Mol Biol Rep (2012) 39:1721–1729
DOI 10.1007/s11033-011-0912-1

Effects of different elicitors on yield of tropane alkaloids in hairy roots of *Anisodus acutangulus*

Guoyin Kai · Sheng Yang · Yan Zhang · Xiuqin Luo · Xueqing Fu · Ang Zhang · Jianbo Xiao

Received: 30 September 2010 / Accepted: 18 May 2011 / Published online: 28 May 2011

Abstract The four tropane alkaloids have played a pivotal role in controlling diseases such as the toxic and septic shock, the organophosphorus poison and the acute lung injury. Here, the elicitation effect of different elicitors on the production of tropane alkaloids and the molecular mechanism of enzyme genes in the pathway was firstly demonstrated in hairy roots of *Anisodus acutangulus*. The results showed ethanol, methyl jasmonate and Ag^{+} could improve the accumulation of tropane alkaloids up to 1.51, 1.13 and 1.08 times after 24 h treatment, respectively ($P < 0.05$), whereas salicylic acid decreased the average content of tropane alkaloids. Furthermore, expression profile analysis results revealed that up-regulation of hyoscyamine-6b-hydroxylase (*AaH6H*) and little regulation of tropinone reducase II (*AaTR2*) elicited by ethanol, increased expression of putrescine *N*-methyltransferase I (*AaPMT1*) elicited by Ag^{+}, elevated expression of tropinone reducase I (*AaTR1*) elicited by methyl jasmonate, respectively, resulted in tropane alkaloids improvement. Our results showed that hairy root culture of *A. acutangulus* in combination with elicitors was a promising way for production of tropane alkaloids in the future.

Keywords *Anisodus acutangulus* · Hairy roots · Tropane alkaloids · Elicitors · Biosynthetic genes

G. Kai (✉) · S. Yang · Y. Zhang · X. Luo · X. Fu · A. Zhang · J. Xiao
Laboratory of Plant Biotechnology, College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, People's Republic of China
e-mail: gykai@shnu.edu.cn; gykai@yahoo.com.cn

Abbreviations

A. acutangulus	*Anisodus acutangulus*
TA	Tropane alkaloids
MeJA	Methyl jasmonate
SA	Salicylic acid
EtOH	Ethanol
SA/EtOH	Salicylic acid dissolved in ethanol
RT-PCR	Reverse transcriptase-polymerase chain reaction
DW	Dry weight
FW	Fresh weight

Introduction

Tropane alkaloids (TA), such as hyoscyamine, scopolamine, anisodamine, and anisodine, are widely used as anticholinergic agents, which act on the parasympathetic nervous system. They have protective effects on acute lung injury induced by oleic acid and microvascular injury of acute renal failure in rats [1, 2]. These compounds are mainly found in Solanaceae plants such as *Anisodus*, *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia* [3, 4]. However, the supply of TA is unable to meet the increasing needs of the pharmaceutical market because of low contents in natural plants. So the alternative methods for TA production such as conventional inter-specific hybridization, chemical synthesis and plant cell cultures were developed. Among these methods, the hairy root technology was found to be a beneficial way [5]. Small scale jar fermenters for several *Solanaceous* species hairy roots have been developed in vitro for production of TA [6, 7].

The use of elicitors including abiotic elicitors such as metal ions, inorganic compounds and biotic elicitors such as fungi, bacteria, viruses, and chemicals which are defined as compounds that not only stimulate any type of defense response, but also induce accumulation of phytoalexins in plants, is very promising and well documented for enhanced production of metabolite from plant cell culture recently [8–10]. In hairy root cultures, the accumulation of TA in several Solanaceae species is a characteristic response to the elicitors [11].

Anisodus acutangulus is a *Solanaceous perennial* plant, which is endemic to China and classified as an endangered species. It contains much TA through HPLC analysis and has been used as an anesthetic herb in Yunnan Province for several hundred years [12, 13]. It is reported that total TA reached above 1.2% DW in wild *A. acutangulus*, which is much higher than the common Solanaceae species, so it has been an attractive resource for the production of TA in China [14].

Lastly, we successfully established hairy root cultures of *A. acutangulus* [7], which provided a new approach to obtain TA. Meanwhile, several important genes involved in TA biosynthetic pathways including *AaH6H*, *AaPMT1, AaPMT2, AaTR1* and *AaTR2* have been isolated from *A. acutangulus* by our laboratory [12–14]. However, there is little information on the relationship between the yield of TA and expression levels of the above genes in *A. acutangulus* when elicited by elicitors. Herein, some elicitors including MeJA, SA/EtOH, Ag^+ and EtOH are firstly used to examine their effects on the yield of four TA and the expression levels of TA biosynthetic genes in hairy roots of *A. acutangulus* (Table 1).

Table 1 Primer pairs employed for the PCR amplification of TA biosynthetic genes *AaPMT1, AaPMT2, AaTR1, AaTR2* and *AaH6H* in *A. acutangulus*

AaPMT1	Forward: 5′-ATGGAGGTCATAAGCAACCAC-3′
	Reverse: 5′-TCAAAATTCAACCAAATCCC-3′
AaPMT2	Forward: 5′-ATGGAGGTCATAAGCAACCA-3′
	Reverse: 5′-TTAATCTAATTCCG ACTCCATC-3′
AaTR1	Forward: 5′-ATGGGAGAATCAAAAGTTTACAT-3′
	Reverse: 5′-TCAAAATCCACCATTAGCTGTGA-3′
AaTR2	Forward: 5′-ATGGCAGGAAGGTGGAATCTTGA-3′
	Reverse: 5′-TTAAAACCCACCATTAGCCATAA-3′
AaH6H	Forward: 5′-ATGGCTACTCTTGTCTCAAATTG-3′
	Reverse: 5′-TAGGCATTGATTTTATATGGC-3′

Primer pairs: KF of *AaPMT1, AaPMT2, AaTR1, AaTR2* and *AaH6H* (forward) and KR of *AaPMT1, AaPMT2, AaTR1, AaTR2* and *AaH6H* (reverse)

Materials and methods

Plant transformation and root cultivation

The hairy roots were obtained according to our previous paper [7]. Seven hairy roots were chosen and inoculated in 150 ml 1/3N6 liquid nutrient media at 27°C with shaking in darkness.

