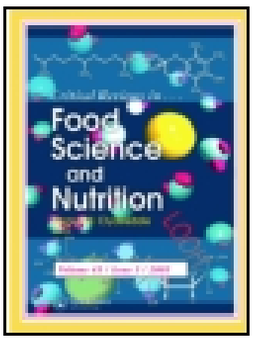

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Bioactivities, biosynthesis and biotechnological production of phenolic acids in *Salvia miltiorrhiza*

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Abstract

Salvia miltiorrhiza (Danshen in Chinese), is a well-known traditional Chinese medicinal plant, which is used as not only human medicine but also health-promotion food. Danshen has been extensively used for the treatment of various cardiovascular and cerebrovascular diseases. As a major group of bioactive constituents from *S.*

miltiorrhiza, water-soluble phenolic acids such as salvianolic acid B possessed good bioactivities including antioxidant, anti-inflammatory, anti-cancer and other health-promoting activities. It is of significance to improve the production of phenolic acids by modern biotechnology approaches to meet the increasing market demand. Significant progresses have been made in understanding the biosynthetic pathway and regulation mechanism of phenolic acids in *S.miltiorrhiza*, which will facilitate the process of targeted metabolic engineering or synthetic biology. Furthermore, multiple biotechnology methods such as *in vitro* culture, elicitation, hairy roots, endophytic fungi and bioreactors have been also used to obtain pharmaceutically active phenolic acids from *S. miltiorrhiza*. In this review, recent advances in bioactivities, biosynthetic pathway and biotechnological production of phenolic acid ingredients were summarized and future prospective was also discussed.

Keywords

Salvia miltiorrhiza, phenolic acids, bioactivities, biosynthetic pathway, biotechnological strategies

1. Introduction

Salvia miltiorrhiza (Danshen in Chinese), belonging to Labiate family (Kai et al. 2011; Shi et al. 2014), is an important traditional Chinese herbal plant with long history for medicine as well as healthy food (**Figure 1**). Its dried root and rhizome are widely used for the treatment of cardiovascular and cerebrovascular diseases in Asia, Europe and the United States (Zhou et al. 2005; Wu et al. 2012). There are many

Danshen-containing preparations including tablet, dripping pill, injection solution, capsule, granule, sprayer, slow release formulation, soft gel and oral liquid in clinical application. Among them, Compound Danshen dripping pills and Fufang Danshen tablets are the two most famous products (Fang et al. 2014; He et al. 2015; Zhao et al. 2015). Notably, the Compound Danshen dripping pills is the first TCM approved by the US Food and Drug Administration for clinical tests and now completed the Phase III clinical trials in the United States (http://clinicaltrials.gov/, NCT01659580). In addition, Danshen Cha (Danshen Tea) is widely used as heathy food to prevent coronary heart disease etc.

According to the pharmacological investigations, the active ingredients of *S. miltiorrhiza* can be divided into two major groups: lipid-soluble (lipophilic) tanshinones including tanshinone I, tanshinone IIA, tanshinone IIB, dihydrotanshnone I and cryptotanshinone (Wang and Wu, 2010; Kai et al. 2011; Shi et al. 2014; Zhou et al. 2017), and water-soluble (hydrophilic) phenolic acids such as danshensu (DSU), caffeic acid (CA), rosmarinic acid (RA), salvianolic acid A (Sal A) and salvianolic acid B (Sal B) (**Figure 2**) (Xing et al. 2015; Zhao et al. 2015). The phenolic acids possess various bioactivities including antioxidant, anticoagulant, anti-thrombotic, antitumor, anti-blood coagulation and anti-HIV activities (Wang et al. 2007). Among the phenolic acids, Sal B is referred as a predominant and marker constituent in the official Chinese Pharmacopoeia. Due to relatively low contents and long growth cycle of cultivated resource plants, the production of phenolic acids cannot meet the fast-growing market needs. It is essential to increase the production

of phenolic acids from *S. miltiorrhiza* through modern biotechnology methods. *S. miltiorrhiza* hairy root cultures in shake-flasks or bioreactor system have been established which may be an alternative for phenolic acid production (Chen et al. 1999a; Zhong et al. 2001; Yuan et al. 2009). Various biotechnological applications including suspension cell, elicitation treatment, transgenic plant, endophytic fungi, metabolic engineering, etc. have been utilized to improve the phenolic acids production.

Recently, the phenolic acids from *S. miltiorrhiza* have become a focus in plant secondary metabolism (Jia et al. 2017; Zhou et al. 2018). This review aims to comprehensively summarize recent advances in the understanding of bioactivities, biosynthetic pathway of phenolic acids, transcriptional regulation mechanism, and various biotechnological strategies for high production of phenolic acids including elicitation treatment and synthetic biology, and the prospects are also discussed.

2. Pharmacological activities of phenolic acids

As a major class of bioactive constituents in *S. miltiorrhiza*, phenolic acids exhibit various clinical application. Numerous studies have demonstrated various bioactivities such as antioxidant, anti-inflammatory, anti-bacteria, antitumor, cardio-protection activities, and its wide application in health-promoting food (Wang et al. 2007; Zhao et al. 2008; Hung et al. 2016; Zhang et al. 2017). Pharmacological effects of salvianolic acid B were listed in **Table 1**.

2.1 Antioxidant Activity

Antioxidant activity plays an important role in fighting against certain diseases and many researches have demonstrated the antioxidant role of phenolic acids (Chen et al. 2013). Sal B which has been proved to show high antioxidant activity could decrease the production of reactive oxygen species and NADPH oxidase activity induced by TNF-alpha (Chen et al. 2013; Zhang and Wang. 2006). Sal B antagonized the high glucose-induced oxidative stress and apoptosis in Schwann cells (Sun et al. 2012). Sal A would undergo methylation metabolism catalyzed by catechol *O*-methyltransferase and protect against acute hepatic damage caused by carbon tetrachloride in rats, which may be responsible for its antioxidant activities (Xu et al. 2014). Salvianolic acid L (Sal L) has antioxidant activity via its free radical scavenging activities for 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide anion radicals (Lu and Foo, 2001).

