

Review

Metabolic regulation and genetic engineering of pharmaceutical component tanshinone biosynthesis in *Salvia miltiorrhiza*

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***Salvia miltiorrhiza* Bunge, called Dan shen in China, is a well-known traditional Chinese herb and has been used widely for the treatment of cardiovascular diseases due to its better performance and fewer side effects as confirmed in the long-time clinical use. Tanshinones, one class of lipid-soluble diterpene quinones from *S. miltiorrhiza*, were found to exhibit various pharmacological activities such as anti-oxidative, anti-inflammatory and anti-tumor properties. Due to the great importance of tanshinone and to its complex chemical nature as well as low content in plants, many efforts have been made to improve tanshinone production including hairy root culture, elicitor treatment and genetic engineering with new advance in recent years. Pharmacological activities of tanshinones and metabolic regulation of tanshinones biosynthesis in *S. miltiorrhiza* were reviewed in this paper.**

Key words: Tanshinones, hairy root, elicitors, metabolic engineering.

INTRODUCTION

Cardiovascular and cerebral vascular diseases are becoming more serious nowadays. Worldwide, over 17.1 million people died each year from cardiovascular and cerebral vascular diseases due to many potential reasons such as tobacco use, unhealthy diets, physical inactivity and harmful use of alcohol (WHO statistics www.who.int/cardiovascular_diseases). However, the shortage of drugs for these chronic diseases makes patients cost much money. So looking for supports from the traditional and alternative medicine became more important. *Salvia miltiorrhiza* Bunge (Dan shen in Chinese) is a well-known traditional Chinese herb and has been used widely in clinical to treat cardiovascular and cerebral vascular diseases (Shi et al., 2005). The bioactive components in *S. miltiorrhiza* can be mainly divided into two groups, the water-soluble phenolic acids derived from caffeic acid and the lipid-soluble

tanshinones (Li, 1998; Chen et al., 2001). Recently, many studies about the elicitors and metabolic engineering strategy based on *S. miltiorrhiza* hairy root systems for tanshinones production provided a novel thought for alleviating the shortages of tanshinones sources.

PHARMACOLOGICAL ACTIVITIES OF TANSHINONES

Tanshinones, including tanshinone I, tanshinone IIA, tanshinone IIB, cryptotanshinone, dihydrotanshinone and the others were lipid-soluble diterpene quinones. Recent studies showed that tanshinones exhibited various pharmacological activities (Hu et al., 2005).

Antibacterial activities

Cryptotanshinone and dihydrotanshinone I showed antibacterial activities to a number of Gram-positive bacteria such as *Bacillus subtilis* KCTC 3069, *B. subtilis*

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ATCC 6633 and so on, but the sensitivity was more evident with cryptotanshinone than dihydrotanshinone I (Lee et al., 1999). In recent study, cryptotanshinone was demonstrated to possess high-efficiency *in vitro* antibacterial activity against all tested 21 *Staphylococcus aureus* strains (Feng et al., 2009).

Antioxidant activity

Tanshinone IIA can prevent liver cell DNA from damaging by inhibiting the association of lipid peroxidation products with DNA, and this effective antioxidant activity is similar to vitamin E and butylated hydroxy-toluene (BHT) (Cao et al., 1996). Not only tanshinone IIA, but also tanshinone I and cryptotanshinone were detected to share the antioxidant effects, which can be used to protect against liver damage induced by carbon tetrachloride (Park et al., 2009).

Anti-cancer

Tanshinone IIA, a phenanthrene quinone extracted from the root of *S. miltiorrhiza*, has been reported to exhibit growth inhibition against human breast cancer cells (Wang et al., 2005), leukemic THP-1 cells (Liu et al., 2009), human lung cancer A549 cells (Chiu et al., 2010), and human cervical cancer cells (Pan et al., 2010). Tanshinone I with similar structure to tanshinone IIA only proved to play an adjunctive role in the treatment of human breast cancer (Nizamutdinova et al., 2008).

Anti-inflammatory

Jang et al (2003) reported that tanshinone IIA may be serve as an effective anti-inflammatory reagent which can be partly explained by inhibiting the production of the pro-inflammatory mediators such as nitric oxide (NO), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α). The inhibitory mechanisms of tanshinone IIA on the production of proinflammatory mediators were subsequently investigated three years later (Jang et al., 2006).

Effects on cardiovascular diseases

Tanshinones was widely used in clinical to treat patients suffering from cardiovascular diseases. Purified tanshinone IIA and IIB are protective against angina pectoris and stroke, as well as neuroprotective in cerebral ischemia and reperfusion (Ji et al., 2000; Lam et al., 2003). Both tanshinone IIA and tanshinone VI (Tan) can attenuate hypertrophy of isolated cardiomyocytes from neonatal rats (Ouyang et al., 2001; Takahashi et al.,

2002; Kawahara et al., 2004).

