the protocol: the DNA template was denatured at 94 °C for 5 min followed by 35 cycles of amplification (94 °C for 45 s, 58 °C for 60 s, 72 °C for 60 s) and inoculated at 72 °C for 10 min.

2.4. RNA isolation and gene expression analysis by RT-PCR

Total RNA was extracted from the hairy root samples using RNA prep pure plant kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. RNase-free DNase I (Tiangen Biotech Co., Ltd.) was used to digest all DNA from the samples following the manufacture's instructions. The quality and concentration of RNA was checked and stored as described before (Kai et al., 2006). Semi-quantitative RT-PCR was carried out to investigate the expression of *SmHMGR*, *SmDXS* and *SmGGPPS* genes in transgenic hairy roots. For each reaction, aliquots of total RNA (0.5 μg) was used as a template to generate a total volume of 30 μL RT reaction. HMGRKF (5′-ATGGATATCCGCCGGAGGCCA-3′) and HMGRKR (5′-TCAGGAGCCAATCTTCGTGAT-3′), DXSKF (5′-ATGGCGTCGTCTTGTGGAGTTA-3′) and DXSKR (5′-TTACAAGTTGTTGATGAGATGA-3′), GGPPSKF (5′-ATGAGATCTATGAATCTGGTG-3′) and GGPPSKR (5′-TTAGTTCTGCCTATGTGCAAT-3′) were used to detect the expression of total (including endogenous and the cDNA we introduced) *SmHMGR*, *SmDXS* and *GGPPS* genes whereas HMGRKF and HMGR3′R (5′-CCAATTCTTGACACCAACCAGT-3′), DXSKF and DXS3′R (5′-TACTGCTTCCGCTTCTGGCTT-3′), GGPPSKF and GGPPS3′R (5′-CACCAAACTTTTCATTAGCAGC-3′) were used only for the measurement of the expression of endogenous *SmHMGR*, *SmDXS* and *SmGGPPS*, respectively. Meanwhile, RT-PCR with primers 18SF (5′-CCAGGTCCAGACATAGTAAG-3′) and 18SR (5′-GTACAAAGGGCAGGGACGTA-3′), designed on the basis of the conserved regions of plant house-keeping genes (18 S rRNA genes), was also performed to normalize equal amounts of RNA among samples as an internal standard (Liao et al., 2009; Kai et al., 2010).

2.5. Determination of tanshinone by HPLC

The dried hairy roots (200 mg) were powdered and extracted with 16 mL of methanol/dichloromethane (3:1, v/v), and then kept at room temperature for 24 h after sonicating for 1 h (Chen et al., 1997). The extract was evaporated under vacuum and the deposition was re-dissolved with 1 mL methanol/dichloromethane (3:1, v/v) (Yan et al., 2005). The solution was filtered through a 0.22 μm filter and subjected to HPLC. HPLC analysis was performed on a HITACHI L2000 apparatus equipped with a photodiode array detector. Separation was carried out on a Waters reversed-phase C18 symmetry column (5 μm, 150 mm × 4.6 mm i.d.). The temperature of column was 30 °C. The mobile phase consisted of acetonitrile–water (65:35, v/v). The detection wavelength was 270 nm and the flow rate was 1.00 mL/min (Shi et al., 2004). The injection volume was 20 μL. Cryptotanshinone (CT), tanshinone I (T1) and tanshinone IIA (T2A) were detected and quantified by comparison with authentic standard curves and retention times. Total tanshinone containing CT, T1 and T2A was shown as TT in this paper. Here we use a photodiode array detector for HPLC to investigate the peak purity (PDA can provide UV–vis scans data). The UV spectra obtained were found to the same as the UV–vis scans of standards.

2.6. Measurement of DPPH free-radical scavenging activity

The free-radical scavenging activity of TT from transgenic hairy roots (H33, G50, HG9 and BC) was measured by the DPPH method

reported by Adedapo et al. (2008) with slight modifications. Briefly, the different concentration (0.25–4 μg/mL) of 1 mL of each sample was added to 1 mL DPPH (0.2 mmol/L) solution in methanol. The tubes with reaction mixture were vortexed thoroughly and incubated at room temperature for 30 min. Spectrophotometer was used to detect the absorbance of the mixture at 517 nm. The DPPH solution in methanol (0.2 mmol/L) was used as the control. Radical scavenging activity of TT was calculated according to the following formula: DPPH radical scavenging activity (%)=[1−absorbance of the sample/absorbance of the control]×100. The IC_{50} value defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% was calculated according to dose–response and used for comparison of DPPH radical scavenging capability from different samples (Zhang and Lin, 2008).

2.7. Statistical analysis

All the experiments including culture of hairy root clones, PCR identification, semi-quantitative RT-PCR, HPLC analysis and measurement of antioxidant activity were repeated three times. Results of tanshinone content are presented as mean values ± S.D. The error bars are due to biological triplicates. The statistical significance of tanshinone difference was analyzed by one sample *t* test and the errors of different hairy root clones were used in the one-way analysis of variance (ANOVA) using SPSS 11.5 software (SPSS, Inc.).

3. Results

3.1. Transformation of S. miltiorrhiza with SmHMGR, SmDXS and SmGGPPS

Four plasmids containing the cDNAs encoding *SmHMGR* and/or *SmGGPPS* as well as *SmDXS* driven by CaMV 35S promoter, were separately introduced into *S. miltiorrhiza* by using disarmed *A. tumefaciens* C58C1 strain. Totally, 43 *SmHMGR* single gene transformed lines (H line), 52 *SmDXS* single gene transformed lines (D line), 60 *SmGGPPS* single gene transformed lines (G line) and 72 *SmHMGR–SmGGPPS* double gene transformed lines (HG line) were generated. Transgenic hairy root lines with abnormal phenotypic characteristics such as short, brown and aged in growth stagnation were discarded (see Fig. S2A), 39 H, 46 D, 55 G and 67 HG hygromycin-resistant hairy root lines with normal phenotype (see Fig. S2B) were remained for further analysis.