Choice of hairy root clone number

After 3 weeks' cultivation, total RNA was extracted from these 7 hairy root clones with RNA pure Plant Kit (Tiangen Biotech Co., Ltd, Beijing, China) according to the manufacturers' handbook. Then it was reversely transcribed by using avian myeloblastosis virus (AMV) reserve transcriptase (Promega, China) to generate cDNA. At last we chose number 2 hairy root clone. This clone was young and its metabolism was active. The genes' expression was a little higher than other clones' (Fig. 1).

Preparation and addition of four elicitors

A solution of 2.4 mol/l SA (Shanghai Zhanyun Chemical Co., Ltd) was prepared by dissolving 2 g SA powder with 6 ml EtOH. MeJA (Sigma, China) was dissolved in DMSO with the concentration of 4.4583 mol/l. $AgNO_3$ (Shanghai Institute of Fine Chemical Materials) was dissolved in distilled water with the concentration of 0.3 mol/l. All solutions were sterilized according to the process of leaching. Sterilized solutions were individually added to 28-day-old hairy root cultures to a final concentration of 100 μmol/l (SA and MeJA), 30 μmol/l (Ag^+) and 6 ml

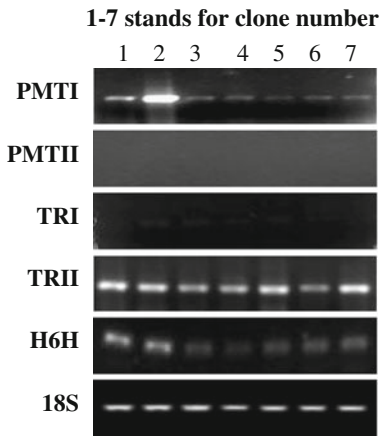

Fig. 1 The expression of five key enzyme genes in biosynthetic pathways of *A. acutangulus* hairy roots analyzed by RT-PCR. Unlike *AaAPMT1*, *AaPMT2* expressed little in roots [12]. The expression of *AaPMT2* was so much weaker than *AaPMT1* expression that we could not see its bands on the electrophoretogram even if we amplified it through RT-PCR

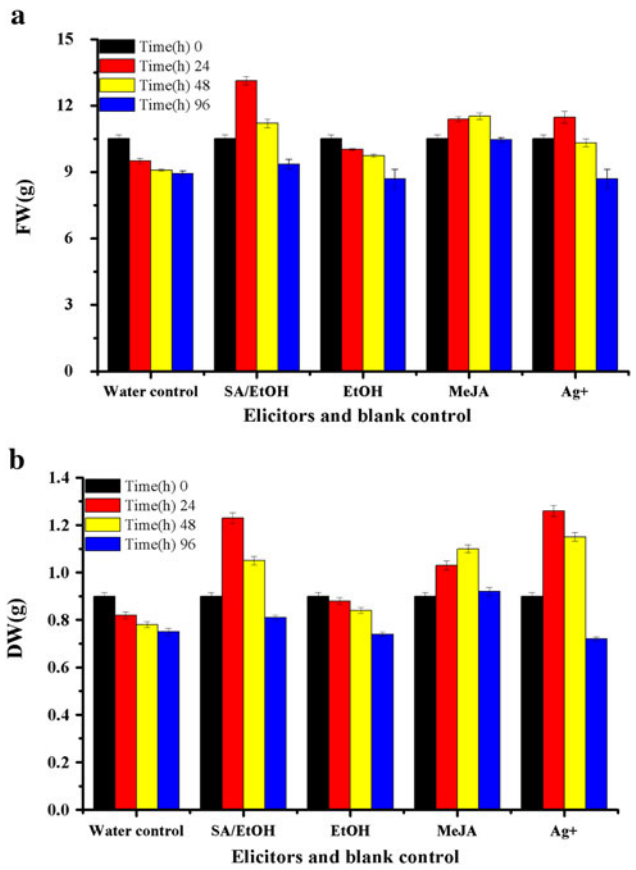

Fig. 2 Effects of elicitors on the fresh growth (**a**) and dry growth (**b**) of hairy roots

(EtOH). The control group was carried out by the addition of water. The effect was measured at 24, 48, and 96 h after elicitation.

RNA extraction and gene expression studies using RT-PCR

When the roots were ready for harvest, the hairy roots in every flask were taken out and weighted. RNA was extracted using RNA pure Plant Kit (Tiangen Biotech Co., Ltd, Beijing, China) following the manufacture's instructions and quantified using spectrophotometric measurements, then kept at −80°C for further analysis. Aliquots of total RNA (1 μg) was used as a template to generate cDNA using avian myeloblastosis virus (AMV) reserve transcriptase (Promega, USA) and then for further semi-quantitative RT-PCR to quantify gene expression profiles of different samples. RT-PCR was carried out using similar procedure as reported before [12, 13].

Extraction and HPLC Analysis of TA

TA was extracted from the hairy roots according to our previous paper [7]. HPLC analyses were performed to determine the contents of anisodamine (National Institutes for Food and Drug Control, China), anisodine (National Institutes for Food and Drug Control, China), hyoscyamine (Sigma, China) and scopolamine (Sigma, China) on a

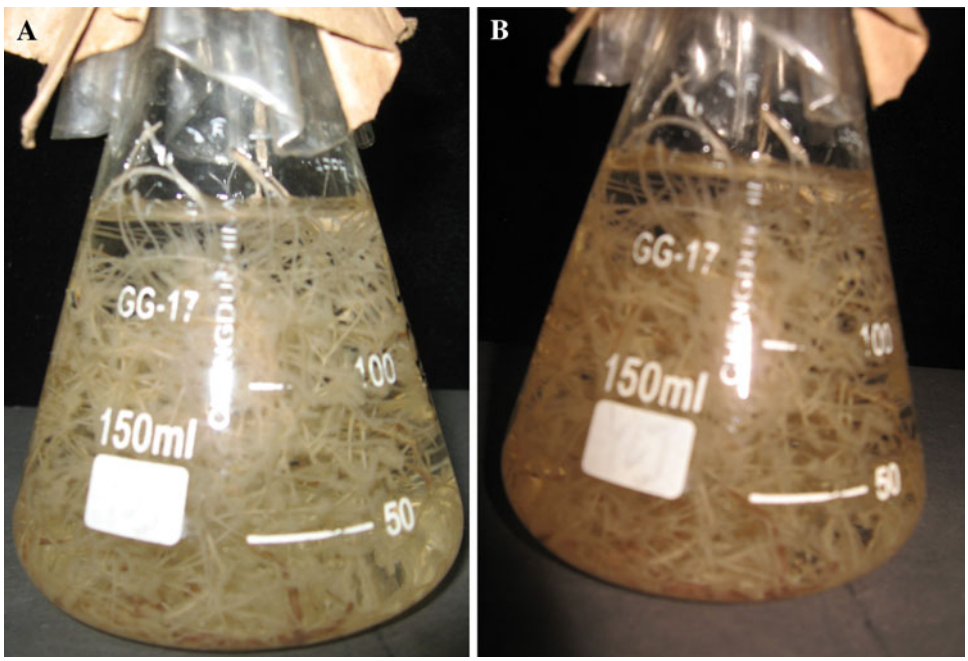

Fig. 3 The morphology of *A. acutangulus* hairy roots. The control group at 96 h (**a**) the group treated by SA/EtOH at 96 h treatment (**b**)