Other pharmacological activities

Tanshinone I can ameliorate learning and memory impairments in mice through the extracellular signal-regulated kinase signalling pathway (Kim et al., 2009). 15, 16-dihydrotanshinone-I and cryptotanshinone extracted from *S. miltiorrhiza* were demonstrated to possess anti-allergic activity *in vitro* (Ryu et al., 1999).

ESTABLISHMENT AND OPTIMIZATION OF HAIRY ROOT CULTURES

Hairy roots have the advantages of high genetic stability, rapid and hormone-free growth and have been widely used for the production of active components from medicinal plants including *S. miltiorrhiza*. Hairy root cultures of *S. miltiorrhiza* were firstly established through infecting sterile plant material with *Agrobacterium rhizogenes* strains LBA 9402, ATCC 15834, TR 105, R 1601 and A 4 1027 in 1993, and seven major tanshinones and ferruginol in both hairy root and liquid medium were simultaneously quantify by a sensitive quantitative High-Performance Liquid Chromatography (HPLC) procedure (Hu and Alfermann, 1993).

It is reported by Zhang et al. (1995) that some hormone such as IAA, NAA, and IBA with appropriate concentration can promote the growth of hairy root of *S. miltiorrhiza* and southern hybridization was firstly used to testify whether the T-DNA was inserted into the genome of *S. miltiorrhiza* by them, which provided molecular evidence for identification of *S. miltiorrhiza* (Zhang et al., 1995). The studies in recent years revealed that acetosyringone (400 μ mol/L) would be beneficial for upgrading transformation frequencies of *S. miltiorrhiza* (Zhou et al., 2007a), and leaves of *S. miltiorrhiza* are more suitable for hairy root induction compared with stem and petiole (Liu et al., 2009).

EFFECTS OF BIOTIC AND ABIOTIC ELICITORS ON TANSHINONES ACCUMULATION IN HAIRY ROOTS

Elicitation or the treatment of cultures with biotic and abiotic elicitors are the specific and most common means for the induction and stimulation of secondary metabolite accumulation in plants (Yan et al., 2005). Many studies in these years showed that the accumulation of tanshinones is also induced by some common elicitors (Table 1). yeast elicitor (YE) was first time employed as a biotic elicitor to stimulate the secondary metabolite production in hairy root culture of *S. miltiorrhiza* by Chen et al. (2001). In that study, a liquid chromatography-mass

Table 1. Studies of effects of elicitors on tanshinone production in *Salvia miltiorrhiza* hairy root cultures in recent years.

| | Author(s) | Elicitor(s) or treatment | Effect on the production of tanshinones |
|------|----------------|---|---|
| 2001 | Chen et al. | YE | Improvement |
| 2004 | Zhang et al. | Ag ⁺ | Improvement |
| 2005 | Ge and Wu (a) | YE; Ag ⁺ ; YE+Ag ⁺ | Improvement |
| 2005 | Ge and Wu (b) | BABA; MeJA; BABA+YE; MeJA+YE | Improvement |
| 2005 | Yan et al. | YE; X-5 adsorption; YE+ X-5 adsorption; repeated medium renewal+YE+ X-5 adsorption | Improvement |
| 2006 | Yan et al. (a) | YE; ligogalacturonides; fungal elicitor; Ag ⁺ ; Co ⁺ ; a-amino isobutyric acid | Improvement |
| 2006 | Yan et al. (b) | YE+ Ag ⁺ ; YE+ Co ⁺ ; YE+ a-amino isobutyric acid | Improvement |
| 2007 | Shi et al. | Sorbitol; YE; sorbitol+YE | Improvement |

YE, Yeast elicitor; BABA, β -aminobutyric acid; MeJA, methyl jasmonate.

Spectrometry (LC-MS) was developed for simultaneous detection of secondary metabolites and the results showed that both phenolic acids and tanshinones were accumulated by YE, which also improved the growth of hairy roots (Chen et al., 2001).