3.2. PCR analysis of engineered hairy roots and establishment of hairy root lines

DNA of all the independent hairy roots was isolated and used for PCR analysis using primers specially designed to overlapping part of the *SmHMGR*, *SmDXS* or *SmGGPPS* and the CaMV 35S promoter sequences. As a positive control (PC), the fragment was also amplified from the 1304^{+}–*SmHMGR*, 1304^{+}–*SmDXS* or 1304^{+}–*SmGGPPS* plasmids. DNA of wild type root was used as a negative control (NC). Another kind of control hairy root lines generated from blank-vector transformations were denoted as BC. In total, the PCR-positive clones amounted for 41.06% (85/207), with 48.72% (19/39) for H, 45.65% (21/46) for D, 23.64% (13/55) for G and 47.76% (32/67) for HG hairy root lines (Table 1). None of the checked DNA bands was detected from NC and BC lines. Parts of the results were shown in Fig. 2. These results indicated that the *SmHMGR*, *SmDXS* and *SmGGPPS* genes were introduced into H, D, and G lines, respectively, while both *SmHMGR* and *SmGGPPS* genes were introduced into HG lines. Here we selected 7 H, 5 D, 6 G and 11 HG of PCR-positive lines for the further experiments (Table 1).

3.3. SmHMGR, SmDXS and SmGGPPS gene expression in transgenic hairy roots

To determine the expression patterns of *SmHMGR, SmDXS* and *SmGGPPS*, total RNA was isolated separately from filtered PCR-positive root lines for semi-quantitative RT-PCR. The results were shown in Fig. 3. The different expression patterns between endogenous *SmHMGR* gene (*HMGR B*) and total *SmHMGR* gene (*HMGR A*) in transgenic HG and H lines showed that all the *HMGR A* transcripts expressed at higher levels, and total *SmGGPPS* gene (*GGPPS A*) also showed higher transcripts than endogenous *SmGGPPS* gene (*GGPPS B*) in transgenic HG and G lines. The expression patterns of *HMGR B* showed similar levels to *HMGR A* in both transgenic G and BC lines, and no obvious diversity was observed in transcriptional level between *GGPPS B* and *GGPPS A* in both transgenic H and BC lines. Compared with G and BC lines, the *HMGR A* transcripts in all of the HG and H lines expressed at higher levels, while higher level of *GGPPS A* mRNA was detected in all HG and G lines compared with H and BC lines. Similarly, the expression of *DXS A* was significantly higher in D lines than in other transgenic lines (HG, H, G and BC) and also dramatically higher than *DXS B* in all of the D lines as well as HG, H, G and BC lines. These results indicated that all the cDNAs of interest were introduced into *S. miltiorrhiza* and expressed in corresponding transgenic lines, but with varied expression levels.

3.4. Tanshinone content in transgenic hairy roots

To determine the tanshinone (including CT, T1 and T2A) production in *S. miltiorrhiza* hairy root lines, HPLC analysis was conducted. One sample *t* test by SPSS 11.5 software was used to analyze significance of difference of tanshinone content between every single line and the control. The capacities of transgenic root lines to synthesize tanshinone are shown in Fig. 4. All the *SmHMGR*-transformed lines (H line) showed higher level of tanshinone ranging from 0.844 to 1.515 mg/g than the control (0.475 mg/g dw) (Fig. 4A and Fig. S3B), implying that overexpression of *SmHMGR* can efficiently promote the accumulation of tanshinone in *S. miltiorrhiza*. The levels of tanshinone in D lines were variable with the range of 1.007–2.320 mg/g dw, which was higher than the control and H lines (Fig. 4B and Fig. S3C). Hairy root lines overexpressing *SmGGPPS*

Table 1
Gene constructs and derived root cultures.

	Number of root lines			
Gene constructs	Total	Hygromycin-resistant	PCR-positive	Established root lines
SmHMGR	43	39	19	H11 H12 H26 H27 H32 H33 H38
SmGGPPS	60	55	13	G28 G39 G41 G50 G53 G56
SmHMGR+SmGGPPS	72	67	32	HG3 HG6 HG9 HG19 HG21 HG43 HG46 HG47 HG49 HG51 HG59
SmDXS	52	46	21	D1 D4 D8 D15 D20

(G line) can also produced higher level of tanshinone with variation from 1.356 to 2.477 mg/g when compared to the control lines (Fig. 4C and Fig. S3D). The highest tanshinone production was detected in line HG9 with the concentration of 2.727 mg/g dw, which was 4.74 fold higher than the control (Fig. 4D and Fig. S3E). Data analysis (Fig. 4) showed that T1 appeared a significant correlation with TT in all the transgenic lines, while CT exhibited a positive relationship with TT to certain degree and T2A has no obvious correlation with TT. Till now, the specific reaction pathways for the different tanshinone (T1, CT and T2A) were unknown, so it is difficult to assess the TI, CT and T2A data further. However, it is certain that all the transgenic lines showed varied but higher TT content compared with the control lines, reflecting that transgenic technology is an effective and promising strategy to improve tanshinone production in *S. miltiorrhiza*.

One-way analysis of variance (ANOVA) was used to detect the difference of average content of total tanshinone among H, D, G, HG, and BC lines to analyze the functional diversity among *SmHMGR*, *SmDXS* and *SmGGPPS* in tanshinone biosynthesis. It showed that the average content of TT was higher in H lines, but much higher in D, G and HG lines compared with BC lines ($P < 0.05$) (Fig. 4E). Furthermore, the content of T2A is apparently related with the phenotype of hairy root in this work. There were two phenotypes (thin and thick) about hairy roots in this study. All the D and HG (except for HG21) lines, line BC2 and BC4 belonged to the first phenotype of thick hairy root (see Fig. S2C), and the lowest content of T2A in these lines was about 0.206 mg/g (line BC4). All the H and G lines, line HG21, BC1, BC3 and BC5, were attributed to another phenotype of thin hairy root (see Fig. S2D), and the highest content of T2A in these lines was about 0.165 mg/g, dw (line G53). So it indicated that the higher content of T2A (about more than 0.2 mg/g) in hairy root may affect its phenotype (thick type) in *S. miltiorrhiza*.

