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ORIGINAL ARTICLE

# Establishment of *Salvia castanea* Diels f. *tomentosa* Stib. hairy root cultures and the promotion of tanshinone accumulation and gene expression with $Ag^+$, methyl jasmonate, and yeast extract elicitation

**Bo Li · Bangqing Wang · Hongyan Li · Liang Peng · Mei Ru · Zongsuo Liang · Xijun Yan · Yonghong Zhu**

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**Abstract** *Salvia castanea* Diels f. *tomentosa* Stib. is an endemic medicinal plant distributed in China, and the notably high content of tanshinone IIA in the root is proven effective for the therapy of heart diseases. Hairy root induction of this *Salvia* species was inoculated with *Agrobacterium rhizogenes* strain ATCC 15834. Transformed hairy root was cultured in 6, 7-V liquid medium for growth kinetics assessment and elicitation. An S curve was present in the hairy root cultures based on the fresh and dry weights with an interval of 3 days. An optimum concentration of the applied elicitors (15 μM $Ag^+$, 200 μM methyl jasmonate, and 200 mg $l^{-1}$ yeast extract elicitor) benefitted both the growth status and tanshinone accumulation in the hairy root cultures. Tanshinone IIA contents were mostly stimulated 1.8-fold and 1.99-fold compared with the control by $Ag^+$ and methyl jasmonate elicitation, respectively. Yeast extract dramatically enhanced dry mass accumulation, while it promoted cryptotanshinone content of 2.84± 0.33 mg $g^{-1}$ dry weight at most in the hairy root cultures. Selected elicitors diversely influenced tanshinone accumulation in the time courses of hairy root cultures within 7 days. Furthermore, transcripts of selected genes in the tanshinone biosynthetic pathway were remarkably upregulated with elicitation. Yeast extract elicitor heightened 13.9-fold of isopentenyl diphosphate isomerase expression level at 12 h, while it increased 16.7-fold of geranylgeranyl diphosphate synthase transcript at 24 h compared with that of the control, which was more effective than $Ag^+$ and methyl jasmonate. This study provided a convenient hairy root culture system of *S. castanea* Diels f. *tomentosa* Stib. for tanshinone production for the first time.

**Keywords** *Salvia castanea* Diels f. *tomentosa* Stib. · Hairy root cultures · Elicitors · Tanshinone · Gene expression

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B. Li · B. Wang · H. Li · L. Peng · Z. Liang
College of Life Sciences, Northwest A&F University, 712100 Yangling, People's Republic of China

B. Wang
Hanzhong Institute of Botany, 723000 Hanzhong, People's Republic of China

L. Peng
College of Pharmacy, Shaanxi University of Chinese Medicine, 712046 Xi'an, People's Republic of China

M. Ru
Institute of Soil and Water Conservation, Chinese Academy of Sciences, 712100 Yangling, People's Republic of China

Z. Liang (✉)
College of Life Sciences, Zhejiang Sci-Tech University, 310018 Hangzhou, People's Republic of China
e-mail: liangzs@ms.iswc.ac.cn

X. Yan · Y. Zhu
Tianjin Tasly Holding Group Co., Ltd., 300410 Tianjin, People's Republic of China

## Introduction

*Salvia castanea* Diels f. *tomentosa* Stib. is an endemic medicinal plant with a folk name of "Linzhi Danshen" in China. The wild type of this species is exclusively distributed in southwestern China, especially in Nyingchi Prefecture, Tibet Autonomous Region at an elevation of 2500 to 3750 m (Wu and

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Li 1977; Ye et al. 2004). *S. castanea* Diels f. *tomentosa* Stib. acts as an ethnomedicinal herb for the therapy of cardiovascular and cerebrovascular diseases for a long time, which plays a consistent purpose with *Salvia miltiorrhiza* Bunge, a well-known traditional Chinese medicine commonly called "Danshen" in China (Cheng et al. 2005; Ye et al. 2004). Belonging to the *Salvia* genus, two groups of effective components are found in *S. miltiorrhiza*, the water-soluble phenolic acids and lipid-soluble tanshinones (Wang et al. 2007). Many other *Salvia* species in Mainland China are also proven rich in tanshinones, such as *S. castanea* Diels f. *tomentosa* Stib., *Salvia przewalskii*, *Salvia yunnanensis*, etc. (Li et al. 2010). Dihydrotanshinone (DT), cryptotanshinone (CT), tanshinone I (T-I), and tanshinone IIA (T-IIA) are the major components of lipid-soluble constituents used for their pharmaceutical purpose. In a previous study, the roots of *S. castanea* Diels f. *tomentosa* Stib. accumulate large amounts of tanshinones (Yang et al. 2009). Several sesquiterpenoids in the aerial parts of *S. castanea* Diels f. *tomentosa* Stib. showed weak inhibition against MCF-7, HeLa, and HepG2 cell lines (Xu et al. 2008).

Tanshinones are a group of diterpenoids with an abietane skeleton present in many *Salvia* species (Li et al. 2010). Biosynthesis of tanshinones in *S. miltiorrhiza* is proven from two pathways: the mevalonate (MVA) pathway which occurred in the cytoplasm and 1-deoxy-D-xylulose 5-phosphate (DXP) pathway located in plastids with some crosstalk and synergistic reaction (Fig. A1) (Laule et al. 2003; Liao et al. 2009). A series of genes involved in tanshinone biosynthetic pathway has been cloned and its function identified, such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), which is the initial and rate-limiting enzyme in the MVA pathway (Liao et al. 2009); 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) plays a key role in the committed steps of the DXP pathway (Yan et al. 2009). Isopentenyl diphosphate isomerase (IPPI) and geranylgeranyl diphosphate synthase (GGPPS) are also important in the midstream of the whole pathway (Kai et al. 2010; Ma et al. 2015). Nevertheless, tanshinone biosynthetic pathway that existed in *S. castanea* Diels f. *tomentosa* Stib. has not been mentioned. Due to the chemotypic homogeneity, a putative coincident metabolic pathway for tanshinone biosynthesis is proposed in both *S. castanea* Diels f. *tomentosa* Stib. and *S. miltiorrhiza*, which are two closely related species of the *Salvia* genus.

Hairy root cultures provide a rapid and sustainable way to obtain secondary metabolites which are precious and trace amounts in the parent plants; this is a traditional approach for the development of the medicinal plants with pharmacological interests. Meanwhile, it is evidently a practical gene transfer system for the study of gene function (Guillon et al. 2006). Hairy root is initiated by infection with *Agrobacterium rhizogenes*, a Gram-negative bacterium that contained a special root-inducing plasmid (pRi), infecting explants by inserting T-DNA into the genomic DNA of the target plant (Thiruvengadam et al. 2014). Wounded sites on different explants are potentially induced for hairy root derivation by coculturing with *A. rhizogenes* (Giri and Narasu 2000). Various hairy root inductions of *Salvia* species have been achieved in a previous study. Hairy root of *Salvia officinalis* induced by *A. rhizogenes* strain ATCC 15834 showed fine growth status and high content of rosmarinic acid (Grzegorczyk et al. 2006); the contents of diterpenoids and triterpenoids in the hairy root culture of *Salvia sclarea* were also investigated by transformation with *A. rhizogenes* LBA 9402 (Kuźma et al. 2006). However, hairy root cultures of *S. castanea* Diels f. *tomentosa* Stib. had not been established in a previous study. For the high contents of tanshinones accumulated in the roots, hairy root induction of this plant will provide an effective way to gain large-scale production of tanshinones in vitro.

