Effects of methyl jasmonate and salicylic acid on tanshinone production and biosynthetic gene expression in transgenic *Salvia miltiorrhiza* hairy roots

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Abstract

Tanshinone is a group of active diterpenes, which are widely used in the treatment of cardiovascular disease. In this study, methyl jasmonate (MJ) and salicylic acid (SA) were used to investigate their effects on tanshinone accumulation and biosynthetic gene expression in the hairy roots of geranylgeranyl diphosphate synthase (*SmGGPPS*) overexpression line (G50) in *Salvia miltiorrhiza*. High-performance liquid chromatography analysis showed that total tanshinone content in G50 was obviously increased by 3.10-fold (11.33 mg/g) with MJ at 36 H and 1.63 times (5.95 mg/g) after SA treatment for 36 H in comparison with their mimic treatment control. Furthermore, quantitative reverse-transcription PCR analysis showed that the expression of isopentenyl-diphosphate delta-isomerase (*SmIPPI*), *SmGGPPS*, copalyl diphosphate synthase (*SmCPS*), and kaurene synthase-like (*SmKSL*) increased significantly with MJ treatment. However, the expression of *SmIPPI* reached the highest level at 144 H, whereas those of *SmGGPPS*, *SmCPS*, and *SmKSL* only increased slightly with SA treatment. The two elicitor treatments suggested that tanshinone accumulation positively correlated to the expression of key genes such as *SmGGPPS*, *SmCPS*, and *SmKSL*. Meanwhile, the study also indicated that it was a feasible strategy to combine elicitor treatment with transgenic technology for the enhancement of tanshinone, which paved the way for further metabolic engineering of tanshinone biosynthesis. Volume 00, Number 0, Pages 1–8, 2014

Keywords: *Salvia miltiorrhiza, transgenic hairy roots, tanshinones, elicitors, metabolic engineering, biosynthesis*

1. Introduction

Salvia miltiorrhiza Bunge, named Danshen or Tanshen in China, is a well-known traditional Chinese medicine herb, which was widely used for the treatment of various cardiovascular diseases such as menstrual disorders, blood circulation disturbance, and inflammation [1–3]. Tanshinones, a group of terpenoids including tanshinone I (T-I), tanshinone IIA (T-IIA), cryptotanshinone (CT), and dihydrotanshinone I (DH-TI), are the main active ingredients in *S. miltiorrhiza* (Fig. 1). Tanshinones have various biological activities including antioxidant, anti-inflammation, antibacterial, and antitumor [4, 5]. Because of the low content, long growth cycle, and serious quality degradation of *S. miltiorrhiza*, investigations on the enhancement of tanshinone content for the increasing clinical needs have become a research spotlight recently [6].

As one kind of diterpene, the biosynthesis of tanshinone can be divided into three stages (Fig. 2). At the first stage,

Abbreviations: *CPS, copalyl diphosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HPLC, high-performance liquid chromatography; IPPI, isopentenyl-diphosphate delta-isomerase; KSL, kaurene synthase-like; MJ, methyl jasmonate; qRT-PCR, quantitative reverse transcription PCR; SA, salicylic acid; Sm, Salvia miltiorrhiza.*

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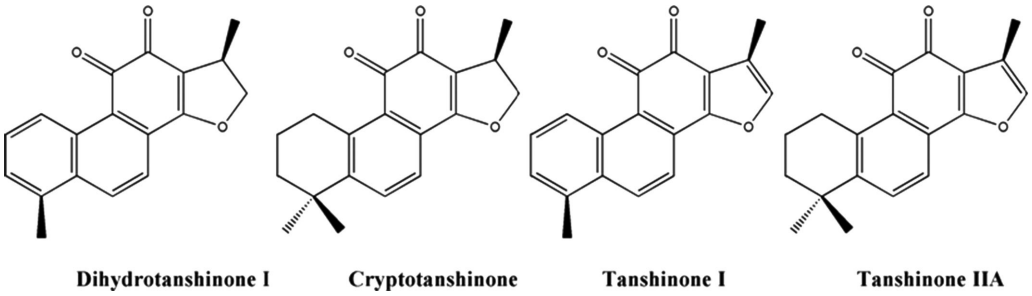

FIG. 1 *The chemical structure of four kinds of tanshinone compounds in Salvia miltiorrhiza.*

the universal isoprene precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate can be synthesized by two distinct isoprenoid biosynthesis pathways, the mevalonate (MVA) pathway in the cytosol and/or the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in the plastids [6]. At the second stage, geranylgeranyl diphosphate synthase (GGPPS) catalyzes the synthesis of the intermediate diphosphate precursor geranylgeranyl diphosphate [4]. At the last stage, diverse terpenoids are synthesized through the catalysis of terpene synthases/cylases including copalyl diphosphate synthase (CPS), kaurene synthase-like (KSL), miltiradiene oxidase CYP76AH1, and other enzymes [7–9].

