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ORIGINAL ARTICLE

# Increased phenolic acid and tanshinone production and transcriptional responses of biosynthetic genes in hairy root cultures of *Salvia przewalskii* Maxim. treated with methyl jasmonate and salicylic acid

**Jie Li[1] · Bo Li[2,3] · Lu Luo[1] · Fulin Cao[1] · Bingyue Yang[1] · Jing Gao[1] · Yonggang Yan[1] · Gang Zhang[1] · Liang Peng[1] · Benxiang Hu[1]**

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## Abstract

The purpose of this study is to reveal the impact of the plant hormone salicylic acid (SA) and methyl jasmonate (MeJA) on the growth, effective components accumulation, and related gene expression of the hairy root of *Salvia przewalskii* Maxim. Various concentrations of SA (0, 25, 50, 100, 200 μM) or MeJA (0, 50, 100, 200, 400, 600 μM) were added to the culture medium of *Salvia przewalskii* Maxim. Low concentrations of SA promoted the growth of hairy root, while a high concentration inhibited it. 0 to 400 μM MeJA promoted the growth of hairy root, but 600 μM MeJA starts to inhibit its growth. 50 μM SA and 400 μM MeJA significantly enhanced the production of caffeic acid, rosmarinic acid, salvianolic acid B, cryptotanshinone, and tanshinone IIA. In general, 50 μM SA can be used to accumulate of tanshinone in hairy roots of *S. przewalskii* with 6 days. 400 μM MeJA can be used to accumulate of phenolic acids in hairy roots of *S. przewalskii* with 3 days. The selected genes in the tanshinone and phenolic acid biosynthetic pathway were upregulated with elicitation. To obtain a higher yield and content of secondary metabolites, it is advisable to use 50 μM SA or 400 μM MeJA as the optimal doses to cultivate the hairy root of *S. przewalskii.* This study provides, for the first time, an efficient tanshinone and phenolic acid production method for *S. przewalskii*.

**Keyword** *Salvia przewalskii* maxim · Methyl jasmonate · Salicylic acid · Phenolic acid · Tanshinone · Gene expression

## Abbreviations

| | |
|---|---|
| SA | Salicylic acid |
| MeJA | Methyl jasmonate |
| FW | Fresh weight |
| DW | Dry weight |
| *PAL* | Phenylalanine ammonia-lyase |
| *TAT* | Tyrosine aminotransferase |
| *HPPR* | 4-Hydroxyphenylpyruvate reductase |
| *4CL1* | 4-Coumaric acid CoA-ligase 1 |
| *RAS* | Rosmarinic acid synthase |
| *CYP98A14* | Acytochrome P450-dependent monooxygenase |
| *HMGR* | 3-Hydroxy-3-methylglutaryl CoA reductase |
| *DXR* | 1-Deoxy-D-xylulose 5-phosphate reductoisomerase |
| *DXS* | 1-Deoxy-D-xylulose 5-phosphate synthase |
| *IPPI* | Isopentenyl-diphosphate delta-isomerase |
| *GGPPS* | Geranylgeranyl diphosphate synthase |
| *CPS* | Copalyl diphosphate synthase |
| *KSL* | Entkaurene synthase like |
| qRT-PCR | Real-time quantitative PCR |

Jie Li and Bo Li contributed equally to this work.

✉ Liang Peng
ppengliang@126.com

✉ Benxiang Hu
hubenxiang1980@163.com

[1] Shaanxi University of Chinese Medicine, Xianyang 712046, China

[2] Shaanxi Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, Shaanxi University of Chinese Medicine, Xianyang 712083, China

[3] State Key Laboratory of Research & Development of Characteristic Qin Medicine Resources (Cultivation), Xianyang 712083, China

## Introduction

*Salvia przewalskii* Maxim. (family Lamiacease), is a herbaceous perennial plant commonly known as Hong Qin Jiao or Gansu Danshen [1, 2], and is endemic to the southwestern and northwestern regions of China [3, 4]. *S. przewalskii* and *S. miltiorrhiza* Bunge belong to the *Salvia* genus, and have a similar chemical composition and pharmacological effects. *S. przewalskii* is used as a substitute for *S. miltiorrhiza* [5–7]. Based on the chemical structures and pharmacological activities, the major constituents in *S. przewalskii* can be divided into two categories; phenolic acid components and tanshinone compounds. The phenolic acid components include rosmarinic acid, caffeic acid, fumaric acid, salvianolic acid B, and danshensu [8, 9], which have a variety of pharmacological properties, such as strong antioxidant activities [10–12]. The tanshinone compounds are abietane-type diterpene pigments-tanshinone, such as tanshinone I, tanshinone IIA, Tanshinone IIB, cryptotanshinone, and dihydrotanshinone [13–15], which have demonstrated antidermatophytic, anti-inflammatory, antioxidant, antimutagenic, and antiplatelet aggregation activities. Furthermore, tanshinones also exhibit cardiovascular effects and are used for the treatment of some coronary heart diseases, and have been shown to exhibit antiproliferative activity against various human tumor cells [16–19]. Among them, tanshinone I and cryptotanshinone prevent the complications of myocardial ischemia [20], tanshinone IIB and cryptotanshinone have bacteriostatic activity against *Staphylococcus aureus* [21], and tanshinone IIA and cryptotanshinone have an inhibitive effect against $H_{37}RV$ [22].

The biosynthesis of phenolic acids can be classified into two pathways, the phenylpropanoid pathway and the tyrosine-derived pathway [23, 24]. The phenylalanine ammonia lyase gene family (*PAL*) plays a key role in initiating the phenylpropanoid pathway, and tyrosine aminotransferase (encoded by the gene *TAT*) is the first enzyme in the tyrosine-derived pathway (Fig. 1). The biosynthesis of tanshinone can be divided into two pathways, the mevalonate (MVA) pathway and the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway, the former occurring in the cytoplasm and the latter in the plastids of the cell [16, 25]. HMG-CoA reductase (encoded by the gene *HMGR*) is an initial and rate-limiting enzyme in the MVP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (*DXS*) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*) are key enzymes in the DXP pathway. Isopentenyl-diphosphate delta-isomerase (*IPPI*) and geranylgeranyl diphosphate synthase (*GGPPS*) also play important roles in the tanshinone biosynthetic pathway (Fig. 2) [26–28].

In recent years, numerous elicitors have been shown to improve the accumulation of various secondary metabolites in many plant species. For example, methyl jasmonate (MeJA) treatment increases tanshinone production in *S. miltiorrhiza* hairy roots [29] and phenolic acid content in *S. miltiorrhiza* [30], salicylic acid (SA) and MeJA were reported to stimulate the tropane alkaloid content in the transgenic *Atropa baetica* [31], and to significantly improve phenolic acid content in *S. miltiorrhiza* cell cultures and *Lithospermum erythrorhizon* suspension cells [32, 33]. Although elicitors have been used in the growth and the production of secondary metabolites of various plants, the influence of elicitors on *S. przewalskii* hairy roots has rarely been investigated. Thus, the purpose of this study was to examine the effects of SA and MeJA on