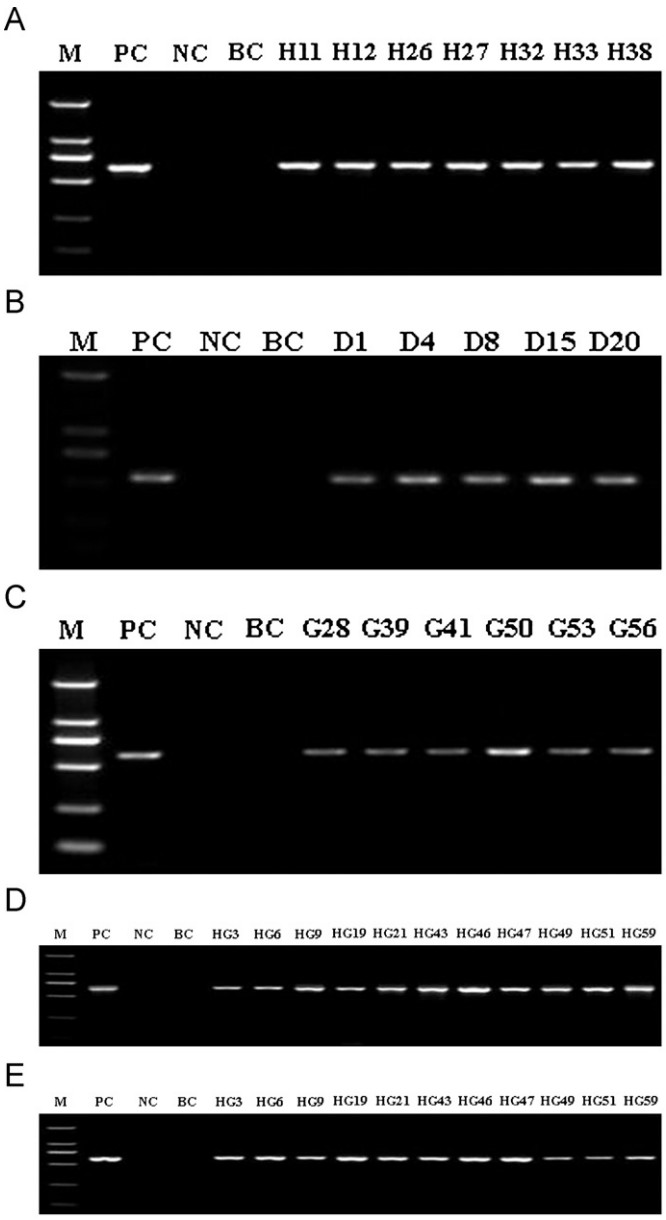

Fig. 2. PCR analyses of transgenic hairy root lines. Representative PCR analyses for the presence of *SmHMGR* (A), *SmDXS* (B) and *SmGGPPS* (C) genes in transgenic H, D and G lines, respectively. (D) and (E) represented PCR analyses for the presence of *SmHMGR* and *SmGGPPS* gene in transgenic HG lines, respectively. M, DL-2000 Marker (100–2,000 bp); PC, positive control (vector contains corresponding gene); NC, negative control (wild type root); BC, blank control (hairy root generate from blank-vector transformation).

3.5. Antioxidant activity analysis of transgenic hairy roots

The DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging activity, based on the reduction of the stable radical DPPH to yellow-colored diphenylpicrylhydrazine in the presence of a hydrogen donor, was widely used to evaluate the antioxidant activity due to its advantage of rapid and simple measure (Wu and Wang, 2009). Since tanshinone possesses the antioxidant activities (Wu et al., 2010), the antioxidant activity of tanshinone from hairy roots was estimated by measuring DPPH radical scavenging in this study. The samples of H12, G50, HG9 and BC were diluted to the varied concentration (0.25–4 μg/mL) of 1 mL with methanol according to their different concentration determined by HPLC and then 1 mL DPPH (0.2 mM) solution in methanol was added. As shown in Fig. 5, all the tested samples were found to possess strong radical scavenging capacity. The IC_{50} values of line H12, G50, HG9 and BC were determined to be 0.3375, 0.2990, 0.3045 and 0.3049 μg/mL, respectively, which suggested that there were no significant differences of antioxidant activity of per weight of tanshinone among these transgenic hairy root lines and the control. The result implied that the transgenic hairy root lines H12, G50 and HG9 owned higher total antioxidant activity of tanshinone due to higher content of total tanshinone.

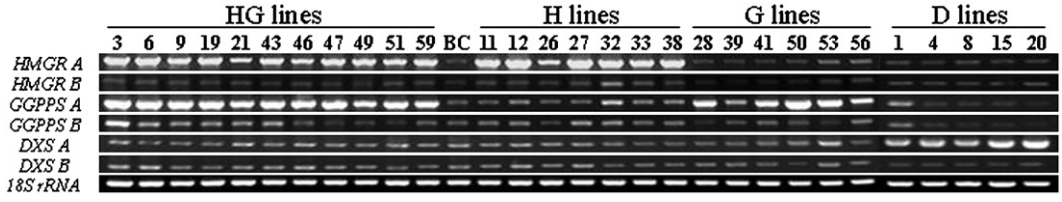

Fig. 3. Effects of transformation on the expression of related genes in the tanshinone biosynthetic pathway during *S. miltiorrhiza* hairy roots culture period. *HMGR A*, *DXS A* and *GGPPS A* represent total gene of *SmHMGR*, *SmDXS* and *SmGGPPS* respectively, while *HMGR B*, *DXS B* and *GGPPS B* represent endogenous *SmHMGR*, *SmDXS* and *SmGGPPS* respectively. *18S rRNA* gene was used as the control to show the normalization of the templates in PCR reactions. The experiment was repeated three times.