In recent years, numerous studies have demonstrated that both biotic and abiotic elicitor such as yeast extract (YE), Ag^{+}, Co^{2+}, α-amino isobutyric acid, β-aminobutyric acid (BABA), methyl jasmonate (MJ), and salicylic acid (SA) can improve the accumulation of tanshinones in *S. miltiorrhiza* [5, 10–14]. Meanwhile, both MJ and SA are able to stimulate secondary bioactive components production in many other species [15–18]. In addition, Zhang et al. [19] reported that MJ treatment increased tropane alkaloids production in transgenic *Hyoscyamus niger* hairy root cultures. SA, acetylsalicylic acid, and MJ were reported to stimulate the tropane alkaloid yields in the transgenic *Atropa baetica*, which overexpressed the *hyoscyamine 6β-hydroxylase* (*h6h*) gene [20].

In order to test whether the production of tanshinones could be further improved by adding elicitors in transgenic *S. miltiorrhiza* hairy root line overexpressing geranylgeranyl diphosphate synthase (*SmGGPPS*), which showed significantly higher tanshinone content (G50) [6], MJ and SA were used in G50 cultivating system. Tanshinone content and the expression of key genes in the biosynthesis pathway after treatment by MJ or SA at different time points were analyzed. High-performance liquid chromatography (HPLC) analysis showed that total tanshinone content in G50 was increased with MJ and SA treatments. Furthermore, quantitative reverse-transcription PCR (qRT-PCR) analysis showed that tanshinone accumulation positively correlated to the expression of *SmGGPPS*, copalyl diphosphate synthase (*SmCPS*), and kaurene synthase-like (*SmKSL*). This study indicates that it was a good strategy to combine elicitor treatment and transgenic technology for further enhancement of tanshinone in hairy root cultures, which provides some useful information for the further increase in tanshinone biosynthesis in *S. miltiorrhiza* hairy root cultures.

2. Materials and Methods

2.1. Hairy root culture

The hairy roots of *SmGGPPS* overexpression line G50 were preserved in our laboratory [6]. The experiments of elicitation were performed in 150 mL Erlenmeyer flasks containing 50 mL 1/2 MS (Murashige and Skoog) liquid medium supplemented with 30% sucrose (pH 5.8) in the orbital shaker at a speed of 100 rpm in the dark at 25 °C. First, ~200 mg of normally growing hairy roots was cultured for 30 days, then divided into 12 equal parts and cultured for another 30 days. The hairy roots were treated by elicitors and harvested at 0 (before treatment), 36, 72, and 144 H after stress treatment for tanshinone extraction and RNA isolation. All treatments were performed in triplicate, and the tanshinone contents were represented with mean ± SD [5].

2.2. MJ and SA preparation

MJ (Sigma, Shanghai, China; W341002) was first dissolved in a small volume of dimethyl sulfoxide and then in distilled water to a storage concentration of 50 mM [5, 13]. SA (Shanghai Zhanyun Chemical Co., Shanghai, China; 0810035) was directly dissolved in distilled water to a storage concentration of 50 mM. All the above-mentioned solutions were sterilized through 0.22-μm filters (Pall Corporation, Shanghai, China) and added to cultures to the final concentrations of 100 μM [13]. Meanwhile, we added 100 μL equivalent volume sterilized water as the mimic treatment control at each time point.

2.3. Extraction and HPLC analysis of tanshinones

Hairy roots were harvested from shake-flask cultures and dried at 45 °C until reaching a constant dry weight. Dry hairy roots (200 mg) were ground with a pestle and mortar, soaked in 16 mL mixture solution (methanol:dichloromethane = 3:1), sonicated for 1 H, and then kept at room temperature for 24 H. The extraction solvent (methanol–dichloromethane) was evaporated under vacuum, and the residue was redissolved in 1.0 mL methanol followed by being filtered through a 0.22-μm filter. HPLC analysis was performed on a Hitachi L2000

