Biosynthesis of bioactive ingredients of *Salvia miltiorrhiza* and advanced biotechnologies for their production

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**ABSTRACT**

This review deals with the progress in the biosynthesis and regulation of tanshinones and salvianolic acids as well as the prospects and challenges for producing such compounds through biotechnology techniques. Tanshinones (lipophilic diterpenoids) and salvianolic acids (hydrophilic phenolic acids) are valuable natural products from danshen (*Salvia miltiorrhiza* Bunge) with remarkable clinical efficacy to treat cardiovascular diseases, with potential application in the treatment of cancer and possibly other disorders. The significant bioactivities of *S. miltiorrhiza* inspired scientists to explore its biosynthetic mechanism that promotes the production of these compounds. Computational and comparative genomics and transcriptomics have been used to analyse a number of pathway genes, transcription factors and microRNAs that are associated with the biosynthesis and regulation of tanshinones and salvianolic acids. At the same time, plant cell and tissue culture, transgenic plants, microbial biotransformation and metabolic engineering have been used to synthesise all these compounds.

**Abbreviations:** AACT: acetyl-CoACacetyltransferase; CMK: 4-(cytidine5-diphospho)-2-C-methylerythritol kinase; CPP: copalyl diphosphate; CPS: copalyl diphosphate synthase; DMAPP: dimethylallyl diphosphate; DXP: 1-deoxy-D-xylulose5-phosphate; DXR: 1-deoxy-D-xylulose5-phosphate reductoisomerase; FPP: farnesyl diphosphate; FPPS: farnesyl diphosphate synthase; GGPP: geranylgeranyl diphosphate synthase; HDR: hydroxy-methylbutenyl 4-diphosphetreductase; HDS: hydroxymethylbutenyl 4-diphosphate synthase; HMGR: HMG-CoA reductase; HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase; IPP: isopentenyl diphosphate; KSL: kaurenesynthase-like; LAA: lathosterolic acid A; LAB: lathosterolic acid B; MCT: MEP cytidyltransferase; MDC: mevalonate 5-diphosphate decarboxylase; MECS: 2-C-methylerythritol-2,4-cyclophosphate synthase; MEP: 2-C-methyl-d-erythritol 4-phosphate; MK: mevalonate kinase; PMK: phophomevalonate kinase; RA: rosmarinic acid; SAB: salvianolic acid B; TA-I: tanshinone I; TA-IIA: tanshinone IIA

**Introduction**

The principal bioactive components in danshen root (radix of *Salvia miltiorrhiza*), a well-known traditional medicinal herb used to treat heart and vascular disorders for thousands of years in Asia, are lipophilic diterpenoids, namely tanshinones, and water-soluble phenolic acids, namely salvianolic acids [1]. About 30 tanshinones have been identified including 15,16-dihydropotanshinone I, 1,2-didehydrotanshinone, methyltanshinonate, cryptotanshinone, TA-I, 1,2-dihydropotanshinone I etc., among which TA-IIA is considered to be a representative compound with remarkable pharmacological activities [2–4]. Salvianolic acids contain about 20 phenylpropanoid derivatives such as danshensu (also named salvianic acid A), salvianolic acid A, B, C, D, E, K, and L. Among all these, SAB (also named LAB) is the main component and the most stable one in regard to the function of danshen radices [5, 6].

Both TA-IIA and SAB exist in the form of injections to treat cardiovascular diseases and both have shown excellent clinical effects. The potential pharmacological applications of TA-IIA, SAB and other tanshinones and salvianolic acids are still under investigation, such as anti-diabetic [7], anti-inflammatory [8], anti-cancer [9], anti-rheumatoid [10] and anti-senile
dementia activities [11]. At present, danshen radices are the only sources for preparation of tanshinones and salvianolic acids. The concentration of SAB ranges from 37.34 mg/g to 68.37 mg/g, whereas that of TA-IIA is from 0.38 mg/g to 0.63 mg/g in danshen radices cultivated in different parts of China [12]. To prepare such compounds, huge amounts of danshen herbs are being consumed every year. Efforts have been made to make pure biosynthetic tanshinones and salvianolic acids and to enhance their production through biological and abiotic methods which would facilitate the production of these compounds [13–19]. This review deals with the advanced knowledge of the biosynthesis of these compounds through various biotechnology techniques with the aim to better understand the available approaches for production of these valuable natural products.

**Biosynthetic pathways of tanshinones and salvianolic acids**

Tanshinones are biosynthesised through the terpenoid pathway, whereas salvianolic acids through the phenylpropanoid metabolism.

**Biosynthetic pathway of tanshinones**

Terpenoids account for the largest proportion of secondary metabolites in nature and are biosynthesised through two different biosynthetic pathways, the MVA pathway and the MEP pathway, in different parts of plant cells. It is generally accepted that tanshinones are mainly biosynthesised through the MEP/DXP pathway [20], while the MVA pathway also contributes to the accumulation of tanshinones in danshen hairy roots [17]. In addition, the MEP pathway is involved in the biosynthesis of monoterpenes, certain sesquiterpenes, carotenoids, the side chains of chlorophylls, plastquinone and diterpenes except tanshinones. Sterols, certain sesquiterpenes and side chains of ubiquinone are mainly synthesized through the MVA pathway.

As in the biosynthesis of terpenoids [21], IPP is the most important intermediate step in the biosynthesis of tanshinones. It is produced by the MVA pathway, starting from two molecules of acetyl-CoA, catalysed through AACT, HMGS, HMGR, MK, PMK and MDC step by step [22–24]. It can also be synthesized through the MEP pathway beginning with one molecule of pyruvate and one molecule of glyceraldehyde-3-phosphate, which are successively catalysed by DXS, DXR, MCT,
CMK, MECPS, HDS and HDR [25–28]. One molecule IPP and one molecule DMAPP are catalysed by GGPPS to form together one molecule of GPP, which is further transformed into monoterpene. FPPS transforms one molecule GPP to FPP, which is the precursor of sesquiterpene. Afterwards, the synthesis of GGPP is catalysed by GGPPS [14]. GGPP can be cyclised by CPS to form CPP or polyterpenes. CPP is catalysed by KSL to form an abietane-type diterpene, miltiradiene, which is the key precursor of tanshinones [29].