As an abiotic elicitor, Ag⁺ can also stimulate *S. miltiorrhiza* hairy root cultures to produce tanshinones but inhibit hairy roots growth, and the effects of the stimulation and inhibition were mainly dependent on the dose of Ag⁺ (Zhang et al., 2004a). In this study, they also demonstrated that sucrose feeding or medium renewal before the addition of Ag⁺ to the culture can effectively prevented the growth inhibition and significantly increased tanshinones yield (Zhang et al., 2004b). Ge and Wu (2005a) examined the accumulation of diterpenoid tanshinones in *S. miltiorrhiza* hairy root cultures induced by combination of a biotic elicitor (YE) and an abiotic elicitor (Ag⁺) with the results that the accumulation of tanshinones in double elicitors-induced hairy root cultures was dramatically higher than each single elicitor-induced one. In the same year, Ge and Wu (2005b) reported that both methyl jasmonate (MeJA) and non-protein amino acid β -aminobutyric acid (BABA) can also enhance tanshinone production, however, BABA is more effective compared with MeJA. Yan et al. (2005) demonstrated that the integration of YE elicitation, *in situ* adsorption by the X-5 resin and semi-continuous operation such as repeated medium renewal, elicitor addition and resin replacement, is an effective strategy for enhanced tanshinones production in hairy root cultures of *S. miltiorrhiza*.

Yan et al. (2006a) reported that some biotic elicitors such as YE, oligogalacturonides and fungal elicitor can selectively enhance cryptotanshinone significantly, while the abiotic elicitors Ag⁺, Co⁺ and a-amino isobutyric acid stimulated the tanshinone I production most remarkably, and these elicitors mentioned above did not inhibit the growth of *S. miltiorrhiza* hairy roots. In the meanwhile,

they also compared the synergistic effects of a biotic elicitor (YE) and one of the different abiotic elicitors (Ag⁺, Co⁺ and a-amino isobutyric acid) on the production of tanshinones in *S. miltiorrhiza* hairy roots with the results that the combination of YE and Ag⁺ produced the highest content of tanshinone I, while the combination of YE and Co⁺ produced the highest tanshinone IIA content, and only the combination of YE and a-amino isobutyric acid can yield higher tanshinone than the single elicitor treatment, which showed that the combination of a biotic and an abiotic elicitor can generate synergistic effects on tanshinone production in *S. miltiorrhiza* hairy root cultures (Yan et al., 2006b).

The results of using of sorbitol (hyperosmotic stress) and YE separately and simultaneously to *S. miltiorrhiza* hairy roots suggested a negative effect of YE and a positive effect of sorbitol on the root growth (dry weight), but all showed effectively enhancement of tanshinone production in hairy-root cultures (Shi et al., 2007).

METABOLIC ENGINEERING

Mapping tanshinone biosynthetic pathway

The specific tanshinone biosynthesis pathway is not fully characterized till now. However, as a kind of diterpene, tanshinones were synthesized both from the pathway of mevalonate (MVA) and methylerythritol phosphate (MEP), which were considered as two distinct but related isoprenoids biosynthesis pathways occurring in the cytosol and plastids respectively (Ge and Wu, 2005a; Yan et al., 2009; Wu et al., 2009) (Figure 1). In MVA pathway, isopentenyl pyrophosphate (IPP) is synthesized from two molecules of acetyl-CoA, through acetyl-CoA C-acetyltransferase (AACT), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), mevalonate kinase (MK),

