

La Dramatically Enhances the Accumulation of Tanshinones in *Salvia Miltiorrhiza* Hairy Root Cultures

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Abstract

The accumulation of tanshinones (tanshinone I, tanshinone II and cryptotanshinone) and the expression of key enzymes (HMGR, HMGS, KSL, DXR, CMK) of tanshinones biosynthetic pathways in *Salvia miltiorrhiza* hairy root cultures induced by La elicitor were investigated in this paper. La at 0.01mmol·L⁻¹ and 0.1mmol·L⁻¹ obviously stimulated the expression of HMGR on day 1 after treatment. The activity of HMGS and KSL was enhanced by La treatment at the earlier stage of the elicitation treatment and declined gradually at later stage. The activity of CMK was significantly improved with the treatment of La at dilution of 0.01mmol·L⁻¹ on day 1 after treatment. La at 0.01mmol·L⁻¹ and 0.1mmol·L⁻¹ improved the activity of DXR by 161.8% and 378.4% respectively on day 3 after treatment. The content of tanshinone I was markedly enhanced by La at 0.01mmol·L⁻¹ and 0.1mmol·L⁻¹ on day 1 after treatment. La at 0.01mmol·L⁻¹ enhanced the content of tanshinone IIA, which was estimated to be 40.9% higher than the control. The content of cryptotanshinone was significantly improved by 92.4% and 71.5% respectively on day 1 and 3 after treatment with La at 0.01mmol·L⁻¹, while it was reduced by La at 0.01mmol·L⁻¹, 0.1mmol·L⁻¹ and 1mmol·L⁻¹ 7 days after treatment. In summary the accumulation of tanshinones and expression of key enzymes of tanshinones biosynthetic pathways were improved by La elicitor with lower dose while inhibited with higher dose. And the effect of La on the accumulation of tanshinones lagged behind its effect on the expression of key enzyme gene.

Keywords: tanshinones, La, *Salvia miltiorrhiza*, isoprenoid pathways

1. Introduction

Salvia miltiorrhizais is a well-known herbal plant, and its roots and rhizomes, called Dan-shen in traditional Chinese medicine (TCM), have been widely used for treatment of cardiovascular and cerebrovascular diseases in China and other countries (Zhou et al., 2005; Dong et al., 2010). The active components of Dan-shen could be classified as lipid-soluble and water-soluble ones, and the former are main tanshinones, such as tanshinone I, tanshinone II and cryptotanshinone (Wang et al., 2007), which show a variety of biological activities such as antioxidant, increasing coronary flow, protecting the myocardium against ischaemia and etc (Li et al., 2008). The ever-increasing demand for Dan-shen in international market has stimulated the improvement of cultivation practices of *S. miltiorrhiza*.

Rare earth elements (REE), referring to elements in the lanthanide series, scandium (Sc) and yttrium (Y), have been widely distributed in China and used in agriculture to improve crop yield and product quality (Wang et al., 2008). There've been many studies that showed REE could improve photosynthesis, nitrogen metabolism and replace calcium (Song et al., 2003; Hong et al., 2002; Zhou et al., 2010; Hong et al., 2005; Liu et al., 2007; Huang et al., 2008). REE could also stimulate the biosynthesis of secondary metabolites in medicinal plants. Our research team has reported that La, an important REE, could improve the accumulation of tanshinones in *S. miltiorrhiza*. However, little is known that how REE mediate the biosynthesis of secondary metabolites in medicinal plants. The mechanism by which La improve the biosynthesis of tanshinones in *S. miltiorrhiza* will be investigated in this paper.

Secondary metabolites are synthesized by a variety of enzymes in medicinal plants. In higher plants, terpenes or

isoprenoids are synthesized via mevalonate (MVA) pathway and 1-deoxy-D-xylulose 5-phosphate (DXP) pathway. HMGS (3-hydroxy-3-methylglutaryl-CoA synthase) and HMGR (3-hydroxy-3-methylglutaryl CoA reductase) catalyze the formation of mevalonate from acetoacetyl-CoA, which is an initial and important step in the MVA pathway. CMK (4-(Cytidine 5-diphospho)-2-C-methylerythritol kinase), DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase) and KSL (Miltiradene synthase) are also important enzymes for the biosynthesis of isoprenoid. Investigating the effects of La on the expression of these key enzymes will allow for a greater understanding of mechanism of La in stimulating the biosynthesis of tanshinones in *S. miltiorrhiza*.