A hypothetical tanshinone biosynthetic pathway from miltiradiene has been proposed [29–31] in which the olefin miltiradiene firstly undergoes aromatization to abietatriene before formation of ferruginol. While the conversion of miltiradiene to abietatriene occurs spontaneously, it is most likely that this aromatization reaction is enzymatically catalysed in the plant. At the same time, the plant biosynthesis of phenolic diterpenoids confines the role of the characterized CYP76AH sub-family members to C12-hydroxylation of the aromatic ferruginol (Figure 1). These results were confirmed when researchers reported that CYP76AH1, CYP76AH3 and CYP76AK1 were candidate genes involved in C12-hydroxylation of the aromatic ferruginol [32–34]. Guo et al. [32] demonstrated that both CYPs are promiscuous via biochemical and RNA interference studies. CYP76AH3 oxidizes ferruginol at two different carbon centers, and CYP76AK1 hydroxylates C-20 of two of the resulting intermediates. Together, these convert ferruginol into 11,20-dihydroxy ferruginol and 11,20-dihydroxy sugiol en route to tanshinones [32].

**Rate-limiting enzymes in tanshinone biosynthesis**

*S. miltiorrhiza* cultivar with a higher tanshinone content displayed a higher gene expression level in the tanshinone biosynthesis pathway [35]. Most of the rate-limiting enzymes in the isoprenoids metabolic pathway have been elucidated. All kinds of isoforms are found and their properties were investigated including the expression patterns in *S. miltiorrhiza* and the corresponding elicitors [36]. Below we will mention a few other reports on rate-limiting enzymes in tanshinone biosynthesis.

Darabi et al. [37] analyzed 23 HMGR protein sequences and found that HMGR has a predictable spatial structure with catalytic regions including L, N and S domains. SmDXR shows extensive homology with DXRs from other plant species [38]. It is constitutively highly expressed in leaves, followed by roots and stems in *S. miltiorrhiza* [38]. Yet, the roles of individual members remain to be determined. SmHMGR, SmDXR, SmDXS and SmCPS are the key enzymes of tanshinone biosynthesis [19, 39, 40]. SmDXS2 plays a more important role than SmDXS1 in channelling the intermediates of the MEP pathway to tanshinone biosynthesis [19]. The expression level of SmKSL1 is associated with the TA-IIA and tanshinone content, whereas that of SmCPS1 is related to the TA-IIA content [41]. SmGGPPS is encoded by multiple analogous genes [42] and overexpression of SmGGPPS1 enhanced tanshinone concentration [43].

**Transcription factors and the microRNAs in regulating tanshinone biosynthesis**

A comprehensive transcriptome profiling of *S. miltiorrhiza* hairy roots after treatment with elicitors identified a total of 375 significantly up-regulated transcription factors (TFs) responsive to MeJA- and YE-mediated elicitation which might participate in the accumulation of tanshinone and salvianolic acids [44]. In the genome of *S. miltiorrhiza*, there are reportedly 127 bHLH transcription factor genes; seven of them are potentially involved in the regulation of tanshinone biosynthesis and SmbHLH37, SmbHLH74 and SmbHLH92 most likely participate in the regulation of tanshinone biosynthesis [45]. APETALA2/ethylene-responsive factors (AP2/ERF) Sm128 and Sm152, two ERF-B3 subgroup members, have also been proposed to regulate tanshinone biosynthesis based on co-expression analysis [46]. Overexpression of SmMYB9b, an R2R3–MYB transcription factor, enhances the tanshinone concentration in *S. miltiorrhiza* hairy roots, indicating that MYB transcription factors are also involved in tanshinone biosynthesis [47]. We also found that maize C1, a heterologous R2R3–MYB, improved the accumulation of tanshinones by comprehensively up-regulating the pathway genes, especially SmMDC and SmPMK through the direct interaction of C1 with its recognition sequences in *S. miltiorrhiza* hairy roots [48]. The fact that so many transcription factors are involved in the biosynthesis of tanshinones implies that the regulation of tanshinone biosynthesis is complicated in *S. miltiorrhiza*.

Regarding miRNAs, at present there are a total of 452 known miRNAs corresponding to 589 precursor miRNAs, and 40 novel miRNAs identified in different tissues of *S. miltiorrhiza* by high-throughput sequencing [49]. Among them, miRS072 cleaves acetyl-CoA C-acetyltransferase and is believed to be involved in the biosynthesis of tanshinones [49]. We have overexpressed SmMiR396b in *S. miltiorrhiza* hairy roots whose growth is inhibited while the concentration of tanshinones in those hairy roots is enhanced (data not
published). The limited amount of reports on the involvement of miRNAs in the regulation of tanshinone biosynthesis in *S. miltiorrhiza* suggests that more attention should be paid on mining such important regulators as potential tools for metabolic engineering of tanshinones in the future.

**Biosynthetic pathway of salvianolic acids**

Phenolic acids are derived from two upstream biosynthetic pathways: the phenylpropanoid pathway and the tyrosine-derived pathway [50, 51]. They are the upstream branches of the biosynthesis pathway of RA. RA biosynthesis begins with L-tyrosine and L-phenylalanine, which are converted into 4-HPLA and 4-coumaroyl-CoA, respectively, through various enzymes. Eventually, the two metabolic intermediates are mutually combined together via a certain biological pathway to generate RA. However, the detailed biosynthetic process from RA to SAB has not been completely described (Figure 2) [52–54].