Elicitors remarkably stimulate and increase both secondary metabolite accumulation and growth of the hairy root cultures (Cheruvathur and Thomas 2014). Elicitors mainly take effect by inducing defense responses and being involved in both extracellular and intracellular signal transduction of plant tissues (Goel et al. 2011; Vasconsuelo and Boland 2007). Two groups of elicitors are commonly applied in hairy root cultures, which are the biotic (yeast extract elicitor, fungal elicitor, etc.) and abiotic (UV, $Ag^+$, etc.) elicitors (Zhao et al. 2010); signal compounds (methyl jasmonate, salicylic acid, etc.) are also widely used in hairy root elicitation (Memelink 2009). Methyl jasmonate (MeJA) is a signal molecule applied for enhancing the production of secondary metabolites in several hairy root cultures (Aftab et al. 2011; Shukla et al. 2010). Yeast extract elicitor (YE) is mainly composed of polysaccharide obtained by steps of water extraction and alcohol precipitation, increases both intracellular and extracellular CT content, and improves the growth of *S. miltiorrhiza* hairy root (Chen et al. 2001). It is affirmed that both tanshinone content and related enzyme activity are enhanced by $Ag^+$ and YE in hairy root cultures of *S. miltiorrhiza*, respectively (Ge and Wu 2005). Furthermore, MeJA applied in the cultures of *S. sclarea* hairy root in a sprinkle bioreactor dramatically enhanced the production of effective diterpenoids (Kuźma et al. 2009). Tanshinone accumulation and expressions of tanshinone biosynthetic related genes of *S. miltiorrhiza* hairy root cultures exposed to MeJA, $Ag^+$, and YE elicitors are also evaluated (Kai et al. 2012). However, the influences of elicitors on the growth and accumulation of secondary metabolites of *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures have not been investigated.

For the huge market demand of tanshinones, the crude drug shortage of Danshen, and long growth period of the original plant of *S. miltiorrhiza*, a large amount of Danshen roots are severely required for treatments (Kai et al. 2011; Liao et al. 2009). Hence, establishment of hairy root cultures of *Salvia*

species with affluent tanshinones is an alternative approach for tanshinone acquisition in vitro. The aims of this study were to establish an effective hairy root culture system of the endemic medicinal plant of *S. castanea* Diels f. *tomentosa* Stib. and to evaluate the influences of $Ag^+$, MeJA, and YE on the growth and active tanshinone accumulation in the hairy root cultures. Furthermore, expression levels of *HMGR*, *DXR*, *IPPI*, and *GGPPS* genes involved in tanshinone biosynthetic pathway of the hairy root cultures with diverse elicitation were also observed.

## Materials and methods

### Plant material

*S. castanea* Diels f. *tomentosa* Stib. seeds were obtained from the Science and Technology Bureau of Nyingchi Prefecture, Tibet, People's Republic of China. The original plant was identified by research fellow Yansheng Chen; the voucher specimen was deposited at the College of Life Sciences, Northwest A&F University. The seeds were washed by running water for 24 h, surface-sterilized with 75 % ethanol (*v*/*v*) for 45 s, and then washed twice by sterile distilled water ($dH_2O$). After that, seeds were immersed into 0.1 % $HgCl_2$ (*w*/*v*) for 15 min, and then the residual disinfectant was rinsed with excess sterile $dH_2O$ five times. The sterile seeds were blot-dried by sterile filter paper and then placed on MS medium (Murashige and Skoog 1962) supplemented with 7.5 g $l^{-1}$ agar and 30 g $l^{-1}$ sucrose for 3 days (25 °C, in the dark). After the seeds germinated, all the culture flasks were transferred into an artificial climate chamber (25 °C) with a photoperiod of 14 h light/10 h dark. Forty-five-day-old seedlings were intended for hairy root induction. Moreover, original plants of *S. castanea* Diels f. *tomentosa* Stib. were cultivated in the medicinal plant garden in the College of Life Sciences, Northwest A&F University, People's Republic of China.

### Hairy root induction and establishment

*A. rhizogenes* strain ATCC 15834 was used for hairy root induction. Bacteria were used in a streak culture on LB solid medium at 28 °C for 2 days, and the single clone was picked, inoculated into 30 ml of YEB liquid medium (Vervliet et al. 1975), and cultured in a gyratory shaker at 180 rpm. After three times of activation, the bacteria were centrifuged at 2770×*g*, suspended in 1/2 MS–$NH_4$ (without $NH_4NO_3$) liquid medium for 30 min, and then prepared for infection with an $OD_{600}$ of 0.5 (UV-1700 spectrophotometer, Shimadzu, Japan).

The leaves of *S. castanea* Diels f. *tomentosa* Stib. aseptic seedlings were cut into 0.5×0.5 cm pieces and wounded with a sterile needle on the surface. After being precultured on 1/2 MS–$NH_4$ medium for 3 days (25 °C, in the dark), the leaf disks were submerged in *A. rhizogenes* suspension prepared above for 30 min (28 °C). The liquid medium containing the bacteria was blotted with sterile filter paper, and then the leaf disks were placed on 1/2 MS–$NH_4$ medium and cocultured for 3 days (25 °C, in the dark). Finally, the leaf disks were washed by sterile $dH_2O$, blot-dried, and transferred to a 1/2 MS–$NH_4$ medium supplemented with 7.5 % agar. Cefotaxime sodium (SinoPharm, China) with an initial concentration of 500 mg $l^{-1}$ was supplemented in the medium at a decrease by degrees (100 mg $l^{-1}$/7 days) to remove bacteria. After 5 weeks of culture, single root lines (>2 cm in length) that originated from the infected sites of explants were excised and transferred into phytohormone- and antibiotic-free 6,7-V liquid medium (Veliky and Martin 1970) supplemented with 30 g $l^{-1}$ sucrose and cultured in a rotary shaker (120 rpm, 25 °C). The hairy root lines showed excellent growth status and degermed thoroughly which were chosen for enlargement cultures and elicitation. For comparison of tanshinone content, the hairy root of *S. miltiorrhiza* induced by our lab was also cultured in 6,7-V liquid medium.

### Confirmation of hairy root induction

Total DNA of the putative *S. castanea* Diels f. *tomentosa* Stib. hairy root lines and the normal root of plant (control) were extracted by the CTAB method with some modification (Porebski et al. 1997). Primers of *rolB* and *rolC* gene fragments for PCR amplification were designed based on a previous work (Królicka et al. 2001) and 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′. Two microliters of DNA template was used in a 50-μl amplification system with 10 μM primers, 25 μl of 2× Premix Taq (Takara, Japan), and 21 μl of $dH_2O$. The PCR procedure was performed on the Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA) and began with a 5-min predenaturation step at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, and finally at 72 °C for 7 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).

### Growth kinetics of hairy root cultures

Optimization of hairy root growth condition was performed in MS and 6,7-V liquid medium after enlargement cultures. In order to investigate the appropriate opportunity for elicitation, a 30-day-long period for hairy root cultures was performed.

Accurately weighed fresh hairy roots (0.3 g) were transferred into 100 ml flasks with 50 ml of 6,7-V liquid medium for subcultures. Fresh and dry weights (FW, DW) of hairy roots were monitored for growth kinetics assessment with an interval of 3 days. Fresh hairy roots were rinsed twice by $dH_2O$, blotted by filter paper, and weighed. After that, the hairy roots were dried at 45 °C to constant weight and weighed separately.

### Preparation of elicitors

Abiotic $Ag^+$, signal molecule MeJA, and biotic YE were applied in the hairy root elicitation. $Ag^+$ was prepared by a mixture of $AgNO_3$ (SinoPharm, China) and $Na_2S_2O_3$ (Xilong, China) at a 1:4 molar ratio to a final concentration of 50 mM and then added into the culture medium after filter sterilization. MeJA (Sigma, USA) was dissolved in ethanol to a concentration of 600 mM. YE was obtained by the method of Ge and Wu (2005). Fifty grams of yeast extract (Sigma, USA) was dissolved in 250 ml of $dH_2O$ and then ethanol (80 % in total) was added (*v*/*v*) for precipitation (4 °C, 4 days); the gummy precipitate was redissolved in 250 ml of $dH_2O$ and reprecipitated by ethanol. Total precipitates were collected, redissolved in 200 ml of $dH_2O$, and autoclaved for 20 min before adding into the medium. The concentration of YE was indicated by the polysaccharide content of 12,855±545 mg $l^{-1}$ and anhydrous glucose was taken as the standard substance (Dubois et al. 1956). To screen the optimum concentration of the elicitors, $Ag^+$ was applied in the hairy root cultures for 7 days, with concentrations ranging from 5, 15, 30, to 45 μM; MeJA was added with concentrations of 50, 100, 200, and 300 μM; and YE was carried out with concentrations of 50, 100, 200, and 300 mg $l^{-1}$; equivoluminal sterile $dH_2O$ was added as control. All the prepared elicitors were added when the hairy roots were cultured in 100 ml flasks with 50 ml of 6,7-V medium for 18 days at the intermediate stage of the whole growth phase. For the time course assay, hairy roots were collected from 0, 0.5, 1, 2, 4, to 7 days after the addition of optimum concentrations of the elicitors. Growth status evaluation, tanshinone content quantification, and gene expression level comparison of different samples were performed simultaneously. For each treatment, three independent biological replicates were used.