Sepax reversed-phase symmetry column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of 22% acetonitrile (HPLC grade) and 78% diethylamin buffer (containing 0.7% diethylamine and adjust pH to 7.2 with orthophosphoric acid). The flow rate was 1.0 ml/min and the injection volume was 40 μl. The chromatogram was monitored at 220 nm on a HITACHI Diode Array Detector L2455. 1 mg/ml of anisodamine, anisodine, hyoscyamine and 0.5 mg/ml of scopolamine were prepared with methanol as the authentic standard. The peak purity of the test samples was determined by comparing their UV spectra to that of the authentic standard. Quantification was based on the peak area of the original sample injected. The recovery rate of extraction was 100% with RSD 0.53% for anisodamine, 99.90% with RSD 0.65% for hyoscyamine, 99.94% with RSD 0.13% for anisodine and 99.96% with RSD 0.92% for scopolamine, respectively. The accuracy and reproducibility of HPLC analysis were quantified using standard curves fit with linear regression, which was liner in the range of 5–40 μg with the correlation coefficient 1.0000 for anisodamine, 5–40 μg with the correlation coefficient 0.9990 for hyoscyamine, 5–40 μg with the correlation coefficient 0.9994 for anisodine, 2.5–20 μg with the correlation coefficient 0.9996 for scopolamine, respectively.

Statistical analysis

Three independent biological samples from both control and elicitor-treated hairy root cultures were analyzed at each time point with SPSS software. A two-factor repeated measure ANOVA was used to identify TA accumulations which showed significant changes in relative abundance, in response to elicitor treatment at different time points. Significance level was set as 5%.

Result

Time courses of hairy roots biomass growth after elicitor treatment

The effects of elicitors used on the biomass of hairy roots were investigated. As compared to control, SA/EtOH, Ag^+ and MeJA could stimulate accumulation of the fresh weight (FW) and the dry weight (DW) before 48 h treatment (Fig. 2a, b). But after 96 h treatment, FW and DW induced by SA/EtOH, Ag^+ and MeJA decreased, which were less than control. Moreover, the hairy roots in this time point looked a little brownish in these groups (Fig. 3). Contrarily, EtOH hardly affected the FW or DW of hairy roots, which was different with other three elicitors (Fig. 2a, b).

Yields of TA elicited by EtOH, MeJA, Ag^+ and SA/EtOH

The production of four TA was analyzed after treated with elicitors for 0, 24, 48 and 96 h, respectively (Fig. 4). EtOH induced a 1.51-fold increase in the level of total TA at 24 h treatment ($P < 0.05$). Specifically, the accumulation of anisodamine and hyoscyamine was increased by 40 percent and 25 percent as compared with the control group ($P < 0.05$) (Fig. 5a, c). The yield of anisodine was found to be 469 μg g DW^{-1}, which was 1.49-fold higher than the control group (314.48 μg g DW^{-1}) ($P < 0.05$) (Fig. 5e). The largest yield of scopolamine was recorded as 659.2 μg g DW^{-1}, which showed a 3.88-fold increase

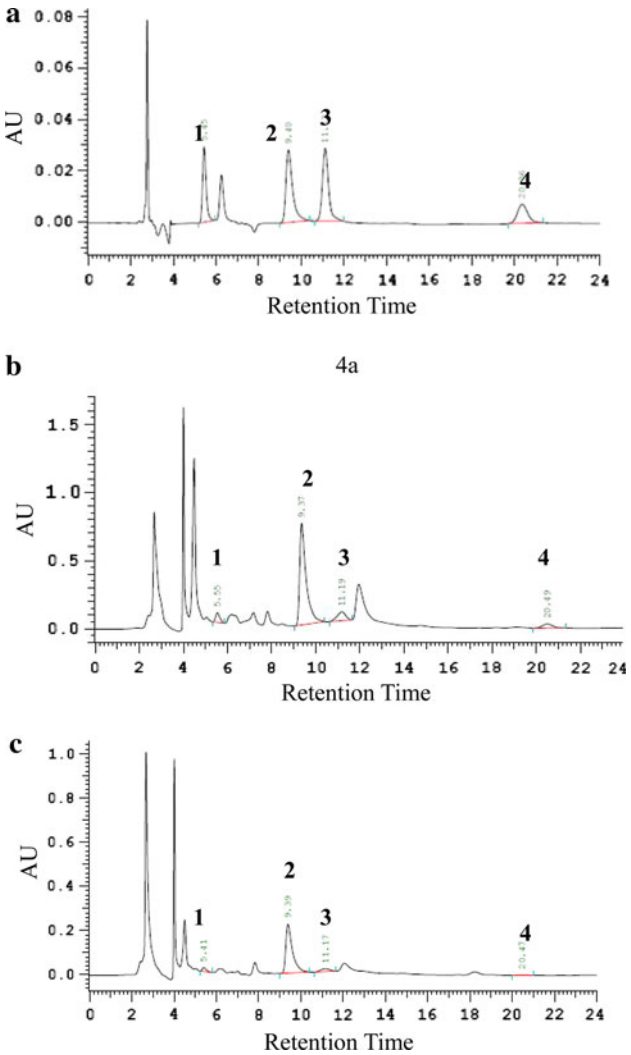

Fig. 4 HPLC chromatograms showing separation of TA from extracts. Separation was carried out on a reversed-phase symmetry 1 column using 22% acetonitrile and 78% water (diethylamine dissolved) and a flow rate of 1 ml/min. (**a**) mixture of standards of TA. (**b**) TA analysis in the samples of hairy roots. (**c**) TA analysis in the control group. *1* anisodamine, *2* hyoscyamine, *3* anisodine, *4* scopolamine

compared with the control group (170 μg g DW^{-1}) ($P < 0.05$) (Fig. 5g).