2.2 Anti-inflammatory Activity

Caffeic acid was able to directly inhibit the enzymes interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK4 which participated in various inflammatory responses, indicating that caffeic acid can suppress inflammatory symptoms (Yang et al. 2013). Sal B could weaken high-fat diet (HFD)-induced inflammation by activation of antioxidant defense system mediated by nuclear factor-erythroid 2-related factor 2 (Wang et al. 2017). Also, Sal B exerted significant anti-inflammatory effects against alcoholic liver injury by downregulation expression of CRP and ChREBP induced by SIRT1 (Zhang et al. 2017).

2.3 Anti-cancer Activity

Sal B is considered as a potential chemopreventive agent for cancer prevention and treatment including human glioma, oral squamous cell carcinoma (OSCC), acute lymphoblastic leukemia (ALL), as well as head and neck squamous cell carcinoma (Wang et al. 2013; Hung et al. 2016; Li et al. 2016; Wu et al. 2016; Yang et al. 2011; Zhao et al. 2010). Sal B can inhibit the growth of head and neck squamous cell carcinoma (HNSCC) by inhibiting cyclooxygenase-2 (COX-2) expression that is associated with increased risk of HNSCC (Hao et al. 2009; Zhao et al. 2010). With the treatment of Sal B, the squamous cell carcinoma (SCC) incidence was decreased in 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis (Zhou et al. 2006). And Sal B attenuated or normalized the gene expression and metabolite levels of inflammation and tumor angiogenesis in DMBA-induced oral dysplasia (Wei et al. 2012). Sal A reversed paclitaxel resistance via suppressing the expression of transgelin 2 that involved in the tumor invasion and metastasis in human breast cancer MCF-7 cells (Cai et al. 2014). DSU inhibited protein expression of MMP-2, -9 and Vascular Endothelial Growth Factor (VEGF), lung metastasis in B16F10 melanoma cell suggested that DSU has anti-cancer activity by affecting tumor angiogenesis and tumor invasion (Zhang et al. 2010). DSU also targeted to a potential anticancer HRas by dual inverse docking (Chen et al. 2014).

2.4 Anti-bacterial Activity

Salvianolic acids have been reported to show anti-bacterial activity on a range of microorganisms. For example, extracts from *S. miltiorrhiza* hairy roots containing rosmarinic acid, danshensu and caffeic acid exhibited weaker inhibition on growth of a series of bacteria including *A.tumefaciens*, *E.coli*, *P.lachrymans*, *R.solanacearum*, *X.vesicatoria*, *B.subtilis*, *S.haemolyticus* and *M.oryzae* (Zhao et al. 2011). Sal B could inhibit meningococcal binding, leading to the prevention of infections by *Neisseria meningitidis* (Huttunen et al. 2016). As a new salvianolic acid, salvianolic acid V together with Levofloxacin or Colistin sulphate revealed an effect against MRSA or *Acinetobacter baumanii* (Zhang et al. 2018).

2.5 Anti-virus Activity

LA and LAB inhibited human immunodeficiency virus type 1 (HIV-1) integrase activity which endowed them as a therapeutic medicine for AIDS based on their high potencies and non-cytotoxicity (Abd-Elazem et al. 2002). RA also directly inhibited the reverse transcriptase (RT) of HIV-1 virus and reduced the serious diseases caused by Japanese encephalitis virus (JEV), thereby acting as a potent antiviral agent against JE (Hooker et al. 2001; Swarup et al. 2007). Magnesium lithospermate B (MLB) and RA have potent antiviral activity against enterovirus 71 (EV71) infections by suppression of EV71 IRES-mediated translation (Chung et al. 2015).

2.6 Anti-fibrotic Activity

Danshen has been testified to possess extensive anti-fibrosis effect. Salvianolic acid A and B exhibited an inhibition on the areca nut extract (ANE)-induced oral

submucous fibrosis (OSF) by activating the AKT, ERK MAPK and TGF-β/Smads pathways (Dai et al. 2015). As an inhibitor of matrix metalloproteinase-9 (MMP-9), Sal A showed strong anti-fibrotic activity in hypertensive fibrosis (Jiang et al. 2013). By blocking collagen accumulations, reducing α-SMA expression and histological alterations induced by bleomycin, Sal B treatment on pulmonary fibrosis was significant (Liu et al. 2015).

2.7 Other Bioactivities

DSU and Sal B can enhance the cell proliferation and increase the production of collagen in Detroit 551 cells and suppress melanin production in B16 cells, indicating their potential as active components in wound healing, cosmetic treatments or hyperpigmentation treatments (Chen et al. 2014). Furthermore, salvianolic acids have other miscellaneous bioactivities including anti-ischemia-reperfusion, anti-hypertension and anti-thrombosis. Although pharmacological activities of phenolic acids have been verified and utilized, the role of various phenolic acids on different symptoms needs more excavation.

3. Biosynthesis and regulation of phenolic acids in *S. miltiorrhiza*

3.1 Biosynthesis of in *S. miltiorrhiza*

Phenolic acids belong to a group of phenylaprapanoid compounds derived from shinimate pathway. The phenolic acids biosynthesis process is not fully characterized, but it could be classified into three stages, starting from the L-tyrosine and L-phenylalanine to synthesize two intermediates 3,4-dihydroxyphenyllactic acid

and 4-coumaroyl-CoA seperately, followed by the condensation of the above two precursors to form rosmarinic acid and other phenolic acids by a series of modifications throught several enzymes involoved in the RA branch pathway (Zhao et al. 2015) (**Figure 3**).

In the tyrosine biosynthetic pathway, L-tyrosine is metabolized to 4-hydroxyphenylpyruvic acid catalyzed by tyrosine amino transferase (TAT), then to form 3,4-dihydroxyphenyllactic acid by 4-hydroxyphenylpyruvate reductase (HPPR) and an unknown cytochrome P450-dependent enzyme. In the process of phenylpropanoid pathway, L-phenylalanine is transformed to 4-coumaroyl-CoA by a series of catalysis by phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and 4-coumarate: CoA ligase (4CL) (Di et al. 2013). The two intermediates 3,4-dihydroxyphenyllactic acid and 4-coumaroyl-CoA are assembled by ester-forming enzyme rosmarinic acid synthase (RAS) to generate 4-coumaroyl-3', 4'-dihydroxyphenyllactic acid. A 3-hydroxyl group is introduced to at C-3 within the aromatic ring of the 4-coumaroyl-3',4'-dihydroxyphenyllactic acid by SmCYP98A14, a cytochrome P450-dependent monooxygenase, to form RA. Other related salvianolic acids are proposed to be the condensation derivatives of RA (Di et al. 2013; Zhao et al. 2015).