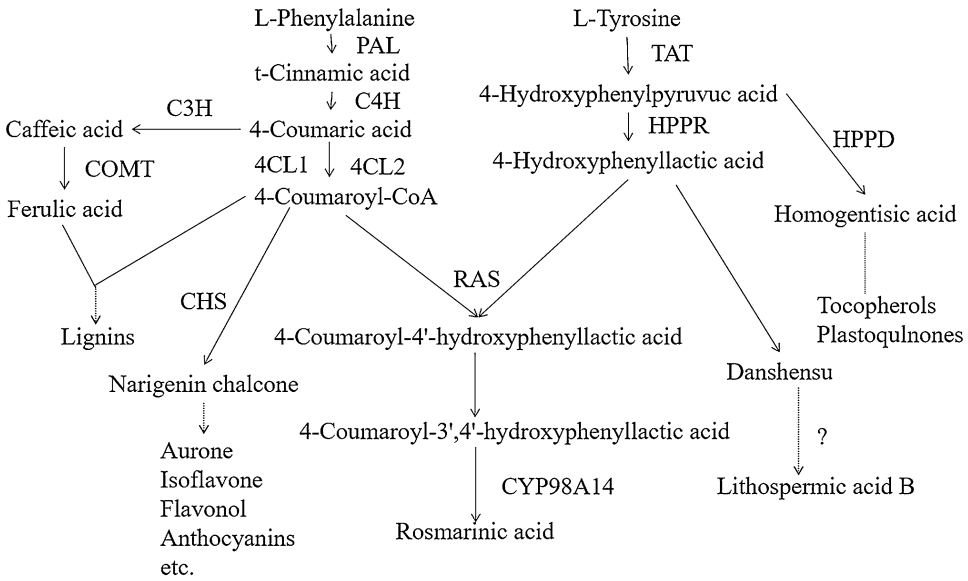



**Fig. 1** The metabolic pathway for phenolic acids

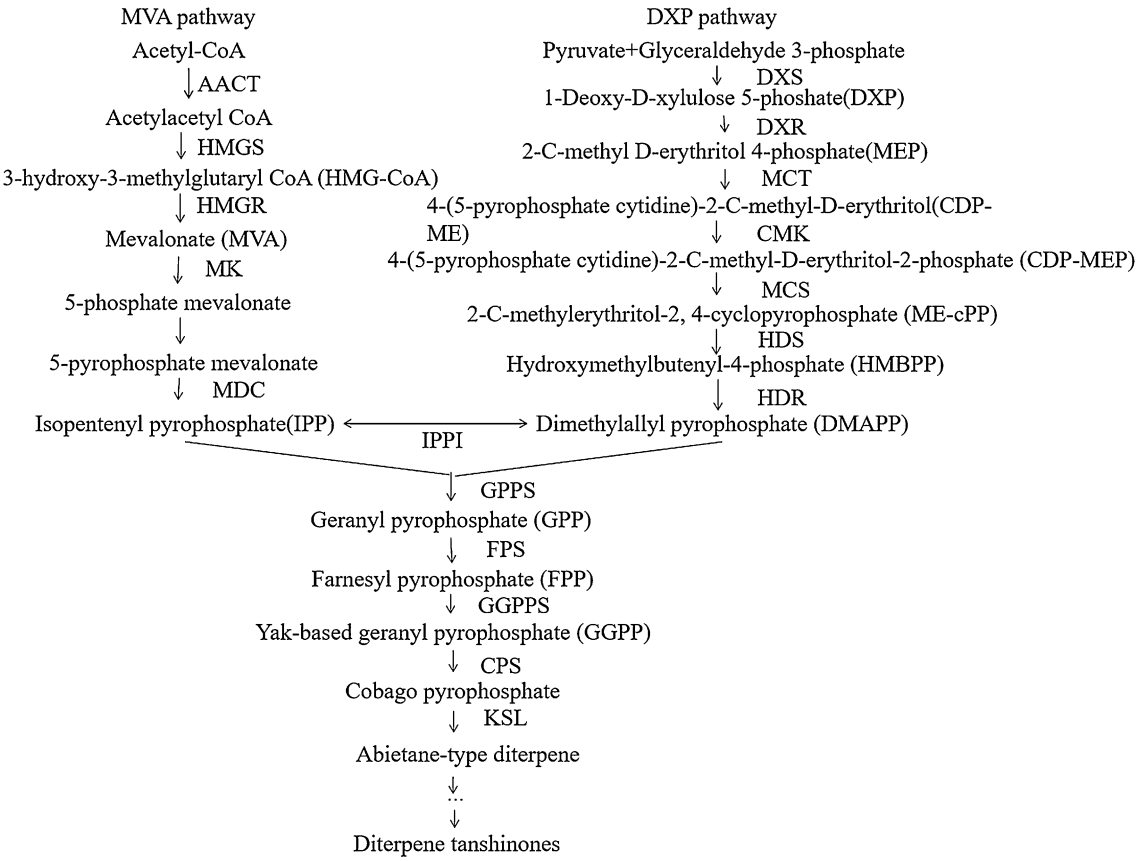



**Fig. 2** The metabolic pathway for tanshinones

the growth, the accumulation of phenolic acid and tanshinone in *S. przewalskii* hairy roots. In addition, we assessed the expression levels of thirteen genes, including *PAL*, *TAT*, *HPPR*, *4CL1*, *RAS*, *CYP98A14*, *HMGR*, *DXR*, *IPPI*, *GGPPS*, *CPS*, *KSL,* and *CYP76AH1*, which are involved in phenolic acids and tanshinone biosynthetic pathway with diverse elicitation.

## Materials and methods

### Plant material

The hairy root of *S. przewalskii* Maxim. was induced by *Agrobacteriom rhizogenes* ATCC 15834 with a modified method from previous work [34]. Hairy root was cultivated in a 100 mL flask containing 50 mL of 6,7-V medium (with 30 g $L^{-1}$ sucrose), and was placed on an gyratory shaker at 25 °C and 120 rpm in the dark [35, 36]. For elicitation experiments, 0.2 g of fresh hairy roots was prepared. Eighteen day cultured hairy roots were applied for MeJA and SA elicitation.

### Confirmation of hairy root induction

Total DNA of the normal root of plant ( control) and the putative *S. przewalskii* hairy root lines were extracted by the CTAB method with some modification [37]. Primers of *rolB* and *rolC* gene fragments for PCR amplification were designed based on aprevious work [38]. It's listed as follows: *rolB,* forward, 5′-GCT CTT GCA GTG CTA GAT TT-3′, and reverse, 5′-GAA GGT GCA AGC TAC CTC TC-3′; *rolC,* forward, 5′-CTC CTG ACA TCA AAC TCG TC-3′, and reverse, 5′-TGC TTC GAG TTA TGG GTA CA-3′. One microliter of DNA template, 1 µL each of the forward and reverse primers (concentration: 10 µM $\mu L^{-1}$), 25 µL of 2X PCR Premix (Tiangen, Beijing), and 22 µL $dH_2O$ were added to the 50 µL amplification system. The conditions of PCR were 5 min for predenaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 50 s, and extension at 72 °C for 1 min, after 35 cycles, at 72 °C for 6 min. The amplified PCR products were subjected to 1.5% (w/v) agarose gel electrophoresis and checked on the Gel Imaging System (Bio-Rad, USA) after stained by ethidium bromide. Predicted products of *rolB* and *rolC* were obtained apart from the control (noninduced root).

## Preparation and application of MeJA and SA

MeJA and SA were applied to the hairy roots elicitation. MeJA (Sigma, USA) and SA ( Sigma, USA) were dissolved in ethanol and filtered through sterile filter, respectively. After 18 days-old hairy roots cultured, MeJA and SA were added to 6,7-V medium to give the final concentrations of 50, 100, 200, 400, 600 μM for MeJA; and 25, 50, 100, 200 μM for SA, hairy root of *S. przewalskii* without elicitation as control, then hairy roots were acquired after adding elicitors for 6 days. For hairy root culture time, the hairy roots were acquired from 0 h, 12 h, 24 h, 3 day, 6 day after adding the optimum concentrations of the elicitors, respectively.