As compared to control, the levels of TA treated with MeJA increased to 1.13-fold at 24 h treatment ($P < 0.05$). But the yield of TA was suddenly lower at 48 h treatment. However, at 96 h treatment, the yield of TA improved again (Fig. 5a, c, e, g). For example, the accumulation of anisodamine was 431.8 μg g DW^{-1}, representing a 1.06-fold increase at 24 h treatment compared with control (407.28 μg g DW^{-1}) ($P < 0.05$). But it reduced to 332.48 μg g DW^{-1}, which was less than the level of the control (396.62 μg g DW^{-1}) at 48 h treatment. However, it increased again to 430.68 μg g DW^{-1}, which improved by 1.18 times compared with the control at 96 h treatment (365.44 μg g DW^{-1}) ($P < 0.05$) (Fig. 5a). The yields of hyoscyamine, anisodine and scopolamine showed similar trends with anisodamine in MeJA group.

Similar to MeJA group, the yield of TA in Ag^+ group enhanced at 24 h treatment, then decreased at 48 h treatment and at last increased at 96 h treatment compared with that in the control group. The highest contents of anisodamine, hyoscyamine, anisodine and scopolamine were 438.28, 1417.84, 340.28 and 211.2 μg g DW^{-1}, which was improved to 1.08, 1.06, 1.08 and 1.24 times, respectively at 24 h treatment in comparison with the control group ($P < 0.05$) (Fig. 5a, c, e g).

In SA/EtOH group, although the contents of hyoscyamine (1351.68 μg g DW^{-1}) at 48 h treatment, anisodamine (380.24 μg g DW^{-1}) and scopolamine (118.4 μg g DW^{-1}) at 96 h treatment were a little higher than that in the control

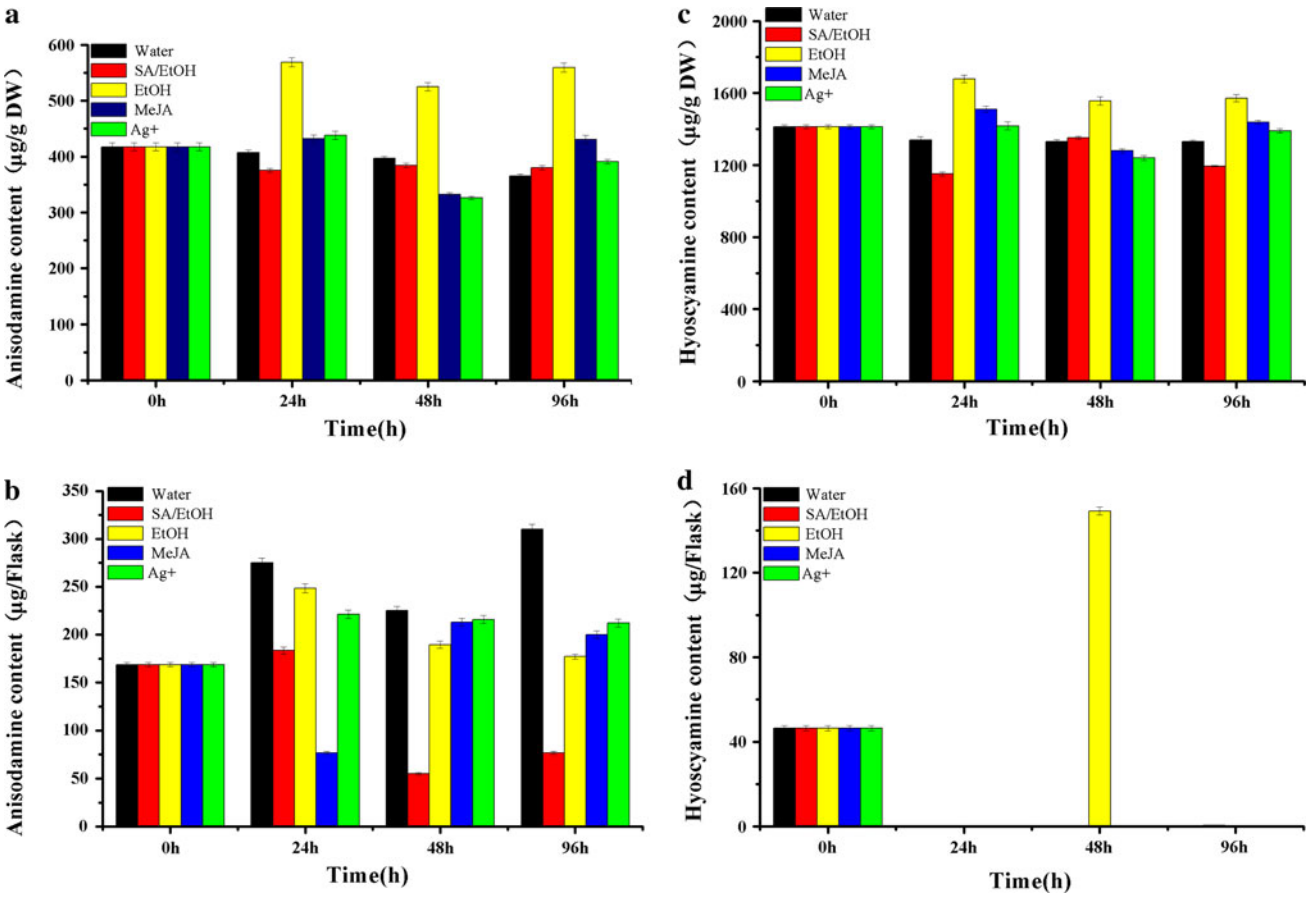

Fig. 5 Anisodamine content in hairy roots (**a**) and in 1/3N6 liquid nutrient medium (**b**) after elicitation of *A. acutangulus* hairy root cultures with EtOH, Ag^+, MeJA and SA/EtOH. Each value is the mean of three replicates standard error. Hyoscyamine content in hairy roots (**c**) and in 1/3N6 liquid nutrient medium (**d**) after elicitation of *A. acutangulus* hairy root cultures with EtOH, Ag^+, MeJA and SA/EtOH. However, we could not detect hyoscyamine at some points in time in 1/3N6 liquid nutrient medium due to the low production. Each value is the mean of three replicates standard error. Anisodine content in hairy roots (**e**) and in 1/3N6 liquid nutrient medium (**f**) after elicitation of *A. acutangulus* hairy root cultures with EtOH, Ag^+, MeJA and SA/EtOH. However, we could not detect anisodine at some points in time in 1/3N6 liquid nutrient medium due to the low production. Each value is the mean of three replicates standard error. Scopolamine content in hairy roots (**g**) and 1/3N6 liquid nutrient medium (**h**) after elicitation of *A. acutangulus* hairy root cultures with EtOH, Ag^+, MeJA and SA/EtOH. However, we could not detect scopolamine in 1/3N6 liquid nutrient medium due to the low production. Each value is the mean of three replicates standard error