3.2 Related genes involved in phenolics acid biosynthetic pathway

With the more extensive application of phenolic acids in medicinal industries and health-promoting food field, *S. miltiorrhiza* has attracted more attention in recent

years and has been regarded as one model traditional Chinese medicine in the biotechnological field. Considerable efforts have been made to elucidate the biosynthesis of phenolic acids in *S. miltiorrhiza*. Several genes involved in the biosynthetic pathways of phenolic acids have been isolated and characterized as described below.

3.2.1 Genes in the tyrosine-derived pathway

TAT (Tyrosine aminotransferase) is the first enzyme in the tyrosine-derived pathway of phenolic acids. Huang et al (2008a) cloned the *SmTAT* gene using the RACE method from seedlings of *S.miltiorrhiza* which has the full-length of 1603bp with an open reading frame of 1233 bp encoding a 410 amino acid protein. Semi-quantitative RT-PCR analysis showed that *SmTAT1* constitutively expressed stronger in stems than that in root, leaf, and responded to SA, MeJA, ABA and UV-B. Two TAT genes designated as *SmTAT2* and *SmTAT3* have been isolated from *S.miltiorrhiza* cDNAs, phylogenetic tree analysis demonstrated *SmTAT3* and *SmTAT1* clustered into the same sub-clade, while *SmTAT2* clustered in another one, revealing that *SmTAT3* and *SmTAT1* may play a similar role in secondary metabolism, and *SmTAT2* may be specialized for others (Wang et al. 2015).

HPPR catalyzes the condensation of 4-hydroxyphenylpyruvic acid to form 4-hydroxyphenyllactic acid. Three HPPR genes named as *SmHPPR1*, *SmHPPR2* and *SmHPPR3* with an ORF of 939bp, 942bp, 960bp were cloned from *S. miltiorrhiza*, respectively. Semiquantitative RT-PCR revealed that *SmHPPR1* expressed highest in

stem, in addition, MJ, SA, GA_3, and ABA treatments could upregulate the *SmHPPR1* transcription level, while its transcription level was downregulated by UV-B and hydrogen peroxide (H_2O_2) (Xiao et al. 2011; Wang et al. 2015).

3.2.2 Genes in the phenylpropanoid pathway

PAL is the first point enzyme involved in the phenylpropanoid pathway playing an important role in mediating primary and secondary metabolism. Recently, three *PALs* named *SmPAL1*, *SmPAL2* and *SmPAL3* have been found from *S. miltiorrhiza* successively (Song and Wang, 2009; Song and Wang, 2011; Hou et al. 2013). The full length of *SmPAL1* was 2827 bp with an open reading frame of 2136 bp encoding a 711-amino acid polypeptide. *SmPAL1* expressed mainly in roots and stems and can be induced by various treatments such as ABA, wounding, dehydration, cold stress and infection of fungus (Song and Wang, 2009). *SmPAL2* encodes a 683-amino acid protein predominately observed in stems (Song and Wang, 2011). *SmPAL3* contains a 2283 bp ORF which encodes 760 amino acid residues presenting high expression level in roots and stems, maybe involved in wound response and salicylic acid (SA)-mediated systemic acquired resistance (SAR). Expression level of *SmPAL1* and *SmPAL2* was drastically elevated at 6h after MJ treatment (Wang et al. 2015).

Cinnamate 4-hydroxylase (C4H) belong to cytochrome P450 enzymes has been confirmed to control the metabolic flow from t-cinnamic acid into 4-coumaric acid. *SmC4H1* containing an open reading frame of 1512 bp encoding a polypeptide of 504 amino acid residues expressed highly in roots and showed sensitivity to MeJA, ABA,

UV-B radiation (Huang et al. 2008b). Compared with *SmC4H1*, *SmC4H2* was short of ER-targeting peptide, thus encoded a polypeptide of 397 amino acid residues. Related motifs including fungal attack and salicylic acid appeared in the promotor region of *SmC4H2*, revealing that it may participate in certain signaling pathway (Wang et al. 2015).

4-coumarate:CoA ligase (4CL), the final enzyme in the phenylpropanoid metabolism controls the formation of 4-coumaroyl-CoA as the precursor to generate rosmarinic acid. Three 4CL genes and seven 4CL-like genes have been characterized from *S.miltiorrhiza*, designated as *Sm4CL1*, *Sm4CL2*, *Sm4CL3*, *Sm4CL-like 1*, *Sm4CL-like 2*, *Sm4CL-like 3*, *Sm4CL-like 4*, *Sm4CL-like 5*, *Sm4CL-like 6*, *Sm4CL-like 7*. All the 4CL and 4CL-like genes except *Sm4CL-like 3*, *Sm4CL-like 4*, *Sm4CL-like 5*, *Sm4CL-like 7* exhibited low relative expression after MeJA induction (Zhao et al. 2006; Wang et al. 2015).

3.2.3 Genes in RA branch pathway

RAS (Rosmarinic acid synthase or Hydroxycinnamoyltransferase) is a rate-limiting enzyme combination of 3,4-dihydroxyphenyllactic acid and 4-coumaroyl-CoA. Until now, seven *SmRASs* including *SmRAS1*, SMil_00025190 and five *SmHCTs* have been dig out (Wang et al. 2015; Zhou et al. 2018). *SmRAS* expressed highly in stem, root and leaf, and can be elicited by MJ, light, and SA, etc. *SmRAS* showed high homology with RAS gene from *C. blumei* and used a dihydroxylated DHPL (3,4-Dihydroxyphenyllactate) as substance to synthesize the

precursor of RA. *SmHCT1* was expressed highly in roots while the rest mainly in stems. All *SmHCTs* exerts sensitivity to MeJA, especially *SmHCT3* and *SmHCT4* (Wang et al. 2015). Knock out of SMil_00025190 utilizing CRISPR/Cas9 decreased the contents of rosmarinic acid and lithospermic acid in *S.miltiorrhiza* hairy root (Zhou et al. 2018).

CYP98A14 encoding a cytochrome P450-dependent monooxygenase regulated the flow from 4-coumaroyl-3', 4'-dihydroxyphenyllactic acid to generate rosmarinic acid (RA) in conjunction with CPR, a NADPH:cytochrome P450 reductase (Di et al. 2013). Full-length ORF of *SmCYP98A14* was 1527 bp with the highest expression level in root, in addition, *SmCYP98A14* can be rapidly induced by ABA and MJ (Wang et al. 2015).