## Total RNA isolation and qRT-PCR

The hairy roots of *S. przewalskii* stored in a − 80 °C refrigerator were used for total RNA isolation. Total RNA of hairy roots was isolated using the Biospin Plant Total RNA Extraction Kit (BioFlux, China). The quality and concentration of RNA were determined by agarose gel electrophoresis and NanoDrop spectrophotometry (Thermo Fisher Scientific Inc, USA). cDNA was synthesized by reverse transcription using the PrimeScrip™ RT reagent Kit (Takara, Japan), initially incubated at 37 °C for reverse transcription (15 min), and then carried out at 85 °C for 5 s to inactivate the reverse transcriptase. The primers of relevant genes used for real-time quantitative PCR (qRT-PCR) are listed in Table 1. *β*-Actin was taken as the reference gene. qRT-PCR assay was performed using SYBR Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara, Japan) on the qTOWER 2.2 Real-time PCR Detection System (Analytik Jena, Germany). The conditions of qRT-PCR were 2 min for predenaturation and 5 s for denaturation at 95 °C, and then 30 s for annealing at 58 °C, 40 cycles in total. Quantification of gene expressions was obtained by a comparative CT method, relative transcripts of related genes in the hairy roots of *S. przewalskii* acquired at 0 day were as 1.

## HPLC determination of phenolic acid and tanshinone

0.1 g of dried hairy roots was weighed accurately and soak for 12 h with 5 mL 70% methanol, then the samples were treated by ultrasound for 45 min and centrifuged at 13,000×*g* for 10 min, the supernatant was filtered through a 0.22 μm millipore filter and analyzed by HPLC system equipped with e2695 separations module and 2998 PDA detector (Waters, USA). HPLC conditions were as follows, column, Symmetry C18 column (250 × 4.6 mm, 5 μm); solvent system, HPLC grade acetonitrile (A)-ultrapure $dH_2O$ with 0.02% phosphoric acid (B) gradient elution: 0–18 min, 10–30% A (v/v); 18–30 min, 30–40% A (v/v); 30–40 min, 40–68% A (v/v); 40–50 min, 68–75%A (v/v); 50–60 min, 75–60%A (v/v); flow rate, 1.0 mL/min; column temperature, 30 °C; detection, 270 nm; Injection volume, 10 μL; HPLC grade acetonitrile (Fisher, Beijing, China) and phosphoric acid (Kermel, Tianjin, China) were used in the this study. Each compounds were confirmed by comparing the retention time with the standard substances, which were purchased from the National Institutes for Food and Drug Control (Beijing, China) under the identical HPLC condition. The standard curve of each component is listed in Table 2.

**Table 1** Primers applied in the qRT-PCR assay

| Oligo name | Forward (5′–3′) |
|---|---|
| *PAL1-F* | ccg agc agc aca acc agg atg |
| *PAL1-R* | acg agg tag gtg gac gac atg ag |
| *TAT-F* | cgc cga cta cca tca cca tta agg |
| *TAT-R* | gca gag cct cca caa cac ctt c |
| *HPPR-F* | cgg aag acg tcg cgg att tg |
| *HPPR-R* | atc gaa tgc ctc tgc tcg ct |
| *4CL1-F* | cag caa gtg gac ggc gag aat c |
| *4CL1-R* | gac acg cag aca gca gag cat c |
| *RAS-F* | ctc cgc tct cca ctt cat caa cac |
| *RAS-R* | agt agg cga tct cgg tgt ctt gg |
| *CYP98A14-F* | tgg cta agg agg tgc tga agg ag |
| *CYP98A14-R* | gga gaa gag gac gac ggt aca gac |
| *HMGR-F* | cct cac caa cgg agt ctt ctt cac |
| *HMGR-R* | acg gaa ttg cgg atc ttc tca cg |
| *DXR-F* | cgg cac aac ctc ctc ctc ctc |
| *DXR-R* | cag caa cta tgt cca gcg tct gag |
| *IPPI-F* | aat gtc gtc ctt gac cag cat cc |
| *IPPI-R* | tag cgg cgg tga atg aag cg |
| *GGPPS-F* | aga gag acg atg cgc cct tc |
| *GGPPS-R* | tca tcg gcg gat tcc tga cc |
| *CPS-F* | gca tga cca cga cgg cac tac |
| *CPS-R* | gta cgg cga cac gct tat tct cc |
| *KSL-F* | agc cag ccg cag aat atg gt |
| *KSL-R* | tag tgc cgt gtc atc gct cc |
| *CYP76AH1-F* | gag cag atg ttc tcc aac cag agc |
| *CYP76AH1-R* | gag gtt gag cgt ggt gat gaa gg |
| *β*-actin-F | ggt gcc ctg agg tcc tgt t |
| *β*-actin-R | agg aac cac cga tcc aga ca |

## Data analysis

Graphics were produced by the OriginPro software version 9.3. Significance analysis were determined by analysis of variance (ANOVA) using the "Statistical Package" for Social Sciences program (SPSS 16.0, SPSS Inc. USA). All the data were expressed as mean ± standard deviation (SD) of three replicates.

**Table 2** Standard curve of the five component

| Composition | Regressive equations | *r* | Linear range (μg) |
|---|---|---|---|
| Caffeic acid | $Y = 2{,}041{,}120.6980X - 5800.7784$ | 0.9992 | 0.207–4.14 |
| Rosmarinic acid | $Y = 910{,}946.7415X - 34041.8643$ | 0.9998 | 0.652–13.04 |
| Salvianolic acid B | $Y = 911{,}600.3611X - 2410.1821$ | 0.9997 | 0.243–4.86 |
| Cryptotanshinone | $Y = 2{,}910{,}393.1200X + 1549.4656$ | 0.9996 | 0.256–5.12 |
| Tanshinone IIA | $Y = 2{,}007{,}944.9640X - 2090.4656$ | 0.9997 | 0.396–7.92 |