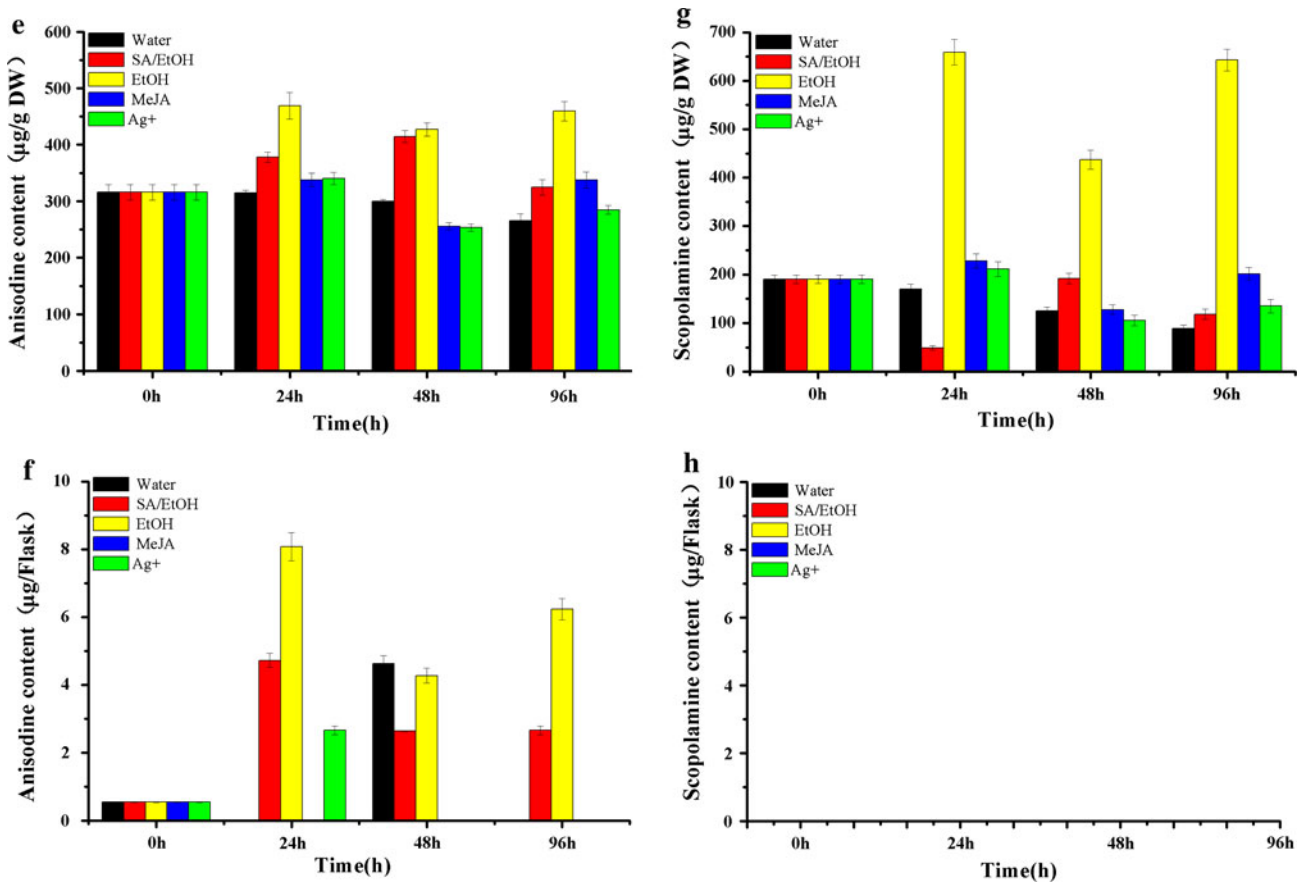

Fig. 5 continued

group (1331.12, 365.44 and 89.2 μg g DW^{-1}), respectively, the yields of them were found to decrease on average (Fig. 5a, c, g). Contrarily, SA/EtOH could improve the production of anisodine all the time, with the highest yield of 414.52 μg g DW^{-1} at 48 h treatment, which showed a 1.38-fold increase (Fig. 5e) ($P < 0.05$).

Effects of EtOH, MeJA, Ag^+ and SA/EtOH on the gene expression

The expression of *AaPMT1* reached the highest level at 24 h treatment in EtOH group. But its levels decreased after 24 h treatment (Fig. 6a, e). *AaTR1* showed little detectable transcript accumulation from 0 h to 96 h (Fig. 6a, e). With respect to *AaH6H*, its expression showed increasingly higher than that in the control group from 0 to 96 h. As for *AaTR2*, the mRNA level gradually decreased from 0 h to 96 h although its expression level was a little higher than the level in the control group after 48 h treatment (Fig. 6a, e).

In MeJA group, mRNA level of *AaPMT1* was a little higher than the level of the control group at 24 h treatment. But the levels decreased and represented a similar trend to the control group after 48 h treatment (Fig. 6b, e). *AaTR1* reached the highest level and was higher than the expression in the control group at 48 h treatment (Fig. 6b, e). The expression of *AaH6H* reached the highest level at 24 h treatment. But its level showed a minor difference from the level in the control group (Fig. 6b, e). *AaTR2* decreased significantly at 24 h treatment. But its level gradually increased at 48 h treatment and reached the highest level at 96 h treatment (Fig. 6b, e).

