Biochemistry, Biosynthesis, and Medicinal Properties of Phenolic Acids in *Salvia miltiorrhiza*

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Abstract

As one group of important bioactive compounds in *Salvia miltiorrhiza*, water-soluble phenolic acids own a variety of bioactivities including anti-oxidation, anti-inflammatory, and anti-cancer. Due to the degradation of genetic resources and low content of phenolic acids in traditionally cultured *S. miltiorrhiza*, limited phenolic acid production cannot meet the increasing market demand. It is extremely important to use modern biotechnology methods to increase the yield of phenolic acids. Here, we summarize pharmacological activities of phenolic acids in *S. miltiorrhiza*, as well as various biological methods including culturing hairy roots, callus, suspension cells, and endophytic fungi for producing phenolic acids and using elicitors treatment, metabolic engineering and transcriptional regulation for increasing the production of phenolic acids.

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11.1 Introduction

Phenolic acids possessing various pharmacological activities such as anti-cancer, anti-oxidant, anti-bacterial, and anti-inflammatory activities are widely distributed in nature, especially in some commonly used traditional Chinese medicines such as *Salvia miltiorrhiza* (Zhou et al. 2011, 2012). *S. miltiorrhiza* has the functions of relieving pain, promoting blood circulation, regulating menstruation, and nourishing the heart so that it is widely used in the treatment of cardiovascular diseases (Wang and Cao 2016). There are more than 20 phenolic acids in *S. miltiorrhiza* including rosmarinic acid (RA), salvianolic acid B (SAB), salvianolic acid A (SAA), danshensu (DSU), caffeic acid, cinnamic acid, ferulic acid, and lithospermic acid (Fig. 11.1) (Xing et al. 2018a, b). SAB and RA in crude *S. miltiorrhiza* account for the largest content (Sun et al. 2016).

In recent years, RA, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, has been proved to be used for prophylaxis and treatment of neuropathic pain for its anti-apoptotic and

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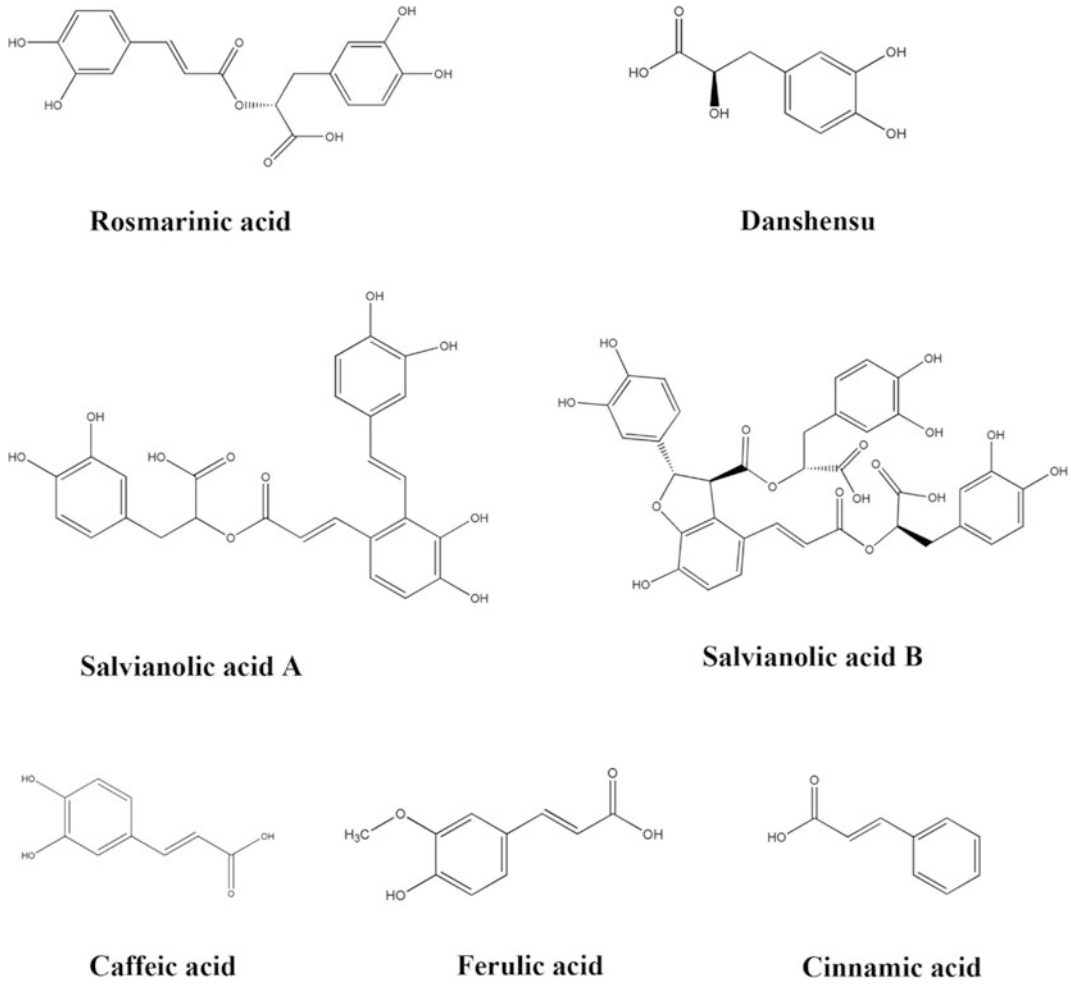