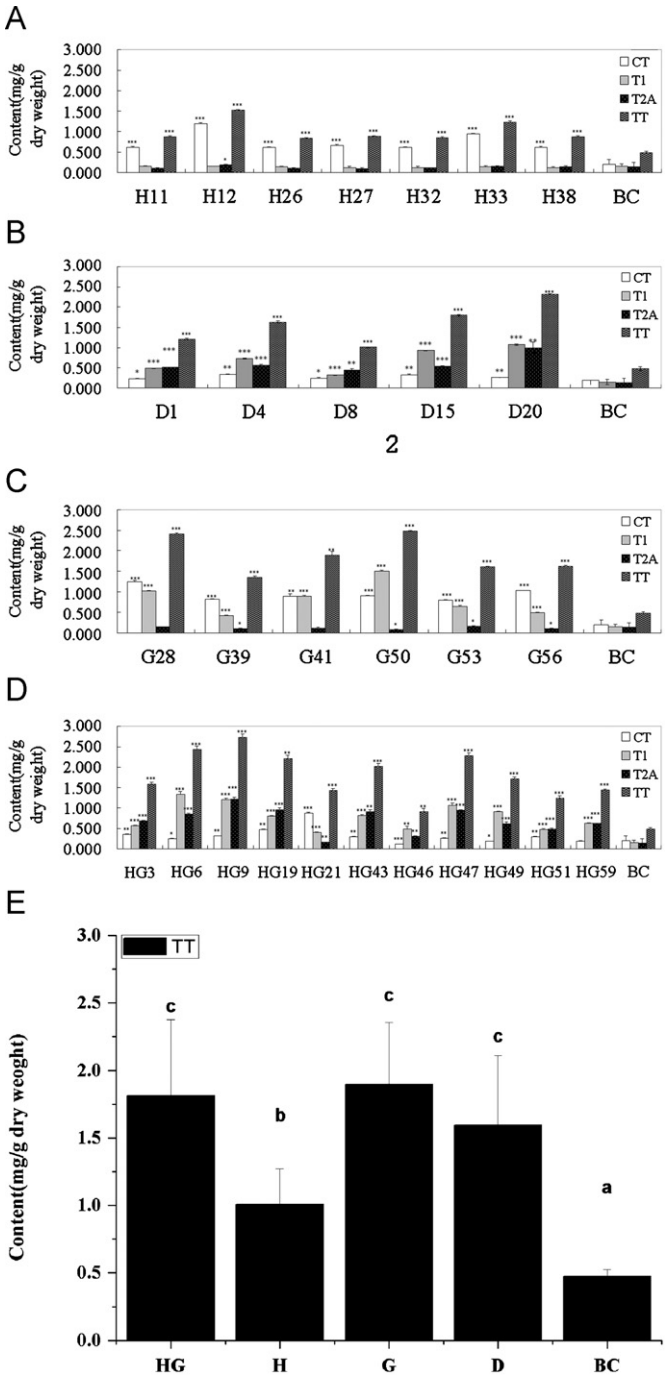

Fig. 4. Tanshinone production analyzed by HPLC from transgenic hairy root lines (A–D) and the differences of average level of total tanshinone production among various transgenic hairy root lines (E). H, *SmHMGR* transgenic hairy root lines; D, *SmDXS* transgenic hairy root lines; G, *SmGGPPS* transgenic hairy root lines; HG, transgenic hairy root lines containing both *SmHMGR* and *SmGGPPS* genes; BC, the control hairy root cultures generate from blank-vector transformation. TT, total tanshinone; CT, cryptotanshinone; T1, tanshinone I; T2A, tanshinone IIA. The values are means ± S.D of triplicate analyses. *, **, and *** Significant difference at $P<0.05$, 0.01, and 0.001 respectively. Different letters indicate significant differences ($P<0.05$). Tamhane is one of multiple comparisons used for the average level of TT in this study.

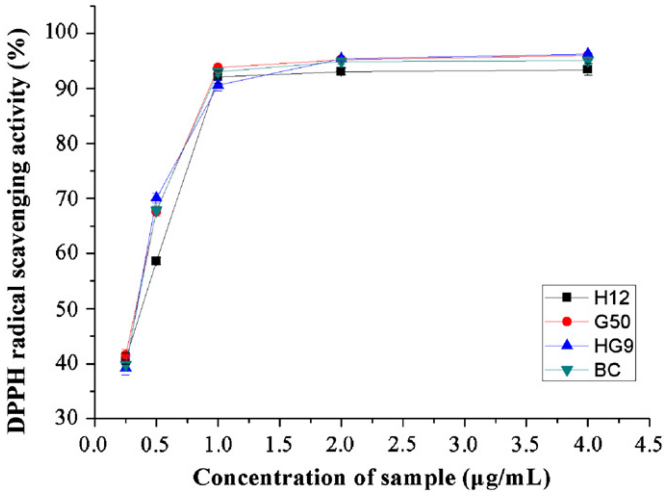

Fig. 5. DPPH scavenging activities of tanshinone from four different hairy root lines (H12, G50, HG9 and BC). Results represent means ± standard deviation ($n=3$).