**Rate-limiting enzymes in salvianolic acid biosynthesis**

The rate-limiting enzymes in phenolic acids biosynthesis have been investigated [36, 55] and the following is some of the progress made so far. SmPAL, SmC4H, Sm4CL and SmHPPD may serve as key enzymes in the regulation of RA accumulation, whereas SmTAT, SmC4H, SmHPPR and SmHPPD may control the biosynthesis of SAB in *S. miltiorrhiza* [56, 57]. There are three SmPALs in *S. miltiorrhiza*. These SmPALs may function redundantly in the biosynthesis of some metabolites, but the roles of each SmPAL could be separate in the biosynthesis of other metabolites [58]. In our work on Sm4CLs, two divergent members were cloned and Sm4CL2 rather than Sm4CL1 was suggested to be responsible for the biosynthesis of SAB in *S. miltiorrhiza* roots according to their different *in vitro* enzyme kinetic properties and promoter functional characteristics [59, 60]. De novo transcriptome sequencing of *S. miltiorrhiza* has identified 5 unigenes encoding 3 TAT genes, 6 unigenes encoding up to 2 HPPR genes and 17 unigenes encoding maximum 14 4CL genes [61]. All the information accumulated so far indicates that each step of the general phenylpropanoid pathway is presumably catalysed by a couple of homologous enzymes of which one is possibly the most related rate-limiting enzyme for the biosynthesis of salvianolic acids.

**Transcription factors in salvianolic acid biosynthesis**

Song et al. [62] comprehensively analysed and categorized the *cis*-acting regulatory elements in the
biosynthetic genes of the RA pathway into five major groups according to their functions. The five major groups included typical consensus elements in eukaryotic promoters as well as ones responsive to light or stress, hormonal regulation and MYB-related roles [62]. On the basis of qRT-PCR analysis, co-expression analysis, and the prediction of cis-regulatory elements in the promoters, Ji et al. [46] proposed that Sm008 and Sm166, which belong to the ERF-B1 and ERF-B4 subgroups, could regulate the biosynthesis of phenolic acid. SmMYC2a and SmMYC2b interact with SmJAZ1 and SmJAZ2, implying that the two MYC2s might function as the switch of jasmonic-acid signalling, through which the regulation of phenolic acid biosynthetic could be achieved [63]. Further studies on such transcription factors will help discover powerful tools regulating the biosynthesis of valuable phenylpropanoid metabolites like rosmarinic acid and salvianolic acid B.

### Biotechnologies for producing tanshinones and salvianolic acids

**Plant cell and tissue cultures**

Tissue culture provided the first bioactive components of *S. miltiorrhiza* in the 1980s. Today, the yield of secondary metabolism has been remarkably improved by polyploid induction, callus culture [64], transformed cell suspension culture and hairy root culture [5]. Wang et al. [65] reviewed the various biotic and abiotic elicitors applied to hairy root cultures and their stimulating effects on the accumulation of secondary metabolites. Here we summarize the common elicitors in *S. miltiorrhiza* hairy root culture and trace them back to the target genes (Table 1).

According to the present literature, it can be concluded that the genes involved in the biosynthesis pathway of *S. miltiorrhiza* which are regulated by elicitors are mainly SmHMGR, SmDXR, SmDXS, SmGGPPS, SmCPS, SmKSL and SmPAL. Xiao et al. [54] reported that silver ions, an abiotic elicitor, did not stimulate RA accumulation but dramatically enhanced SAB. This reminds us to explore whether the same elicitor has the same effect on different bioactive components. Cheng et al. [66] examined the influences of various combined elicitors on the expression of SmCPS and the production of tanshinones in *S. miltiorrhiza* hairy root cultures. The contents of cryptotanshinone and dihydrotanshinone I were enhanced by treatment using YE + Ag⁺, Ag⁺ + MJ, and YE + Ag⁺ + MJ. Besides, yeast extract contributes more to tanshinone biosynthesis than Ag⁺ does [67]. The results point to the significance of the synergistic effect. The mechanism of metabolism in specific species is differential. *S. miltiorrhiza* produces higher levels of dihydrotanshinone I and SAB but lower levels of cryptotanshinone, TA-IIA, caffeic acid and RA than other species of genus *Salvia* [68]. Wang et al. [69] biosynthesised a tanshinone derivative named 2-(N-pyrrolidine-alkyl)

### Table 1. Common elicitors in *S. miltiorrhiza* hairy root culture.

| Elicitor type | Elicitor agents | Secondary metabolites | Target gene(s) | References |
|--------------|-----------------|----------------------|----------------|------------|
| Abiotic elicitors | | | | |
| Heavy metal ions | Ag⁺ (Ag₂S₂O₃) | Tanshinone | SmHMGR; SmDXS | [64] |
| Light and UV | UV-B | Tanshinone | SmHMGR; SmGGPPS | [16] |
| Osmotic stress | Sorbitol | Tanshinone and phenolic acids | SmHMGR | [91] |
| Proteins | β-cryptogein | Phenolics | | [92] |
| Temperature shift | Low temperature | Tanshinone and salvianolic acid | SmAOC | [93] |
| Light and UV | UV | | | |
| Small molecules | MeJA | Tanshinone | SmHMGR; SmDXR; SmDXS; SmPAL | [20,58] |
| Small molecules | PEG; ABA; MeJA | Tanshinone | SmHMGR | [17] |
| Small molecules | MEV | Tanshinone | SmHMGR | [16] |
| Small molecules | FOS | SmDXR; SmDXS | | |
| Small molecules | MeJA; NO; SNP | Tanshinone | SmHMGR; SmDXR | [66] |
| Small molecules | ABA; polyamine | Salvianolic acid | SmPAL | [69] |
| Small molecules | Non-ionic surface active agent (TWEEN 20, TRITON X-10) | Tanshinone and salvianolic acid | | [73] |
| Small molecules | MeJA; SA | Tanshinone | SmGGPPS; SmCPS; SmKSL | [13] |
| Fungal elicitors | Trichoderma atroviride D16 (EM; PSF) | Tanshinone and phenolic acids | SmHMGR; SmDXR; SmGGPPS; SmCPS; SmKSL | [15] |
| Live bacteria | Bacillus cereus | Tanshinone | | |
| Live bacteria | Streptomyces pactum Act12 | Tanshinone | SmHMGR; SmDXR; SmDXS; SmGGPPS | [78] |
| Yeast elicitor | Yeast extract | Tanshinone and phenolic acids | SmHMGR; SmDXR; SmDXS | [91] |
| Yeast elicitor | Yeast extract | Tanshinone and phenolic acids | SmPAL | [79] |
| Polysaccharides | Polysaccharides from Bacillus cereus cells | Tanshinone | SmPAL | [18] |
| Glycoproteins | Polysaccharide-protein fraction of Bacillus cereus cells | | | |

Note: UV-B, ultraviolet-B; MeJA, methyl jasmonate; PEG, polyethylene glycol; ABA, abscisic acid; MEV, mevinolin; FOS, fosmidomycin; NO, nitric oxide; SNP, sodium nitroprusside; SA, salicylic acid; EM, mycelium; PSF, polysaccharide fraction; YE, yeast elicitor.
tanshinone, which was considered as a potential candidate antibacterial agent. This new compound obtained in tissue culture must be taken into future consideration.