In Ag^+ group, the expression level of *AaPMT1* increased from 0 h and approached to a maxim value at 48 h treatment, which was significantly higher than the value in the control group. However, the transcript levels of *AaPMT1* dramatically decreased at 96 h treatment (Fig. 6c, e). *AaTR1* transcript showed a little higher accumulation than that in the control group at 48 h treatment in Ag^+ group (Fig. 6c, e). *AaH6H* transcript gradually decreased from 24 to 96 h, whose expression level displayed a minor difference from the level in the control group (Fig. 6c, e). *AaTR2* gradually increased after 0 h and reached the highest level at 48 h treatment. Then its expression levels decreased at 96 h treatment (Fig. 6c, e).

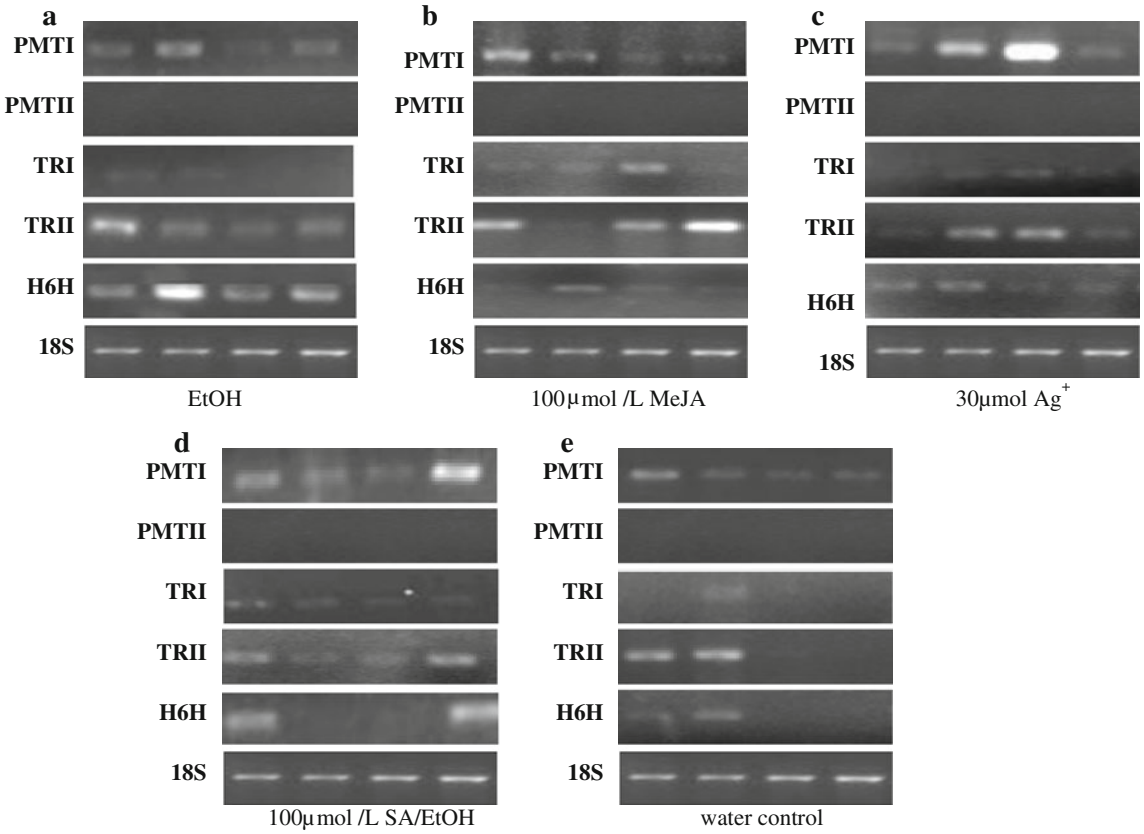

Fig. 6 Expression profiles of five key enzyme genes in biosynthetic pathways of *A. acutangulus* hairy roots analyzed by RT-PCR at 0, 24, 48 and 96 h. The total RNAs used in RT-PCR were isolated from the number 2 hairy roots that exposed to EtOH (**a**), 100 μmol/l MeJA (**b**), 30 μmol/l Ag^+ (**c**), 100 μmol/l SA/EtOH (**d**) and water control (**e**) for the indicated times

In SA/EtOH group, mRNA level of *AaPMT1* was similar to the control group before 48 h treatment, but its level reached the highest at 96 h treatment (Fig. 6d, e). *AaTR1* transcript showed a little higher accumulation than that in the control group at 48 h treatment (Fig. 6d, e). *AaH6H* decreased from 24 to 48 h, but dramatically increased and reached the highest level at 96 h treatment (Fig. 6d, e). *AaTR2* showed little detectable transcript accumulation before 48 h treatment. But its expression level reached the highest after 96 h treatment and higher than that in the control group (Fig. 6d, e).

Discussion

The *A. acutangulus* hairy root cultures can be a valuable tool to study plant second metabolism, since it can be assessed in strictly controlled conditions [5, 15]. Our study reported EtOH, MeJA and Ag^+ could enhance the production of four TA on average in *A. acutangulus* hairy root cultures. Furthermore, TA accumulation was also discharged into the medium (Fig. 5b, d, f). This may be because the produced TA had some damage to the hairy roots. Therefore, the addition of elicitors may commonly cause negative effects on the growth of hairy roots such as tissue browning and decomposition [16–20]. Meanwhile, the effect of elicitors on the gene expression was also studied because of the importance of integration of gene transcripts and metabolites for the study of secondary metabolism [21]. In our work, the accumulation of TA may depend on the antagonistic effect between positive genes such as the *AaPMT1*, *AaTR1* and *AaH6H* and the negative gene *AaTR2* (Fig. 7).

The extent of EtOH-induced TA accumulation was the highest in the elicitor used, which indicated the powerful ability of EtOH to stimulate TA biosynthesis. Similar stimulatory effects of EtOH were also reported in *Escherichia coli* and *Glycine max* [22, 23]. Furthermore, EtOH hardly affected the growth of hairy roots (Fig. 4a). Therefore, this elicitor will hold a potential value in improving TA production. The effect of EtOH on the yield of TA may be because it plays a role of an activator that could stimulate the genes involved in TA production. Analysis of gene expression revealed that EtOH led to little expression level of *AaTR2*, a branching point in the biosynthesis of TA in *A. acutangulus* [13], while obviously