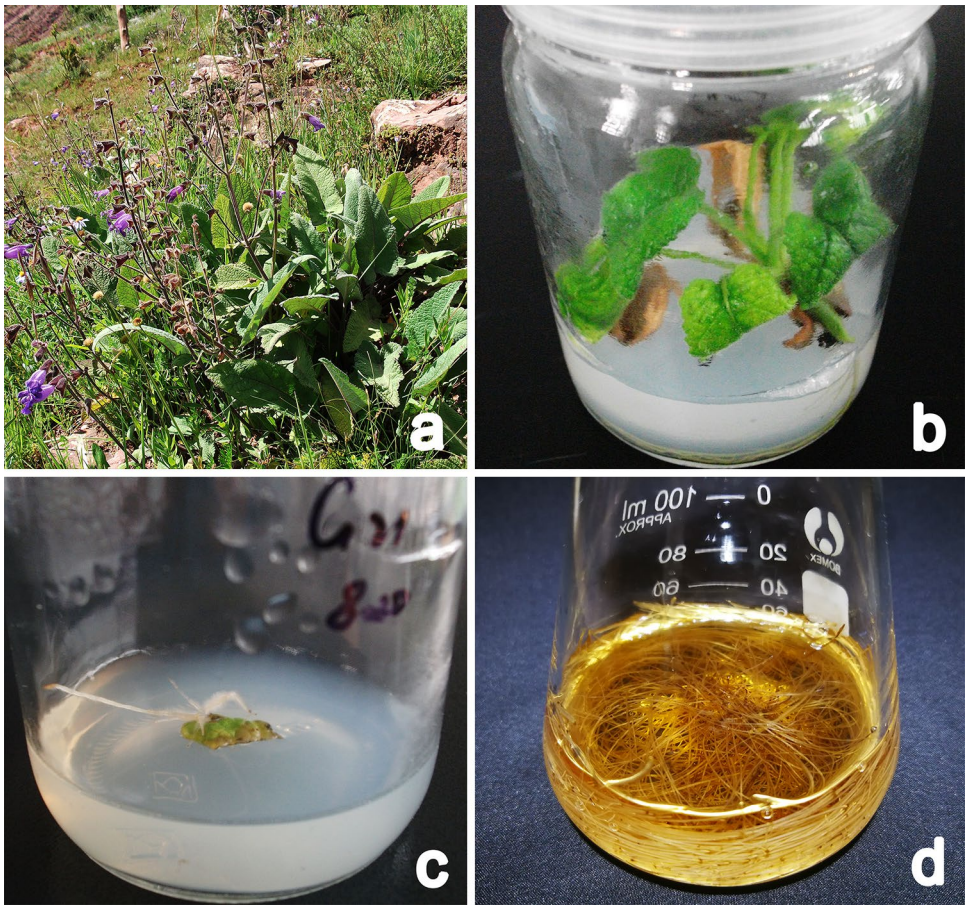



**Fig. 3** Hairy Root Induction of *S. przewalskii*. **a** Original plant of *Salvia przewalskii* Maxim. was cultivated in the field; **b** aseptic seedlings was cultured on MS solid medium; **c** hairy root induction was implemented on medium; **d** hairy root line of *Salvia przewalskii* Maxim. was cultured in 6,7-liquid medium

## Results

### Induction and confirmation of hairy root cultures

As shown in Fig. 3, the leaf explants of *S. przewalskii* Maxim. responded to induction of *Agrobacteriom rhizogenes* ATCC 15,834 and formed hairy roots finally. About 400 bp band for *rolB* and 600 bp band for *rolC* were obtained from the putative hairy root lines, while no band was detected in the root sample of nontransformed contral plant (Fig. 4). Thus, *rolB* and *rolC* genes in the plasmid (pRi) of the bacteria were integrated into the explants successfully and induced hairy roots of *S. przewalskii* completely.

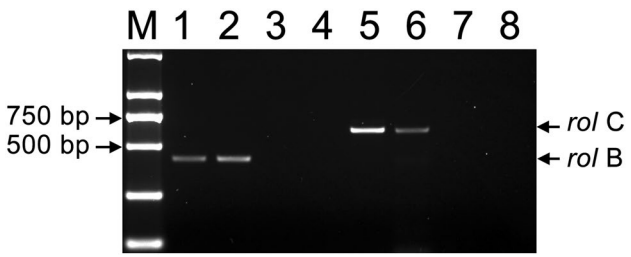



**Fig. 4** rolB and rolC gene fragments of putative hairy root lines amplified by PCR. Lane M: D2000 DNA marker (Tiangen, Beijing), lane 1, 5: Hairy root sample 1 of *S. preziwalskii*, lane 2, 6: Hairy root sample 2 of *S. przewalskii*, lane 3, 7: root sample 1 of nontransformed control plant, lane 4, 8: root sample 2 of nontransformed control plant

## Effect of elicitor concentration on hairy root cultures

The effect of different concentrations of SA and MeJA on *S. przewalskii* hairy root is shown in Figs. 5 and 6. SA and MeJA affect the growth and the production of phenolic acid and tanshinone in *S. przewalskii* hairy root. As shown in Table 3, fresh weight and dry weight increased initially and then decreased with increasing concentrations of SA. SA (50 µM) markedly promoted the fresh weight and dry weight of hairy root with 61.69% and 7.17% increment, respectively. As shown in Fig. 7a, the addition of 50 µM SA promoted the content of caffeic acid, rosmarinic acid, salvianolic acid B, cryptotanshinone, and tanshinone IIA by 1.82-, 1.41-, 2.67-, 1.38-, and 2.61-fold, respectively. Thus, 50 µM SA was appropriate for *S. przewalskii* hairy root growth and the accumulation of chemical components.

As shown in Table 3, treatment with MeJA promoted the fresh weight and dry weight of hairy root culture with increasing concentrations, but it decreased when then concentration was 600 MeJA. Treatment with 400 µM enhanced the fresh weight and dry weight of hairy root with 64.92% and 105.68% increment, respectively. As shown in Fig. 7b, addition of 400 µM MeJA increased caffeic acid, rosmarinic acid, salvianolic acid B, cryptotanshinone and tanshinone IIA contents significantly by 1.78-, 1.27-, 8.14-, 3.00- and 11.04-fold, respectively. Thus, 400 µM MeJA was the

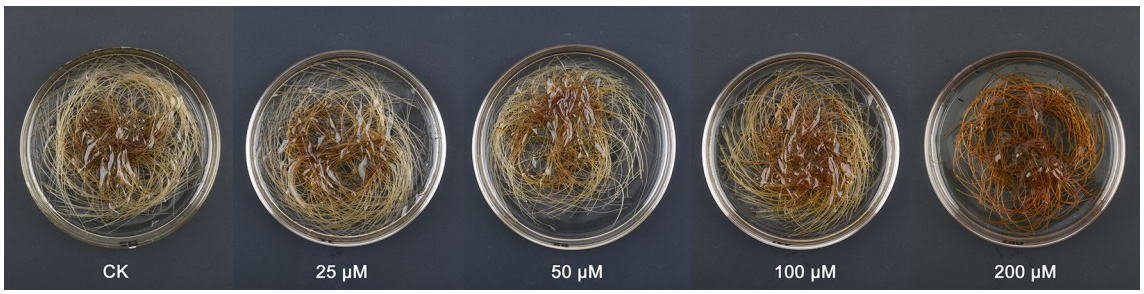



**Fig. 5** Different concentrations of SA on *S. przewalskii* hairy root cultures

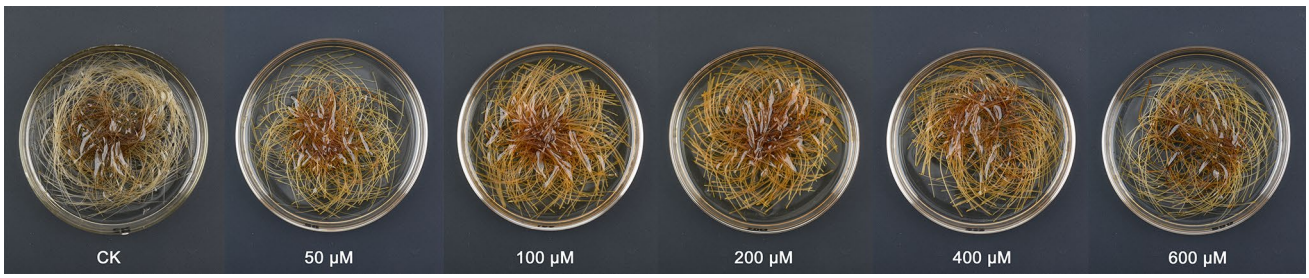



**Fig. 6** Different concentrations of MeJA on *S. przewalskii* hairy root cultures

**Table 3** Fresh and dry weights of hairy roots treated by different concentrations of elicitors

| | Elicitors | A | B | C | D | E | F |
|---|---|---|---|---|---|---|---|
| Fresh weight (g) | SA | 1.5780 ± 0.23d | 1.6649 ± 0.15c | 2.5515 ± 0.33a | 1.9841 ± 0.33b | 1.3217 ± 0.61e | |
| | MeJA | 1.5780 ± 0.23e | 1.6360 ± 0.44d | 1.8334 ± 0.53c | 2.0860 ± 0.23b | 2.6024 ± 0.62a | 1.7239 ± 0.21cd |
| Dry weight (g) | SA | 0.1091 ± 0.02d | 0.1385 ± 0.00c | 0.1886 ± 0.01a | 0.1483 ± 0.03b | 0.1057 ± 0.04e | |
| | MeJA | 0.1091 ± 0.02f | 0.1222 ± 0.01e | 0.1549 ± 0.04d | 0.1768 ± 0.02c | 0.2244 ± 0.04a | 0.1916 ± 0.02b |
| Dry rate (%) | SA | 7.0603 ± 1.67e | 8.3533 ± 0.60a | 7.4430 ± 0.57d | 7.4624 ± 0.16c | 8.1176 ± 0.46b | |
| | MeJA | 7.0603 ± 1.67d | 7.7208 ± 1.44c | 8.4778 ± 0.30b | 8.4951 ± 0.41b | 8.6769 ± 0.37b | 11.1442 ± 0.37a |

