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MINI REVIEW

Biosynthesis of Tanshinones and Metabolic Regulation in Danshen (*Salvia miltiorrhiza*)

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Tanshinones, a group of diterpenoids, are the important bioactive components of Danshen (*Salvia miltiorrhiza* Bunge) roots used as a well-known Chinese traditional medicine. They are detected to have many biological functions, such as antimicrobial, antiinflammatory, antioxidant, antineoplastic *etc.* and are widely used in the treatment of cardiovascular and cerebral vascular diseases. In the present paper, we reviewed the morphological characteristics, active components and chemical uses of Danshen in China and then summarize the pathways of tanshinones biosynthesis and biological functions. The metabolic regulation involved enhancing tanshinone production in Danshen is discussed, in which it will provide a new insight into understanding mechanism of biosynthesis of tanshinones.

Key Words: Danshen, Tanshinones, Biosynthesis pathways, Mmetabolic regulation.

INTRODUCTION

S. miltiorrhiza Bunge, also known as "Danshen", is a deciduous perennial plant with branching stems (Fig. 1A). The height of a mature Danshen plant usually ranged from 35 to 80 cm, with odd-pinnate leaves. The length of petiole ranged from 1.3 to 7.5 cm. The inflorescences are covered with hair and sticky glands. Flowers grow in whorls, with light purple to lavender blue corollas that are 2.5 cm long, with a dark purple calyx. In general, it flowers from May to September every year in China. Its root with cylindrical shape is rich in fibers. The colour of the tuberous roots from outside to inside: milky and brick-red (Fig. 1B). Danshen prefers well draining soil, with about half a day of sunlight. It is hardy to -10 ºC.

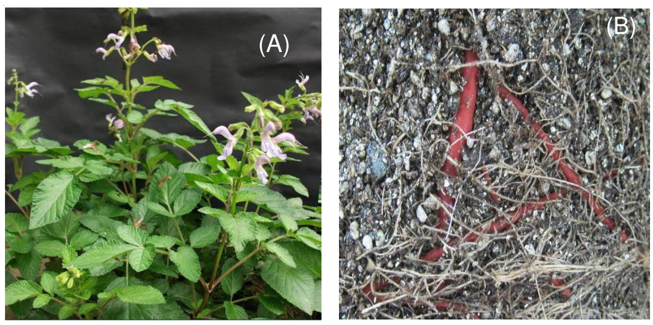

Fig. 1. Overground part (A) and the roots (B) of Danshen

The dried root of Danshen is a well-known Chinese traditional medicine used in China for thousands of years. It is widely used in the clinical diagnosis of cardiovascular and cerebral diseases[1-5]. To date, it has been listed in the official Chinese Pharmacopoeia for medical treatment of menstrual disorder and blood circulation diseases and for prevention of inflammation (The Pharmacopoeia Commission of People's Republic of China, 2005). Danshen receives people's favour in China because its root has relatively high content of tanshinones, which are a class of important bioactive components. The biological activities of tanshinones were demonstrated through many *vivo* and/or *vitro* tests. Cryptotanshinone (CT), tanshinone IIA (T2A) and tanshinone I (T1) are three major diterpene compounds of tanshinones in Danshen. So far, more than 30 tanshinones and related diterpenoid quinones have been isolated from Danshen and structurally characterized[6]. In addition to their functions in cardiovascular systems, tanshinones have been recently shown to possess some activities against human cancer cells[7]. More and more people having cardiovascular and cerebral vascular diseases reported from WHO resulted in short supply of tanshinones. Danshen has been widely used to extract tanshinones to produce a number of traditional Chinese medicine preparations for clinical application[8]. Danshen has been developed to be a potential model medicinal plant because of its relatively small genome size (600 Mb), short life cycle, undemanding growth requirement and significant medicinal value[9]. A total of 13 genes regarding tanshinones biosynthesis have been clone from Danshen and several key genes, such as SmDXR, SmHMGR, SmHMGS and SmGGPPS, have been characterized[10-13]. With the start of genome sequencing programme of Danshen and the determination of 611208 contigs representing 92 % of the entire Dashen genome and 96 % of the protein-coding genes[9]. It will provide an insight to understand the pathway of transhinones synthesis. Moreover, a dominant gene for male sterility in *S. miltiorrhiza* Bunge have been clone and DNA markers linked to the male sterile gene in a segregating population using bulked segregant analysis (BSA) and amplified fragment length polymorphism (AFLP) techniques, which provides useful material for breeding[14]. In this paper, the biosynthesis of transhinones and metabolic regulation in Danshen were reviewed.