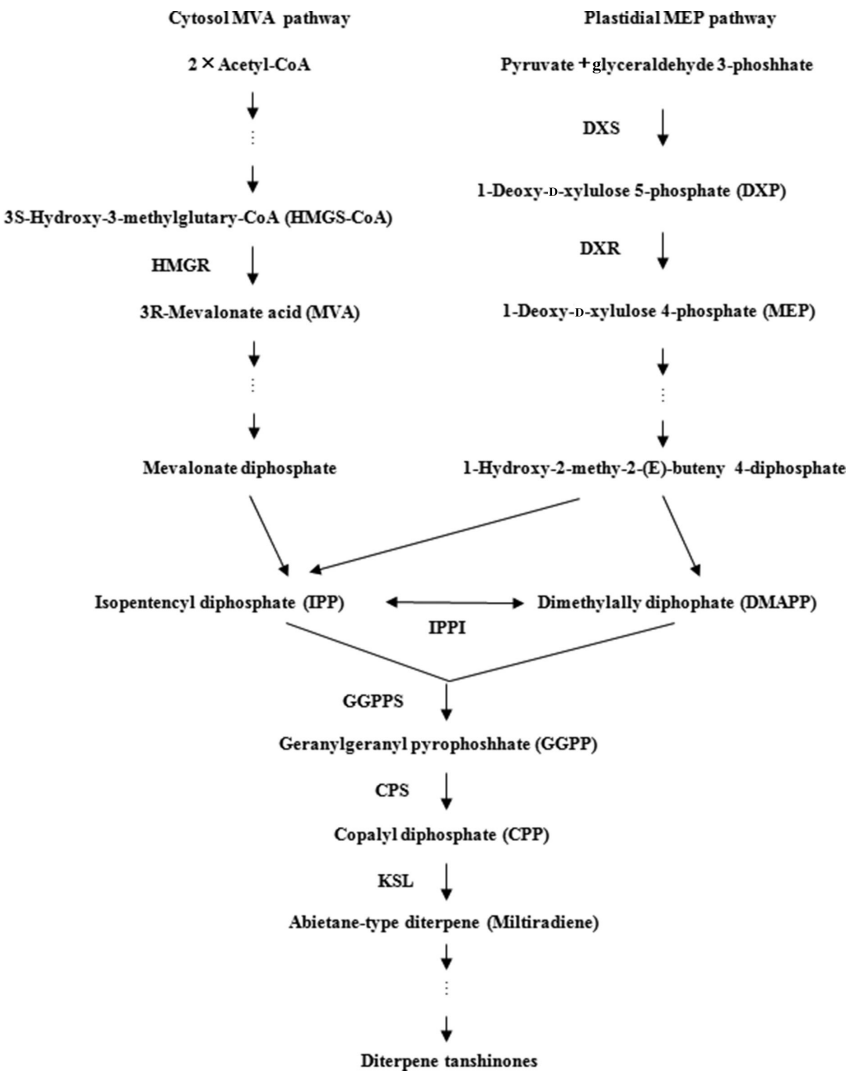

FIG. 2 *The metabolic pathway for tanshinones. 3-Hydroxy-3-methylglutaryl-CoA reductase (SmHMGR), 1-deoxy-D-xylulose-5-phosphate synthase (SmDXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (SmDXR), isopentenyl diphosphate isomerase (SmIPPI), geranylgeranyl diphosphate synthase (SmGGPPS), geranylgeranyl diphosphate synthase (SmCPS), and kaurene synthase-like (SmKSL) were used to study in this work.*

apparatus together with a Waters (Shanghai, China) reversed-phase C18 symmetry column at 30 °C. The mobile phase consisted of acetonitrile–water at 65:35 (v/v) and UV detection wavelength was 270 nm. The flow rate was 1.0 mL/Min, and the injection volume was 10 μL [5]. Four tanshinone constituents including DH-TI, CT, T-I, and T-IIA were detected and quantified with authentic standards. Total tanshinone represented the sum of DH-TI, CT, T-I, and T-IIA contents. Because tanshinones are lipophilic and barely soluble in aqueous solution, tanshinone contents (dissolved) in the medium were negligible and not calculated [2].

2.4. RNA isolation and qRT-PCR analysis

G50 hairy roots with various treatments at selected time points (0, 36, 72, and 144 H) were collected for RNA isolation. Total RNA was isolated from the hairy root samples using RNA prep pure plant kit (Tiangen Biotech Co., Beijing, China). DNase I (Tiangen Biotech Co.) was used to remove all DNA from the RNA samples. The quality and concentration of the extracted RNA were detected as described before [3].