The values are mean of three replicates followed by standard deviation (±SD). The lowercase letters represent significance at 0.05 level ($P < 0.05$)

A, Control, hairy root of *S. przewalskii* without elicitation; B, 25 µM SA and 50 µM MeJA; C, 50 µM SA and 100 µM MeJA; D, 100 µM SA and 200 µM MeJA; E, 200 µM SA and 400 µM MeJA respectively; F, 600 µM MeJA

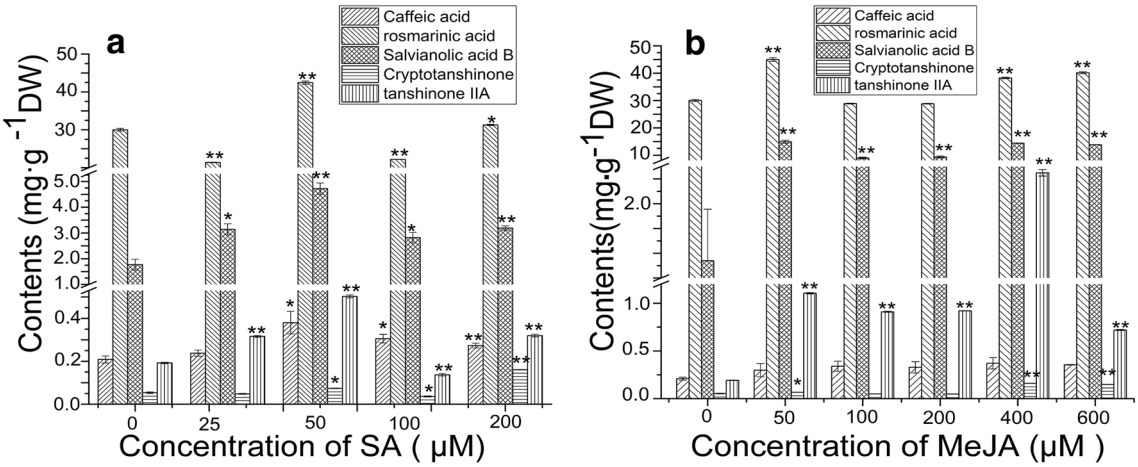



**Fig. 7** Effects of different concentration of elicitors on phenolic acid and tanshinone accumulation in the hairy root cultures of *S. przewalskii*. The hairy root without elicitation was used as control. *$P<0.05$, **$P<0.01$

optimal concentration for *S. przewalskii* hairy root growth and effective components accumulation.

## Effect of induction time on hairy root cultures

In Table 4, the fresh weight and dry weight of hairy root at different culture time were listed. Fresh weight showed a gradual increase in response to treatment with SA (50 μM) and MeJA (400 μM) within 6 days. SA inhibited caffeic acid content within 12 h, and then showed a significant enhancement at 24 h. Finally, a 0.07-fold ($0.2506 \pm 0.03$ mg·g$^{-1}$ DW) increase of caffeic acid was acquired at 6 days compared with the control after SA treatment (Fig. 8a). Caffeic acid accumulation showed a gradually increase and reached its maximum ($0.3071 \pm 0.01$ mg·g$^{-1}$ DW) at 6 days after the addition of 400 μM MeJA (Fig. 8a).

The amount of rosmarinic acid increased after treatment with SA and MeJA for 12 h (Fig. 8b). The highest concentration of rosmarinic acid was observed at three days after SA ($44.0306 \pm 0.08$ mg·g$^{-1}$ DW) and MeJA ($67.1273 \pm 0.41$ mg g$^{-1}$ DW) treatment; however, it decreased significantly on the sixth day. Therefore, SA and MeJA consistently enhanced rosmarinic acid accumulation in the hairy root cultures of *S. przewalskii* during the first three days.

The contents of salvianolic acid B showed a significant increase during the first three days after SA treatment

**Table 4** Fresh and dry weights of hairy roots treated by optimum concentrations of elicitors in time courses

| Elicitors | Day | | | | |
|---|---|---|---|---|---|
| | 0 | 0.5 | 1 | 3 | 6 |
| Fresh weight (g) | | | | | |
| Control[a] | 1.2486 ± 0.06 | 1.3146 ± 0.28b | 1.4438 ± 0.25b | 1.6098 ± 0.11a | 1.6980 ± 0.14c |
| SA[b] | 1.2486 ± 0.06 | 1.3299 ± 0.05a | 1.4545 ± 0.14a | 1.5616 ± 0.14b | 1.8392 ± 0.05a |
| MeJA[c] | 1.2486 ± 0.06 | 1.2643 ± 0.24c | 1.4186 ± 0.03c | 1.4516 ± 0.24c | 1.8362 ± 0.05b |
| Dry weight (g) | | | | | |
| Control | 0.0907 ± 0.01 | 0.1013 ± 0.01a | 0.1119 ± 0.01a | 0.1139 ± 0.01a | 0.1205 ± 0.00c |
| SA | 0.0907 ± 0.01 | 0.0923 ± 0.00b | 0.1111 ± 0.01b | 0.1109 ± 0.00b | 0.1313 ± 0.00a |
| MeJA | 0.0907 ± 0.01 | 0.0915 ± 0.01c | 0.1027 ± 0.02c | 0.1109 ± 0.01b | 0.1237 ± 0.00b |
| Dry rate (%) | | | | | |
| Control | 7.2887 ± 1.03 | 7.9669 ± 1.56a | 7.8974 ± 1.15a | 7.0961 ± 0.58c | 7.1345 ± 0.58b |
| SA | 7.2887 ± 1.03 | 6.9470 ± 0.39c | 7.7324 ± 1.35b | 7.1555 ± 0.79b | 7.1374 ± 0.14a |
| MeJA | 7.2887 ± 1.03 | 7.3690 ± 1.15b | 7.2543 ± 1.22c | 7.8564 ± 1.75a | 6.7375 ± 0.24c |

The values are mean of three replicates followed by standard deviation (±SD). The lowercase letters represent significance at 0.05 level ($P<0.05$)

a, control, hairy root of *S. przewalskii* without elicitation; b, 50 μM SA; c, 400 μM MeJA