4. Discussion

Metabolic engineering can be used to produce large amounts of valuable metabolites by genetic manipulation. The "push–pull" strategy has been successfully applied to significantly increase provitamin A, flavonols, limonene and artemisinin yields in several plants such as *Oryza sativa*, *Solanum lycopersicum*, *Lavandula latifolia* and *Artemisia annua,* respectively (Ye et al., 2000; Muir et al., 2001; Muñoz-Bertomeu et al., 2008; Aquil et al., 2009), while for enhanced scopolamine, tropane alkaloid and terpenoid indole alkaloid accumulation in hairy roots of *H. niger*, *Anisodus acutangulus* and *Catharanthus roseus* (Zhang et al., 2004b; Kai et al., 2009; Peebles et al., 2010). To our knowledge, there are no reports about enhancement of tanshinone content in *S. miltiorrhiza* by means of metabolic engineering. Recently several genes such as *SmHMGS*, *SmHMGR*, *SmGGPPS* and *SmDXR* involved in the tanshinone biosynthesis pathways have been cloned by our laboratory (Zhang et al., 2010; Liao et al., 2009; Yan et al., 2009; Kai et al., 2010) as well as *SmDXS*, which provides the possibilities to enhance the yield of tanshinone by metabolic engineering. In the present study, *Agrobacterium*-mediated metabolic engineering of hairy roots of the plant *S. miltiorrhiza* was successfully performed with significantly improved tanshinone yield. *SmHMGR*, *SmDXS* and *SmGGPPS* may be considered as three of the key enzymes from early stages in tanshinone biosynthesis in *S. miltiorrhiza.*

HMGR is a rate-limiting enzyme of the mevalonate (MVA) biosynthetic pathway in plants (Liao et al., 2009). Up-regulation of an N-terminal truncated HMGR enhances production of essential oils and sterols in transgenic *L. latifolia* (Muñoz-Bertomeu et al., 2007). Recently, overexpression of the HMGR gene leads to enhanced artemisinin biosynthesis in transgenic *A. annua* plants (Aquil et al., 2009). The above reports indicated the contribution of MVA pathway to monoterpene and sesquiterpene biosynthesis by genetic manipulation, but there are no reports for diterpenes including tanshinone in plants. In the present study, the expression levels of the *HMGR* varied from line to line but correlated with their corresponding tanshinone content apparently in most of H lines. Our results showed that overexpression of *SmHMGR* can lead to enhanced tanshinone production (up to 2.2-fold higher) than the control (Fig. 4A), which suggested that MVA pathway also play an important role in diterpene biosynthesis at least in *S. miltiorrhiza.*

1-Deoxy-D-xylulose-5-phosphate synthase (DXS) catalyzes the first committed step of the MEP pathway for isoprenoid biosynthesis, which is an alternative isoprenoids biosynthetic pathway that has been recently discovered in plants. Overexpression of DXS gene resulted in enhanced isoprenoids production in *Arabidopsis thaliana*,

S. lycopersicum and *L. latifolia* (Estévez et al., 2001; Enfissi et al., 2005; Munoz-Bertomeu et al., 2006). Here, the content of tanshinone in these transgenic lines exhibited a positive correlation with the expression of *SmDXS* gene, and the *SmDXS* single-gene transgenic hairy root lines obviously showed higher levels of tanshinone (up to 3.9-fold higher) than the control (Fig. 4B), which implied that *SmDXS* is an effective regulatory site for the biosynthesis and accumulation of tanshinone. Furthermore, *SmDXS* showed much more powerful pushing effect than *SmHMGR* in tanshinone production. Our results indicated that genetic manipulation of the MEP pathway resulted in significantly higher yields than MVA pathway enhancement in *S. miltiorrhiza*, which was in good agreement with that previous deduced viewpoint that the tanshinone accumulation was mainly synthesized via the MEP pathway (Ge and Wu, 2005a).

Geranylgeranyl diphosphate synthase (GGPPS) catalyzes the biosynthesis of geranylgeranyl diphosphate (GGPP), which is a key precursor for diterpenes including tanshinone (Kai et al., 2010). Although isopentenyl pyrophosphate (IPP) is biosynthesized from both MVA pathway in the cytosol and MEP pathway in the plastids, it is necessary to increase the metabolic flux towards GGPP. In order to produce much more tanshinone, the sufficient precursor geranylgeranyl diphosphate is necessary. It is reported that the taxol production showed a positive correlation with the expression level of *GGPPS* gene, which demonstrated that *GGPPS* is the important regulatory target in the diterpene biosynthetic pathway (Hefner et al., 1998; Engels et al., 2008). GGPPS was considered as a key enzyme in the forskolin (diterpene) biosynthetic pathway in *Coleus forskohlii* (Engprasert et al., 2004), but the role of GGPPS in *S. miltiorrhiza* was never identified. In this study, the levels of tanshinone in most of G lines were variably corresponded to its transcript level of *GGPPS* gene. Furthermore, tanshinone production in *SmGGPPS* single-gene transgenic hairy root lines (G lines) was much higher than the control lines (Fig. 4C), testifying that *SmGGPPS* was a key regulatory site for the biosynthesis of tanshinone for metabolic engineering. *SmGGPPS* exhibited a more powerful pulling effect than did *SmHMGR* and *SmDXS*, which may be due to *SmGGPPS* is closer in the reaction pathway to tanshinone (Fig. 4E).

Manipulating multiple genes at multiple control sites in target metabolites production may achieve desired metabolic flux alterations (Nims et al., 2006). In this study, the highest tanshinone production (2.727 mg/g dw) was observed in HG line (HG9) (Fig. 4D), which was about 5.74, 1.80, 1.18 and 1.11 times as that of the control group (0.475 mg/g dw), H12 (the highest H line, 1.515 mg/g dw), D20 (the best D line, 2.320 mg/g dw) and G50 (highest tanshinone-producing G line, 2.477 mg/g dw), respectively. This result showed that co-expression of *SmHMGR* and *SmGGPPS* can produce a positive cooperative effect on stimulating tanshinone accumulation. To our knowledge, this is the first report on enhancement of isoprenoids production using *HMGR* and *GGPPS* in plants by means of presented "push–pull" strategy. The present results (Fig. 4E) showed that *SmGGPPS* and *SmDXS* exhibited more powerful pulling effects in tanshinone production than does *SmHMGR*. Hence, the strategy of co-overexpression of these genes (*SmGGPPS* and *SmDXS*, or even all the three genes) in *S. miltiorrhiza* would be more promising for further investigation in the future. Alternatively, introduction of a global transcription factor that positively regulates the pathway is more effective than modifying multiple biosynthetic pathway genes (Peebles et al., 2009). Recently, it has been reported that considerable increase in scopolamine was found in the transgenic *H. niger* hairy roots after MJ treatment (Zhang et al., 2006). Thus, combination of transgenic technology with elicitor treatments may be another feasible strategy to further improve tanshinone yield in *S. miltiorrhiza* hairy roots in the near future.