Active components in Danshen: There are two main active components in Danshen, one is the hydrophilic including polyphenolic acids such as caffeic acid, lithospermic acid A, B, C, rosmarinic acid, *etc.*, another is the lipophilic containing diterpene quinines as showed (Fig. 2), such as cryptotanshinone, tanshinone I, tanshinone IIA, dihydrotanshinone I, tanshinone IIB, *etc.*[3,15,16]. Two major active components, as valuable traditional medicine, have indicated significant therapeutic effects on various diseases through many *in vivo* and *in vitro* tests[1,5]. The formulation of hydrophilic polyphenolic acids extracted from Danshen mainly include many kinds of injections consisting of Danshen, tanshinol and Danshen compound, respectively, paste and syrup comprised by Danshen compound, *etc*. The formulation of lipophilic tanshinones in Danshen mainly includes tanshinone tablets, capsule, ointment, shuxin capsule, *etc.* (Fig. 3).

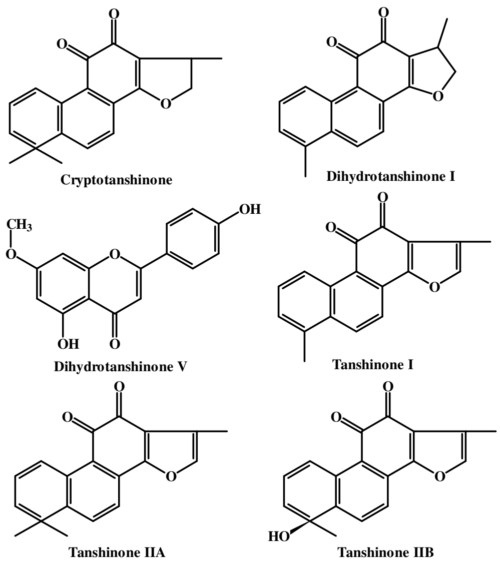

Fig. 2. Chemical structures of tanshinones, including cryptotanshinone, dihydrotanshinone I, dihydrotanshinone V, tanshinone I, tanshinone IIA, tanshinone IIB

Fig. 3. Different formulations of Danshen including lipophilictanshinones and hydrophilic polyphenolic acids in China

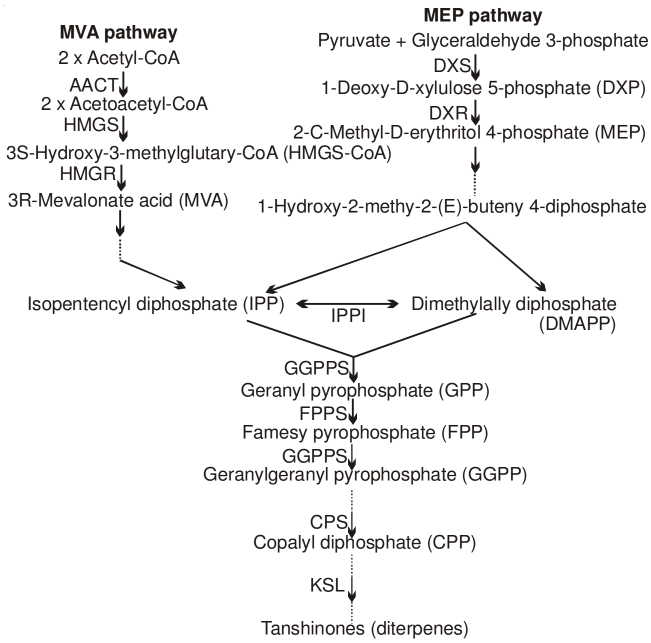

Fig. 4. Proposed pathways of terpenoid biosynthesis in Danshen (according to Kai *et al.*[12] with slight modification)

Pathways of biosynthesis of tanshinone: As shown in Fig. 4, the biosynthesis of tanshinones, in general, consists of two pathways, which are mevalonate (MVA) and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways. Both isopentenl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP) are synthesized *via* two different MVA and MEP pathways in separate cellular compartments. The MVA pathway occurring in the cytosol and the MEP pathway in the plastids[17]. The conversion between chemical compounds IPP and DMAPP is catalyzed by IPPI. To investigate further tanshinone biosynthesis, Ma *et al.*[9] had identified 40 tanshinone biosynthesis-related genes in Danshen, these genes are members of 19 families, which encode all of the enzymes involved in the biosynthesis of IPP and DMAPP. Some of the key enzymes, such as DXS, HDR, HMGR and GGPPS, are encoded by multiple gene members with different expression patterns and subcellular localizations, suggesting the complexity of tanshinone biosynthesis in Danshen[9].