Secondary metabolites can be synthesized by elicitors in hairy root culture, which has been considered as stable and efficient materials for studying the mechanism of the accumulation of secondary metabolites (Xiao et al., 2010). This work was carried out to investigate the effects of La on the expression of the key enzymes (HMGR, HMGS, DXR, CMK, KSL) involved in terpenes or isoprenoids biosynthesis and the accumulation of tanshinones (tanshinone I, tanshinone II and cryptotanshinone) in *S. miltiorrhiza* hairy root cultures for the purpose of understanding the mechanisms by which La improve tanshinones production in *S. miltiorrhiza*.

2. Materials and Methods

2.1 Hairy Root Culture

Seeds of *S. miltiorrhiza* were grown in the gardens of Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences. The plants were identified and voucher specimens were deposited in the Herbarium, the Institute of Chinese Materia Medica.

S. miltiorrhiza hairy root culture was derived after infection of plantlets with a Ri T-DNA bearing *Agrobacterium rhizogenes* bacterium (ACCC10060). Hairy root culture was cultivated in 6, 7-V suspending medium on an orbital shaker set at 110 rpm and 25°C in the dark (Ge et al., 2005). La^{3+} at concentration of 0.01 mmol·L⁻¹, 0.1 mmol·L⁻¹ and 1 mmol·L⁻¹ were treated on day 18 post-inoculation, and hairy root culture with no La^{3+} was designed as control. The hairy roots were harvested on 0 day (before treatment), 1 day, 3 day, 7 day, 15 day, 21 day respectively. About 0.1 g of them, which were used for RNA isolation, were immediately frozen in liquid nitrogen and the other were dry by paper towels and dried at -70°C until constant dry weight for analysis of metabolites.

2.2 RNA Isolation and Real-time Quantitative PCR Analysis

Total RNA from *S. miltiorrhiza* hairy roots was extracted using TRIzol Reagent (GIBCO BRL) according to the manufacturer's instruction and it was reversely transcribed to generate cDNA using avian myeloblastosis virus (AMV) reverse transcriptase (Takara, Japan). Primers used for real-time PCR and the length of target fragments were listed in Table 1. The Real-time PCR was performed according to manufacturer's instruction (Takara, Japan) under the following condition: 10 min pre-denaturation at 95°C, 1 cycle; 15 s denaturation at 95°C, 40 cycles; 1 min denaturation at 60°C, 1 cycles. The products of real time quantitative PCR were run on 1.5% agarose gelelectrophoresis and showed an equal-sized band as predicted. Quantification of the gene expression was done with comparative CT method (Xiao et al., 2009).

Table 1. List of primers used for the real-time quantitative PCR

Gene	Primer
<i>β-Actin</i>	AGGAACCAACCGATCCAGACA GGTGCCCTGAGGTCCTGTT
<i>HMGR</i>	GCAACATCGTCTCCGCCGTCTACA GATGGTGGCCAGCAGCCTGGAGTT
<i>HMGS</i>	GATGCCGACTACTTTGTATTTC CTCGACTTCAACTTCTCTGAA
<i>CMK</i>	ATGAGAAAAGAAGAAGGGGATATCA TCAGGAAAAAGACAGGGGTTG
<i>DXR</i>	GAGAATCTACTGCTCCGAGA CTGGTCGTAGTGGATGATCT
<i>KSL</i>	CTTCCCAAGACAATGCAAAGAT ATTTCCTCTCACATTATTAGC

2.3 Determination of Tanshinones

Quantitative analysis of tanshinones (tanshinone I, tanshinone II and cryptotanshinone) in *S. miltiorrhiza* hairy root was performed according to modifications of the methods by Liu et al. (2007). The dried hairy root (200 mg) was ground into power, immersed with methyl alcohol (50 ml) and sonicated (300 W, 25 kHz) for 40 min. The extract was filtered through a 0.45 μm organic membrane filter and 10 μl was injected for each HPLC analysis. The tanshinones were determined on a Waters RP-C₁₈ (3.9 mm $\times$ 150mm, 5 μm) column. The mobile phase consisted of mobile phase A (water) and B (acetonitrile), using a gradient of 45% A at 0-35 min, 45%-15% A at 35-38 min, 15% A at 38-48 min. The flow rate was 1.0 ml $\cdot$ min⁻¹, the detection wavelength was set at 270 nm, and temperature of column component was maintained at 30 °C.