**Transgenic plant materials**

The advantages of synthesizing bioactive components by plant bioreactors are the lower cost, easier manufacturing, more accurate expression and higher stability than chemical synthesis. Zhang et al. [70] transduced the Arabidopsis transcription factor called Production of Anthocyanin Pigment 1 (AtPAP1) into S. miltiorrhiza. The content of SAB was strongly improved in 1-month-old transgenic plantlets, whereas in the growth stage, there was no significant induction. Liu et al. [71] transduced the Arabidopsis thaliana-enhanced drought tolerance 1 (AtEDT1) into S. miltiorrhiza. Interestingly, they observed that the overexpression of the AtEDT1 transgene showed an augmentation in salvianolic acids synthesis, such as RA, lithospermic acid, SAB, whereas tanshinone synthesis was decreased. The effects of MeJA and SA on tanshinone productivity and biosynthetic gene expression in hairy roots of transgenic S. miltiorrhiza have been explored and described previously [13]. The results from gene expression experiments are consistent with those for non-transgenic S. miltiorrhiza.

CRISPR/Cas9 is a gene-editing tool gaining popularity in recent years. Li et al. [72] knocked out the committed diterpene synthase gene (SmCPST) involved in tanshinone biosynthesis in S. miltiorrhiza by CRISPR/Cas9. The metabolomic analysis revealed that tanshinones, especially cryptotanshinone, TA-IIA and tanshinone I, are completely missing in homozygous mutants, without influencing other phenolic acid metabolites. By contrast, tanshinones are decreased but still detectable in chimeric mutants. Thus paving the way for large-scale genome editing, S. miltiorrhiza is important for pathway elucidation of secondary metabolites, quality improvement and, at the same time, yield increase for this valuable traditional Chinese medicinal herb. At present, this technology is not mature yet. Published reports in this field are quite rare and it is becoming the focus of future research.

**Microbial biotransformation of Danshen bioactive ingredients**

Microbial transformation has the characteristics of abundant enzyme system, strong selectivity, mild reaction conditions [73], low cost and ease of manufacture. Plant microbiomes are a big gene reservoir for secondary metabolism in medicinal plants. Chaetomium globosum D38 mainly colonized S. miltiorrhiza hairy roots, which showed significant enhancement in the contents of tanshinones and salvianolic acids and benefit to the growth of the hairy roots [74]. Alternaria sp. A13 can enhance the accumulation of total phenolic acid, LAA and LAB [75]. Chen et al. [76] had analysed and clarified the core microbiome of the seeds of S. miltiorrhiza.

Some non-endophytic fungi can transform the precursors into bioactive components of S. miltiorrhiza. The aqueous extract of S. miltiorrhiza was transformed by Fusarium graminearum in a bioreactor containing phosphate buffer, in which RA was transformed into danshensu and caffeic acid and the yield of SAB was higher than 85% [77]. Sometimes microbial biotransformation can produce some stronger pharmacological compounds to expand the derivative group of a certain active compound. He et al. [78] found that the fungus Hypocre a sp. (AS 3.17108) could transform TA-IIA into a new antibacterial agent tanshisorbicin. Sun et al. [79] transformed cryptotanshinone into three new products by Cunninghamella elegans (AS 3.2082). These biotransformed metabolites were proved to be identical to three of the minor hydroxylated metabolites in vivo. Microbial transformation of TA-IIA by Cunninghamella elegans gave two new glycosylated derivatives [80]. These studies suggest that microbial biotransformation is a feasible approach for the preparation of pharmacologically stronger or more easily absorbed derivatives.

**Synthetic biology-based approaches for producing Danshen bioactive ingredients**

Synthetic biology-based heterologous biosynthesis of plant natural products in microorganisms is an attractive approach. On the other hand, in reality, the supply of natural products cannot meet the increasing demand. As many biosynthetic pathways of natural products have been revealed, the enzymes involved provide powerful tools for biosynthesis of bioactive compounds from precursor-directed substances [81]. In fact, Hamano et al. [23] reported that the GGPDS, MK, MDPD, PMK, IPP isomerase and HMG-CoA synthase could be expressed in Escherichia coli as early as 2001. Kwon et al. [82] confirmed that both HDR enzymes could complement an E. coli HDR deletion mutant. Zhou et al. [83] indicated that GPPS is an essential regulation point in balancing a recombinant
geraniol synthesis pathway. What is more, the GPPS-based regulation approach could be applied to optimize the microbial production of other monoterpenes. Zhang et al. [84] demonstrated that TwGGPPS1, TwGGPPS4 and TwGGPPS5 of all six TwGGPPS genes can participate in miltiradiene biosynthesis in recombinant E. coli. These results confirmed that many genes involved in the tanshinone biosynthetic pathway are able to function in E. coli, which, no doubt, is paving the way for biosynthesis of tanshinones in E. coli. There are many other strains that could be used besides E. coli. For example, Dai et al. [85] produced miltiradiene by metabolically engineered Saccharomyces cerevisiae and finally increased the production of miltiradiene from 5.4 to 488 mg/L.

As for phenolic acids, recent progress in metabolic engineering shows that several L-tyrosine-derived phenolic acids, including caffeic acid, gallic acid, RA and salvianolic acid can be biosynthesised in microbial systems [86–88]. Jiang et al. [89] successfully biosynthesised RA by feeding caffeic acid thus constructing an artificial pathway for 3,4-dihydroxyphenyllactic acid. Furthermore, unnatural compounds such as caffeoyl-phenyllactate were biosynthesised in engineered E. coli, too. Interestingly, a broad range of RA analogues were biosynthesised in E. coli through incubation of the recombinant E. coli strain BLRA1 with exogenously supplied phenyllactic acid and its analogues as acceptor substrates, coumaric acid and its analogues as donor substrates [78]. Our team has constructed a de novo biosynthetic pathway of RA in Saccharomyces cerevisiae, which produced milligram-scale RA (data not published).