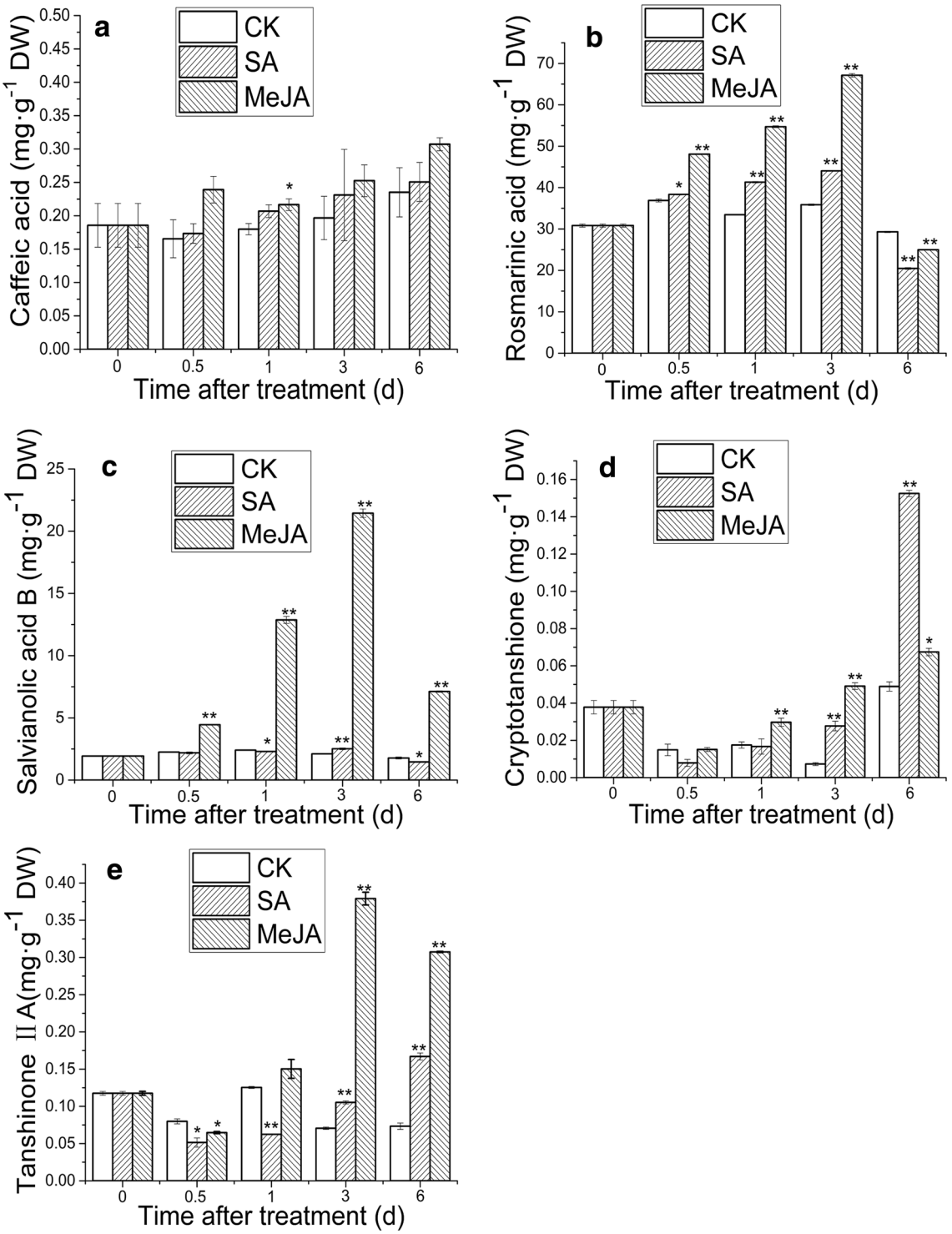



**Fig. 8** Phenolic acid and tanshinone accumulation in the hairy root cultures of *S. przewalskii* by SA and MeJA treatment. SA concentration, 50 μM; MeJA concentration, 400 μM; CK, the hairy root without elicitation. $^{*}P < 0.05$, $^{**}P < 0.01$

and reached its maximum ($2.5138 \pm 0.07$ mg $g^{-1}$ DW) at three days, yet a sharp decrease was observed at six days. After the addition of 400 μM MeJA, the concentration of salvianolic acid B increased at first then decreased, and reached its maximum ($21.4448 \pm 0.34$ mg $g^{-1}$ DW) at three days after treatment (Fig. 8c).

50 μM SA treatment resulted in an inhibition of cryptotanshione and tanshinone IIA concentrations during the first 3 days, while a sharp increment was observed at 6 day. The treatment with 400 μM MeJA led to a short inhibition of cryptotanshione and tanshinone IIA concentrations at first, while a sharp increase appeared, the concentration of cryptotanshione reached its maximum ($0.0674 \pm 0.00$ mg $g^{-1}$ DW) at 6 day after treatment (Fig. 8d), while the concentration of tanshinone IIA reached its maximum ($0.3791 \pm 0.00$ mg $g^{-1}$ DW) at 3 day after treatment and slightly decreased after six days (Fig. 8e).

### Effect of elicitors on expression of genes involved in the phenolic acid biosynthetic pathway

*PAL* expression levels showed a significant increase and reached its maximum on the sixth day after treatment with 50 μM SA, which was a 7.3-fold increase as compared with the control (Fig. 9a). *4CL1* expression levels showed a slight inhibition at 12 h after treatment with 50 μM SA, and then gradually increased and reached its maximum at 6 days after treatment, and the maximum increment of 4.2-fold was acquired after three days as compared with the control (Fig. 9b). SA promoted the expression levels of *TAT* and *HPPR* and reached the maximum expression at 3 day after the addition of the elicitor, which resulted in a 4.1- and 4.9-fold increase compared with control, respectively (Fig. 9c, d). SA had a significant inhibitory effect on the expression levels of *RAS* during the first three days, while a promotional effect was shown on the sixth day, which was 1.4-fold higher than that of the control (Fig. 9e). *CYP98A14* expression levels increased during the first three days and reached its maximum of 2.8-fold at 3 day after treatment with SA, while it showed a relative decrease at 6 day after the treatment (Fig. 9f).

Elicitation with 400 μM MeJA led to significant inhibition of *PAL* expression levels at 12 h, then it showed a moderate promotion within 3 days and reached its maximum of a 5.4-fold increase compared with the control, yet it showed a slight inhibition after six days (Fig. 9a). *4CL1, TAT,* and *RAS* responded to MeJA elicitation sensitively at 12 h, about 29.8-, 4.2-, and 3.3-fold higher expression levels than that of the control were observed, respectively, and then showed a gradual decrease (Fig. 9b, c, e). *HPPR* expression levels were promoted after the addition of MeJA, which showed a relatively higher level of 7.5-fold after three days compared with the control (Fig. 9d). *CYP98A14* expression levels increased during the first three days and reached its maximum of 1.7-fold at 3 day after treatment with MeJA, and then it showed a slight inhibition compared with the control at 6 day (Fig. 9f).

### Effect of elicitors on genes expression of tanshinone biosynthetic pathway

SA (50 μM) caused enhancement of *HMGR* expression levels and reached its maximum of 17.3-fold higher level at three days (Fig. 10a). *DXR, IPPI, GGPPS, KSL,* and *CYP76AH1* expression levels showed a gradual increase and reached the maximums at 6 day. *DXR* and *KSL* indicated a 5.3- and 12.1-fold increase on 6 day, respectively (Fig. 10b, f). The expression levels of *IPPI* and *CYP76AH1* showed 10.1- and 3.9-fold increments compared with the control at 3 day, respectively (Fig. 10c, g). *GGPPS* indicated an 11.7-fold increase compared with the control after 12 h (Fig. 10d). *CPS* expression levels were significantly inhibited within first three days and then showed a sharp enhancement on 6 day, which was 9.3-fold higher than that of the control (Fig. 10e).

After the addition of 400 μM MeJA, *HMGR* expression levels were slightly inhibited (Fig. 10a). *DXR* expression levels were slightly inhibited at 12 h, then showed a slight promotion within 3 days. After 6 days of treatment, *DXR* expression levels were slightly inhibited again (Fig. 10b). *IPPI* expression levels gradually increased during the first three days and then slightly decreased on the sixth day, with a relatively higher level of 17.2-fold compared with the control on 3 days (Fig. 10c). *GGPPS* expression levels were slightly inhibited at 12 h, and then showed a gradual increase and reached its maximum at 6 day, which was 1.2-fold higher than that of the control (Fig. 10d). *CPS* expression levels showed significant inhibition at 24 h, while increased and reached 5.9-fold higher levels than controls at 6 day (Fig. 10e). 400 μM MeJA caused the promotion of *KSL* expression levels and reached its maximum at 3 day, which was 13.6-fold higher than that of the control (Fig. 10f). *CYP76A* expression levels showed a gradual increase and reached the maximum at 6 day, which was 1.5-fold higher than that of the control (Fig. 10g).