### Analyses of tanshinones by high-performance liquid chromatography method

Contents of DT, CT, T-I, and T-IIA in all samples were determined simultaneously by the high-performance liquid chromatography (HPLC) method of Yang et al. (2009); 0.02 g of dried and powdered hairy root samples were extracted by 4 ml of 70 % methanol for 12 h; for comparison, 0.1 g of normal roots of *S. castanea* Diels f. *tomentosa* Stib. and *S. miltiorrhiza* were soaked in 25 ml of extraction solvent. The samples were treated by ultrasound for 45 min and centrifuged at 9820×*g* for 10 min, and then the supernatants were filtered with 0.45 μm Millipore filter and injected into the HPLC system. HPLC-grade acetonitrile (Tedia, NJ, USA) and ultrapure $dH_2O$ with 0.02 % HPLC-grade phosphoric acid (Kermel, Tianjin, China) were used in the present study. Tanshinone analysis was performed on the HPLC system (Waters, USA) equipped with a 1252 binary HPLC pump, 2487 dual λ absorbance detector, and 2707 autosampler. A Waters Symmetry C18 column (250×4.6 mm, 5 μm) was applied in the gradient elution procedure (Table A1). All the tanshinones were detected at 270 nm. Confirmation of each compound was by comparing the retention time with the standard substance 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 A2.

### Transcripts of selected genes in tanshinone biosynthetic pathway performed by quantitative real-time RT-PCR method

Hairy roots of *S. castanea* Diels f. *tomentosa* Stib. stored in a −80 °C refrigerator were used in quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay. Relative expression levels of *HMGR*, *DXR*, *IPPI*, and *GGPP*S genes in tanshinone biosynthetic pathway were achieved by the qRT-PCR method, and *β-actin* was taken as the reference gene. Total RNA of hairy root was obtained by Biospin Plant Total RNA Extraction Kit (DNA-Free) (BioFlux, China); the quality and concentration of RNA were examined by agarose gel electrophoresis and NanoDrop spectrophotometer (Thermo Fisher Scientific Inc, USA). cDNA was achieved by the protocol of PrimeScript™ 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. qRT-PCR assay was performed on the CFX96™ Real-Time PCR Detection System (Bio-Rad, USA) with SYBR Premix Ex Taq™ II (Tli RNaseH Plus) Kit (Takara, Japan). qRT-PCR conditions were as follows: 30 s at 95 °C for predenaturation, 5 s at 95 °C for denaturation, and 30 s at 58 °C for annealing, at 40 cycles. qRT-PCR primers of tanshinone biosynthetic related genes were designed which were cloned and identified by its closely related species of *S. miltiorrhiza* (Yang et al. 2012) (Table A3). Quantification of gene expressions was performed by a comparative CT method, transcripts of related genes in the hairy roots of *S. castanea* Diels f. *tomentosa* Stib. harvested at the initial of elicitors treatment (0 day) were as 1.

### Data analyses

Graphics were performed by the OriginPro software version 9.1, and analyses of significance were determined by analysis of variance (ANOVA) using the SPSS software version 16.0,

taking $P<0.05$ and $P<0.01$ as significant and highly significant, respectively. qRT-PCR data were acquired and processed by Bio-Rad CFX Manager (Bio-Rad, USA). All the data were expressed as mean±standard deviation (SD).

## Results

### Induction and establishment of hairy root cultures

The leaf explants of *S. castanea* Diels f. *tomentosa* Stib. responded to induction of *A. rhizogenes* ATCC 15834 and formed hairy roots with the frequency of 3.92 to 8 % after 5 weeks of culture. Hairy roots were generated from wounded sites of the explants on 1/2 MS–$NH_4$ medium with a decreasing concentration of cefotaxime sodium after 2–3 weeks (Fig. 1); however, no root or callus was formed on the control leaf disks (without infection). 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 untransformed root (Fig. 2). Thus, *rolB* and *rolC* genes in the plasmid (pRi) of the bacteria were integrated into the explants successfully and induced hairy roots of *S. castanea* Diels f. *tomentosa* Stib. completely.

### Tanshinone production and growth kinetics of hairy root cultures

Tanshinone contents of individual hairy root lines, hairy roots cultured with diverse substrates, normal roots of *S. castanea* Diels f. *tomentosa* Stib., and *S. miltiorrhiza* were analyzed by HPLC (Fig. 3). The result showed that line 1 of the hairy root was of high tanshinone content, while the 6,7-V medium benefitted both the growth status and T-IIA accumulation in these hairy root cultures. Moreover, the content of T-IIA was notably higher in the hairy root of *S. castanea* Diels f. *tomentosa* Stib. than in *S. miltiorrhiza* (Danshen) hairy root (Fig. 3), which was consistent with the situation in their parent plants confirmed by a previous study (Yang et al. 2009). Therefore, hairy root culture of *S. castanea* Diels f. *tomentosa* Stib. is a practical and effective way for tanshinone production, and hairy root line 1 of this plant cultured in 6,7-V medium was chosen for elicitation.

Hairy roots cultured in 6,7-V medium were sampled with an interval of 3 days, and a typical "S" curve was exhibited in the growth kinetics assessment based on the fresh and dry weights (Fig. 4). The fresh hairy root showed short growth retardation in the first 3 days and then grew gradually and an "S" growth phase appeared owing to the rapid cell division and excess nutrients, yet a coming plateau phase was present at the end of the curve. After 30-day-long cultures, the hairy root showed a dense lump. About 1.59±0.18 g FW and 0.09±0.006 g DW of hairy roots were harvested at 30 days, and the dry to fresh mass ratio of 5.66 % indicated a less dry matter accumulation of the hairy root cultures. Besides, the hairy root cultures were extended to 60 days and 2.98±0.09 g FW and 0.25±0.01 g DW were obtained finally. However, hairy roots in this period showed dark brown and lacked vitality, and a little medium remained in the flasks. Thus, 18-day-old hairy

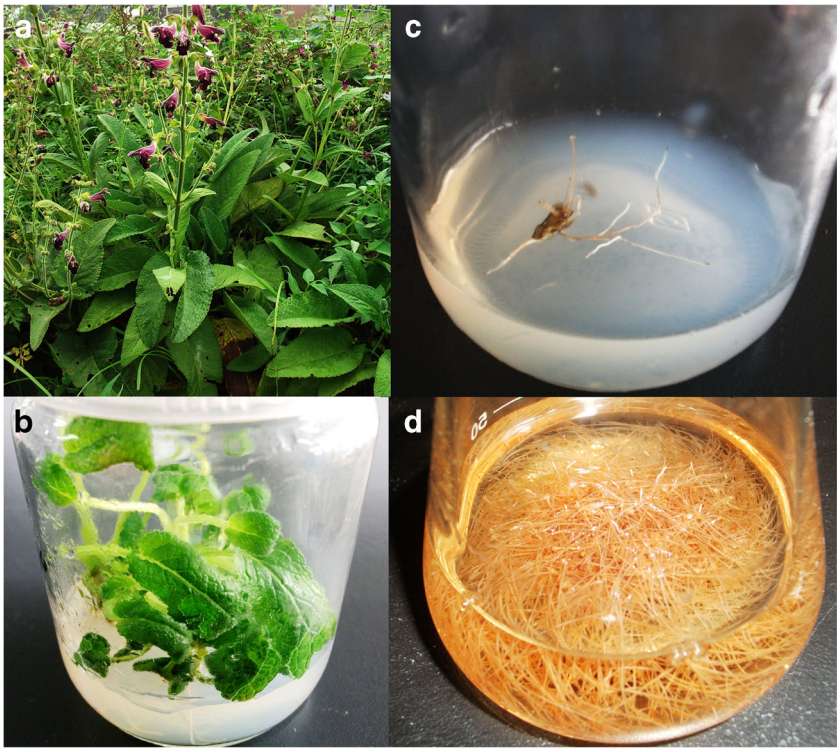


**Fig. 1** Growth and hairy root induction of *S. castanea* Diels f. *tomentosa* Stib. **a** Original plant of *S. castanea* Diels f. *tomentosa* Stib. was cultivated in the field. **b** Aseptic seedlings was cultured on MS solid medium for 45 days. **c** Hairy root induction was implemented on 1/2 MS–$NH_4$ (without $NH_4NO_3$) medium supplemented with cefotaxime sodium. **d** Hairy root line 1 of *S. castanea* Diels f. *tomentosa* Stib. was cultured in 6,7-V liquid medium for 30 days

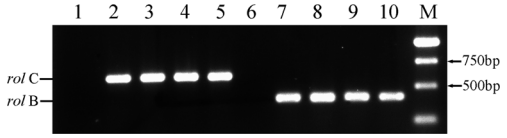


**Fig. 2** *rolB* and *rolC* gene fragments of putative hairy root lines amplified by PCR. *Lanes 1* and *6*: roots from nontransformed control plant (normal root). *Lanes 2*, *3*, *7*, and *8*: hairy root line 1 of *S. castanea* Diels f. *tomentosa* Stib. induced by *A. rhizogenes*. *Lanes 4*, *5*, *9*, and *10*: hairy root line 2 induced by *A. rhizogenes*. *M*, DL5000 DNA marker (Takara, Japan)

roots at the intermediate growth stage were favorable for elicitation.