First-strand cDNA was synthesized using reverse transcriptase (RT) (TaKaRa, Dalian, China) from 2 μg total RNA. qRT-PCR was performed with SYBR® PrimeScript™ RT-PCR Kit (TaKaRa) on ABI StepOne™ Real-Time PCR System Thermal Cycling Block (Applied Biosystems, Foster City, CA, USA). Specific primers of *S. miltiorrhiza* genes including 3-hydroxy-3-methylglutaryl-CoA reductase (*SmHMGR*), 1-deoxy-D-xylulose-5-phosphate synthase I (*SmDXS I*), 1-deoxy-D-xylulose-5-phosphate synthase II (*SmDXS II*), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*SmDXR*), isopentenyl-diphosphate delta-isomerase (*SmIPPI*), *SmGGPPS*,

TABLE 1 *S. miltiorrhiza genes and primer pairs used for qRT-PCR*

Gene	*GeneBank accession*	*Primer name*	*Primer sequence*
HMGR	EU680958	*SmHMGR*-982F	5′-TCGTTTTCAATAAGTCGAGTAGA-3′
		SmHMGR-1142R	5′-ATTCTGAAGGAAGTCCAAAACAT-3′
DXS I	EU670744	*SmDXS1*-1428F	5′-CGACCAGGTAGTGCACGACG-3′
		SmDXS1-1589R	5′-TCATCTGAAGGAGCCATCACCAC-3′
DXS II	FJ643618	*SmDXS2*-1828F	5′-TTGGAGATTGGGAAGGGAAGGAT-3′
		SmDXS2-1980R	5′-AGGCTTGCAGAATCTCGCATCAG-3′
DXR	DQ991431	*SmDXR*-1248F	5′-CGACGAGAAAATCGGATACCTGG-3′
		SmDXR-1424R	5′-CATACAAGAGCAGGACTCGAACCG-3′
IPPI	EF635967	*SmIPPI*-1422F	5′-GCAACGATCCACAACTAAGGT-3′
		SmIPPI-1572R	5′-ATGCCGAGTTCATCCAACAG-3′
GGPPS	FJ643617	*SmGGPPS*-603F	5′-GCTGTGCTCGCAGGGGATG-3′
		SmGGPPS-774R	5′-ATCGCCGGTGCAGTTCAGG-3′
CPS	EU003997	*SmCPS*-459F	5′-GATCGCCTCGTCAATACCAT-3′
		SmCPS-609R	5′-TTCGAACCCACAAGTCATGT-3′
KSL	EF635966	*SmKSL*-1480F	5′-GTGTGACCCTTCTGCTAGCA-3′
		SmKSL-1630R	5′-TGCATTGTCTTGGGAAGATG-3′

SmCPS, and *SmKSL* are listed in Table 1. The reference gene was *Ubiquitin*, and the primer sequences were *Ubiquitin*F (5′-ACCCTCACGGGGAAGACCATC-3′) and *Ubiquitin*R (5′-ACCACGGAGACGGA GGACAAG-3′). The qRT-PCR procedure was 95 °C for 5 Min followed by 40 cycles of amplification (95 °C for 30 Sec, 60 °C for 30 Sec, 72 °C for 30 Sec), and then one cycle of amplification (95 °C for 15 Sec, 60 °C for 1 Min, 95 °C for 15 Sec). Relative expression values were calculated with the comparative Ct method. Experiments were performed in triplicate, and the results were represented by the mean ± SD.

3. Results

3.1. Effects of the elicitors on tanshinone accumulation in *S. miltiorrhiza* hairy roots

Our previous study showed that the overexpression of *SmGGPPS* in hairy roots line G50 enhanced the content of tanshinones significantly. To investigate the effect of phytohormones on tanshinone accumulation, 60-day-old *S. miltiorrhiza* G50 transgenic hairy roots were treated with MJ and SA, and the content of tanshinones was further investigated. The effect of MJ and SA on tanshinone contents is described in Figs. 4 and 5. Four tanshinone components, DH-TI, CT, T-I, and T-IIA, were tested.

The total tanshinone content (only detected T-I, T-IIA, and CT) in transgenic *S. miltiorrhiza* hairy root line G50 was about 2.48 mg/g [6], whereas the content of total tanshinone was about 3.55 mg/g for the detected four tanshinones (T-I, T-IIA, CT, and DH-TI) in this study (Fig. 3). Meanwhile, the content of total tanshinones of the mimic treatment control with equivalent volume sterilized water changed slightly at each time point, varying from 3.55 to 4.10 mg/g (Fig. 3).

The content of total tanshinones was obviously elevated by MJ treatment and reached the highest point (11.33 mg/g) at 36 H and then fell to 6.68 mg/g at 144 H after the treatment (Fig. 4). For DH-TI and CT, the content at 36 H was similar to that at 72 H; in detail, the content of DH-TI was 5.36 mg/g at 36 H and 5.43 mg/g at 72 H, and the content of CT was 5.06 mg/g at 36 H and 5.09 mg/g at 72 H. Both DH-TI and CT contents fell down to 3.00 mg/g at 144 H after MJ treatment. On the other hand, the contents of T-I and T-IIA were quite low, even at their highest level 36 H after MJ treatment, the T-I content was 0.18 mg/g, whereas T-IIA content was 0.74 mg/g.