2.4 Statistics

The results were represented with their mean $\pm$ standard error (SE). Six independent biological samples from both control and La-treated hairy root cultures were analyzed at each time point with SPSS 17.0 software. Significant differences were determined by one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1 Examination of the Quality and Concentration of RNA in *S. miltiorrhiza* Hairy Root Culture

As shown in Figure 1, the result of agarose gel electrophoresis and spectrophotometer analysis of the total RNA showed three clearly distinguishable bands at the 28 s, 18 s and 5.8 s and the ratio of 28 s and 18 s is between 1.8-2.0, which meets the requirements of antitranscription of cDNA.

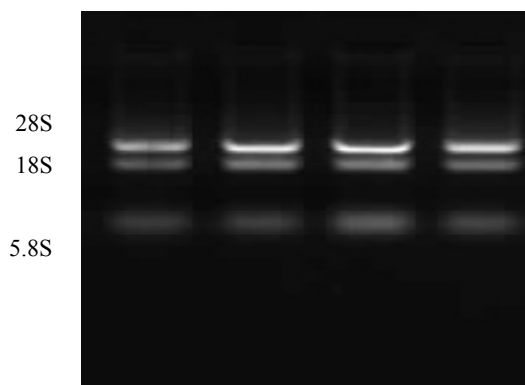


Figure 1. RNA electrophoresis

3.2 Effects of La Elicitor on the Expression of Key Enzymes of Tanshinone Biosynthetic Pathways

Treatment of La elicitor caused a substantial change in the expression of key enzyme (HMGR, HMGS, KSL, DXR, CMK) of tanshinone biosynthetic pathways (Figure 2). The activity of HMGR in hairy roots rose rapidly to the maximum values on day 1 after treatment by La at dilutions of 0.01mmol $\cdot$ L⁻¹ and 0.1mmol $\cdot$ L⁻¹, which were estimated to be 1.5-fold and 1.4-fold higher than the control respectively ($p < 0.05$), while it showed no significant difference by treatment of La at 1mmol $\cdot$ L⁻¹ (Figure 2A). HMGR is the first key enzyme in the MVA pathway, which is responsible for the synthesis of many terpenoids such as sesquiterpenes and triterpenes. The induction of HMGR activity by La elicitor suggested that *S. miltiorrhiza* partly resort to the MVA pathway for the biosynthesis of tanshinone. The activity of HMGS was enhanced by La at 0.01mmol $\cdot$ L⁻¹, 0.1mmol $\cdot$ L⁻¹ and 1mmol $\cdot$ L⁻¹ at the earlier stage of the elicitation treatment and declined gradually at later stage (Figure 2B). The promotion effect of La treatment on the expression of KSL was prominent at earlier stage of the elicitation treatment. KSL activity was enhanced to the peak by La at 0.01mmol $\cdot$ L⁻¹ and 0.1mmol $\cdot$ L⁻¹, and more rapidly and dramatically by La at 0.01mmol $\cdot$ L⁻¹ (Figure 2C). The activity of CMK was significantly improved with the treatment of La at dilution of 0.01mmol $\cdot$ L⁻¹ on day 1 after treatment (Figure 2D). La at 0.01mmol $\cdot$ L⁻¹ and 0.1mmol $\cdot$ L⁻¹ improved the activity of DXR by 161.8% and 378.4% respectively on day 3 after treatment. The promotion effect of La on the activity of DXR was observed later than that of HMGR, HMGS and KSL (Figure 2E). In general the expression of key enzyme gene was stimulated by La elicitor with lower dose.