## Discussion

Generally, hairy root is obtained by infection of plants with *Agrobacteriom rhizogenes* and it is characterized by a high growth rate and genetic stability. Compared with the original plants, the hairy root can produce higher levels and more valuable secondary metabolites, which could be used as the continuous resource for the practical production [39, 40]. In the present study, the application of hairy root cultures to produce the active substances required in cosmetics or pharmaceuticals is reported, such as peanut [41], *Catharanthus roseus* [42], *Valeriana wallichii* DC [43], *Atropa belladonna* [44] and *S. miltiorrhiza* [45].

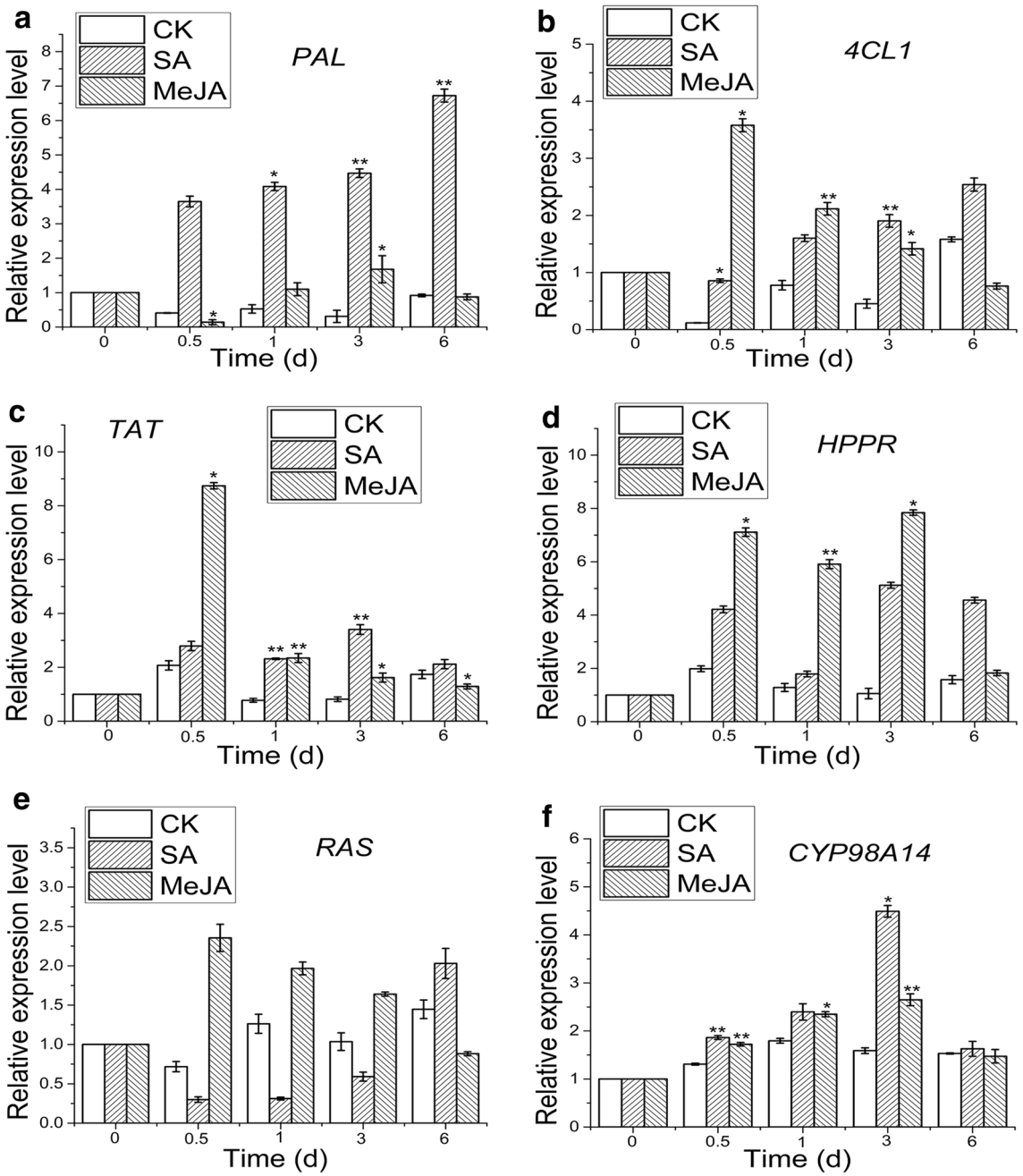



**Fig. 9** Relative expression levels of phenolic acid biosynthetic related genes in the hairy root culture of *S. przewalskii* by SA and MeJA treatment. SA concentration, 50 μM; MeJA concentration 400 μM; CK, the hairy root without elicitation. Gene expressions of the control at the initial treatment (0 day) were as relative 1. $*P<0.05$, $**P<0.01$

SA and MeJA, the effective elicitors, have been widely used to regulate growth and increase the accumulation of secondary metabolites in the medicinal plants. For instance, SA and MeJA have been confirmed to improve the content of total phenols in *Bletilla striata* seedings [46]. MeJA also promotes total flavonoid and phenolic contents of the callus of *Phyllanthus pulcher* [47]. SA increases the accumulation of phenolic acids in *S. miltiorrhiza* cell culture [32]. In this study, SA and MeJA were used to induce the hairy root growth and secondary metabolites of *S. przewalskii*.

Elicitation with SA is known to inhibit the growth of the hairy root cultures of *Silybum marianum* [48]. In this study, however, low concentrations of SA promoted the growth of hairy roots of *S. przewalskii*. a relative high concentration of SA (200 μM) had an inhibitory effect on the growth of the hairy root. This may be due to the variable tolerance of

the plant species. In addition, SA is known to enhance the production of withanolide A, withanone, and withaferin A in the hairy root cultures of *Withania somnifera* (L.) Dunal [49]. In this study, the remarkable accumulation of caffeic acid, rosmarinic acid, salvianolic acid B, and tanshinone IIA were observed by using 50 μM SA treatment.