Under the treatment of SA, the total tanshinone content also reached the highest level (5.95 mg/g) at 36 H and then dropped to 5.60 mg/g at 144 H (Fig. 5). For the specific four compounds, DH-TI and CT contents reached the highest level (2.78 and 2.64 mg/g, respectively) at 36 H. The variation

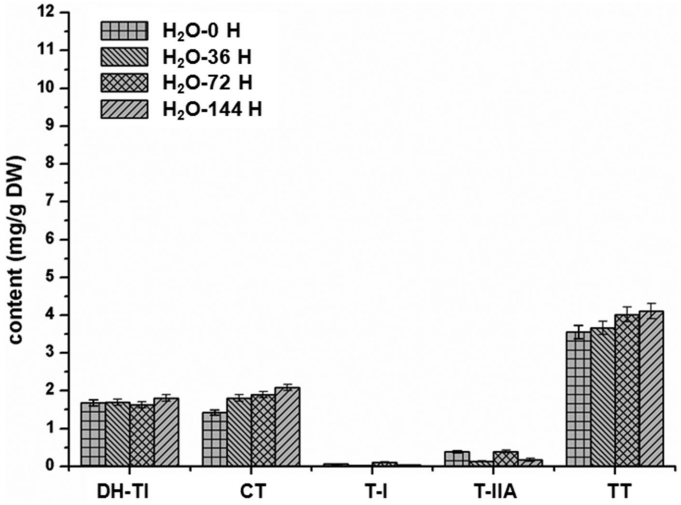

FIG. 3 *Effects of H_2O on tanshinone accumulation of S. miltiorrhiza hairy roots. DH-TI, dihydrotanshinone-I; CT, cryptotanshinone; T-I, tanshinone-I; T-IIA, tanshinone-IIA; TT, total tanshinone. (Error bars for SDs, n = 3.)*

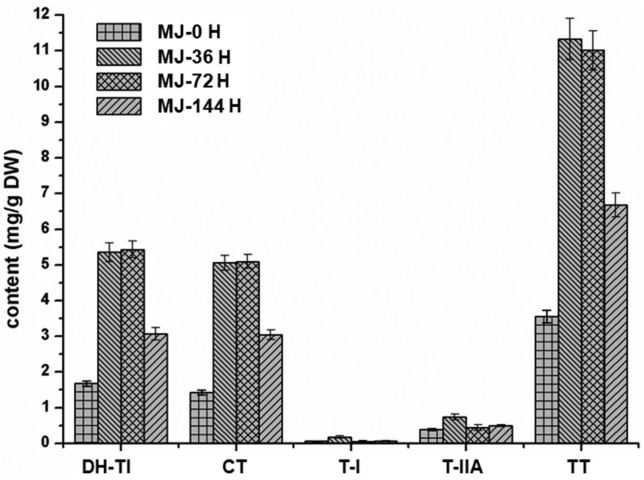

FIG. 4 *Effects of MJ on tanshinone accumulation of S. miltiorrhiza hairy roots. DH-TI, dihydrotanshinone-I; CT, cryptotanshinone; T-I, tanshinone-I; T-IIA, tanshinone-IIA; TT, total tanshinone. (Error bars for SDs, n = 3.)*

trend of T-I and T-IIA after SA treatment was similar with MJ treatment; the highest content of T-I and T-IIA was only 0.14 and 0.66 mg/g, respectively, 72 H after SA treatment.

It can be concluded that the best condition for further enhancement of tanshinone in transgenic *S. miltiorrhiza* hairy roots line G50 was MJ treatment at 36 H, which reached the highest level of total tanshinone (11.33 mg/g, 3.10-fold of the mimic treatment control). In addition, the best condition for further enhancement of tanshinone by SA in transgenic *S. miltiorrhiza* hairy roots line G50 was at 36 H, the total tanshinone increased only 1.63-fold of the mimic treatment control.

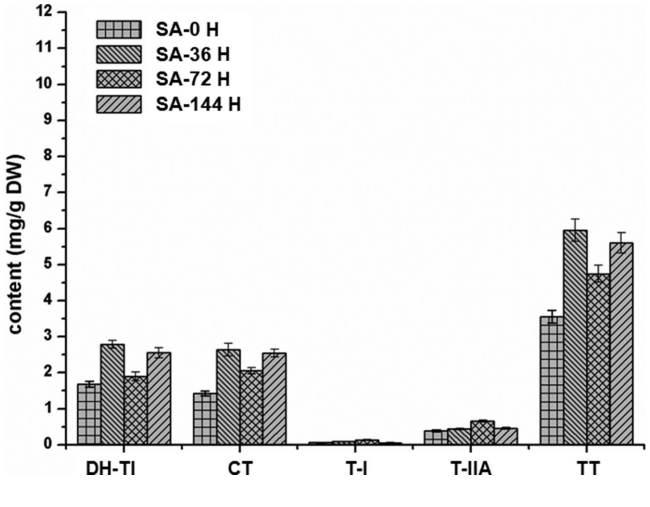

FIG. 5 *Effects of SA on tanshinone accumulation of S. miltiorrhiza hairy roots. DH-TI, dihydrotanshinone-I; CT, cryptotanshinone; T-I, tanshinone-I; T-IIA, tanshinone-IIA; TT, total tanshinone. (Error bars for SDs, n = 3.)*