The current study provides good basement and helpful information for commercial large-scale production of tanshinones by means of the hairy roots system in future. This report on engineering *SmHMGR* and *SmGGPPS* simultaneously into *S. miltiorrhiza* results in significantly improved tanshinone accumulation in cultured transgenic hairy root lines. Furthermore, the content of tanshinone in the intact plant was within the range of 1.7–9.7 mg/g according to different region (Li et al., 2000). In this study, the content of tanshinone in the control hairy root is about 0.475 mg/g, which was much lower than that in the plant. However, the average content of tanshinone in the transgenic hairy root lines were significantly enhanced (2.727 mg/g in the best line HG9). Transgenic lines have reached to similar order magnitude as the plant. Furthermore, it also can be concluded from the DPPH radical scavenging activity analysis that total antioxidant activities of tanshinone from the tested transgenic hairy root lines were also higher than the control, due to their stable antioxidant activity of per weight of tanshinone and higher total tanshinone content compared with the control in this study, which indicated the transgenic hairy root cultures was an attractive and promising system for tanshinone production in the future. To our knowledge, this is the first report on significant enhancement of tanshinone production and antioxidant activity achieved through metabolic engineering in *S. miltiorrhiza*, which serves as a good first step in tanshinone-rich lines for commercial application. However, tanshinone yield need to be an order of magnitude higher in the hairy roots than in the plant for promise for commercial larger-scale production. Thus, a more detailed study of the genes (especially those unknown key genes in the specific steps) involved in the tanshinone biosynthesis pathway as well as a deep understanding of the different regulation mechanism between *S. miltiorrhiza* plants and hairy roots would be helpful for further improving tanshinone production in hairy root cultures by metabolic engineering.

Acknowledgments

This work was supported by National Natural Science Fund (30900110), Shanghai Science and Technology Committee Project (10JC1412000, 09QH1401900, 06QA14038, 08391911800, 073158202, 075405117, 065458022, 05ZR14093), Project from Ministry of Science and Technology of China (NC2010AE0075, NC2010AE0372), Zhejiang Provincial Natural Science Fund (Y2080621), Shanghai Education Committee Fund (09ZZ138, 06DZ015, J50401), Fujian Science and Technology Committee Key Special Project (2008NZ0001-4), National Transgenic Organism New Variety Culture Key Project (2009ZX08012-002B), Project from Shanghai Normal University. Miss Pin Liu's (Shanghai Jiaotong University, China) kind assistance with HPLC analysis is also acknowledged.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2011.02.003.

References

Adedapo, A.A., Jimoh, F.O., Koduru, Srinivas., Afolayan, A.J., Masika, P.J., 2008. Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of *Calpurnia aurea*. BMC Complem. Alt. Med. 8, 53.

Aquil, S., Husaini, A.M., Abdin, M.Z., Rather, G.M., 2009. Overexpression of the HMG-CoA reductase gene leads to enhanced artemisinin biosynthesis in transgenic *Artemisia annua* plants. Planta Med. 75, 1453–1458.

Chappell, J., 1995. Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 521–547.

Chen, H., Chen, F., Chiu, F.C.K., Lo, C.M.Y., 2001. The effect of yeast elicitor on the growth and secondary metabolism of hairy root cultures of *Salvia miltiorrhiza*. Enzym. Microb. Technol. 28, 100–105.

Chen, H., Yuan, J.P., Chen, F., Zhang, Y.L., Song, J.Y., 1997. Tanshinone production in Ti-transformed *Salvia miltiorrhiza* cell suspension cultures. J. Biotechnol. 58, 147–156.

Dong, X.R., Dong, J.H., Peng, G., Hou, X.H., Wu, G., 2007. Growth-inhibiting and apoptosis-inducing effects of Tanshinone IIA on human gastric carcinoma cells. J. Huazhong Univ. Sci. Technol. 27, 706–709.

Doyle, J.J., Doyle, J.L., 1990. A rapid total DNA preparation procedure for fresh plant tissue. Focus 12, 13–15.

Enfissi, E.M.A., Fraser, P.D., Lois, L.M., Boronat, A., Schuch, W., Bramley, P.M., 2005. Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. Plant Biotech. J. 3, 17–27.

Engels, B., Dahm, P., Jennewein, S., 2008. Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab. Eng. 10, 201–206.

Engprasert, S., Taura, F., Kawamukai, M., Shoyama, Y., 2004. Molecular cloning and functional expression of geranylgeranyl pyrophosphate synthase from *Coleus forskohlii* Briq. BMC Plant Biol. 4, 18.

Estévez, J.M., Cantero, A., Reindl, A., Reichler, S., Leon, P., 2001. 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J. Biol. Chem. 25, 22901–22909.

Ge, X.C., Wu, J.Y., 2005a. Tanshinone production and isoprenoid pathways in *Salvia miltiorrhiza* hairy roots induced by Ag^+ and yeast elicitor. Plant Sci. 168, 487–491.

Ge, X.C., Wu, J.Y., 2005b. Induction and potentiation of diterpenoid tanshinone accumulation in *Salvia miltiorrhiza* hairy roots by β-aminobutyric acid. Appl Microbiol. Biotechnol. 68, 183–188.

Guillon, S., Trémouillaux-Guiller, J., Pati, P.K., Rideau, M., Gantet, P., 2006. Hairy root research: recent scenario and exciting prospects. Curr. Opin. Plant Biol. 9, 341–346.

Hefner, J., Ketchum, R.E.B., Croteau, R., 1998. Cloning and functional expression of a cDNA encoding geranylgeranyl diphosphate synthase from *Taxus canadensis* and assessment of the role of this prenyltransferase in cells induced for Taxol production. Arch. Biochem. Biophys. 360, 62–74.