4. Biotechnological approaches for phenolic acids production

Because of the scarce natural source and relatively low contents in the cultivated parent-plant roots, effective alternatives including cell culture, hairy root system and endophytic fungi, etc have been developed to supplement the production of desired compounds extracted from wild or cultivated plants. The above systems exhibited different advantages, for example, cell culture is insensitive to the external environment and produce metabolites rapidly. Hairy root system possesses more advantages including relatively fast growth rates and high genetic stability, which is considered as a kind of promising system (Kai et al. 2011; Shi et al. 2014). Endophytic fungi is a novel production system originated from their host plants

which can rapidly produce metabolites (Ming et al. 2012). All of them have been utilized to produce phenolic acids compounds (**Table 2**). Mass culture of *S.miltiorrhiza* hairy roots of 50-day-old in 75 L airlift bioreactor can yield protocatechualdehyde and Sal B which occupy 0.0203% and 0.1176% of the root dry weight (Qiu et al. 2004). However, production of phenolic acids using the above strategies in large scale with appropriate bioreactors need to be conducted further.

4.1 Production of phenolic acids compounds in cell culture

Due to low contents of phenolic acids in *S. miltiorrhiza*, in vitro cell culture has been explored for production of phenolic acids which has been confirmed a sustainable and efficient strategy. For example, transformed cell cultures of *S. miltiorrhiza* established by infecting sterile plantlets with *Agrobacterium tumefaciens* strain C58 were able to produce LAB (85.0 mg/L) and RA (481.5 mg/L) cultivated in flasks for 20 days (Chen et al. 1999b). A callus culture system derived from *S. miltiorrhiza* leaves or stems could produce enhanced RA and Sal B, and extracts from callus stem is higher than that from leaf callus (Wu et al. 2016). At present, studies on the cell culture for production of phenolic acids are limited which need more concentration (Wu et al. 2016).

4.2 Metabolic engineering of biosynthetic pathway of phenolic acids

With the successful isolation of key enzyme genes catalyzing committed steps involved in phenolic acids biosynthesis, rational metabolic engineering of biosynthesis pathway is a feasible strategy to improve phenolic acids accumulation

with different combinations of key genes. For example, introduction of single gene including *C4H, TAT* or *HPPR* enhanced the production of RA and LAB. Besides, simultaneous manipulation of *TAT* and *HPPR* in *S.miltiorrhiza* hairy root cultures resulted in an abundance of RA and LAB, which were 906 mg/L and 992 mg/L, respectively (Xiao et al. 2011). The overexpression of allene oxide cyclase (*SmAOC*) also significantly enhanced the yields of RA and LAB in *S. miltiorrhiza* hairy roots via regulating the biosynthesis of plant hormone jasmonates (Gu et al., 2012). Apart from engineering the committed steps, blocking competitive branching pathway sharing the common metabolic precursor was also an effective method for production of targeted natural products. Commonly, 4-hydroxyphenylpyruvic acid can be metabolized by both HPPR and HPPD, while HPPD competitively transformed 4-hydroxyphenylpyruvic acid to homogentisic acid which was the substrate of vitamin E (Xiao et al. 2011; Kim and Petersen, 2002). Suppression of HPPD led to an increased accumulation of 4-hydroxyphenylpyruvic acid, which may result from the shift of metabolic flux (Xiao et al. 2011). Down-regulation of the cinnamoyl CoA reductas (*SmCCR1*) in the lignin pathway strongly increased the biosynthesis of phenolic acids in transgenic plants (Wang et al. 2012). By combing the RNAi-mediated silencing of chalcone synthase gene (CHS) of flavonoid pathway with SA treatment enhanced contents of phenolic acids in *S. miltiorrhiza* hairy roots (Zhang et al. 2015).

In addition to genetic manipulation of key enzyme genes to regulate the production of desired products, ectopic expression of transcription factors has been

testified an effective approach which have been conducted in many plant species (Zhao et al. 2015; Zhang et al. 2015). For production of phenolic acids, *Arabidopsis PAP1* transcription factor (Production of Anthocyanin Pigment 1) was heterologously expressed in *S. miltiorrhiza* plantlets, transgenic plants exhibited obviously induced salvianolic acid B contents consistent with other constituents derived from phenylpropanoid and tyrosine-derived pathways such as anthocyanin and lignin (Zhang et al. 2010), revealing that the metabolic flux may be converted into both phenolic acid and lignin biosynthesis. Based on the previous reports, combinational strategy consisting of *AtPAP1* and two lignin biosynthesis genes (*SmCCR/SmCOMT*) was carried out. Heterologous expression of the *AtPAP1* and suppression of *SmCCR/SmCOMT* led to enhanced production of Sal B and reduced lignin (Zhang et al. 2014). To our knowledge, sometimes ectopic transcription factors may result in negative effect for some biosynthetic pathway. Take an example, Zhao et al (2015) reported that introduction of maize transcription factor C1 in hairy roots of Danshen inhibited the yield of phenolic acids compared with the control by downregulation of *SmTAT*. Generally, stacking some endogenous functional structural genes or engineering of transcription factors as well as combination of them was a valid way for natural products biosynthesis.

4.3 Elictation treatment

Elicitation treatment is one of the most common method to stimulate the plant cells to produce high-valued secondary metabolites with a variety of inducers (Afrin