3.2. MJ and SA induced the expression of tanshinone biosynthetic genes

To investigate the effect of phytohormones on key genes in tanshinone biosynthesis, the expressions of tanshinone biosynthetic genes including *SmHMGR*, *SmDXS I*, *SmDXS II*, *SmDXR*, *SmIPPI*, *SmGGPPS*, *SmCPS*, and *SmKSL* were measured by qRT-PCR.

The expression pattern of tanshinone biosynthetic genes in the mimic treatment control group is shown in Fig. 6. The expression of *SmHMGR*, *SmDXS I*, *SmDXS II*, *SmDXR*, *SmIPPI*, *SmGGPPS*, and *SmKSL* changed slightly and only the expression of *SmCPS* increased slightly.

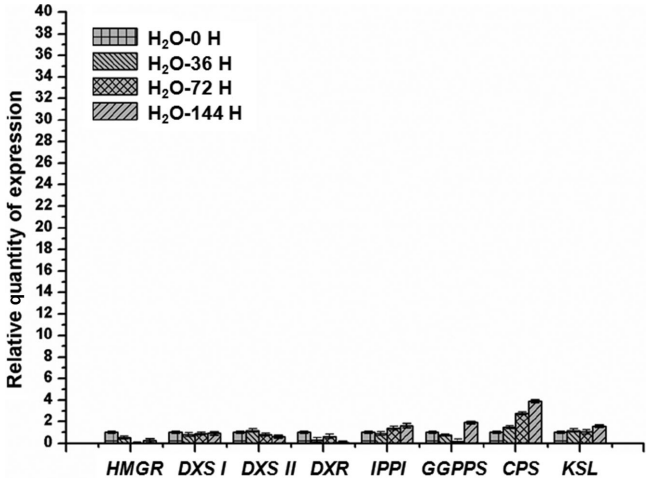

FIG. 6 *The expression levels of tanshinone biosynthesis genes after treated with H_2O at different times. (Error bars for SDs, n = 3.)*

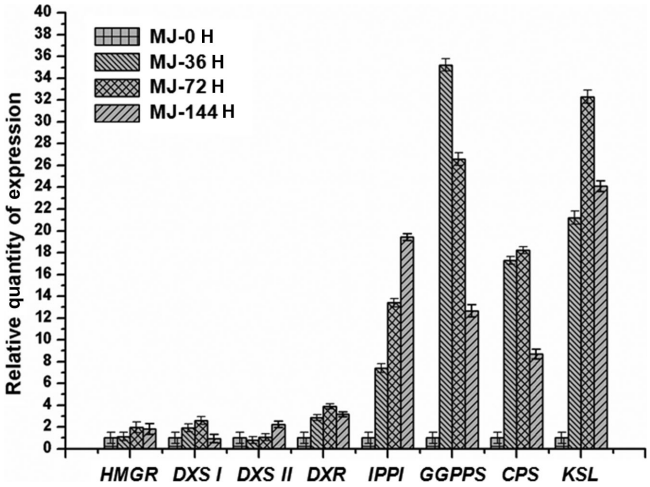

FIG. 7 *The expression levels of tanshinone biosynthesis genes after treated with MJ at different times. (Error bars for SDs, n = 3.)*

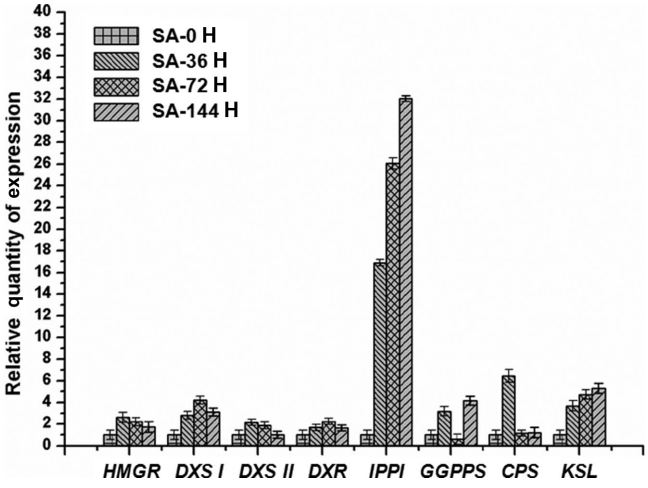

FIG. 8 *The expression levels of tanshinone biosynthesis genes after treated with SA at different times. (Error bars for SDs, n = 3.)*