## Effect of elicitor dosage on hairy root cultures

$Ag^+$ influenced both the growth status and tanshinone production in the hairy root cultures of *S. castanea* Diels f. *tomentosa* Stib. $Ag^+$ apparently promoted the fresh weight of hairy root with 23.5 % increment over the control at 15 μM. However, a rapid decline of both fresh and dry weights was noticed followed by an elevated concentration of $Ag^+$ (Table 1). $Ag^+$ (15 μM) was also proven to cause significantly a 1.8-fold of T-IIA accumulation compared with the control, yet the CT content showed an increase initially and then decreased to 0.78-fold than the control with gradually enhanced $Ag^+$ (Fig. 5a). It has been reported that $Ag^+$ can stimulate the total tanshinone accumulation with 1.2-fold increment over the control in the

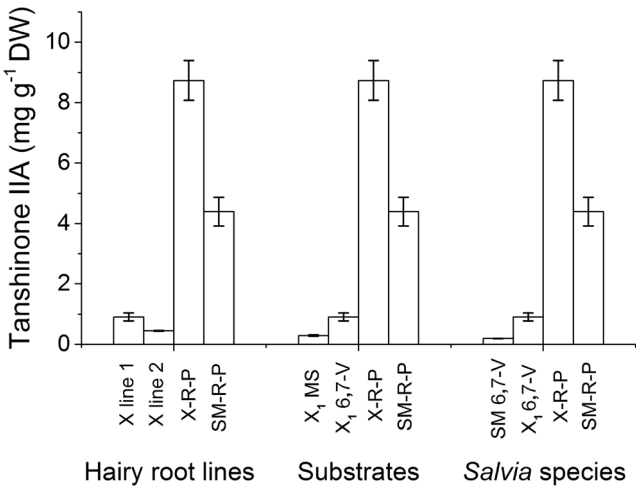


**Fig. 3** Comparison of T-IIA contents from different hairy root lines, hairy root cultured with different substrates, and normal roots of the parent plants. X line 1 and X line 2, two individual hairy root lines of *S. castanea* Diels f. *tomentosa* Stib. induced by *A. rhizogenes*, were cultured in 6,7-V liquid medium for 30 days. $X_1$ MS and $X_1$ 6,7-V, selected hairy root line (X line 1), were cultured in MS and 6,7-V liquid medium for 30 days, respectively. SM 6,7-V, hairy root of *S. miltiorrhiza* was cultured in 6,7-V liquid medium for 30 days. X-R-P and SM-R-P, normal roots of *S. castanea* Diels f. *tomentosa* Stib. and *S. miltiorrhiza*, respectively, were cultivated in the field. T-IIA content of the dry root from Danshen (SM-R-P) was used as a positive control. *DW* dry weight

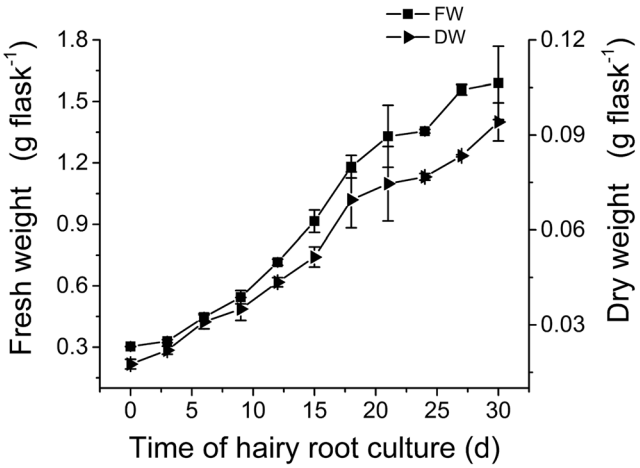


**Fig. 4** Growth kinetics of *S. castanea* Diels f. *tomentosa* Stib. hairy root cultured in 6,7-V liquid medium. *FW* fresh weight, *DW* dry weight; 0.3 g of fresh hairy roots were cultured in 100 ml flasks with 50 ml of 6,7-V liquid medium for 30 days and sampled with an interval of 3 days

hairy root of *S. miltiorrhiza* (Ge and Wu 2005). Thus, 15 μM $Ag^+$ was appropriate for *S. castanea* Diels f. *tomentosa* Stib. hairy root growth and improvement of T-IIA content than that of control.

In contrast to $Ag^+$, exogenous MeJA showed suppression on the fresh weight of hairy root cultures in pace with the increasing concentration, and 300 μM MeJA was confirmed to decrease 16 % of fresh weight at most than that of the control (Table 1). However, the dry weight of hairy root presented a gradual promotion with MeJA treatment. Meanwhile, MeJA improved the T-IIA content intensively (1.8± 0.11 mg $g^{-1}$ DW at most) in the hairy root cultures, and 200 μM MeJA was recommended to be the optimum concentration by causing a 1.99-fold of T-IIA accumulation than that of the control (Fig. 5b). Furthermore, MeJA favored the CT content slightly from 0.34±0.04 to 0.51±0.02 mg $g^{-1}$ DW, which reached its maximum of 1.5-fold enhancements by elicitation of 200 μM MeJA (Fig. 5b). Other tanshinones were proven not to be influenced by exogenous MeJA in the present study. Totally, MeJA dramatically improved both the dry weight and T-IIA content of *S. castanea* Diels f. *tomentosa* Stib. hairy root cultured in 6,7-V medium. We therefore chose 200 μM MeJA for tanshinone accumulation and gene expression investigation within different days.

After YE elicitation for 7 days, a deep red color was present on both the hairy root of *S. castanea* Diels f. *tomentosa* Stib. and the culture medium. The fresh weight showed a bit decrease at the low level of elicitor and then recovered by the elevated concentration of YE. However, the dry weight accumulation of the hairy root was acutely promoted by YE, and a 1.73-fold increase at most was found by treatment with 200 mg $l^{-1}$ YE than that of the control (Table 1). YE is obviously the optimum elicitor for obtaining more dry matter excelling MeJA and $Ag^+$ in the hairy root cultures. Moreover,

**Table 1** Fresh and dry weights of hairy roots treated by different concentrations of elicitors

| FW, DW (g) | Elicitors | Control[a] | C1[b] | C2[c] | C3[d] | C4[e] |
|---|---|---|---|---|---|---|
| Fresh weight | $Ag^+$ | 1.49±0.09bc | 1.61±0.05b | 1.84±0.13a | 1.46±0.05bc | 1.40±0.12c |
| | MeJA | 1.49±0.09a | 1.25±0.07b | 1.36±0.06ab | 1.30±0.04b | 1.26±0.12b |
| | YE | 1.49±0.09 | 1.36±0.04 | 1.44±0.02 | 1.51±0.21 | 1.47±0.17 |
| Dry weight | $Ag^+$ | 0.09±0.01 | 0.09±0.00 | 0.10±0.01 | 0.09±0.00 | 0.09±0.00 |
| | MeJA | 0.09±0.01c | 0.10±0.01b | 0.12±0.01a | 0.11±0.01ab | 0.11±0.00ab |
| | YE | 0.09±0.01c | 0.11±0.00b | 0.13±0.00ab | 0.15±0.02a | 0.14±0.02a |
| Dry rate (%) | $Ag^+$ | 5.73±0.39ab | 5.36±0.18b | 5.48±0.38b | 5.94±0.21ab | 6.16±0.48a |
| | MeJA | 5.73±0.39c | 8.08±0.39b | 8.65±0.07ab | 8.61±0.27ab | 9.16±0.45a |
| | YE | 5.73±0.39c | 8.48±0.37b | 8.97±0.08b | 9.88±0.28a | 9.73±0.40a |