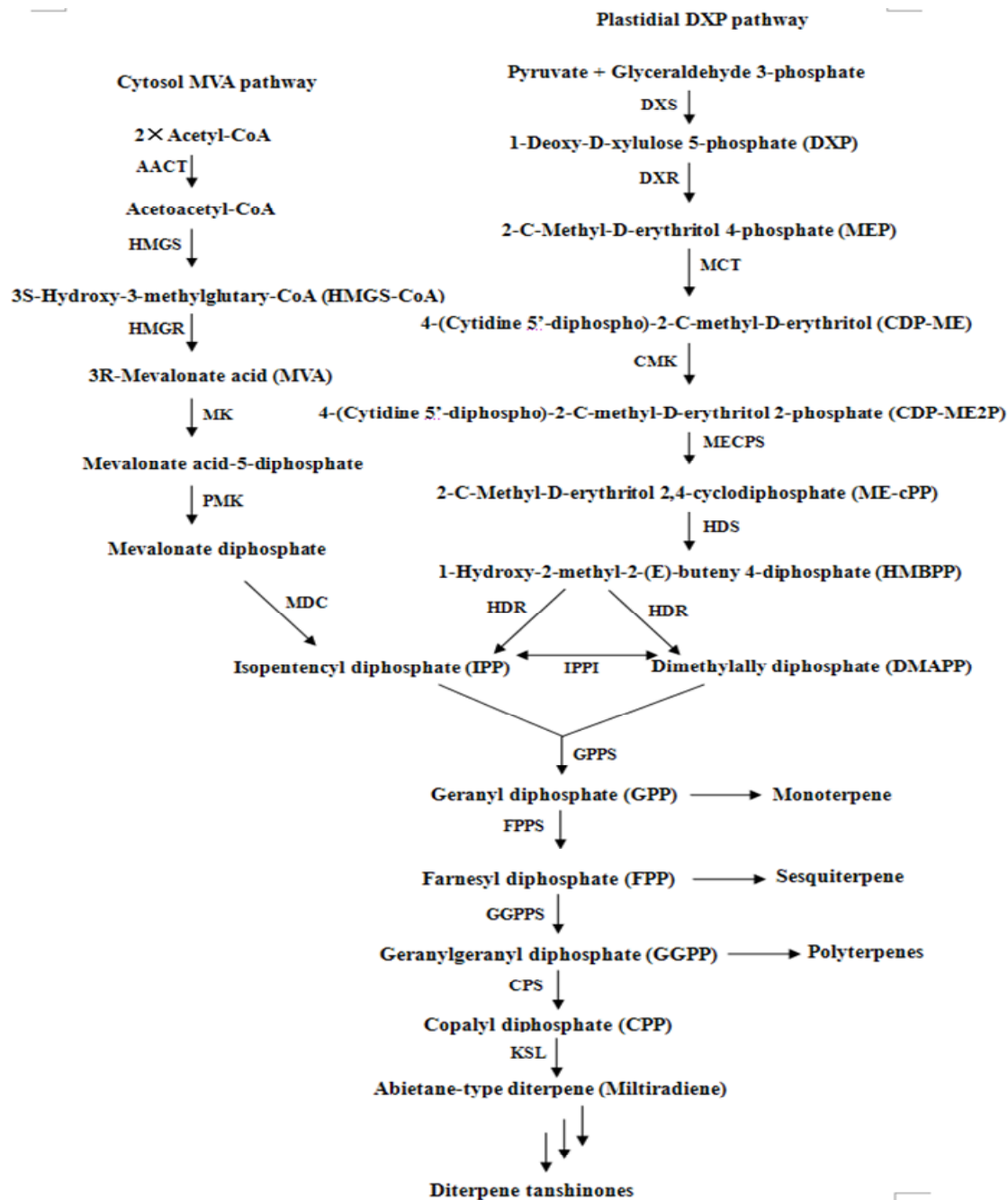


Figure 1. Tanshinone biosynthetic pathway in *S. miltiorrhiza*.

phophomevalonate kinase (PMK) and mevalonate 5-diphosphate decarboxylase (MDC) step by step (Tsay and Robinson, 1991; Igual et al., 1992; Dhe-Paganon et al., 1994; Lluch et al., 2000; Ha et al., 2003; Nagegowda et al., 2004). Furthermore, IPP can be also synthesized from one molecule of pyruvate and one molecule of glyceraldehyde-3-phosphate through MEP pathway, through catalyzing by 1-deoxy-D-xylulose5-phosphate synthase (DXS), 1-deoxy-D-xylulose5-phosphate reductoisomerase (DXR), MEP cytidyltransferase (MCT),

4-(cytidine5-diphospho)-2-C-methylerythritol kinase (CMK), 2-C-methylerythritol 2,4-cyclodiphosphate synthase (MECPS), hydroxymethylbutenyl 4-diphosphate synthase (HDS) and hydroxymethylbutenyl 4-diphosphate reductase (HDR) gradually (Baker et al., 1992; Takahashi et al., 1998; Rohdich et al., 2000; Herz et al., 2000; Estévez et al., 2001; Steinbacher et al., 2003; Eisenreich et al., 2004). Geranyl Diphosphate Synthase (GPPS) catalyses one molecule of IPP and one molecule of dimethylallyl pyrophosphate (DMAPP) to form 10-carbon

geranyl pyrophosphate (GPP), farnesyl diphosphate synthase (FPPS) catalyses one molecule of GPP and the second molecule of IPP to form 15-carbon farnesyl diphosphate (FPP), then 20-carbon geranylgeranyl diphosphate (GGPP) is synthesized from one molecule of FPP and the third molecule of IPP by geranylgeranyl diphosphate synthase (GGPPS) (Wang and Ohnuma, 1999; Engprasert et al., 2004; Liao et al., 2009). GGPP would be cyclized to form normal copalyl diphosphate (CPP) by copalyl diphosphate synthase (CPS), then normal CPP would be catalyzed into an abietane-type diterpene named miltiradiene by kaurene synthase-like (KSL) through further cyclization and rearrangement (Gao et al., 2009).

Gene cloning

So far most genes involving in the tanshinones biosynthesis have been cloned by the classical approaches such as homology-based method and differential screening as follows.