In the MVA pathway, 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) catalyzes 3-hydroxy-3-methylglutaryl CoA to produce MVA. The HMGR is the rate-limiting enzyme of the MVA pathway[13]. It is reported that expression of SmHMGR in Danshen root was the highest, followed by stem and then leaf[11]. Overexpression of SmHMGR in Danshen hairy root can improve tanshinones production[12].

In the MEP pathway, both compounds pyruvate and glyceraldehyde 3-phosphate synthetize 1-deoxy-D-xylulose-5-phosphate (DXP) catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS), which is encoded by SmDXS1 and SmDXS2. The higher expression level of SmDXS1 locate at leaf, followed by stem and root while SmDXS2 in root only (unpublished data). 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) is a key enzyme in MEP pathway, in which it catalyzes DXP to MEP. The higher expression level of SmDXR in Danshen plant locates at leaf, followed by stem and root[10]. The DXS and DXR are the key enzymes of the MEP pathway and they are both very important to isoprenoid synthesis in some plants.

Geranylgeranyl diphosphate synthase (GGPPS) is a key enzyme in the downstream of tanshinones synthesis pathway. It catalyzes the consecutive condensation of the DMAPP with three IPP to produce geranylgeranyl diphosphate (GGPP), the universal precursor of diterpenes biosynthesis,such as tanshinones[18,19]. SmGGPPS was used as an important regulatory factor in the tanshinones synthetic pathway, with the higher expression level in Danshen root[11] and it was reported that overexpression of SmGGPPS in hairy root of Danshen can increase tanshinones content. The result is almost equal to the co-expression of SmHMGR and SmGGPPS in Danshen[12]. With the genes encoded the enzymes in tanshinone-synthesis pathway, such as, SmAACT (acetyl-CoA: acetyl-CoA C-acetyltransferase), SmHMGS (3-hydroxy-3-methylglutaryl -CoA synthase), SmIPPI (isopentenyl pyrophosphate isomerase), SmFPPS (farnesyl diphosphate synthase); SmCMK(4-(cytidine5-diphospho)-2-C- methylerythritol kinase), SmCPS (copalyl diphosphate synthase) and SmKSL (ent-kaurene synthase like) being cloned in Danshen[11,20-23], it will be helpful in understanding the mechanism of diterpenes tanshinones biosynthesis and then developing the useful biotechnologies to increase the tanshinones accumulation in Danshen.

Biological functions of tanshinones: Tanshinones have been detected to show many biological functions, such as antimicrobial, antiinflammatory, antioxidant, antineoplastic *etc.*[3,5,24]. It was reported that tanshinone I can enhance learning ability and memory and ameliorates memory impairment *via* the regulation of extracellular signal-regulated kinase signaling pathway in mice[25]. The growth-inhibitory and apoptosis-inducing effects of tanshinone IIA on leukemia cells have recently been reported. Such as, tanshinone IIA induced apoptosis in human leukemia cell lines HL-60, K562 and U-937 through the activation of caspase-3[26,27]. In acute promyelocytic leukemia cells NB4, tanshinone IIA could promote cell differentiation and apoptosis with elevated C/EBP β and CHOP[28]. Additionally, tanshinone IIA could reduce BT-20 breast cancer cell viability and induce apoptosis[29]. Recent studies have demonstrated that tanshinone IIA has protective effects against focal cerebral I/R injury through inhibition of macrophage migration inhibitory factor[30-32]. Cryptotanshinone has very strong activity of antimicrobial functions. It can resist *Staphylococcus aureus* and its drug-resistance strain strongly *in vitro*, also it can resist *Streptococcus*[33]. It inhibits low-density lipoprotein oxidation and attenuates hypertrophy induced by angiotensin II in cultured neonatal rat cardiac cells, which have strong effect on cardiovascular diseases *in vitro*[34,35]. It also has cytotoxic function against human carcinoma cells[36-38] as well as human breast cancer cells[39] and moreover, its anti-inflammatory function responded to radiation-induced microglia BV-2 cells was reported in details by Dong *et al.*[40]. Additionally, a purified extract isolated from Danshen, enriched with tanshinone I, tanshinone IIA and cryptotanshinone, can protect liver against hepatocyte injury *in vitro* and *in vivo*[41]. These results suggested that tanshinones might have protective effects.