Eighteen-day-old hairy roots cultured in 6,7-V medium were treated by different elicitors for 7 days. 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, 18-day-old hairy root of *Salvia castanea* Diels f. *tomentosa* Stib. cultured for 7 days without elicitation was used as control

[b] C1, concentrations of elicitors, 5 μM $Ag^+$, 50 μM MeJA, and 50 mg $l^{-1}$ YE, respectively

[c] C2, concentrations of elicitors, 15 μM $Ag^+$, 100 μM MeJA, and 100 mg $l^{-1}$ YE, respectively

[d] C3, concentrations of elicitors, 30 μM $Ag^+$, 200 μM MeJA, and 200 mg $l^{-1}$ YE, respectively

[e] C4, concentrations of elicitors, 45 μM $Ag^+$, 300 μM MeJA, and 300 mg $l^{-1}$ YE, respectively

an evident activation of DT production was obtained which grew from nothing to a high level of 1.95±0.09 mg $g^{-1}$ DW; however, no DT was detected from the control (Fig. 5c). The CT content was improved to 2.84±0.33 mg $g^{-1}$ DW intensively (8.37-fold increments) than that of the control by 200 mg $l^{-1}$ YE treatment (Fig. 5c). This is consistent with an earlier work which showed that YE can dramatically stimulate the content of CT in *S. miltiorrhiza* hairy root cultures (Shi et al. 2007). YE also played a crucial role in T-IIA accumulation that we observed, and the content of T-IIA was polished up to 2.77-fold than that of the control by adding 200 mg $l^{-1}$ YE (Fig. 5c), which exceeded the standard of T-IIA content (2 mg $g^{-1}$ DW) in the dry root of *S. miltiorrhiza* (National Pharmacopoeia Committee 2010). Our results showed that 15 μM $Ag^+$, 200 μM MeJA, and 200 mg $l^{-1}$ YE were appropriate for time course assay of *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures separately.

## Time courses of hairy root cultures with diverse elicitation

In the time course assay, accumulation of tanshinones showed notable diversity according to the elicitor type within 7 days; fresh weight of hairy root cultured with elicitation at selected time points is listed in Table 2. CT was susceptible to both positive and negative regulations with selected elicitors. $Ag^+$ (15 μM) played an inconspicuous role in CT accumulation despite of the time elapsed. A similar result was achieved from the suitable $Ag^+$ dosage investigation above. CT accumulation showed a gradual increase and reached its maximum at 7 days with the addition of 200 μM MeJA (Fig. 6a). However, YE showed a short inhibition on CT content within 24 h, and then a sharp enhancement was perceived by YE addition for 2 days. Finally, a noticeable 8.8-fold (3±0.08 mg $g^{-1}$ DW) increase of CT content was obtained at 7 days compared with the control (0 day) (Fig. 6a). Consequently, YE is an effective elicitor for the promotion of CT yield in *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures.

In this promising hairy root culture system, the T-I content was slightly responsive to elicitation at different time points. YE promoted a high yield of T-I from 0.68±0.01 to 0.83±0.03 mg $g^{-1}$ DW progressively compared with $Ag^+$. $Ag^+$ (15 μM) still displayed inhibition on T-I accumulation in 2 days though recovered to a relatively high level later (Fig. 6b). Contents of CT and T-I achieved in *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures presented an apparent decline by adding $Ag^+$ into the medium immediately, and then slowly coming promotions were observed in the following days. Hence, the hairy root of this plant is affirmed to be sensitive to elicitation and responded to the regulation of biosynthesis in the form of releasing different secondary metabolites.

The contents of T-IIA revealed identical increases along with different addition of elicitors for 12 h (Fig. 6c). Most of T-IIA accumulation was observed at 2 days by $Ag^+$ treatment (2.19±0.09 mg $g^{-1}$ DW) and at 4 days after MeJA addition (2.23±0.21 mg $g^{-1}$ DW), yet a mild decrease was apparently present at 7 days in pace with the hairy root growth. Moreover, the content of T-IIA showed a sustainable promotion treated with 200 mg $l^{-1}$ YE, and 2.52±0.67 mg $g^{-1}$ T-IIA at most was obtained at 7 days after YE addition (Fig. 6c), and this is consistent with the assay of YE dosage effect in the hairy root cultures. Therefore, all three elicitors consistently enhanced tanshinone accumulation in the hairy root cultures of *S. castanea* Diels f. *tomentosa* Stib. at different time points.

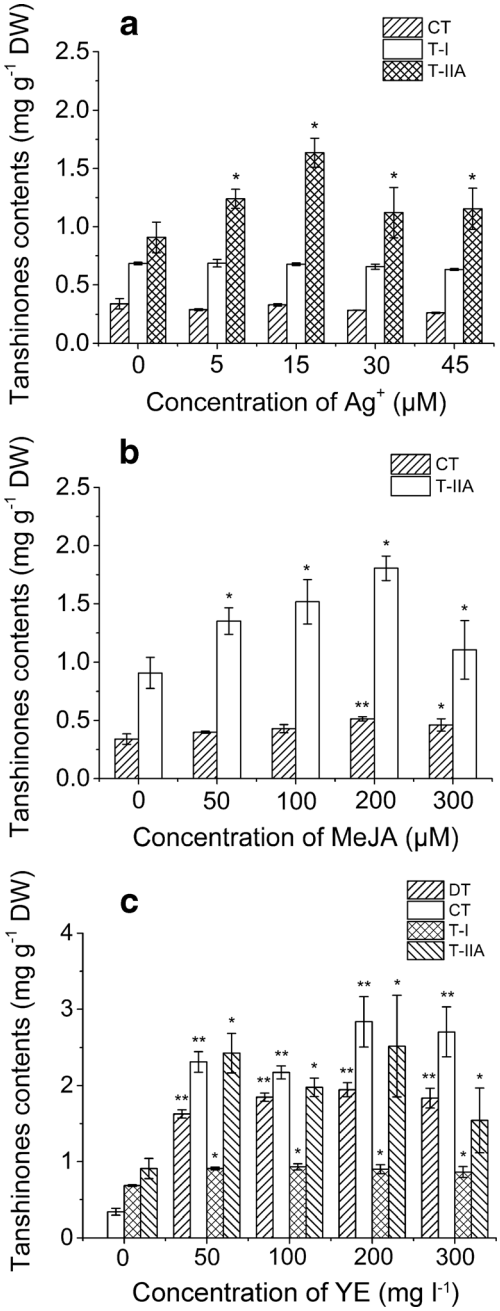


**Fig. 5** Effects of different concentration of elicitors on tanshinone accumulation in hairy root cultures. *S. castanea* Diels f. *tomentosa* Stib. hairy roots were treated by different concentrations of $Ag^+$ (**a**), MeJA (**b**), and YE (**c**) for 7 days; 18-day-old hairy root cultured for 7 days without elicitation was used as control. *DT* dihydrotanshinone, *CT* cryptotanshinone, *T-I* tanshinone I, *T-IIA* tanshinone IIA, *DW* dry weight. **P*<0.05, ***P*<0.01

## Influences of selected elicitors on the expressions of tanshinone biosynthetic related genes

$Ag^+$ (15 μM) caused enhancement of all gene expressions within 12 h and then reached their maximums at 24 h after the addition of elicitors except *IPPI*, which still showed a relatively higher level of 3-fold compared with the control (without eliCitation) at 4 days in the hairy root cultures (Fig. 7). Maximum expression levels of *HMGR*, *DXR*, and *GGPPS* with 15 μM $Ag^+$ elicitation indicated 3-fold, 2-fold, and 4.6-fold increases compared with the control, respectively (Fig. 7). It is obvious that selected genes responded to $Ag^+$ stimulation which was almost completed within 24 h, yet accumulation of tanshinones appeared mostly posterior to related gene expressions. Therefore, evident time-response effects of involved genes were achieved completely by applying $Ag^+$ in the medium of *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures.