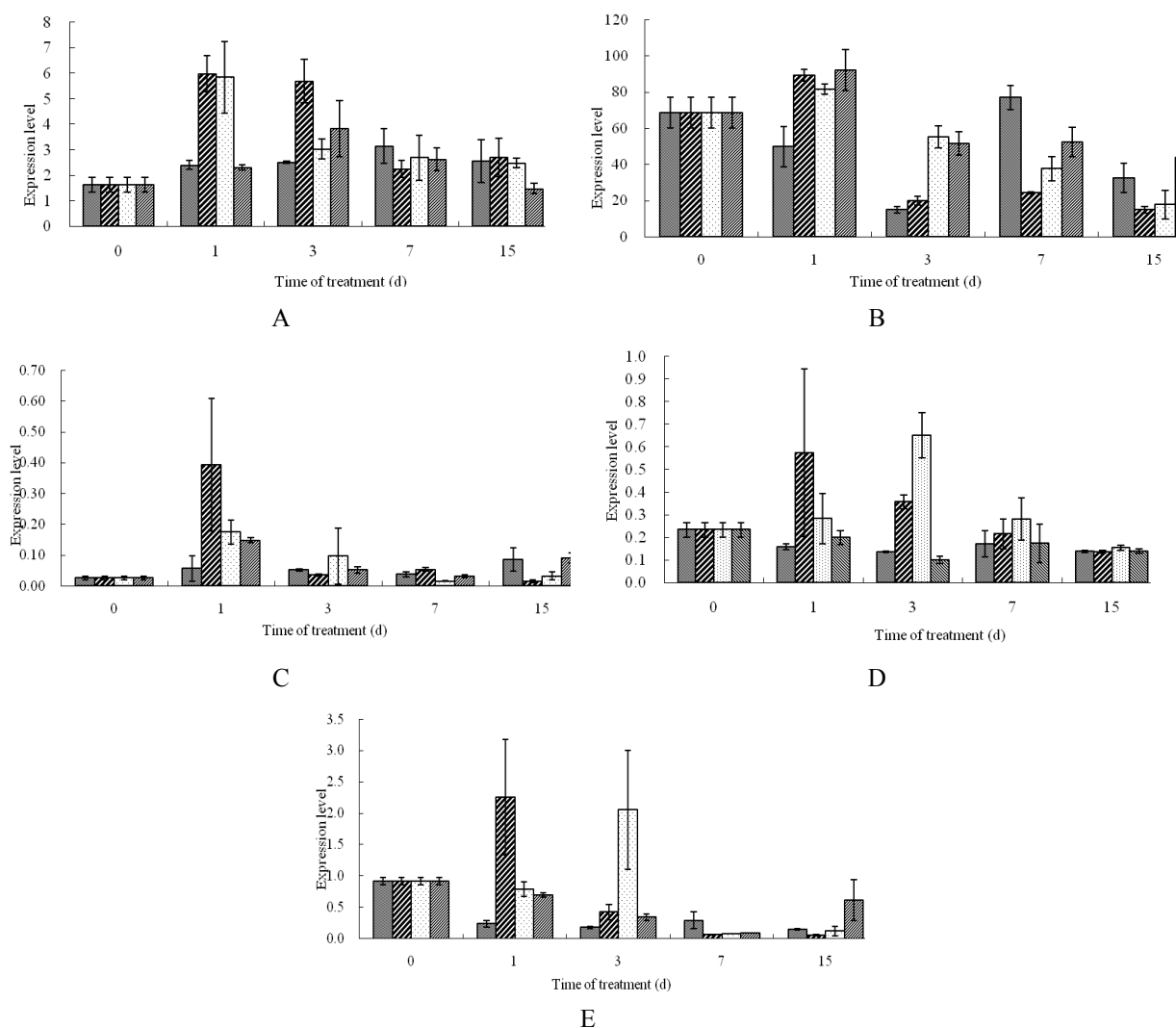


Figure 2. Effects of La elicitor on the expression of key enzyme HMGR (A), HMGS (B), KSL (C), DXR (D) and CMK (E) gene transcripts during *S. miltiorrhiza* hairy roots culture after treatment. Bars represent the mean $\pm$ SE (n=6)