Honda, G., Koezuka, Y., Tabata, M., 1988. Isolation of an antidermatophytic substance from the root of *Salvia miltiorrhiza*. Chem. Pharm. Bull. 36, 408–411.

Hu, P., Luo, G.A., Zhao, Z.Z., Jiang, Z.H., 2005. Quality assessment of *Radix Salviae Miltiorrhizae*. Chem. Pharm. Bull. 53, 481–486.

Jang, S.I., Jeong, S.I., Kim, K.J., Kim, H.J., Yu, H.H., Park, R., You, Y.O., 2003. Tanshinone IIA from *Salvia miltiorrhiza* inhibits expression of inducible nitric oxide synthase and production of TNF-α, IL-1β and IL-6 in activated RAW 264.7 cells. Planta Med. 69, 1057–1059.

Jang, S.I., Kim, H.J., Kim, Y.J., 2006. Tanshinone IIA inhibits LPS-induced NF-κB activation in RAW 264.7 cells: possible involvement of the NIK–IKK, ERK1/2, p38 and JNK pathways. Eur. J. Pharmacol. 542, 1–7.

Kai, G.Y., Li, L., Jiang, Y.X., Yan, X.M., Zhang, Y., Lu, X., Liao, P., Chen, J.B., 2009. Molecular cloning and characterization of two tropinone reductases in *Anisodus acutangulus* and enhancement of tropane alkaloid production in AaTRI-transformed hairy roots. Biotechnol. Appl. Biochem. 54, 177–186.

Kai, G.Y., Liao, P., Zhang, T., Zhou, W., Wang, J., Xu, H., Liu, Y.Y., Zhang, L., 2010. Characterization, expression profiling, and functional identification of a gene encoding geranylgeranyl diphosphate synthase from *Salvia miltiorrhiza*. Biotechnol. Bioprocess Eng. 15, 236–245.

Kai, G.Y., Miao, Z.Q., Zhang, L., Zhao, D.L., Liao, Z.H., Sun, X.F., Zhao, L.X., Tang, K.X., 2006. Molecular cloning and expression analyzes of a new gene encoding 3-hydroxy-3-methylglutaryl-CoA synthase from *Taxus media*. Biol. Plant. 50, 359–366.

Kim, D.H., Kim, S., Jeon, S.J., Son, K.Ho., Lee, S., Yoon, B.H., Cheong, J.H., Ko, K.Ho., Ryu, J.H., 2009. Tanshinone I enhances learning and memory, and ameliorates memory impairment in mice via the extracellular signal-regulated kinase signalling pathway. Br. J. Pharmacol. 158, 1131–1142.

Laule, O., Fürholz, A., Chang, H.S., Zhu, T., Wang, X., Heifetz, P.B., Gruissem, W., Lange, B.M., 2003. Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. U. S. A. 100, 6866–6871.

Lee, D.S., Lee, S.H., Noh, J.G., Hong, S.D., 1999. Antibacterial activities of cryptotanshinone and dihydrotanshinone |from a medicinal herb, *Salvia miltiorrhiza* Bunge. Biosci. Biotechnol. Biochem. 63, 2236–2239.

Leech, M.J., May, K., Hallard, D., Verpoorte, R., Luca, V.D., Christou, P., 1998. Expression of two consecutive genes of a secondary metabolic pathway in transgenic tobacco: molecular diversity influences levels of expression and product accumulation. Plant Mol. Biol. 38, 765–774.

Li, L., Lou, Z.Y., Chen, W.S., Zhang, W.D., Qiao, C.Z., 2000. Contents variety of three kinds of tanshinone among intraspecies of *Salvia miltiorrhiza* Bunge. Acad. J. Second Mil. Med. Univ. 21, 753–755 (in Chinese).

Li, L.N., 1998. Biologically active components from traditional Chinese medicines. Pure Appl. Chem. 70, 547–554.

Liao, P., Zhou, W., Zhang, Lin., Wang, J., Yan, X.M., Zhang, Y., Zhang, R., Li, L., Zhou, G.Y., Kai, G.Y., 2009. Molecular cloning, characterization and expression analysis of a new gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase from *Salvia miltiorrhiza*. Acta. Physiol. Plant. 31, 565–572.

Liu, J.J., Lin, D.J., Liu, P.Q., Huang, M., Li, X.D., Huang, R.W., 2006. Induction of apoptosis and inhibition of cell adhesive and invasive effects by tanshinone IIA in acute promyelocytic leukemia cells in vitro. J. Biomed. Sci. 13, 813–823.

Lois, L.M., Rodríguez-Concepción, M., Gallego, F., Campos, N., Boronat, A., 2000. Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. Plant J. 22, 503–513.

Muir, S.R., Collins, G.J., Robinson, S., Hughes, S., Bovy, A., Ric De Vos, C.H., van Tunen, A.J., Verhoeyen, M.E., 2001. Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. Nat. Biotechnol. 19, 470–474.

Munoz-Bertomeu, J., Arrillaga, I., Ros, R., Segura, J., 2006. Up-regulation of 1-deoxy-dxylulose-5-phosphate synthase enhances production of essential oils in transgenic spike lavender. Plant Physiol. 142, 890–900.

Muñoz-Bertomeu, J., Ros, R., Arrillaga, I., Segura, J., 2008. Expression of spearmint limonene synthase in transgenic spike lavender results in an altered monoterpene composition in developing leaves. Metab. Eng. 10, 166–177.

Muñoz-Bertomeu, J., Sales, E., Ros, R., Arrillaga, I., Segura, J., 2007. Up-regulation of an N-terminal truncated 3-hydroxy-3-methylglutaryl CoA reductase enhances production of essential oils and sterols in transgenic *Lavandula latifolia*. Plant Biotechnol. J. 5, 746–758.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Nims, E., Dubois, C., Roberts, S.C., Walker, E.L., 2006. Expression profiling of genes involved in paclitaxel biosynthesis for targeted metabolic engineering. Metab. Eng. 8, 385–394.