Sometimes, the well-established parts of pathways in recombinant bacteria require specialized environments or compartments for optimal function. Thus it is difficult to combine the partial pathways together in one cell. Zhou et al. [90] solved this problem through co-culture of engineered organisms, each of which contains the part of the pathway that is best suited to hosting. Stable co-culture in the same bioreactor was achieved by designing a mutualistic relationship between two species in which a metabolic intermediate produced by one species, such as E. coli, is used and functionalized by the other species, like yeast. Such consortium has been applied to produce tanshinone precursors [90].

**Conclusions**

A number of unigenes encoding rate-limiting enzymes, miRNAs and transcription factors in the biosynthesis of bioactive compounds have been identified through transcriptome sequencing. Co-expression analysis with the known functional genes, comparative transcriptomics and efficient screening methods could be applied to narrow down the candidate genes obtained from various transcriptomes. Based on the depth of all the above researches, the biosynthetic pathways and regulation mechanisms of tanshinones and salvianolic acids has been explained in more and more detail. Although the general biosynthetic pathways of tanshinones and salvianolic acids have been basically elucidated, the detailed steps from RA to SAB and those from miltiradiene to tanshinone still need to be confirmed. Meanwhile, more and more biotechnologies have been invented and the existing ones have gradually matured. Plant tissue culture, microbial biotransformation and transgenic plants have developed early in time, which are common techniques alongside mature biotechnologies at present. Using elicitors to induce transgenic hairy roots had been accomplished. In future, researchers could be devoted to combining the elicitors with engineered bacteria. Synthetic biology-based metabolic engineering, that is, using meticulously designed and genetically engineered microbial organisms as microbial cell factories, is a biotechnology under development. No doubt, the emergence of new biotechnologies such as CRISPR/Cas9 will promote the study of the biosynthesis of pharmacologically active compounds in traditional medicinal herbs in the future.

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**References**

[1] Zhou LM, Zuo Z, Chow MSS. Danshen: An overview of its chemistry, pharmacology, pharmacokineti, and clinical use. J Clin Pharmacol. 2005;45:1345–1359.
[2] Maione F, De Feo V, Caiazzo E, et al. Tanshinone IIA, a major component of Salvia miltiorrhiza Bunge, inhibits platelet activation via Erk-2 signaling pathway. J Ethnopharmacol. 2014;155:1236–1242.

[3] Sun DD, Shen M, Li JY, et al. Cardioprotective effects of tanshinone IIA pretreatment via kinin B2 receptor-Akt-GSK-3 beta dependent pathway in experimental diabetic cardiomyopathy. Cardiovasc Diabetol. 2011;10:4–12.

[4] Tan XQ, Yang Y, Cheng J, et al. Unique action of sodium tanshinone II-A sulfonate (DS-201) on the Ca2+ dependent BKCa activation in mouse cerebral arterial smooth muscle cells. Eur J Pharmacol. 2011;656:27–32.

[5] Gao SL, Zhu DN, Cai ZH, et al. Autotetraploid plants from colchicine-treated bud culture of Salvia miltiorrhiza Bge. Plant Cell Tiss Organ Cult. 1996;47:73–77.

[6] Wang J, Xiong XJ, Feng B. Cardiovascular effects of salvianolic acid B. Evid Based Complement Alternat Med. 2013;2013:247948–247964.

[7] Leng J, Chen MH, Zhou ZH, et al. Triterpenoids-enriched extract from the aerial parts of Salvia miltiorrhiza regulates macrophage polarization and ameliorates insulin resistance in high-fat fed mice. Phytother Res. 2017;31:100–107.

[8] Ma SL, Zhang DW, Lou HX, et al. Evaluation of the anti-inflammatory activities of tanshinones isolated from Salvia miltiorrhiza var. alba roots in THP-1 macrophages. J Ethnopharmacol. 2016;188:193–199.

[9] Ye YT, Zhong W, Sun P, et al. Apoptosis induced by the methanol extract of Salvia miltiorrhiza Bunge in non-small cell lung cancer through PTEN-mediated inhibition of PI3K/Akt pathway. J Ethnopharmacol. 2017;200:107–116.

[10] Yoon JJ, Sohn EJ, Kim JH, et al. Anti-rheumatoid arthritis effect of Kaejadan via analgesic and antiinflammatory activity in vivo and in vitro. Phytother Res. 2017;31:418–424.

[11] Habtemariam S. Molecular pharmacology of rosmanin and salvianolic acids: Potential seeds for Alzheimer’s and vascular dementia drugs. JMS. 2018;19:458–483.

[12] Cheng HT, Li XL, Li XR, et al. Simultaneous quantification of selected compounds from Salvia herbs by HPLC method and their application. Food Chem. 2012;130:1031–1035.

[13] Hao XL, Shi M, Cui LJ, et al. Effects of methyl jasmonate and salicylic acid on tanshinone production and biosynthetic gene expression in transgenic Salvia miltiorrhiza hairy roots. Biotechnol Appl Bioc. 2015;62:24–31.

[14] Liao P, Zhou W, Zhang L, et al. Molecular cloning, characterization and expression analysis of a new gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase from Salvia miltiorrhiza. Acta Physiol Plant. 2009;31:565–572.

[15] Ming QL, Su CY, Zheng CJ, et al. Elicitors from the endophytic fungus Trichoderma atroviride promote Salvia miltiorrhiza hairy root growth and tanshinone biosynthesis. J Exp Bot. 2013;64:5687–5694.

[16] Wang CH, Zheng LP, Tian H, et al. Synergistic effects of ultraviolet-B and methyl jasmonate on tanshinone biosynthesis in Salvia miltiorrhiza hairy roots. J Photoch Photobio B. 2016;159:93–100.

[17] Yang DF, Du XH, Liang X, et al. Different roles of the mevalonate and methylerythritol phosphate pathways in cell growth and tanshinone production of Salvia miltiorrhiza hairy roots. Plos One. 2012;7:e46797. [cited 2018 Apr 27] Doi: 10.1371/journal.pone.0046797.