The cDNA encoding HMGR, which catalyzes the conversion of HMG-CoA to mevalonate (MVA) and was considered as the first committed step in MVA pathway, has been cloned from *S. miltiorrhiza* by rapid amplification of cDNA ends (RACE) (Liao et al., 2009). *SmHMGR* was highly expressed in the root of *S. miltiorrhiza*, followed by stem and leaf. And the expression of *SmHMGR* could be up-regulated by methyl jasmonate (MeJA) and salicylic acid (SA). The full-length cDNAs of DXS (DXS1, DXS2), catalyzes the first rate-limiting step in the DXP biosynthetic pathway were also isolated from *S. miltiorrhiza* recently (Kai et al., unpublished data). Followed with DXS, DXR catalyzes the second step in the MEP biosynthetic pathway, which converts DXP to MEP. Over-expressing DXR in *Arabidopsis* could increase accumulation of MEP-derived plastid isoprenoids such as chlorophylls and carotenoids, which showed DXR, may play an important role in regulating the MEP pathway (Carretero-Paulet et al., 2006). The cDNA of DXR from *S. miltiorrhiza* has been cloned and its function was also complemented in *Escherichia coli* (Yan et al., 2009; Wu et al., 2009).

GGPPS is an important branch point prenyltransferase enzyme which catalyses the consecutive condensation of a DMAPP with three molecules of IPP to produce GGPP, a universal diterpenes precursor (Engprasert et al., 2004). Kai et al. (2010) reported on the isolation of full-length cDNA encoding GGPPS from *S. miltiorrhiza*. *SmGGPPS* was also demonstrated to be a functional protein and could improve carotenoid accumulation in *E. coli*. The expression of *SmGGPPS*, which expressed higher in leaves and roots but weaker in stems, could be induced by SA in leaves but all inhibited by methyl jasmonate (MeJA) in leaves, stems and roots. Many other genes, such as *SmAACT*, *SmHMGS*, *SmCMK*,

SmIPPI, *SmFPPS*, *SmCPS* and *SmKSL*, which may be related to tanshinone production in *S. miltiorrhiza* has also been cloned in recent years (Wang et al., 2008; Gao et al., 2008; Wang et al., 2009; Gao et al., 2009; Cui et al., 2010; Kai et al., unpublished data), providing possibilities to enhance the yield of tanshinones by metabolic engineering. Actually, there may exist several unknown genes involved in the unknown specific tanshinone biosynthetic pathway after *SmKSL*. And more studies should be done to isolate and identify these unknown genes for better understanding the specific tanshinone biosynthetic pathway by biological technologies, such as cDNA microarray, mRNA differential display etc.

Genetic transformation

Compared to elicitor treatment, metabolic engineering is a more effective approach of metabolites improvement and has been successfully used in several plants (Muir et al., 2001; Zhang et al., 2004b; Aquil et al., 2009). In our previous study, the cDNAs of *SmHMGR*, *SmDXS* and *SmGGPPS*, which may be considered as three of the key enzymes from early stages in tanshinone biosynthesis in *S. miltiorrhiza*, were introduced on a recombinant plasmid pCAMBIA1304⁺ into *S. miltiorrhiza* leaves by the disarmed *A. tumefaciens* strain C58C1. It showed that over-expression of *SmGGPPS*, *SmDXS* or *SmHMGR* in transgenic hairy root lines results in significant enhancement of tanshinone accumulation with different levels than control and co-expression of *SmGGPPS* and *SmHMGR* can produce a synergistic effect on stimulating tanshinones production (Kai et al., 2010 unpublished data).

CONCLUSION

Many approaches such as traditional breeding, tissue culture, elicitor treatments and over expression of genes encoding the key enzymes in the biosynthetic pathway can be used to improve the production of tanshinones (Zhou et al., 2007b). Compared to elicitors treatment, the transgenic hairy roots can bring the modified hereditary information to the offspring specifically. Despite over-expression of the upstream genes (*SmHMGR*, *SmDXS* and *SmGGPPS*) could significantly improved tanshinones, it remains a necessary task to exploit the specific pathway of tanshinone biosynthesis.

Zhang et al. (2007) reported that MeJA treatment could increase tropane alkaloids production in transgenic *Hyoscyamus niger* hairy root cultures over-expressing Putrescine *N*-methyltransferase. So the strategy of combination of metabolic engineering and elicitor treatments may be a more promising mean to stimulate tanshinone production in the future.

With the advance in mapping the biosynthetic pathway and identifying the key genes, it is possible to isolate upstream transcription factors to enhance production of target medicinal natural products by over expression of transcription factors alone or in combination with biosynthetic key genes.

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