et al. 2015). Different suitable elicitors including biotic and abiotic elicitors have been utilized to enhance the accumulation of phenolic acids in *S. miltiorrhiza* in cell or hairy roots cultures, such as silver ions (Ag^+), yeast extract (YE), salicylic acid (SA), hydrogen peroxide (H_2O_2), nitric oxide (NO), abscisic acid (ABA), gibberellic acid (GA), ethylene, polyamines, Tween 20, Triton X-100, methyl jasmonate (MJ) and endophytic bacteria (**Table 3**). Generally, different elicitors would stimulate a variety of key genes involved in the biosynthesis pathway. For example, Ag^+ could promote the expression level of a series of phenolic acids biosynthesis genes including the upstream genes (*PAL, C4H, 4CL, TAT, HPPR*) and the down-stream genes (*RAS* and *CYP98A14*), led to significantly promoted ferulic acid, rosmarinic acid and caffeic acid (Xing et al. 2014). Moreover, Xiao et al (2010) reported that Ag^+ obviously enhanced LAB by activating several genes. Moreover, multiple phytohormones such as ABA, MJ, SA, ethylene has been applied for production of phenolic acids, the results revealed that increased expression levels of *PAL* and *TAT* were observed along with the promoted production of phenolic acids (Liang et al. 2013). Previous studies reported that endophytic microorganism is one kind of well-behaved elicitors to influence the metabolites in hairy roots (Wang et al. 2002). Five endophytic bacteria *P.brassicacearum* sub sp. *neoaurantiaca*, *R.radiobacter*, *P.thivervalensis*, *P.frederiksbergensis*, *N.resinovorum* have been testified to significantly decrease the expression of *SmPAL* and *SmTAT* and resulted in a significant decrease in RA and Sal B content in *S. miltiorrhiza* hairy roots (Yan et al. 2014). In addition to hairy roots system, H_2O_2 and NO have been used to elicite the

(SA)-induced *S.miltiorrhiza* cell cultures, which would induce the accumulation of Sal B (Guo et al. 2014). Although multiple elicitors have been proved to be efficient in accumulation of secondary metabolites, however, specific mechanism remained unclear which need more investigation.

4.4 Transcriptional regulation of phenolic acids in *S. miltiorrhiza*

Multiple key enzyme genes involved in biosynthetic pathway of phenolic acids have been successfully isolated from *S. miltiorrhiza* in recent years, most of them have been reported to be responsive to different elicitors including MJ, SA, ABA, etc, revealing that these genes are proposed to be regulated by some transcriptional factors participating in certain signaling pathway. With the rapid evolution of sequencing technology, transcriptome and genome sequencing have been completed, and various transcription factors or transcriptional regulators belong to different families have been found and studied, for instance, MYB transcription factors, bHLH gene family and Jasmonate ZIM-Domain proteins (JAZ).

Methyl jasmonate (MeJA) has been testified to be an efficient elicitor to promote the production of phenolic acids in *S. miltiorrhiza* (Xiao et al. 2009; Hao et al. 2015). Transcriptome of *S. miltiorrhiza* hairy roots induced by MeJA have been conducted revealing a large amount of transcription factors or transcription regulators in response to MeJA (Zhou et al. 2017). The biosynthesis mechanism of phenolic acids is being clarified gradually. JAZ proteins and MYC2 transcription factors are key components in JA signal processes. Two novel JAZ genes responsive

to MeJA named *SmJAZ3* and *SmJAZ9* endowed a suppression on the accumulation of tanshinones in *S. miltiorrhiza* hairy roots implying that they acted as negative regulators in JA signaling pathway (Shi et al. 2016). Besides, RNA interference of *SmJAZ8* which showed the strongest expression by treatment with MeJA compared with other *SmJAZs* could up-regulate the concentration of phenolic acids in the transgenic hairy roots treated by MeJA. Meanwhile, the transcript levels of other *SmJAZ* genes were altered by *SmJAZ8* suggesting a crosstalk existing in JAZ-regulated secondary metabolism and transcriptome analysis presented that a primary-secondary metabolism balance was regulated by *SmJAZ8* (Pei et al. 2018). Generally, JAZ family repress MYC2 transcription factors in JA signaling, and degradation of JAZ makes MYC2 transcription factor released rendering activation or suppression of biosynthetic genes. Zhou et al (2016) reported that two novel JA-inducible genes designated as SmMYC2a and SmMYC2b interacted with SmJAZ1 and SmJAZ2, and RNA interference of *SmMYC2a* and *SmMYC2b* led to reduced accumulation of phenolic acids. Further studies revealed that SmMYC2a could bind with *SmHCT6* and *SmCYP98A14*, and *SmCYP98A14* was the target to SmMYC2b, thereby regulated the phenolic acids biosynthesis, and there was an interaction between SmJAZ8 and SmMYC2a, and repressing expression of *SmMYC2a* in overexpressing *SmJAZ8* line (Pei et al. 2018). Also, Yang et al (2017) reported that introduction of SmMYC2 in transgenic *S.miltiorrhiza* plants increased the production of phenolic acids especially for Sal B content, furthermore, genes participating in the phenylpropanoid biosynthesis pathway were up-regulated.

R2R3-MYB transcription factors are the largest group of MYB transcription factor families playing important roles in various plant activities (Li and Lu, 2014). A new R2R3-MYB transcription factors *SmMYB36* were characterized. Overexpression of *SmMYB36* inhibited accumulation of phenolic acids and flavonoids by targeting with *C4H1*, *4CL2*, *HPPR1* as the targets. In addition, primary metabolism in *S. miltiorrhiza* hairy roots was also influenced which implied the multidimensional manipulating mechanism (Ding et al. 2017). It is generally supposed that R2R3-MYB may function together with bHLH and WD-repeat (WDR) transcription factors to regulate metabolite synthesis, for example, *SmPAP1*, a R2R3-type MYB transcription factor identified from white flowered *S. miltiorrhiza* Bge. f. alba has been reported to be able to interact with SmMYC2 and increase activities of *SmPAL* and *SmC4H* promoters. Similarly, a bHLH-binding motif was found existed in *SmMYB36*, an interaction was speculated to occur between them while need to be studied further (Ding et al. 2017). Transcriptional metabolism of phenolic acids in *S. miltiorrhiza* is exhibited here (**Figure 4**), for that *S. miltiorrhiza* is one model herbal plant with high value which provide extensive reference values for other medicinal plants. Nevertheless, crosstalk of multiple networks should be excavated deeper in the future.

4.5 Endophytic fungi

Plant endophytic fungi are an important and novel resource of natural bioactive compounds. Some endophytes can produce the same or similar bioactive

compounds as those originated from their host plants (Ming et al. 2012; Lou et al. 2013). Recent studies have been carried out to isolate endophytic fungi from *S. miltiorrhiza* to obtain the secondary metabolites production such as phenolic acids, which is an alternative method partly in place of *S. miltiorrhiza* plants.