[18] Zhao JL, Zhou LG, Wu JY. Promotion of Salvia miltiorrhiza hairy root growth and tanshinone production by polysaccharide-protein fractions of plant growth-promoting rhizobacterium Bacillus cereus. Process Biochem. 2010;45:1517–1522.

[19] Zhou W, Huang FF, Li S, et al. Molecular cloning and characterization of two 1-deoxy-D-xylulose-5-phosphate synthase genes involved in tanshinone biosynthesis in Salvia miltiorrhiza. Mol Breeding. 2016;36:124–136.

[20] Yang DF, Ma PD, Liang X, et al. PEG and ABA trigger methyl jasmonate accumulation to induce the MEP pathway and increase tanshinone production in Salvia miltiorrhiza hairy roots. Physiol Plantarum. 2012;146:173–183.

[21] Yang L, Yang CQ, Li CY, et al. Recent advances in biosynthesis of bioactive compounds in traditional Chinese medicinal plants. Sci Bull. 2016;61:13–17.

[22] Ahumada I, Cairo A, Hemmerlin A, et al. Characterisation of the gene family encoding acetocetyl-CoA thiolase in Arabidopsis. Functional Plant Biol.. 2008;35:1100–1111.

[23] Hamano Y, Daini T, Yamamoto M, et al. Cloning of a gene cluster encoding enzymes responsible for the mevalonate pathway from a terpenoid-antibiotic-producing Streptomyces strain. Biosci Biotech Bioch. 2001;65:1627–1635.

[24] Liao P, Wang H, Hemmerlin A, et al. Past achievements, current status and future perspectives of studies on 3-hydroxy-3-methylglutaryl-CoA synthase (HMGs) in the mevalonate (MVA) pathway. Plant Cell Rep. 2014;33:1005–1022.

[25] Cordova B, Salmi M, Leon P. Unravelling the regulatory mechanisms that modulate the MEP pathway in higher plants. J Exp Bot. 2009;60:2933–2943.

[26] Liang CW, Zhang W, Zhang XW, et al. Isolation and characterization of methyl- D-erythritol 4-phosphate synthase genes involved in tanshinone biosynthesis in Salvia miltiorrhiza hairy roots. Physiol Plantarum. 2012;146:173–183.

[27] Nishimura H, Azami Y, Miyagawa M, et al. Biochemical characterization of the gene family encoding deoxy-D-xylulose-5-phosphate synthases from Haematococcus pluvialis. J Appl Phycol. 2016;28:209–218.

[28] Singh N, Cheve G, Avery MA, et al. Targeting the methyl erythritol phosphate (MEP) pathway for novel antimalarial, antibacterial and herbicidal drug discovery: Inhibition of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) enzyme. CPD.. 2007;13:1161–1177.