MeJA (200 μM) showed multiple effects on gene expression levels which were different from $Ag^+$. *HMGR* responded to MeJA elicitation sensitively; about 3.1-fold higher level of *HMGR* expression was observed at 12 h than the control and then reached its maximum of 5.8-fold at 2 days by MeJA treatment (Fig. 8). *DXR* expression level showed a moderate increase within 7 days; a maximum of 2.1-fold increase was obtained at 4 days compared with the control. A sustainable increment of *IPPI* expression level was confirmed remarkably higher than that of the control; about 4.9-fold promotion at most was achieved at 4 days by MeJA addition. Transcript of *GGPPS* was enhanced within 24 h and then reached its maximum of 3.6-fold compared with the control in the whole period (Fig. 8).

Noticeable transcript improvements of different key genes were perceived within 7 days by 200 mg $l^{-1}$ YE treatment. Severe upregulation of *IPPI* was noticed with 13.9-fold enhancement by YE addition immediately (Fig. 9). Moreover, *HMGR* and *GGPPS* expression levels efficiently approached their maximums of 5.6- and 16.7-fold relative to the control at 24 h, respectively; a 5.9-fold increment of *DXR* expression level higher than the control was achieved at 4 days by YE treatment (Fig. 9). The considerable upregulations of *IPPI* and *GGPPS* genes in the tanshinone biosynthetic pathway were attractive by adding YE into the medium of *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures.

## Discussion

Hairy root induction of *S. castanea* Diels f. *tomentosa* Stib. indicated a relatively low transformation rate compared with 56.7 % of *S. officinalis* hairy root induction (Grzegorczyk et al. 2006) and 12.5 % transformation efficiency in the establishment of *Physalis minima* hairy root cultures (Azlan et al. 2002). A previous study confirms that the transformation rate of hairy root induction depends on multiple factors, such as the bacterial strain specificity, types of induced explants, and the presence of acetosyringone (Georgiev et al. 2007). Moreover, the integration sites of the T-DNA from *A. rhizogenes* plasmid (pRi) in the target plant genomic DNA often show randomness, while several copies of T-DNA may be

**Table 2** Fresh weight of hairy roots treated by optimum concentrations of elicitors at different time points

| Elicitors | Day | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1 | 2 | 4 | 7 |
| $Ag^{+a}$ | 1.18±0.06c | 1.25±0.02c | 1.36±0.06c | 1.32±0.04c | 1.54±0.13b | 1.71±0.16a |
| MeJA | 1.18±0.06b | 1.26±0.05ab | 1.27±0.1ab | 1.34±0.11a | 1.37±0.13a | 1.36±0.07a |
| YE | 1.18±0.06e | 1.20±0.05de | 1.28±0.02d | 1.37±0.05 c | 1.47±0.06b | 1.60±0.03a |
| Control[b] | 1.18±0.06c | 1.19±0.11c | 1.19±0.05c | 1.25±0.13bc | 1.40±0.08ab | 1.49±0.09a |

The hairy roots were sampled at 0, 0.5, 1, 2, 4, and 7 days after the addition of selected elicitors. Values are mean of three replicates followed by standard deviation (±SD). The lowercase letters represent significance at 0.05 level ($P<0.05$)

[a] Applied concentrations of elicitors, 15 μM $Ag^{+}$, 200 μM MeJA, and 200 mg $l^{-1}$ YE

[b] Eighteen-day-old hairy root of *Salvia castanea* Diels f. *tomentosa* Stib. cultured without elicitation within 7 days was used as control

integrated at a single locus of the host plant genome (Gelvin 2003). Thus, considerable uncertainty and preference still remain in the procedure of *Agrobacterium*-mediated hairy root transformation.

In earlier works, leaves and shoot tips of *Salvia austriaca* Jacq were infected by *A. rhizogenes* A4, and several abietane-type diterpenoids were achieved from the hairy root culture (Kuźma et al. 2011). Moreover, two new and several known diterpenes were previously isolated from the transformed roots of *Salvia broussonetii* (Fraga et al. 2005), which showed selective insect cytotoxic effects. In the present study, affluent tanshinones were obtained from hairy root cultures of *S. castanea* Diels f. *tomentosa* Stib., which are also attributed to diterpenoids. These investigations reveal the outstanding capability of production of effective diterpenes by hairy root cultures of diverse *Salvia* species.

Elicitation of $Ag^{+}$ is affirmed to associate with ethylene signal transduction which regulates the secondary metabolism for defense responses in plant tissues (Khalili et al. 2010; Zhang and Wu 2003). Moreover, $Ag^{+}$ is validated to cause increased ion fluxes and production of reactive oxygen species (ROS), as well as tanshinone accumulation in *S. miltiorrhiza* hairy root cultures (Zhang et al. 2004). In the present study, T-IIA content of *S. castanea* Diels f. *tomentosa* Stib. hairy root was improved apparently by 15 μM $Ag^{+}$ than that of the control, and it has been proven that a low level of $Ag^{+}$ plays a positive role in cell suspension cultures of *Silybum marianum* (Ashtiani et al. 2010), while a high concentration of $Ag^{+}$ showed inhibition on the growth of hairy root cultures (Table 1). Furthermore, enhanced *HMGR*, *DXR*, and *GGPPS* expressions at the initial elicitation period were achieved, which is consistent with a previous work where $Ag^{+}$ enhances several gene expressions of tanshinone metabolism in *S. miltiorrhiza* hairy root cultures (Kai et al. 2012). Thus, $Ag^{+}$ is supposed to participate in the promotion of gene expressions in tanshinone biosynthetic pathway of *S. castanea* Diels f. *tomentosa* Stib. hairy root and, therefore, results in affluent tanshinone accumulation.

In previous works, MeJA is confirmed to decrease dry weight yet induce the secretion of terpene indole alkaloids and promote related gene transcriptions in *Catharanthus roseus* hairy root cultures (Ruiz-May et al. 2009; Zhou et al. 2010). Decreased fresh weight of hairy root cultures was noticed more severely by MeJA addition than $Ag^{+}$ and YE treatments in the present investigation (Table 2), and it could be explained that exogenous MeJA negatively regulated hairy root growth of *S. castanea* Diels f. *tomentosa* Stib. As the methyl ester of jasmonic acid (JA), the effect of MeJA is revealed by involving in signal transduction, activating relevant promoters and transcription factors, and producing a series of secondary metabolites, such as polyphenol, alkaloid, and even terpenoids in plants (Memelink 2009; Zhao et al. 2005). It is validated that endogenous JA and MeJA were induced by several elicitors and improved the production of secondary metabolites; besides, the effects of elicitation on the accumulation of secondary metabolites and the transcripts of related genes were abrogated once JA/MeJA biosynthesis was cut off (Memelink et al. 2001). MeJA also enhanced triterpene saponin contents and promoted related gene expression in hairy root cultures of ginseng (Kim et al. 2009). Our study achieved similar results where T-IIA content was markedly heightened by MeJA treatment and confirmed that MeJA regulates tanshinone biosynthesis in *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures.

YE is considered to be the effective biotic elicitor widely used in plant cell and tissue cultures for its intensive regulation on secondary metabolism (Shi et al. 2007); 200 mg $l^{-1}$ YE is confirmed to be the most recommendable elicitor for dry matter accumulation and active tanshinone enhancement in *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures, which has more advantages than $Ag^{+}$ and MeJA. In a previous study, notable CT accumulation is present from 0.24 to 0.54 % DW by adding yeast extract into the culture medium of *S. miltiorrhiza* cell suspension transformed by *Agrobacterium tumefaciens* C58 (Chen and Chen 2000). High yields of DT and CT are also obtained in *S. castanea* Diels f. *tomentosa*