The expression pattern of tanshinone biosynthetic genes after MJ treatment is shown in Fig. 7. The expression of *SmIPPI*, *SmGGPPS*, *SmCPS*, and *SmKSL* increased significantly. In detail, the expression of *SmIPPI* increased 7.4 times at 36 H and further increased to 19.4 times of the untreated control at 144 H. The expression of *SmGGPPS* reached the highest level at 36 H (35.2-fold of the untreated control), and then decreased gradually at 72 and 144 H, while still maintained a much higher level than the untreated control. The expression of *SmCPS* and *SmKSL* at 72 H was 18.1 and 32.2 times of the untreated control and maintained a much higher level than the untreated control at other time points, respectively. Slight upregulation was observed for *SmDXS I* at 36 and 72 H and for *SmDXR* at 36, 72, and 144 H, whereas the expression of *SmHMGR* was slightly increased at 72 and 144 H but not at 36 H and *SmDXS II* was only upregulated moderately at 144 H.

The expression of tanshinone biosynthetic genes by SA treatment is shown in Fig. 8. The expression of *SmIPPI* increased significantly during SA treatment, and reached the highest level (32.0 times of the untreated control) at 144 H, but the expression of other tested genes was slightly elevated. The expression of *SmHMGR*, *SmDXS II*, and *SmCPS* reached the highest level at 36 H, and then decreased at 72 H. *SmGGPPS* was upregulated at 36 H, downregulated at 72 H but upregulated at 144 H again. The expression of *SmDXS I* and *SmDXR* reached the highest level at 72 H, and then decreased at 144 H; the expression of *SmKSL* increased gradually and reached the highest level at 144 H. These results showed that most tested genes in the tanshinone biosynthetic pathway were responsive to SA elicitation positively at transcription level with slight upregualtion, coinciding with the slight induction of SA on improving the tanshinone production at 36 H.

When we combine results from MJ and SA treatments, it is interesting that the expression of *SmIPPI*, *SmGGPPS*, *SmCPS*, and *SmKSL* increased significantly and the expression of *SmGGPPS* reached the highest level (35.2-fold of the control) at 36 H after MJ treatment. The expression of *SmIPPI* increased obviously from 36 H and reached the highest level (32.0 times of the control) at 144 H under the treatment of SA. These data suggested that tanshinone accumulation positively correlated to expression of *SmGGPPS*, *SmCPS*, and *SmKSL*.

4. Discussion

4.1. Combination elicitation with transgene for enhancement of tanshinone was feasible

Our previous work showed that overexpression of *SmGGPPS*, *SmHMGR*, and *SmDXS* in hairy roots line could enhance the production of tanshinones significantly, while *SmGGPPS* plays a more important role in stimulating tanshinone accumulation than *SmHMGR* or *SmDXS* [6]. Coexpression of *SmHMGR* and *SmGGPPS* resulted in the highest production of tanshinone (about 2.73 mg/g) in line HG9, which was about 4.74-fold higher than that of the control (0.48 mg/g) [6]. Therefore, it is useful to enhance the tanshinone content in *S. miltiorrhiza* by metabolic engineering.

In addition, previous studies showed that tanshinone accumulation in *S. miltiorrhiza* hairy roots could be stimulated by various kinds of treatments such as biotic (YE) and abiotic elicitors (MJ, Ag^{+}, α-amino isobutyric acid, and BABA) [5, 10–14]. In recent years, elicitors such as MJ and SA were shown to play an important role in promoting the production of secondary metabolites including phenylpropanoids [21], terpenes [22], isoflavonoid [23, 24], and phytosterols [25]. As a plant stress-signaling factor, SA has been confirmed as an effective elicitor to influence plant resistance to pathogens by activating certain plant defense responses such as the biosynthesis of special secondary metabolites and by inducing the expression of pathogenesis-related genes [16, 26, 27].

Likewise, MJ also acts as another important elicitor for the accumulation of various groups of secondary metabolites including alkaloids in hairy roots [28].

Our study showed that total tanshinone content in transgenic *S. miltiorrhiza* hairy root line G50 was obviously increased by 3.10 and 1.63 times (reached to 11.33 and 5.95 mg/g, respectively) after MJ and SA treatments for 36 H, respectively. The results proved that it was a more effective strategy to improve tanshinone content in *S. miltiorrhiza* hairy roots by combining elicitor treatments with transgenic technology.