Fig. 11.1 Chemical structure of main phenolic acids including rosmarinic acid (RA), danshensu (DSU), salvianolic acid A (SAA), salvianolic acid B (SAB), caffeic acid, ferulic acid, and cinnamic acid in *S. miltiorrhiza*

anti-inflammatory effects (Pezeshki and Petersen 2018; Rahbardar et al. 2018). SAB and DSU isolated from *S. miltiorrhiza* are extremely effective anti-oxidants and have stronger oxygen free radical scavenging activity than vitamin C. Moreover, SAB shows better anti-oxidant activity than DSU (Zhao et al. 2008). It has been reported that SAB has a protective effect on emphysema-like lung cell death and protect against ischemia/reperfusion-induced cerebral injury (Dhapare and Sakagami 2018; Fan et al. 2018). SAB can effectively inhibit the growth of cultured MDA-MB-231 cells and tumor xenografts via a ceramide-mediated pathway. SAB also enhances apoptosis by regulating ceramide glycosylase and reduces TNBC cell proliferation (Sha et al. 2018). SAA possesses extensive pharmacological activities like treating liver disease, which prevents chronic ethanol-induced liver damage via SIRT1-mediated autophagosome–lysosome fusion recovery (Shi et al. 2018).

With the rapid development of biotechnology, the biosynthetic pathway of phenolic acids in *S. miltiorrhiza* has been gradually revealed (Petersen and Simmonds 2003; Di et al. 2013; Ma et al. 2015). Due to its wide medicinal value, the depletion of wild resources and the low yield of phenolic acid, genetic engineering and metabolic

engineering have been used to enhance the production of phenolic acids in *S. miltiorrhiza*, which has become a research hotspot. Here we summarize the research progress of the main substances, pharmacological activities, biosynthetic pathways, and in vitro synthesis of phenolic acids in *S. miltiorrhiza*, in order to more efficiently produce pharmacological phenolic acids and discuss their prospects.

11.2 Medicinal Properties of Phenolic Acids

Phenolic acids are the major active components in *S. miltiorrhiza*, which exerts a variety of pharmacological activities including anti-oxidant, cardio-protective, neuro-protective, anti-platelet, anti-cancer, anti-inflammatory, reno-protective, anti-diabetic properties (summarized in Fig. 11.2). The pharmacological activities of SAA and SAB are listed in Table 11.1.