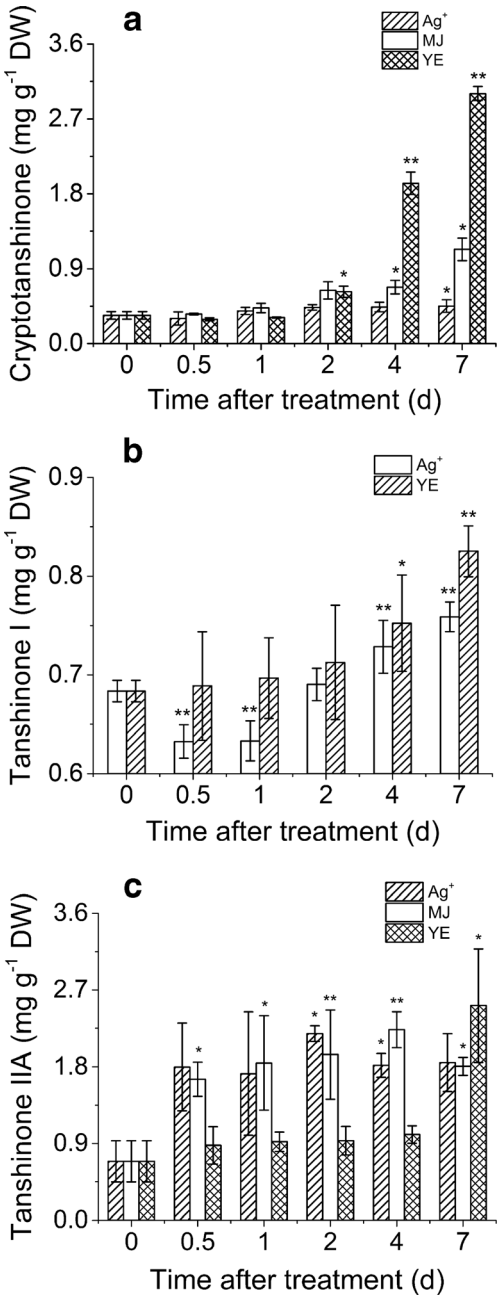


**Fig. 6** Tanshinones accumulation in hairy root cultures at different time points exposed to selected concentrations of elicitors. Cryptotanshinone (**a**), tanshinone I (**b**), and tanshinone IIA (**c**) contents of *S. castanea* Diels f. *tomentosa* Stib. hairy roots were treated by 15 μM $Ag^+$, 200 μM MeJA, and 200 mg $l^{-1}$ YE within 7 days, respectively; 18-day-old hairy roots were applied in the initial of time courses. *DW* dry weight. **P*<0.05, ***P*<0.01

Stib. hairy root cultures by YE treatment, and other elicitors that did not stimulate the DT production might be attributed to the diverse participation ways of elicitors in the regulation of tanshinone secondary metabolism. Actually, accumulation of CT is presumed to be a defensive response to elicitation in *S. miltiorrhiza* cell cultures (Zhao et al. 2010). T-IIA content is also strongly enhanced by 200 mg $l^{-1}$ YE in this hairy root culture, and it is the striking active compound we focus on. Improvement of T-IIA content benefits the production in vitro due to its impactful purpose. Therefore, YE is predicated to be positively involved in the regulation of tanshinone biosynthesis in the hairy root cultures, and the abundant tanshinone accumulation is supposed to be an inductive response to exogenous polysaccharide. Interestingly, several new unknown peaks were present in the tanshinone HPLC profile of this hairy root culture with YE elicitation, which might be ascribed to tanshinone relatives (Fig. 10), and this result is in accordance with a previous study on *S. miltiorrhiza* cell cultures (Zhao et al. 2010).

Overall, three selected elicitors exhibited pronounced improvements on the growth status and tanshinone contents of *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures, and apparent dosage effects of three elicitors were also achieved. Many studies previously reported that diverse elicitors play a remarkable role in the accumulation of interested metabolites in different hairy root cultures, and the concentration of a specific elicitor is elucidated to be a factor strongly influencing the intensity of the response of plant tissues exposed to elicitation (Vasconsuelo and Boland 2007). Meanwhile, the effect of a selected elicitor varied with different culture systems. Besides the commonly investigated $Ag^+$, MeJA, and YE, several other elicitors are also applied in practice, which could be performed in the future. Thus, the application of elicitors in the hairy root culture systems of medicinal plants has the potential toward improvements of the levels of effective components.

Plenty of genes and transcription factors in the secondary metabolism of plants responded to and are regulated by several factors, such as abiotic and biotic stress, as well as exogenous elicitors (Broun et al. 2006). *HMGR*, *DXR*, *IPPI*, and *GGPPS* genes involved in tanshinone biosynthetic pathway of *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures were enhanced by diverse elicitors almost at the initial time courses; however, the most striking increments of tanshinones were observed at the end of the treatment period, which are closely related to the elicitor types and culture time. *HMGR* and *DXR* are the important genes at the early stages of two parallel tanshinone biosynthetic pathways (Ge and Wu 2005); the simultaneous increments of these two gene expressions by 15 μM $Ag^+$, 200 μM MeJA, and 200 mg $l^{-1}$ YE elicitation indicated that tanshinone biosynthesis in *S. castanea* Diels f. *tomentosa* Stib. hairy root is accomplished by collaboration of the two pathways. In a previous study, genetic manipulation of related genes in the DXP pathway is affirmed more effective for tanshinone production than that of the MVA pathway in *S. miltiorrhiza* (Kai et al. 2011). However, *HMGR* showed a relatively higher expression level than *DXR* in this hairy root culture by $Ag^+$ and MeJA treatments (Figs. 7 and 8). It is interpreted that the sensitivities of *HMGR* and *DXR* in *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures are

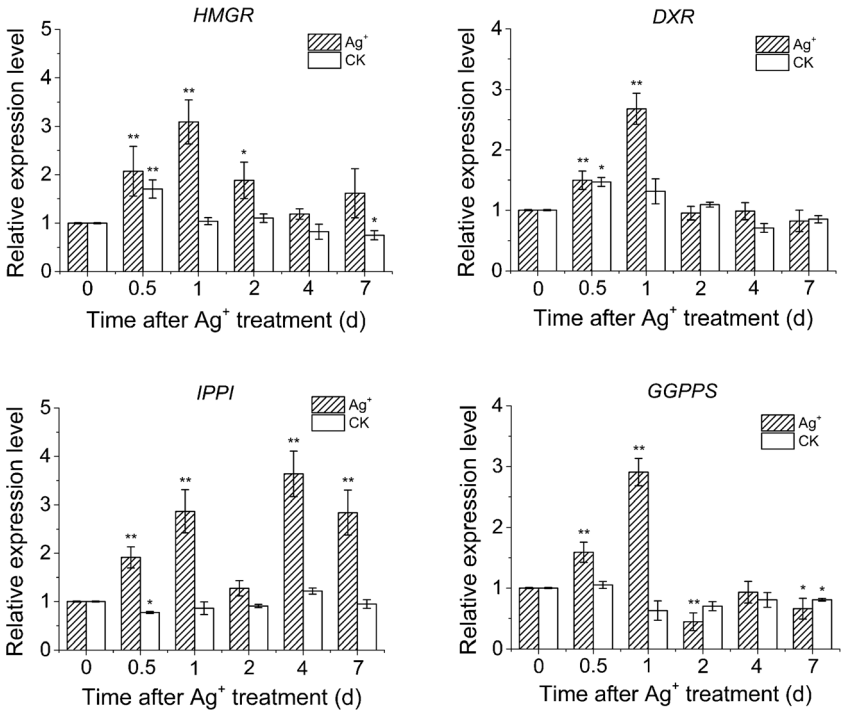


**Fig. 7** Relative expression levels of four key genes in hairy root cultures by 15 μM $Ag^+$ treatment; 15 μM $Ag^+$ was applied in the hairy root cultures of *S. castanea* Diels f. *tomentosa* Stib. within 7 days. All the hairy roots were sampled at 0, 0.5, 1, 2, 4, and 7 days after $Ag^+$ addition. Eighteen-day-old hairy root cultured without elicitation within 7 days was used as control (CK); gene expressions of control hairy root at the initial treatment (0 day) were as 1. **P*<0.05, ***P*<0.01

different from *S. miltiorrhiza* when exposed to various elicitors. *IPPI* and *GGPPS* also played key roles in the middle stage of tanshinone biosynthetic pathway (Ma et al. 2015). Overexpression of *GGPPS* and high production of tanshinone in *S. miltiorrhiza* hairy root illustrated that *GGPPS* is an important regulatory site due to its location in the tanshinone biosynthetic pathway (Kai et al. 2011). In the present study, transcripts of all selected genes were activated and promoted by MeJA at different time points, and *HMGR* and *IPPI* showed preferable upregulations than *DXR* and *GGPPS*; however, the maximum *IPPI* expression level showed notable hysteresis compared with the other genes, which is mainly due to diverse intensity and sensitivity of selected genes that responded to MeJA. Notably, higher expression levels of *IPPI*