A total of 14 species of endophytic fungi have been isolated from 216 tissue segments including roots, stems, leaves and flowers of *S. miltiorrhiza* Bge.f.alba, which is a white flowered species, and the antioxidant activity was measured by FRAP and DPPH method (Li et al. 2015). *F. proliferatum* SaR-2 among 14 tested strains exhibited higher yield of total phenolic acids and displayed a stronger antioxidant activity compared with others (Li et al. 2015). *Chaetomium globosum* D38, an endophytic fungus isolated from the *S. miltiorrhiza* roots, was an efficient inducer both live fungus and its mycelia extract to stimulate tanshinones production, as well as one high-quality biotic fertilizer which improved the contents of salvianolic acids in *S. miltiorrhiza* seedlings (Zhai et al. 2018). Up to now, only a small number of endophytic fungi species could produce phenolic acids but with low content, which was not suitable for industrial production on a large scale. Therefore, optimization of culture conditions should be conducted to enhance the production of phenolic acids.

5. Synthetic biology of phenolic acids

Traditionally, phenolic acids are mainly extracted and purified from roots of

S.miltiorrhiza while with low contents, for example, the salvianic acid A (SAA) accounted for less than 0.1% in whole *S. miltiorrhiza* plants (Lam et al. 2007) which have limited the medicinal or industrial use on a large scale. As the downstream biosynthetic pathways of some natural compounds are continuously excavated, engineering biosynthesis pathways in microorganism such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica* provide a valid platform for production of desired products (Harder et al. 2016; Cao et al. 2016). Research of the synthetic biology strategies on phenolic acids has become a hot topic. The L-tyrosine pathway furnished a variety of aromatic compounds to produce substrates for production of salvianic acids (Yao et al. 2013). For SAA biosynthesis, a *Lactobacillus pentosus D-LDH* gene and endogenous hydroxylase complex *HpaBC* from *E. coli* were integrated into the L-tyrosine biosynthetic pathway to catalyze 4-hydroxyphenylpyruvate (4HPP) to form SAA with the yield of 7.1 g/L (Yao et al. 2013). Generally, plasmid-mediated system needed more inducers and antibiotics to produce SAA, to make up for this deficiency, engineering *E. coli* chromosome by integration of biosynthetic genes produced 5.6 g/L of SAA after fed-batch fermentation for 60 h supplemented without antibiotics or inducers (Zhou et al. 2017). To obtain high concentration of rosmarinic acid (RA) in *E. coli*, an artificial pathway consisted of rosmarinic acid synthase (RAS), 4-coumarate: CoA ligase (4CL), 4-hydroxyphenyllactate 3-hydroxylase (HpaBC) and D-lactate dehydrogenase (LDH) coming from *Coleus blumei*, *Arabidopsis thaliana*, *E. coli* and *Lactobacillus pentosus* separately was introduced into *E. coli* strain

over-producing L-tyrosine (Jiang et al. 2016). The engineered *E. coli* strain yielded about 130 mg/L RA together with 55 mg/L caffeoyl-phenyllactate, which provided a new insight into RA biosynthesis in *E. coli* (Jiang et al. 2016). Furthermore, the above constructed *E. coli* strain containing *At4CL* and *CbRAS* was supplied with a series of acceptor or donor substrates to produce 18 RA analogues (Zhuang et al. 2016). However, production of other phenolic acids such as caffeic acid and salvianolic acid B etc have not been tested yet.

6. Conclusions and prospects

S. miltiorrhiza is an important model herbal which has been widely used in treatment of cardiovascular and cerebrovascular diseases, as well as a healthy plant that could be used as tea etc. As one of the two main types of bioactive compounds in *S. miltiorrhiza*, phenolic acids exhibit good curative effect and pharmacological activities with increasing market demands. Due to low content in mother plants, a series of biotechnological approaches such as elicitation, hairy root culture, endophytic fungi, genetic engineering, transcription regulation and synthetic biology, have been carried out to expendably enhance phenolic acids production (**Figure 5**).

Manipulating known key enzyme genes including engineering one single gene or combination of several genes in transformed hairy root culture system has been proved to be a feasible way for phenolic acids accumulation. It is noteworthy that the biosynthesis pathway of phenolic acids remained not fully clear, and

biosynthesis mechanism regulated by transcription factors is still limited. Currently, only several MYB, bHLH and JAZ etc transcription factors or regulators have been studied. With the information of whole genome of *S. miltiorrhiza*, more functional genes, gene family or transcription regulators will be identified and isolated by combination of various analytical tools, maybe "transcription factor-to-biosynthetic gene-to-metabolite network" based on the available information will help to rationally design better strategies for further improvement of phenolic acids production.

Engineering microorganism such as *E.coli* has been verified to be a practicable approach for production of targeted bioactive products on a large scale. At present, salvianic acid A and rosmarinic acid have been successfully produced in *E.coli* which is suitable for fermentation, more factors affecting fermentation need to be examined in order to produce them in a large scale. Moreover, other excellent microorganisms could be developed and reconstruction of biosynthesis pathways for different phenolic acids such as caffeic acid and salvianolic acid B need to study further.

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Figure 1 Various organs of two-year-old *S. miltiorrhiza* grown in the experimental field and related medicines or health products. (A) Aerial part *S. miltiorrhiza* plants; (B) Roots and rhizome; (C) Flowers and leaves; (D) Compound Danshen Dripping Pills; (E) Danshen Cha (Tea)