4.2. Tanshinone accumulation was positively correlated to the expression of *SmGGPPS*, *SmCPS*, and *SmKSL*

Our results indicated that *SmIPPI*, *SmGGPPS*, *SmCPS*, and *SmKSL* were highly upregulated and the expression of *SmGGPPS* reached the highest level at 36 H (35.2 times of the control) when treated with MJ. The expression of *SmHMGR*, *SmDXS I*, *SmDXS II*, and *SmDXR* were moderately upregulated when treated with MJ. This showed that *SmIPPI*, *SmGGPPS*, *SmCPS*, and *SmKSL* responded to MJ at much higher level than *SmHMGR*, *SmDXS I*, *SmDXS II*, and *SmDXR* in transgenic *S. miltiorrhiza* hairy root line G50. Although only *SmIPPI* increased significantly (reached the highest level, 32.0 times of the control, at 144 H) during SA treatment, the expression of other tested genes including *SmKSL*, *SmCPS*, *SmGGPPS*, *SmHMGR*, *SmDXS I*, *SmDXS II*, and *SmDXR* at different stages was also slightly elevated even the increased level is less than that for *SmIPPI* with the same treatment. This result suggested that *SmIPPI* responded to SA at much higher level than *SmKSL*, *SmCPS*, *SmGGPPS*, *SmHMGR*, *SmDXS I*, *SmDXS II*, and *SmDXR* in transgenic *S. miltiorrhiza* hairy root line G50. Taken together, our results from MJ and SA treatments suggested that tanshinone accumulation was positively correlated to the expression of most key genes tested, especially *SmGGPPS*, *SmCPS*, and *SmKSL*.

GGPPS is an important target for metabolic regulation in diterpenoid biosynthesis [29]. Our previous work demonstrated that *SmGGPPS* played an important role in promoting carotenoid pathway flux by color complementation assay in *E. coli* [30], and overexpression of *SmGGPPS* in hairy roots line enhanced the production of tanshinones significantly [6]. Both of these studies showed that the expression of *SmGGPPS* was dramatically induced by MJ, suggesting that there is a tight relationship between the expressions of *SmGGPPS* and tanshinones accumulation in *S. miltiorrhiza* hairy roots [5]. Our work here also showed that *SmGGPPS* was highly upregulated and reached the highest level (35.2 times of the control) at 36 H by MJ (Fig. 7), consistent with highest increase of total tanshinone content 36 H after MJ treatment (Fig. 4). Also, *SmGGPPS* expression increased threefolds to fourfolds at 36 and 144 H by SA (Fig. 8), which is coincided with the increase of total tanshinone at least at 36 and 144 H after SA treatment (Fig. 5). Hence, *SmGGPPS* was considered as one of the key genes in further promoting tanshinone biosynthesis in transgenic *S. miltiorrhiza* hairy root line G50.

In recent years, *SmCPS* has been isolated and confirmed to be an important enzyme in the late specific tanshinone biosynthesis pathway [8]. The expression of *SmCPS* was found to have a positive relationship with tanshinone accumulation under both the treatments of MJ and YE–Ag^+ in *S. miltiorrhiza* hairy roots [8]. This result and our previous work showed the similar elicitation under the treatment of MJ [5]. Furthermore, the expression of *SmCPS* increased significantly by MJ treatment at all corresponding points and by SA at least at 36 H in the transgenic *S. miltiorrhiza* hairy root line G50 (Figs. 7 and 8). At the same time, total tanshinone increased to the highest level at 36 H after MJ and SA treatments. Therefore, *SmCPS* was also considered as another key gene in further promoting tanshinone biosynthesis in transgenic *S. miltiorrhiza* hairy root line G50.

Meanwhile, *SmKSL* has also been isolated and identified as another diterpene synthase in tanshinone biosynthesis pathway [8]. Furthermore, both MJ and YE–Ag^+ treatments induced the expression of *SmKSL* and resulted in increased content of T-IIA in *S. miltiorrhiza* hairy roots [8]. Our results (Figs. 7 and 8) coincided with these results and indicated that *SmKSL* was also important in further promoting tanshinone biosynthesis in transgenic *S. miltiorrhiza* hairy root line G50.

5. Conclusions

Our study indicated that it is feasible and a good strategy to combine elicitor treatments such as MJ or SA with transgenic technology for highly enhancement of tanshinones in *S. miltiorrhiza* hairy roots. In addition, our study also demonstrated that tanshinone accumulation was positively correlated to the expression of key genes including but not limited to *SmGGPPS*, *SmCPS*, and *SmKSL* in transgenic *S. miltiorrhiza* hairy root line G50. These results paved the way for metabolic engineering of tanshinone biosynthesis in hairy root cultures in future.

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7. References

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