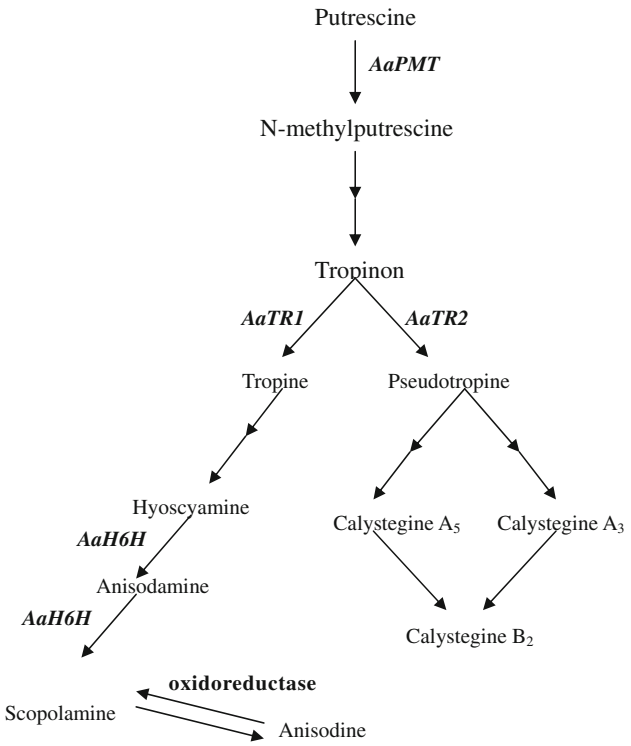

Fig. 7 Scheme of the main biosynthetic tropane alkaloid pathway in *A. acutangulus*

increased the expression level of *AaH6H*, which catalyzes the last committed step in the biosynthesis of TA [14], in the whole time. These dual positive effects could account for the fact that EtOH improved the yield of TA largely. Analogous effects of EtOH were also found in *Atropa baetica* hairy root cultures [11].

MeJA played a pivotal role in the elicitation process leading to gene activation coded for the enzymes involved in pathways of secondary metabolites [24–26]. Our work showed MeJA augmented the production of TA by means of inhibiting *AaTR2* at 24 h treatment, providing tight molecular evidence for improving TA content. At 48 h treatment, the up-regulation of *AaTR2* seems to be more effective than *AaTR1,* the second committed step in TA biosynthetic pathway [13], which led to weakening the production of TA. However, the up-regulation of *AaTR2* at 96 h treated with MeJA did not seem to be enough to explain the high yield of TA, which showed TA production induced by MeJA could be independent from the transcriptional activation of *AaTR2*. This may be due to the activation of different signaling pathways or the direct activation of other genes [11]. Similar conclusion was got in *Artemisia annua* suspension cultures [27].

Ag^+ is supposed to play a role in blocking ethylene which had a negative effect on TA production in the plant [9]. It indeed improved TA yield in our study although the production was not high. Parallel result was also reported in *Salvia miltiorrhiza* hairy root cultures [28, 29]. Integrating expression levels and TA production, Ag^+ could stimulate the accumulation of TA by means of high expression of *AaPMT1* gene, which catalyzes the first committed step in TA biosynthetic pathway. However, up-regulation of *AaPMT1* just improved the precursor of TA named *N*-methylputrescine. There were still two steps to generate hyoscyamine (Fig. 7), which could explain why Ag^+ improved a little yield of TA on average. Similar conclusions were also reported in *Atropa belladonna* and *Duboisia* hybrid [30, 31].

SA/EtOH, led to the decrease of the mean production of TA except anisodine because of its resistible effect, which was consistent to the result reported in *Atropa belladonna* root cultures or in plants of cotton [32, 33]. From the analysis of expression profiles, there was little expression on *AaPMT1* and *AaH6H* gene before 48 h, coinciding with the low production of hyoscyamine, anisodamine and scopolamine, which agreed to the result reported in *Atropa baetica* [11]. On the contrary, SA could elicit the production of secondary metabolites like scopolamine in other Solanaceae plants [26, 34]. Taking together the results above, the plant response to SA/EtOH seemed to be species-related. Moreover, that the production of anisodine was always higher than the control in SA/EtOH group attracted our attention (mechanism is not clear). Therefore, studies on biotransformation among different compounds of TA in *A. acutangulus* hairy roots have been performed to find the metabolic pathway of anisodine in our future work.

Acknowledgments This work was supported by National Natural Science Fund (30900110), Shanghai Science and Technology Committee Project (10JC1412000, 09QH1401900, 06QA14038, 08391911800, 073158202, 075405117, 065458022, 05ZR14093), Shanghai Education Committee Fund (09ZZ138, 06DZ015, J50401), Zhejiang Provincial Natural Science Fund (Y2080621), Fujian Science and Technology Committee Key Special Project (2008NZ0001-4), National Transgenic Organism New Variety Culture Key Project (2009ZX08012-002B), Project from Shanghai Normal University (SK200830, CH030). Miss Pin Liu's (Shanghai Jiaotong University, China) kind assistance with HPLC analysis is also acknowledged.

References

1. Wang H, Zhang SH, Huang LS, Zhao RH, Wei HP (1999) Protective effects of anisodine and anisodamine on acute lung injury induced by oleic acid in rats. Chin J Crit Care Med 19(11):663–664
2. Wu GL, Zheng QY, Tian N (2002) Protective effects of anisodine, buscopan, anisodamine on acute renal failure with damages of renal microvessels in rat. Chin J Microcirc 12(1):6–8
3. Zayed R, Wink M (2004) Induction of tropane alkaloid formation in transformed root cultures of *Brugmansia suaveolens* (Solanaceae). Z Naturforsch C 59(11–12):863–867
4. Zhang L, Ding R, Chai YR, Bonfill M, Piñol MT, Xu TF, Pi Y, Wang ZN, Zhang HM, Kai GY, Liao ZH, Sun XF, Tang KX (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus*