Peebles, C.A.M., Hughes, E.H., Shanks, J.V., San, K.Y., 2009. Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time. Metab. Eng. 11, 76–86.

Peebles, C.A.M., Sander, G.W., Hughes, E.H., Peacock, R., Shanks, J.V., San, K.Y., 2010. The expression of 1-deoxy-D-xylulose synthase and geraniol-10-hydroxylase or anthranilate synthase increases terpenoid indole alkaloid accumulation in *Catharanthus roseus* hairy roots. Metab. Eng.. doi:10.1016/j.ymben.2010.11.005.

Shi, Z.H., He, J.T., Chang, W.B., 2004. Micelle-mediated extraction of tanshinone from *Salvia miltiorrhiza* bunge with analysis by high-performance liquid chromatography. Talanta. 64, 401–407.

Wang, A.M., Sha, S.H., Lesniak, W., Schacht, J., 2003. Tanshinone (*Salviae miltiorrhizae* Extract) Preparations attenuate aminoglycoside-induced free radical formation in vitro and ototoxicity in vivo. Antimicrob. Agents Chemother., 1836–1841.

Wang, K., Ohnuma, S., 1999. Chain-length determination mechanism of isoprenyl diphosphate synthases and implications for molecular evolution. Trends Biochem. Sci. 24, 445–451.

Wu, D.F., Jiang, X.H., Wu, S.H., 2010. Direct purification of tanshinone from *Salvia miltiorrhiza* Bunge by high-speed counter-current chromatography without presaturation of the two-phase solvent mixture. J. Sep. Sci. 33, 67–73.

Wu, J.Y., Shi, M., 2008. Ultrahigh diterpenoid tanshinone production through repeated osmotic stress and elicitor stimulation in fed-batch culture of *Salvia miltiorrhiza* hairy roots. Appl. Microbiol. Biotechnol. 78, 441–448.

Wu, S.J., Shi, M., Wu, J.Y., 2009. Cloning and characterization of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase gene for diterpenoid tanshinone biosynthesis in *Salvia miltiorrhiza* (Chinese sage) hairy roots. Biotechnol. Appl. Biochem. 52, 89–95.

Wu, Y.L., Wang, D.N., 2009. A New class of natural glycopeptides with sugar moietydependent antioxidant activities derived from *Ganoderma lucidum* fruiting bodies. J. Proteome Res. 8, 436–442.

Xu, H., Zhang, L., Zhou, C.C., Xiao, J.B., Liao, P., Kai, G.Y., 2010. Metabolic regulation and genetic engineering of pharmaceutical component tanshinone biosynthesis in Salvia miltiorrhiza. J. Med. Plant Res. 4, 2591–2597.

Yan, Q., Hu, Z.D., Tan, R.X., Wu, J.Y., 2005. Efficient production and recovery of diterpenoid tanshinone in *Salvia miltiorrhiza* hairy root cultures with in situ adsorption, elicitation and semi-continuous operation. J. Biotechnol. 119, 416–424.

Yan, X.M., Zhang, L., Wang, J., Liao, P., Zhang, Y., Zhang, R., Kai, G.Y., 2009. Molecular characterization and expression of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) gene from *Salvia miltiorrhiza*. Acta. Physiol. Plant. 31, 1015–1022.

Yang, R.F., Liu, A., Ma, X.J., Li, L., Su, D.F., Liu, J.G., 2008. Sodium Tanshinone IIA sulfonate protects cardiomyocytes against oxidative stress–mediated apoptosis through inhibiting JNK activation. J. Cardiovasc. Pharmacol. 51, 396–401.

Ye, X., Al-Babili, S., Klöti, A., Zhang, J., Lucca, P., Beyer, P., Potrykus, I., 2000. Engineering the provitamin A (ß-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287, 303–305.

Zhang, C.H., Yan, Q., Cheuk, J., 2004a. Enhancement of tanshinone production in *Salvia miltiorrhiza* hairy root culture by Ag^+ and nutrient feeding. Planta Med. 70, 147–151.

Zhang, L., Ding, R.X., Chai, Y.R., Bonfill, M., Moyano, E., Oksman-Caldentey, K.-M., Xu, T.F., Pi, Y., Wang, Z.N., Zhang, H.M., Kai, G.Y., Liao, Z.H., Sun, X.F., Tang, K.X., 2004b. Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc. Natl. Acad. Sci. U. S. A. 101, 6786–6791.

Zhang, L., Yan, X.M., Wang, J., Li, S.S., Liao, P., Kai, G.Y., 2010. Molecular cloning and expression analysis of a new putative gene encoding 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) from *Salvia miltiorrhiza*. Acta Physiol. Plant. doi:10.1007/s11738-010-0627-2.

Zhang, L., Yang, B., Lu, B.B., Kai, G.Y., Wang, Z.N., Xia, Y., Ding, R.X., Zhang, H.M., Sun, X.F., Chen, W.S., Tang, K.X., 2006. Tropane alkaloids production in

transgenic *Hyoscyamus niger* hairy root cultures over-expressing Putrescine *N*-methyltransferase is methyl jasmonate-dependent. Planta 225, 887–896.
Zhang, L.L., Lin, Y.M., 2008. Tannins from *Canarium album* with potent antioxidant activity. J. Zhejiang Univ. Sci. B. 9, 407–415.
Zhao, J., Matsunaga, Y., Fujita, K., Sakai, K., 2006. Signal transduction and metabolic flux of b-thujaplicin and monoterpene biosynthesis in elicited *Cupressus lusitanica* cell cultures. Metab. Eng. 8, 14–29.
Zhou, L.M., Zuo, Z., Chow, M.S.S., 2005. Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. J. Clin. Pharmacol. 45, 1345–1359.
Zhou, W., Shen, Y.F., Chen, J.F., Dai, L.M., Kai, G.Y., Zhou, G.Y., 2007. Research and development of biotechnology of *Salvia miltiorrhiza* Bunge. J Biol. 24, 48–51 (in Chinese).