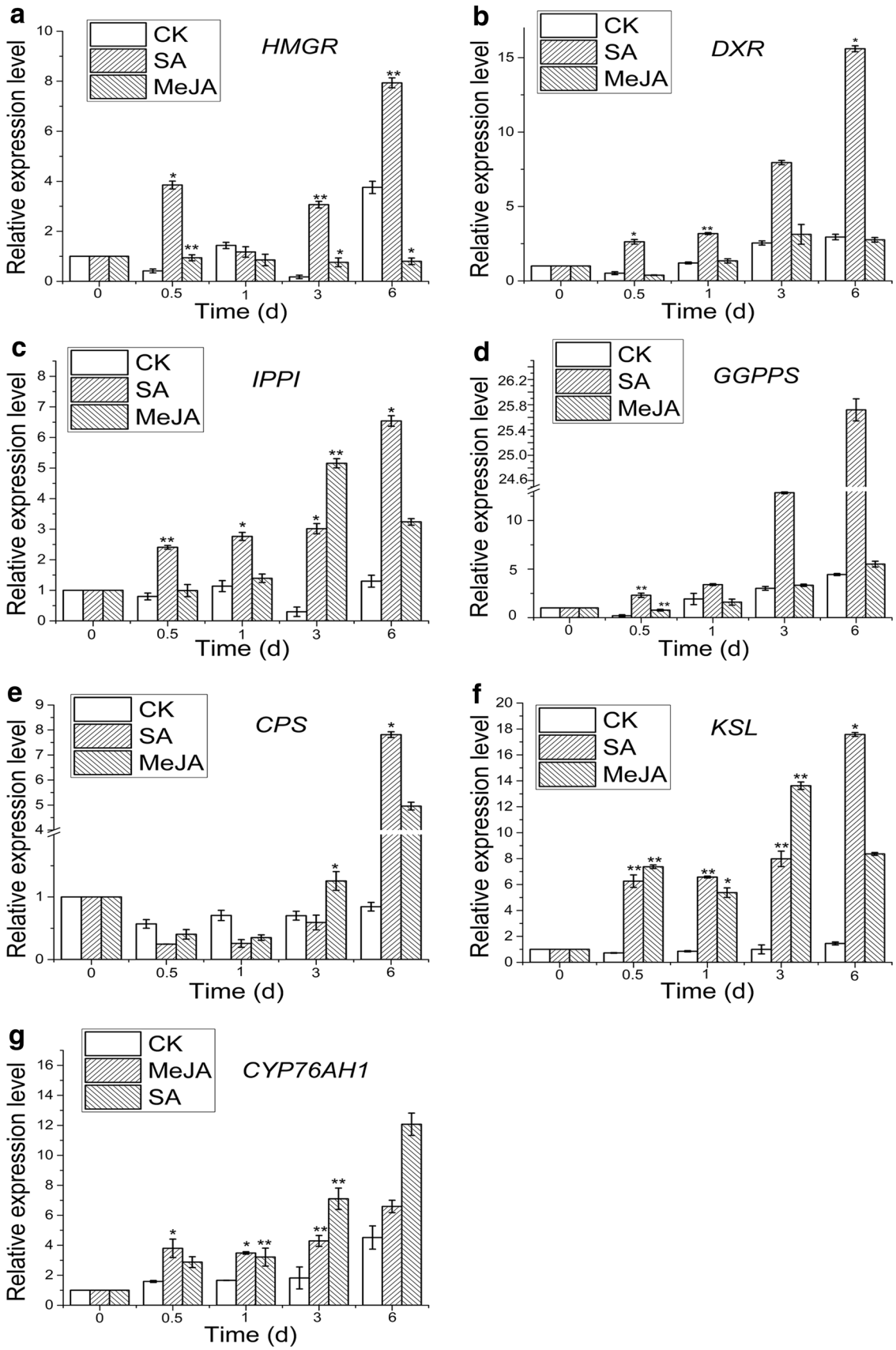
According to a previous report, MeJA was found to be the most effective elicitor for astragaloside biosynthesis of *Astragalus membranceus* hairy root [50], and 100 μM MeJA could enhance the massive accumulation of anthraquinones in the hairy root culture of *Rubia tinctorum* [51]. Additionally, MeJA was found to improve the fresh weight and tanshinone IIA contents of hairy root cultures of *S. castanea* Diels f. *tomentosa* Stib [34]. In our study, we achieved a similar result with variable concentrations of MeJA treatment, which promoted the growth of hairy roots, and the promotion effect was stronger in pace with increasing elicitor concentrations. When the concentration of MeJA was 400 μM, the growth rate reached its maximum. Furthermore, the content of caffeic acid, salvianolic acid B, cryptotanshinone, and tanshinone IIA reached the highest value under the same conditions.

After addition 50 μM SA, the content of caffeic acid, cryptotanshione and tanshinone IIA reached there maximum at 6 days, rosmarinic acid and salvianolic acid B reached the highest values at 3 days. After addition 400 μM MeJA, the content of caffeic acid and cryptotanshione reached there maximum at 6 days, tanshinone IIA, rosmarinic acid and salvianolic acid B reached the highest values at 3 days. The experimental results show that in the treatment group, the chemical components accumulated under the optimal time node are higher than those in the control group. In general, 400 μM MeJA has a greater effect on the accumulation of phenolic acids than 50 μM SA, but 50 μM SA has a greater effect on the accumulation of tanshinones than 400 μM MeJA. Therefore, 50 μM SA can be used to accumulate of tanshinone in hairy roots of *S. przewalskii* with 6 days. 400 μM MeJA can be used to accumulate of phenolic acids in hairy roots of *S. przewalskii* with 3 days.

*PAL* and *TAT* are the key genes at the early stages of two parallel phenolic acids biosynthetic pathways, and the expression of *PAL* is related to the biosynthesis of caffeic acid, lignin, anthocyanidin, and other phenolic compounds [52]. *4CL1* and *HPPR* play key roles in the middle stages of the phenolic acid biosynthetic pathway. *RAS* and *CYP98A14* are the rate-limiting enzymes in the final stages of the rosmarinic acid biosynthetic pathway [23, 24]. In this study, the expression levels of rate-limiting enzyme *CYP98A14* were increased on 3 day with 50 μM SA and 400 μM MeJA treatments respectively, meanwhile, the content of rosmarinic acid was also observed to reach the maximum.

In the two biosynthetic pathways of tanshinone, HMGR is a crucial enzyme in the early MVA pathway, and DXR is a key enzyme in the early DXP pathway. *IPPI* and *GGPPS* are also critical genes in the middle stages of the tanshinone biosynthetic pathway. *CPS*, *KSL,* and *CYP76AH1* are the rate-limiting enzymes in the downstream of tanshinone biosynthetic pathway [26–28]. In our study, with 50 μM SA treatment, *HMGR*, *DXR*, *IPPI*, *GGPPS*, *CPS*, *KSL,* and *CYP76AH1* gene expression levels were higher at the end of the treatment period, and the most striking increments of cryptotanshinone and tanshinone IIA were also observed. These genes are involved in tanshinone biosynthetic pathway of *S. przewalskii* hairy root cultures. With the application of 400 μM MeJA in the hairy root culture, the expression levels of *CPS* and *CYP76AH1* were enhanced at the end of the treatment stage, while *KSL* expression level was enhanced at the mid-term of the hairy culture. Additionally, cryptotanshinone content achieved the maximum at the end of the treatment stage, while the accumulation of tanshinone IIA was found markedly at the mid-term period.

As an effective tool for regulating plant secondary metabolites, elicitors have been widely used in the regulation of secondary metabolites in *S. miltiorrhiza*. However, the effects of elicitors on the synthesis of secondary metabolites in the hairy roots of *S. przewalskii* are rarely reported. In this work, the growth, phenolic acids and tanshinones accumulation of hairy root treated by different concentration of SA and MeJA were studied. It is conclude that low concentration of SA promoted the growth of hairy root while high concentration inhibited. MeJA promoted the growth of hairy root. 50 μM SA and 400 μM MeJA significantly enhanced the production of caffeic acid, rosmarinic acid, salvianolic acid B, cryptotanshinone and tanshinone IIA. The selected genes in the tanshinone and phenolic acids biosynthetic pathways were remarkably upregulated with the elicitation. This study provide a reference for the selection of elicitors to improve the industrial production of phenolic acids and tanshinone in the hairy root culture of *S. przewalskii*.


a
HMGR
CK
SA
MeJA
Relative expression level
Time (d)
b
DXR
c
IPPI
d
GGPPS
e
CPS
f
KSL
g
CYP76AH1

◂**Fig. 10** Relative expression levels of tanshinone biosynthetic related genes in the hairy root culture of *S. przewalskii* by SA and MeJA treatment. SA concentration, 50 μM; MeJA concentration 400 μM; CK, the hairy root without elicitation. Gene expressions of the control at the initial treatment (0 day) were as relative 1. $*P<0.05$, $**P<0.01$

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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