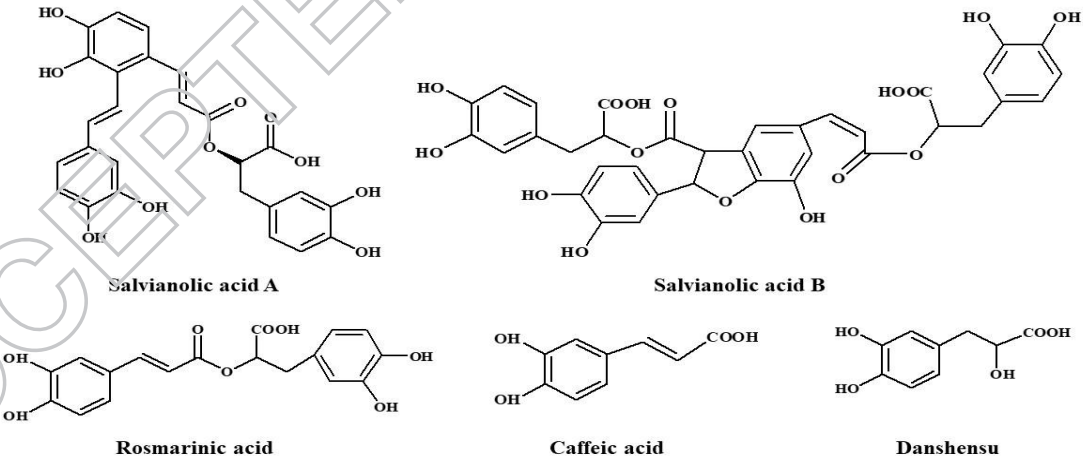

Figure 2 Chemical structure of several phenolic acids in *S. miltiorrhiza*

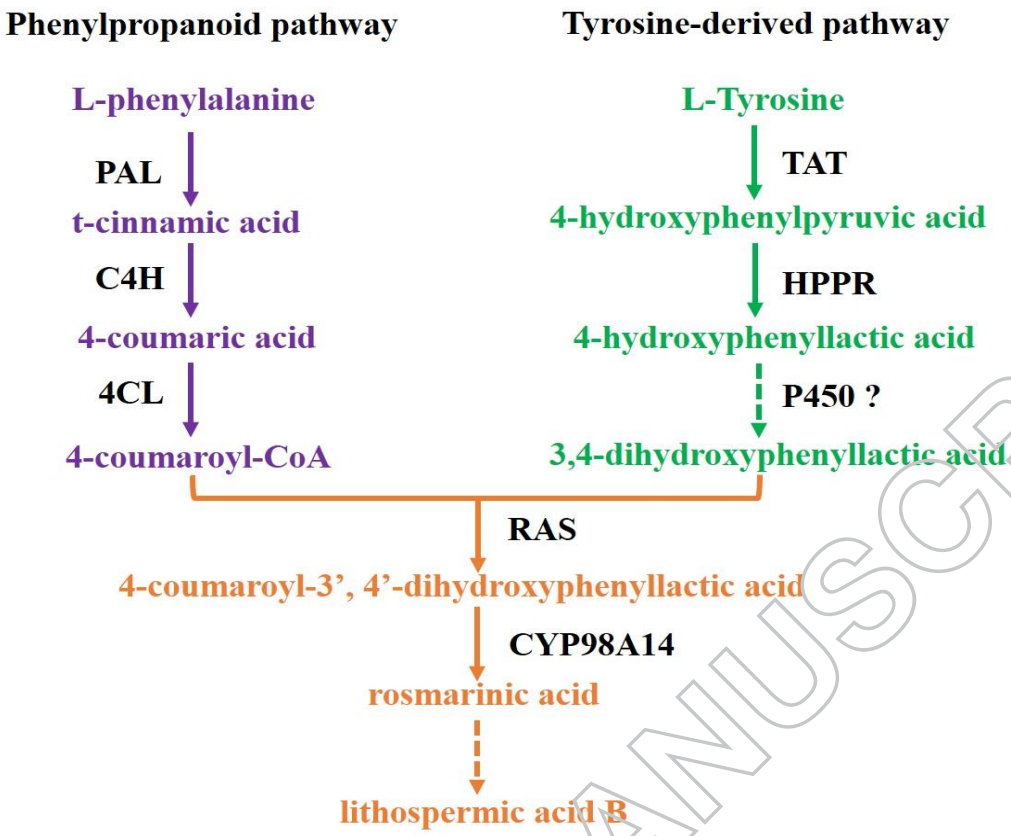

Figure 3 The proposed phenolic acids biosynthesis pathway in *S. miltiorrhiza*. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; TAT, tyrosine amino transferase; HPPR, 4-hydroxyphenylpyruvate reductase; RAS, rosmarinic acid synthase; CYP98A14, a cytochrome P450-dependent monooxygenase

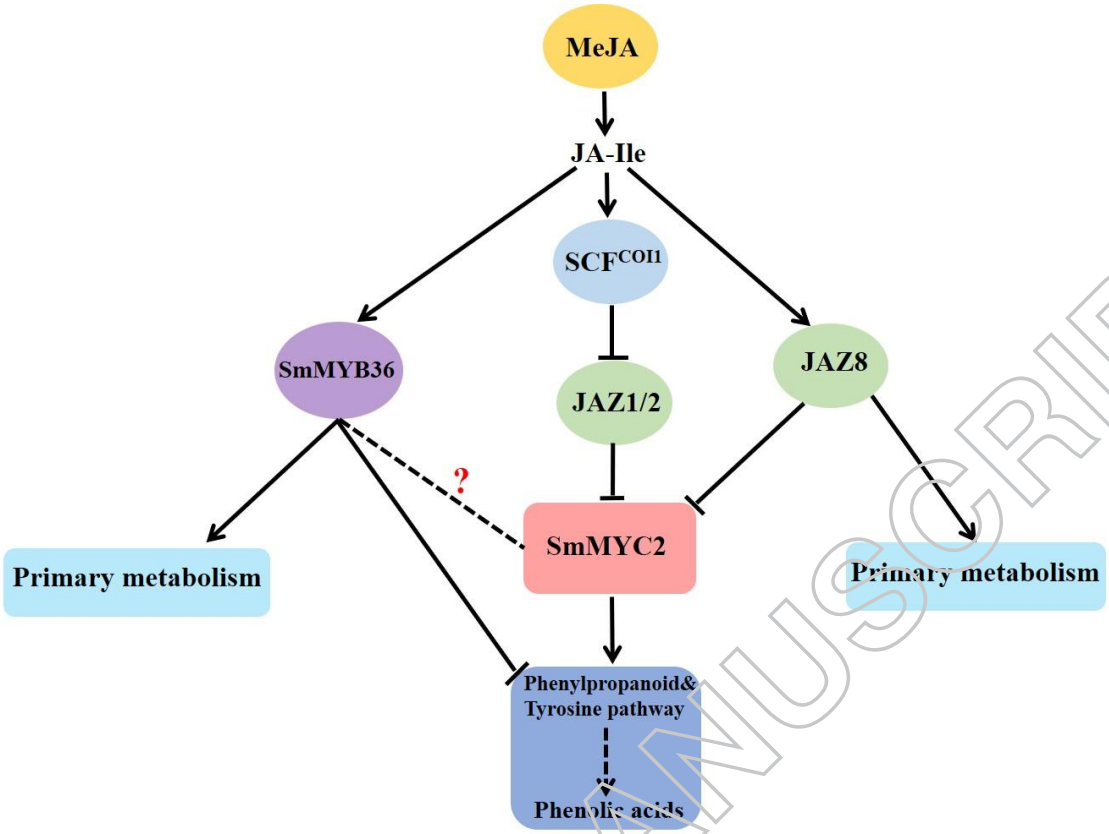

Figure 4 Transcription mechanisms of phenolic acids in *S. miltiorrhiza* reported recently