[29] Wang JW, Wu JY. Tanshinone biosynthesis in Salvia miltiorrhiza and production in plant tissue cultures. Appl Microbiol Biotechnol.. 2010;88:437–449.
[30] Gao W, Hillwig ML, Huang L, et al. A functional genomics approach to tanshinone biosynthesis provides stereochemical insights. Org Lett. 2009;11:5170–5173.
[31] Zi JC, Peters RJ. Characterization of CYP76AH4 clarifies phenolic diterpenoid biosynthesis in the Lamiaceae. Org Biomol Chem. 2013;11:7650–7652.
[32] Guo J, Ma XL, Cai Y, et al. Cytochrome P450 promiscuity leads to a bifurcating biosynthetic pathway for tanshinones. New Phytol. 2016;210:525–534.
[33] Guo J, Zhou YJ, Hillwig ML, et al. CYP76AH1 catalyzes turnover of miltiradiene in tanshinones biosynthesis and enables heterologous production of ferruginol in yeasts. P Natl Acad Sci USA. 2013;110:12108–12113.
[34] Ma Y, Ma XL, Meng FY, et al. RNA interference targeting CYP76AH1 in hairy roots of Salvia miltiorrhiza reveals its key role in the biosynthetic pathway of tanshinones. Biochem Bioph Res Co. 2016;477:155–160.
[35] Song ZQ, Wang JH, Li XF. Expression profiles of genes involved in tanshinone biosynthesis of two Salvia miltiorrhiza genotypes with different tanshinone contents. J Genet. 2016;95:433–439.
[36] Ma XL, Ma Y, Tang JF, et al. The biosynthetic pathways of tanshinones and phenolic acids in Salvia miltiorrhiza. Molecules. 2015;20:16235–16254.
[37] Darabi M, Farhadi-Nejad H. Study of the 3-hydroxy-3-methylglotaryl-coenzyme a reductase (HMGR) protein in Rosaceae by bioinformatics tools. Caryologia. 2013;66:351–359.
[38] Yan XM, Zhang L, Wang J, et al. Molecular characterization and expression of 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR) gene from Salvia miltiorrhiza. Acta Physiol Plant. 2009;31:1015–1022.
[39] Cheng QQ, Su P, Hu YT, et al. RNA interference-mediated repression of SmCPS (copalylidiphosphate synthase) expression in hairy roots of Salvia miltiorrhiza causes a decrease of tanshinones and sheds light on the functional role of SmCPS. Biotechnol Lett. 2014;36:363–369.
[40] Shi M, Luo XQ, Ju GH, et al. Increased accumulation of the cardio-cerebrovascular disease treatment drug tanshinone in Salvia miltiorrhiza hairy roots by the enzymes 3-hydroxy-3-methylglutaryl CoA reductase and 1-deoxy-D-xylulose 5-phosphate reductoisomerase. Funt Integr Genomics. 2014;14:603–615.
[41] Bai ZQ, Liu JL, Zhang CL, et al. Coding single nucleotide polymorphisms and SmCPS1 and SmKSL1 subcellular localization are associated with tanshinone biosynthesis in Salvia miltiorrhiza Bunge roots. Acta Physiol Plant. 2018;40:6–17.
[42] Ma YMA, Yuan LC, Wu B, et al. Genome-wide identification and characterization of novel genes involved in terpenebiosynthesis in Salvia miltiorrhiza. J Exp Bot. 2012;63:2809–2823.
[43] Kai G, Xu H, Zhou C, et al. Metabolic engineering tanshinone biosynthetic pathway in Salvia miltiorrhiza hairy root cultures. Metab Eng. 2011;13:319–327.
[44] Zhou W, Huang Q, Wu X, et al. Comprehensive transcriptome profiling of Salvia miltiorrhiza for discovery of genes associated with the biosynthesis of tanshinones and phenolic acids. Sci Rep-Uk. 2017;7:10554–10566.
[45] Zhang X, Luo HM, Xu ZC, et al. Genome-wide characterization and analysis of bHLH transcription factors related to tanshinone biosynthesis in Salvia miltiorrhiza. Sci Rep-Uk. 2015;5:11224–11256.
[46] Ji AJ, Luo HM, Xu ZC, et al. Genome-wide identification of the AP2/ERF gene family involved in active constituent biosynthesis in Salvia miltiorrhiza. Plant Genome-U. 2016;9:10–14.
[47] Zhang JX, Zhou LB, Zheng XY, et al. Overexpression of SmMYB9b enhances tanshinone concentration in Salvia miltiorrhiza hairy roots. Plant Cell Rep. 2017;36:1297–1309.
[48] Zhao SJ, Zhang JJ, Tan RH, et al. Enhancing diterpenoid concentration in Salvia miltiorrhiza hairy roots through pathway engineering with maize C1 transcription factor. EXBOTJ. 2015;66:7211–7226.
[49] Xu XB, Jiang QH, Ma XY, et al. Deep sequencing identifies tissue-specific microRNAs and their target genes involving in the biosynthesis of tanshinones in Salvia miltiorrhiza. Plos One. 2014;9:e111679. [cited 2018 Apr 27]DOI: 10.1371/journal.pone.0111679
[50] Wang ZJ, Cui LJ, Chen C, et al. Downregulation of Cinnamoyl CoA reductase affects lignin and phenolic acids biosynthesis in Salvia miltiorrhiza Bunge. Plant Mol Biol Rep. 2012;30:1229–1236.
[51] Zhang Y, Yan YP, Wu YC, et al. Pathway engineering for phenolic acid accumulations in Salvia miltiorrhiza by combinational genetic manipulation. Metab Eng. 2014;21:71–80.
[52] Ferrer JL, Austin MB, Stewart C, et al. Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiol Biochem.. 2008;46:356–370.
[53] Petersen M, Hausler E, Karwatzki B, et al. Proposed biosynthetic-pathway for rosmarinic acid in cell-cultures of Coleus-Blumei Benth. Planta. 1993;189:10–14.
[54] Xiao Y, Gao SH, Di P, et al. Lithospermic acid B is more responsive to silver ions (Ag+) than rosmarinic acid in Salvia miltiorrhiza hairy root cultures. Biosci Rep.. 2009;30:33–40.
[55] Xiao Y, Di P, Chen JF, et al. Characterization and expression profiling of 4-hydroxyphenylpyruvate dioxygenase gene (Smhpd) from Salvia miltiorrhiza hairy root cultures. Mol Biol Rep. 2009;36:2019–2029.
[56] Song ZQ, Li XF. Expression profiles of rosmarinic acid biosynthesis genes in two Salvia miltiorrhiza lines with differing water-soluble phenolic contents. Ind Crop Prod. 2015;71:24–30.
[57] Xiao Y, Zhang L, Gao SH, et al. The c4h, tat, hppr and hppd Genes Prompted Engineering of Rosmarinic Acid Biosynthetic Pathway in Salvia miltiorrhiza Hairy Root Cultures. Plos One. 2011;6:e29713. [cited 2018 Apr 27]DOI: 10.1371/journal.pone.0029713
[58] Hou XM, Shao FJ, Ma YM, et al. The phenylalanine ammonia-lyase gene family in Salvia miltiorrhiza: genome-wide characterization, molecular cloning and expression analysis. Mol Biol Rep. 2013;40:4301–4310.
[59] Jin XQ, Chen ZW, Tan RH, et al. Isolation and functional analysis of 4-coumarate:coenzyme A ligase gene promoters from Salvia miltiorrhiza. Biol Plant.. 2012;56:261–268.
[60] Zhao SJ, Hu ZB, Liu D, et al. Two divergent members of 4-coumarate: Coenzyme A ligase from Salvia miltiorrhiza bunge: cDNA cloning and functional study. J Integrative Plant Biology. 2006;48:1355–1364.

[61] Hua WP, Zhang Y, Song J, et al. De novo transcriptome sequencing in Salvia miltiorrhiza to identify genes involved in the biosynthesis of active ingredients. Genomics. 2011;98:272–279.

[62] Song J, Ji YY, Xu K, et al. An integrated analysis of the rosmarinic acid-biosynthetic genes to uncover the regulation of rosmarinic acid pathway in Salvia miltiorrhiza. Acta Physiol Plant. 2012;34:1501–1511.

[63] Zhou YY, Sun W, Chen JF, et al. SmMYC2a and SmMYC2b played similar but irreplaceable roles in regulating the biosynthesis of tanshinones and phenolic acids in Salvia miltiorrhiza. Sci Rep. 2016;6:22852–22864.

[64] Siu KC, Wu JY. Enhanced release of tanshinones and phenolics by nonionic surfactants from Salvia miltiorrhiza hairy roots. Eng Life Sci. 2014;14:685–690.

[65] Pei DQ, Xu JY, Zhuang QA, et al. Induced pluripotent stem cell technology in regenerative medicine and biology. Adv Biochem Eng Biotechnol.. 2010;123:127–141.

[66] Cheng QQ, He YF, Li G, et al. Effects of combined elicitors on tanshinone metabolic profiling and SmCPS expression in Salvia miltiorrhiza hairy root cultures. Molecules. 2013;18:7473–7485.

[67] Yang DF, Fang YM, Xia PG, et al. Diverse responses of tanshinone biosynthesis to biotic and abiotic elicitors in hairy root cultures of Salvia miltiorrhiza and Salvia castanea Diels f. tomentosa. Gene. 2018;643:61–67.