niger hairy root cultures. Proc Natl Acad Sci USA 101: 6786–6791
5. Hashimoto T, Yamada Y (1987) Purification and characterization of hyoscyamine 6 beta-hydroxylase from root cultures of *Hyoscyamus niger* L. Hydroxylase and epoxidase activities in the enzyme preparation. Eur J Biochem 164:277–285
6. Oksman-Caldentey KM, Kivel O, Hiltunen R (1991) Spontaneousshoot organogenesis and plant regeneration from hairy root cultures of *Hyoscyamus muticus*. Plant Sci 78:129–136
7. Li L, Wang J, Wang W, Lu Y, Wang YL, Zhou GY, Kai GY (2008) Optimization of induction and culture conditions and tropane alkaloid production in hairy roots of *Anisodus acutangulus*. Biotechnol Bioprocess Eng 13:606–612
8. Boitel-Conti M, Laberche JC, Lanoue A, Ducrocq C, Sangwan-Norreel BS (2000) Influence of feeding precursors on tropane alkaloid production during an abiotic stress in *Datura innoxia* transformed roots. Plant Cell Tissue Organ Cult 60:131–137
9. Pitta-Alvarez SI, Spollansky TC, Giulietti AM (2000) The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of *Brugmansia candida*. Enzyme Microb Technol 26(2–4):252–258
10. Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333
11. El Jaber-Vazdekis N, Barres ML, Ravelo AG, Zarate R (2008) Effects of elicitors on tropane alkaloids and gene expression in *Atropa baetica* transgenic hairy roots. J Nat Prod 71(12): 2026–2031
12. Kai GY, Zhang Y, Chen JF, Li L, Yan XM, Zhang R, Liao P, Lu X, Wang W, Zhou GY (2009) Molecular characterization and expression analysis of two distinct putrescine N-methyltransferases from roots of *Anisodus acutangulus*. Physiol Plant 135: 121–129
13. Kai GY, Li L, Jiang YX, Yan XM, Zhang Y, Lu X, Liao P, Chen JB (2009) Molecular cloning and characterization of two tropinone reductases in *Anisodus acutangulus* and enhancement of tropane alkaloids production in *AaTRI*-transformed hairy roots. Biotechnol Appl Biochem 54(3):177–186
14. Kai GY, Chen JF, Li L, Zhou GY, Zhou LM, Zhang L, Chen YH, Zhao LX (2007) Molecular cloning and characterization of a new cDNA encoding hyoscyamine 6β-hydroxylase from roots of *Anisodus acutangulus*. J Biochem Mol Biol 40(5):715–722
15. Cardillo AB, María Otalvaro Alvarez A, Calabró Lopez A, Enrique Velásquez Lozano M, Rodríguez Talou J, María Giulietti A (2009) Anisodamine production from natural sources: seedlings and hairy root cultures of Argentinean and Colombian *Brugmansia candida* plants. Planta Med 76(4):402–405
16. Chen H, Chen F (2000) Effect of yeast elicitor on the secondary metabolism of Ti-transformed *Salvia miltiorrhiza* cell suspension cultures. Plant Cell Rep 19:710–717
17. Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol 42:185–209
18. Hirano H, Shimomura K, Yamakawa T (2003) Responses of transformed roots of *Atropa belladonna* to treatment with salicylic acid and other phenolic compounds. Plant Biotechnol 20(1): 97–100
19. Suresh B, Thimmaraju R, Bhagyalakshmi N, Ravishankar GA (2004) Polyamine and methyl jasmonate-influenced enhancement of betalaine production in hairy root cultures of *Beta vulgaris* grown in a bubble column reactor and studies on efflux of pigments. Process Biochem 39:2091–2096
20. Wang YD, Wu JC, Yuan YJ (2007) Salicylic acid-induced taxol production and isopentenyl pyrophosphate biosynthesis in suspension cultures of *Taxus chinensis* var. *mairei*. Cell Biol Int 31:1179–1183
21. Rischer H, Oresic M, Seppanen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Van Montagu MC, Inze D, Oksman-Caldentey KM, Goossens A (2006) Gene-tometabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proc Natl Acad Sci USA 103:5614–5619
22. Steczko J, Donoho GA, Dixon JE, Sugimoto T, Axelrod B (1991) Effect of ethanol and low-temperature culture on expression of soybean lipoxygenase L-1 in *Escherichia coli*. Protein Expr Purif 2(2–3):221–227
23. Kuo HF, Tsai YF, Young LS, Lin CY (2000) Ethanol treatment triggers a heat shock-like response but no thermotolerance in soybean (*Glycine max* cv. Kaohsiung No. 8) seedlings. Plant Cell Environ 23:1099–1108
24. Gundlach H, Mfiller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci USA 89:2389–2393
25. Guo BH, Kai GY, Gong YF, Jin HB, Wang YC, Miao ZQ, Sun XF, Tang KX (2007) Molecular cloning and heterologous expression of a 10-deacetylbaccatin III-10-O-acetyl transferase cDNA from *Taxus x media*. Mol Biol Rep 34:89–95
26. Kang SM, Jung HY, Kang YM, Yun DJ, Bahk JD, Yang JK, Choi MS (2004) Effects of methyl jasmonate and salicylic acid on the production of tropane alkaloids and the expression of PMT and H6H in adventitious root cultures of *Scopolia parviflora*. Plant Sci 166:745–751
27. Caretto S, Quarta A, Durante M, Nisi R, De Paolis A, Blando F, Mita G (2009) Methyl jasmonate and miconazole differently affect arteminisin production and gene expression in *Artemisia annua* suspension cultures. Plant Biol 13:51–58
28. Xiao Y, Gao S, Di P, Chen J, Chen W, Zhang L (2009) Lithospermic acid B is more responsive to silver ions (Ag+) than rosmarinic acid in *Salvia miltiorrhiza* hairy root cultures. Biosci Rep 30(1):33–40
29. Xiao Y, Di P, Chen J, Liu Y, Chen W, Zhang L (2009) Characterization and expression profiling of 4-hydroxyphenylpyruvate dioxygenase gene (Smhppd) from *Salvia miltiorrhiza* hairy root cultures. Mol Biol Rep 36(7):2019–2029
30. Sato F, Hashimoto T, Haciya A, Tamura K, Choi K-B, Morishige T, Fujimoto H, Yamada Y (2001) Metabolic engineering of plant alkaloid biosynthesis. Proc Natl Acad Sci USA 98:367–372
31. Moyano E, Fornalé S, Palazón J, Cusidó RM, Bagni N, Piñol MT (2002) Alkaloid production in *Duboisia* hybrid hairy root cultures overexpressing the pmt gene. Phytochemistry 59:697–702
32. Lee KT, Hirano H, Yamakawa T, Kodama T, Igarashi Y, Shimomura K (2001) Responses of transformed root culture of *Atropa belladonna* to salicylic acid stress. J Biosci Bioeng 91:586–589
33. Xu YH, Wang JW, Wang S, Wang JY, Chen XY (2004) Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-δ-cadinene synthase-A. Plant Physiol 135:507–515
34. Frankfater CR, Dowd MK, Triplett BA (2009) Effect of elicitors on the production of gossypol and methylated gossypol in cotton hairy roots. Plant Cell Tissue Organ Cult 98:341–349