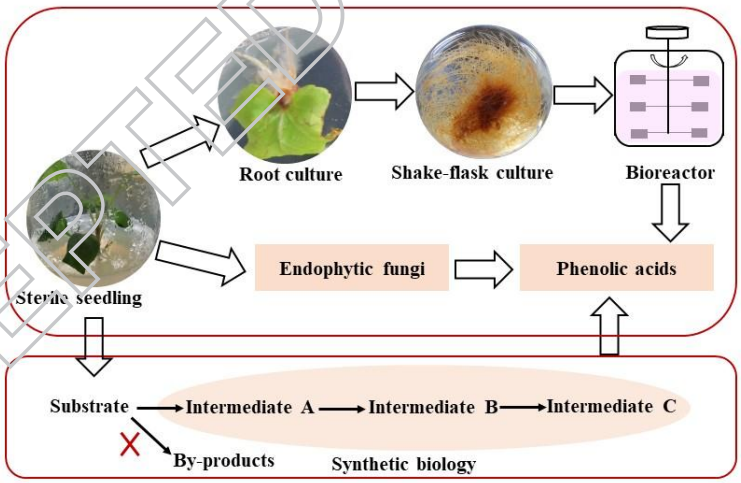

Figure 5 Illustration of biotechnologies approaches for phenolic acids production

Table 1 Pharmacological effects of salvianolic acid B

Phenolic acid	Bioactivities	Targets	Reference
Sal B	Anti-oxidant	Schwann cells	Sun et al. 2012
		human aortic smooth muscle cells	Zhang and Wang, 2006
		renal ischemic reperfusion injury	Ma et al. 2017
	Anti-inflammatory	high-fat diet (HFD)-induced inflammation	Wang et al. 2017
		alcoholic liver injury	Zhang et al. 2017
	Anti-cancer	human glioma U87 cells	Wang et al. 2013
		oral squamous cell carcinoma	Zhou et al. 2006
		acute lymphoblastic leukemia	Wu et al. 2016
		breast cancer	Ding et al. 2016
		head and neck cancer	Li et al. 2016
		colorectal cancer	Jing et al. 2016
	Anti-bacteria	*N.meningitidis*	Huttunen et al. 2016
	Anti-virus	Enterovirus 71	Chung et al. 2015
	Anti-fibrosis	oral submucous fibrosis	Dai et al. 2015
		pulmonary fibrosis	Liu et al. 2015

Table 2 Various culture systems of *S. miltiorrhiza* for production of phenolic acids

Culture type	Related genes or strains	Sal B	RA	Sal C	Reference
Suspension cells		85.0 mg/L	481.5 mg/L		Chen et al. 1999b
		Detectable	Detectable		Huang et al. 2000
Callus (stem)		0.87±0.20%	1.27±0.38%		Wu et al. 2016
Callus (leaf)		0.07±0.03%	0.28±0.02%		
		Detectable	Detectable		Tan et al. 2014
	SmC4H	584mg/L	201mg/L		

Hairy root	*SmHPPR*	669mg/L	616 mg/L		Xiao et al. 2011
	SmTAT-SmHPPR	992 mg/L	906 mg/L		
	SmHPPD	334 mg/L	542 mg/L		
	SmAOC	19.0 mg/g DW	2.8 mg/g DW		Gu et al. 2012
	SmSnRK2.6	Increased	Increased		Jia et al. 2017
	SmAREB1	Increased	Increased		
	Maize C1	Total phenolics (Down-regulated)			Zhao et al. 2015
Endophytic fungi	*F. proliferatum SaR-2*	Total phenolics (21.75 mg/g)			Li et al. 2015
	P.glomerata D14			Detectable	Li et al. 2016

Table 3 Elicitors applied for stimulation of phenolic acids in *S.miltiorrhiza* hairy roots or cell cultures

Elicitors	Concentration	Culture system	Treatment time	Phenolic acids	Contents or increased fold	References
Ag^+	15 μM	Hairy roots	6 d	RA	15.27 mg/g DW	Xing et al. 2014
				CA	0.37 mg/g DW	
				ferulic acid	0.11 mg/g DW	
Ag^+	15 μM	Hairy roots	18 d	LAB	188 mg/g DW	Xiao et al. 2010
YE	200 mg/L	Hairy roots	4 d	RA	43.8 mg/g DW	Yan et al. 2006
SA	6.25 mg/L	Cell culture	8 h	CA, SalB	~2 folds	Dong et al. 2010
H_2O_2		SA-elicited cells	2 d	SalB	9.37 folds	Guo et al. 2014
NO					9.39 folds	
Putrescine	50 mg/L	*S.miltiorrhiza* Bge.f.alba hairy	12 d	SalA, SalB	3.95, 12.13 mg/g DW	Hao et al. 2012
Spermidine	50 mg/L		12 d	SalA, SalB	4.01, 11.34 mg/g DW	

Putrescine+ Spermidine	50 mg/L+50 mg/L	roots	10, 12d	SalA (10 d), SalB (12 d)	4.21, 17.11 mg/g DW	
Endophytic bacteria	0.025%	Hairy roots	9 d	RA, SalB	significant decrease	Yan et al. 2014
Tween 20	1%	Hairy roots	1 d	Total phenolics	60-70% release	Siu and Wu, 2014
Triton X-100	1%		6 d			
MJ	0.1 mM	Hairy roots	6 d	RA	60.2 mg/g DW	Xiao et al. 2009
				LAB	192 mg/g DW	
ABA	50 μM	Hairy roots	6 d	CA, RA, SAB	1.96, 7.45, 26.3 mg/g	Liang et al. 2013
GA	100 μM			RA, SAB	~1 and 3 folds	
Ethylene	50 μM			CA, RA, SAB	0.90, 23.12, 25.13 mg/g	
ABA+GA	50 μM+100 μM			CA	Obviously increased	
ABA+Ethylene	50 μM+50 μM			CA, RA, SAB	9.2 folds, 13.4 mg/g, ~2 folds	
GA+Ethylene	100 μM+50 μM			RA, SAB	improved largely	