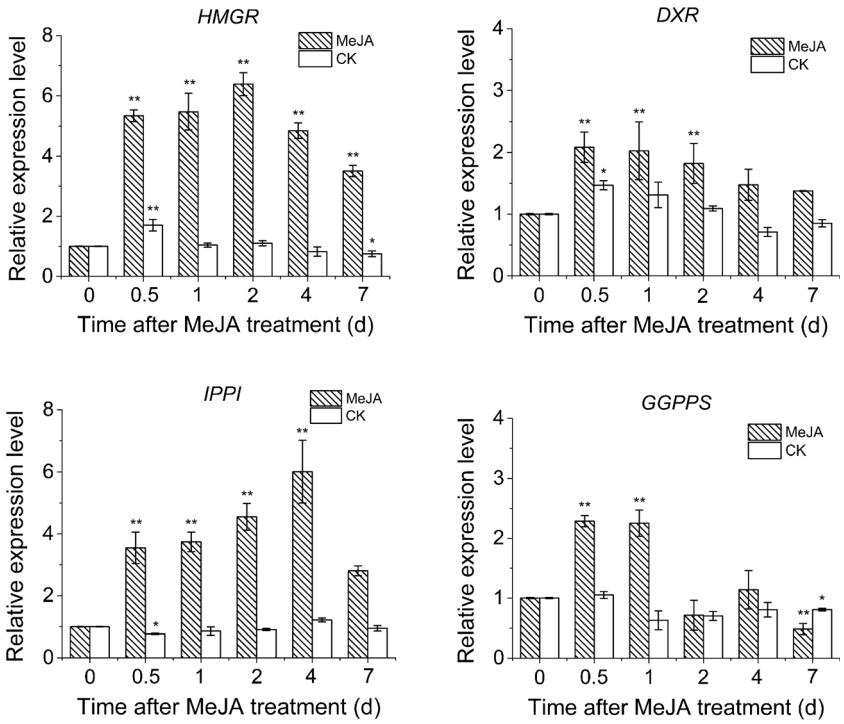


**Fig. 8** Relative expression levels of four key genes in hairy root cultures exposed to 200 μM MeJA; 200 μM MeJA was added in the hairy root cultures of *S. castanea* Diels f. *tomentosa* Stib. within 7 days. All the hairy roots were sampled at 0, 0.5, 1, 2, 4, and 7 days after MeJA addition. Eighteen-day-old hairy root cultured without elicitation within 7 days was used as control (CK); gene expressions of control hairy root at the initial treatment (0 day) were as 1. **P*<0.05, ***P*<0.01

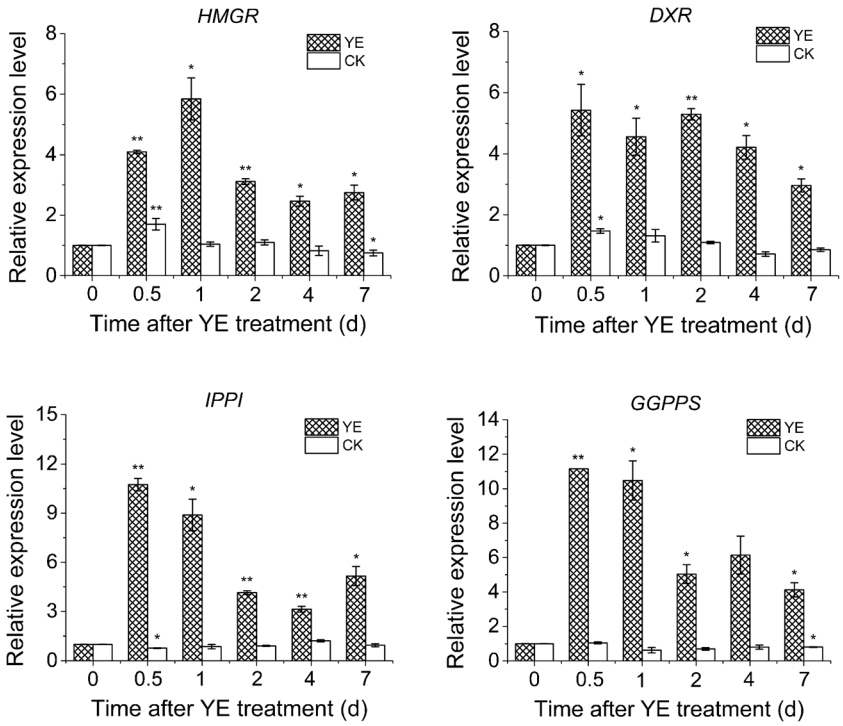


**Fig. 9** Relative expression levels of four key genes in hairy root cultures exposed to 200 mg $l^{-1}$ YE; 200 mg $l^{-1}$ YE was applied in the hairy root cultures of *S. castanea* Diels f. *tomentosa* Stib. within 7 days. All the hairy roots were sampled at 0, 0.5, 1, 2, 4, and 7 days after YE addition. Eighteen-day-old hairy root cultured without elicitation within 7 days was used as control (CK); gene expressions of control hairy root at the initial treatment (0 day) were as 1. **P*<0.05, ***P*<0.01

and *GGPPS* genes were also observed in *S. castanea* Diels f. *tomentosa* Stib. hairy root cultured with YE elicitor, which has more advantages than treatments using other elicitors (Fig. 9). Meanwhile, the addition of YE promoted tanshinone accumulation intensely (Fig. 5c). Our results demonstrated a tight relationship between promoted *IPPI* and *GGPPS* expressions and affluent tanshinone accumulation in *S. castanea* Diels f. *tomentosa* Stib. hairy root cultures with exposure to YE. Furthermore, these two genes are confirmed to be more sensitively and effectively regulated by elicitors especially YE. The signal transduction of the polysaccharide elicitor, such as YE, is confirmed to be initiated by the receptors on the cell plasma membrane of plant tissues (Savitha et al. 2006). Totally, the optimum concentration of the three elicitors showed substantially positive improvements on tanshinone biosynthetic related gene expressions in this hairy root culture.

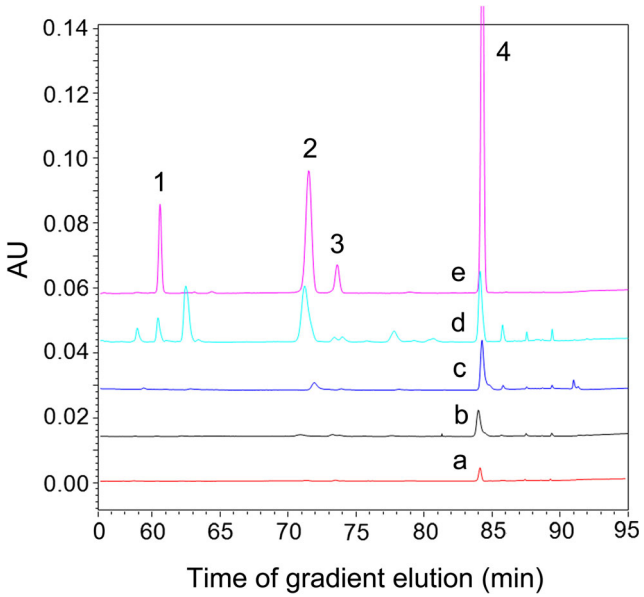


**Fig. 10** HPLC chromatographic profiles of *S. castanea* Diels f. *tomentosa* Stib. hairy roots treated by different elicitors. Peak 1, dihydrotanshinone; peak 2, cryptotanshinone; peak 3, tanshinone I; peak 4, tanshinone IIA. *a* Hairy root control without elicitation. *b* Hairy root treated by $Ag^+$. *c* Hairy root exposed to MeJA. *d* Hairy root treated by YE. *e* Mixed standard substances

In conclusion, this is the first study on the establishment of hairy root cultures of endemic *S. castanea* Diels f. *tomentosa* Stib. The growth and tanshinone accumulation of hairy root treated by different concentrations of elicitors were evaluated. YE elicitor enhanced both tanshinone contents and gene expressions in the hairy root cultures intensively, which is better than $Ag^+$ and MeJA. Furthermore, the expression levels of selected tanshinone biosynthetic related genes were promoted apparently prior to tanshinone accumulation in the time courses of hairy root cultures when exposed to different elicitors. Our study provides an effective and convenient approach to gain more tanshinones in the hairy root cultures of *S. castanea* Diels f. *tomentosa* Stib. and elucidates the relationship between tanshinone accumulation and gene expression in this hairy root culture with diverse elicitation.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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