Tanshinones in cell culture and hairy root system: Due to the important functions of tanshinones in regulating bioactivities, many researchers tried to produce tanshinones using biotechnologies. Cell suspension cultures of Danshen were studied more systematically by a group of Japanese scientists for production of tanshinones and related diterpenes[17]. Tissue and cell suspension cultures of crown galls induced by wild tumor-inducing plasmids have been studied for production of secondary metabolites from various plants[42]. The content of tanshinones produced from cell culture was so low even cannot be detected under five different basal medium[43].

In order to further improve tanshinones production, Hu and Alfermann[44] firstly established Danshen hairy root system. It was widely used as a more efficient and stable culture system to produce secondary metabolite in plants[17,44-46]. Although there is no significant difference on the chemical constituents between the hairy roots collected from successive subculture and normal roots of plant[47], hairy roots have a lot of advantages such as high genetic stability, fast growth and hormone-free by the infection of wounded plant tissues with Ri plasmid of *Agrobacterium rhizogenes*[48,49]. Compared to cell suspension culture and chemical synthesis, metabolic engineering. It is an alternative optimal strategy to improve secondary metabolites. A typical and successful example is that overexpression of both SmHMGR and SmGGPPS in Danshen hairy roots could enhance tanshinones contents and the results revealed that the tanshinones content in HG lines increase by 4.47-fold compared with control[12].

Elicitation for enhancing tanshinones production: The elicitor strategy has been developed for many years and has been used to not only stimulate defense response, but also induce accumulation of phytoalexins in plants, which is feasible and well documented for enhanced production of metabolite from plant cell culture[50,51]. It has been reported that biotic or abiotic elicitors could induce Danshen hairy roots to enhance bioactive compounds[16,52-55]. Biotic elicitor yeast extract, a kind of polysaccharide, has been proved to increase the accumulation of tanshinones in various tissue culture system of Danshen. In liquid culture of a tumor-inducing transformed Danshen cell line, yeast extract treatment increased the intracellular content of cryptotanshinone (CT) in roots from 0.24-5.4 mg/g DW and the extracellular concentration of cryptotanshinone from 12.5-24.2 mg/L[16,52]. Abiotic elicitors, such as Ag^+ was also used to enhance the yields of tanshinones and phenolic acids in Danshen[53]. Total tanshinones content in Danshen hairy roots treated with Ag^+ for 2-3 days was more than twice as control and Xiao *et al.*[54] also reported that Ag^+ could significantly boost the content of lithospermic acid B. Recently, there is another new elicitor, sodium nitroprusside (SNP), a donor of NO, was developed. Using the elicitor may result in a significant increase of tanshinones production in Danshen[55].

Elicitation with different elicitors for the suspension cells, the whole plant or hairy roots was widely used to stimulate tanshinones production. Hairy-root elicitation system seems to be more stable to produce tanshinones, Combination of elicitation with metabolic engineering was a impactful methods to increase tropane alkaloids production in transgenic Glycyrrhiza uralensis hairy root system[56], in which it will gives us a useful clue that combination of both strategies will be the best choose in increasing tanshinones contents in the near future. In addition, Wang *et al.*[56] reported that overexpression of G10H and ORCA3 in the hairy roots of *Catharanthus roseus* could improve catharanthine accumulation; with the genes in tanshinones biosynthesis pathway being cloned, it is possible to isolate and overexpression of the transcription factors or/and the genes' special promoter to enhance tanshinones contents in Danshen.

Conclusion

Being many biological functions such as antimicrobial, antiinflammatory, antioxidant, anti-neoplastic, as well as the other unknown functions of tanshinones, more and more researchers try to use many approaches such as molecular breeding, tissue culture, cell suspension culture, hairy root culture and elicitor treatments to regulate tanshinones biosynthesis and then increase tanshinones production in Danshen. Morever, the genes in the diterpenoid tanshinones biosynthetic pathways in Danshen were also successfully cloned and validated, which will provide an opportunity to improve tanshinone accumulation in Danshen using transgenic technology. It is inevitable that metabolic engineering will become an alternative strategy for increasing tanshinones production with further regulation of tanshinones biosynthesis pathways in the near future.

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