3.3 Effects of La Elicitor on the Accumulation of Tanshinone (Tanshinone I, Tanshinone II and Cryptotanshinone)

ANOVA analysis showed a significant difference in the accumulation of tanshinone I, tanshinone II and cryptotanshinone in response to La treatment during hairy root culture period (Figure 3A). The content of tanshinone I was markedly enhanced by 74.9 % and 18.3% significantly higher than the control respectively with the treatment of La at dilutions of $0.01\text{mmol}\cdot\text{L}^{-1}$ and $0.1\text{mmol}\cdot\text{L}^{-1}$ ($p<0.05$) on day 1 after treatment, while treatment with La at dilutions of $1\text{mmol}\cdot\text{L}^{-1}$ showed no significant difference. Maximum values of tanshinone I for La treatment at $0.01\text{mmol}\cdot\text{L}^{-1}$ and $0.1\text{mmol}\cdot\text{L}^{-1}$ were observed on day 1 and 3 after treatment respectively. As shown in Figure 3B, the content of tanshinone IIA was markedly enhanced with the treatment of La at dilutions of $0.01\text{mmol}\cdot\text{L}^{-1}$, which was estimated to be 40.9% higher than the control while it showed no significant difference with the treatment of La at dilutions of $0.1\text{mmol}\cdot\text{L}^{-1}$ and $1\text{mmol}\cdot\text{L}^{-1}$ on day 1 after treatment. The effect of La elicitor on the accumulation of cryptotanshinone was shown in Figure 3C. Treatment with La at dilution of $0.01\text{mmol}\cdot\text{L}^{-1}$ improved significantly the content of cryptotanshinone by 92.4% and 71.5% on day 1 and 3 after treatment respectively, while it was reduced with the treatment of La at $0.01\text{mmol}\cdot\text{L}^{-1}$, $0.1\text{mmol}\cdot\text{L}^{-1}$ and $1\text{mmol}\cdot\text{L}^{-1}$ 7 days after treatment, which were estimated to be 28.6%, 57.9% and 58.6% respectively. In summary the accumulation of tanshinones I, tanshinone IIA and cryptotanshinone in the seedlings of *S. miltiorrhiza* was improved by La elicitor with lower dose while inhibited with higher dose and the effect of La on the accumulation of secondary metabolites lagged behind its effect on the expression of key enzyme gene.

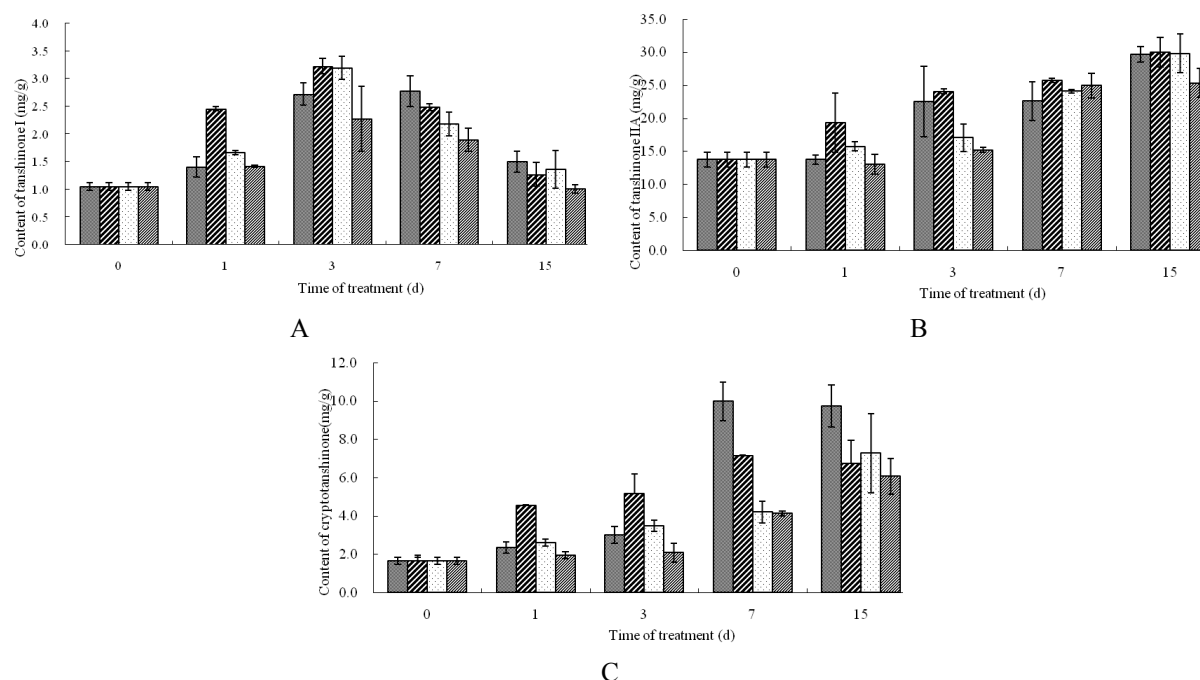


Figure 3 Effects of La elicitor on the content of tanshinone I (A), tanshinone II (B) and cryptotanshinone (C) during *S. miltiorrhiza* hairy roots culture after treatment. Bars represent the mean $\pm$ SE (n=6)

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