[68] Fang YM, Hou ZN, Zhang XD, et al. Diverse specialized metabolism and their responses to lactalbumin hydrolysate in hairy root cultures of Salvia miltiorrhiza Bunge and Salvia castanea Diels f. tomentosa Stib. Biochem Eng J. 2018;131:58–69.

[69] Wang DD, Zhang WX, Wang TT, et al. Unveiling the Mode of Action of Two Antibacterial Tanshinone Derivatives. Int J Mol Sci. 2015;16:17668–17681.

[70] Zhang YA, Yan YP, Wang ZZ. The Arabidopsis PAP1 Transcription Factor Plays an Important Role in the Enrichment of Phenolic Acids in Salvia miltiorrhiza. J Agric Food Chem.. 2010;58:12168–12175.

[71] Liu Y, Sun G, Zhong ZH, et al. Overexpression of AtEDT1 promotes root elongation and affects medicinal secondary metabolite biosynthesis in roots of transgenic Salvia miltiorrhiza. Protoplasma. 2017;254:1617–1625.

[72] Li B, Cui GH, Shen GA, et al. Targeted mutagenesis in the medicinal plant Salvia miltiorrhiza. Sci Rep. 2017;7:43320–43329.

[73] Cheng JR, Liu XM, Chen ZY, et al. Mulberry anthocyanin biotransformation by intestinal probiotics. Food Chem. 2016;213:721–727.

[74] Zhai X, Luo D, Li XQ, et al. Endophyte Chaetomium globosum D38 promotes bioactive constituents accumulation and root production in Salvia miltiorrhiza. Front Microbiol. 2018;9:2694–2707.

[75] Zhou LS, Tang K, Guo SX. The plant growth-promoting fungus (PGPF) Alternaria sp A13 markedly enhances Salvia miltiorrhiza root growth and active ingredient accumulation under greenhouse and field conditions. JAPS. 2018;19:270–284.

[76] Chen H, Wu H, Yan B, et al. Core Microbiome of Medicinal Plant Salvia miltiorrhiza Seed: A Rich Reservoir of Beneficial Microbes for Secondary Metabolism?. JAPS. 2018;19:672–687.

[77] Kan SD, Li J, Huang WY, et al. Microsphere resin chromatography combined with microbial biotransformation for the separation and purification of salvianolic acid B in aqueous extract of roots of Salvia multiorrhiza Bunge. J Chromatogr A. 2009;1216:3881–3886.

[78] He WN, Liu MM, Li XL, et al. Fungal biotransformation of tanshinone results in [4 + 2] cycloaddition with sorbicillinol: evidence for enzyme catalysis and increased antibacterial activity. Appl Microbiol Biotechnol. 2016;100:8349–8357.

[79] Sun JH, Yang M, Ma XC, et al. Microbial biotransformation of cryptotanshinone by Cunninghamella elegans and its application for metabolite identification in rat bile. J Asian Nat Prod Res. 2009;11:482–489.

[80] Liang WF, Li ZW, Ji S, et al. Microbial glycosylation of tanshinone IIa by Cunninghamella elegans AS 3.2028. Rsc Adv. 2015;5:63753–63756.

[81] Mora-Pale M, Sanchez-Rodriguez SP, Linhardt RJ, et al. Metabolic engineering and in vitro biosynthesis of phytochemicals and non-natural analogues. Plant Sci. 2013;210:10–24.

[82] Kwon M, Shin BK, Lee J, et al. Characterization of Burkholderia glumae BGR1 4-Hydroxy-3-methylbut-2-enyl Diphosphate Reductase (HDR), the Terminal Enzyme in 2’-C-Methyl-D-erythritol 4-Phosphate (MEP) Pathway. J Korean Soc Appl Biol Chem.. 2013;56:35–40.

[83] Zhou J, Wang CL, Yang LY, et al. Geranyl diphosphate synthase: An important regulation point in balancing a recombinant monoterpenic pathway in Escherichia coli. Enzyme Microb Tech. 2015;68:50–55.

[84] Zhang M, Su P, Zhou YJ, et al. Identification of geranylgeranyl diphosphate synthase genes from Tripterygium wilfordii. Plant Cell Rep. 2015;34:2179–2188.

[85] Dai ZB, Liu Y, Huang LQ, et al. Production of mitiradiene by metabolically engineered Saccharomyces cerevisiae. Biotechnol Bioeng. 2012;109:2845–2853.

[86] Muir RM, Ibanez AM, Uratsu SL, et al. Mechanism of gallic acid biosynthesis in bacteria (Escherichia coli) and walnut (Juglans regia). Plant Mol Biol. 2011;75:555–565.

[87] Munoz AJ, Hernandez-Chavez G, De Anda R, et al. Metabolic engineering of Escherichia coli for improving L-3,4-dihydroxyphenylalanine (L-DOPA) synthesis from glucose. J Ind Microbiol Biotechnol. 2011;38:1845–1852.

[88] Yao YF, Wang CS, Qiao JJ, et al. Metabolic engineering of Escherichia coli for production of salvianic acid A via an artificial biosynthetic pathway. Metab Eng. 2013;19:79–87.

[89] Jiang JJ, Bi HP, Zhuang YB, et al. Engineered synthesis of rosmarinic acid in Schisandra chinensis resulting production of a new intermediate, caffeoyl-phenyllactate. Biotechnol Lett. 2016;38:81–88.
[90] Zhou K, Qiao KJ, Edgar S, et al. Distributing a metabolic pathway among a microbial consortium enhances production of natural products. Nat Biotechnol. 2015;33:377–386.

[91] Wu JY, Ng J, Shi M, et al. Enhanced secondary metabolite (tanshinone) production of Salvia miltiorrhiza hairy roots in a novel root-bacteria coculture process. Appl Microbiol Biotechnol. 2007;77:543–550.

[92] Yan Y, Zhang SC, Yang DF, et al. Effects of streptomyces pactum Act12 on Salvia miltiorrhiza hairy root growth and tanshinone synthesis and its mechanisms. Appl Biochem Biotechnol. 2014;173:883–893.

[93] Hasanloo T, Sepehrifar R, Rahnama H, et al. Evaluation of the yeast-extract signaling pathway leading to silymarin biosynthesis in milk thistle hairy root culture. World J Microbiol Biotechnol. 2009;25:1901–1909.