11.2.1 Anti-oxidant Activity

Phenolic acids have been found to possess potent anti-oxidative ability due to their polyphenolic structure. SAA could enhance the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase in an established 5/6 nephrectomized (5/6Nx) rat model, and decreased reactive oxygen species (ROS) in H_2O_2-induced HK-2 cells. The protective effects of SAA on oxidative stress were suggested to be related to the modulation of Akt/GSK-3β/Nrf2 and the NF-κB signaling pathway (Zhang et al. 2019a). In addition, SAA decreased malondialdehyde (MDA) but increased SOD level in angiotensin II-incubated macrophages (Li et al. 2016a). SAB protected against subarachnoid hemorrhage-induced oxidative damage in vivo through activating the SIRT1 and Nrf2 signaling pathway (Zhang et al. 2018a). The levels of antioxidase SOD and GPx were decreased in patients with cysteine stone, which were associated with oxidative stress. Inversely, SAB could

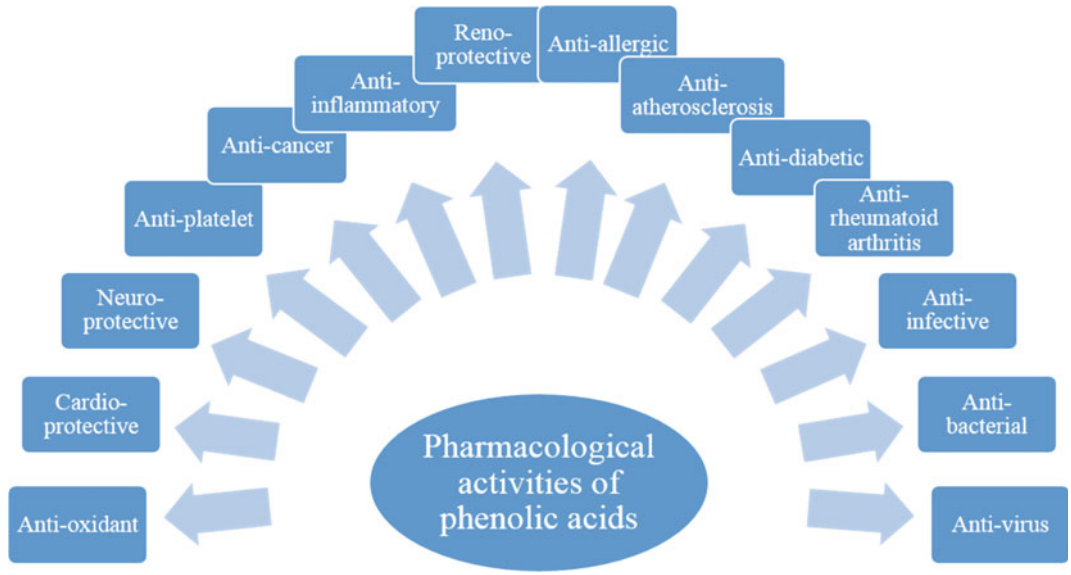

Fig. 11.2 Pharmacological activities of phenolic acids in *S. miltiorrhiza*

Table 11.1 Pharmaceutical effects of SAA and SAB

Phenolic acids	Bioactivities	Targets	References
SAA	Anti-oxidant	5/6 nephrectomized rat	Zhang et al. (2019a, b)
		Angiotensin II-induced murine peritoneal macrophages	Li et al. (2016a)
	Cardio-protective	Myocardial ischemia/reperfusion injury	Qian et al. (2019)
		Arsenic trioxide-induced cardiac injury	Zhang et al. (2018b, c)
	Neuro-protective	Spinal cord injury rats	Yu et al. (2017)
		Middle cerebral artery occlusion mice	Mahmood et al. (2017)
		ROS-induced neuronal damage	Zhang et al. (2012)
	Anti-platelet	Human platelet and $LDLR^{-/-}$ mice	Huang et al. (2010)
		Rat and human platelet, arterio-venous shunt thrombosis rats	Fan et al. (2010)
	Anti-cancer	Acute myeloid leukemia	Pei et al. (2018)
		Oral squamous cell cancer	Fang et al. (2018)
		Lung cancer	Bi et al. (2013), Tang et al. (2017)
		Angiogenesis	Yang et al. (2019)
		Breast cancer	Wang et al. (2015a, b), Cai et al. (2014), Zheng et al. (2015)
	Anti-inflammatory	Ischemia reperfusion-induced rat brain damage	Zhang et al. (2018d)
		LPS-induced macrophages	Liu et al. (2018)
	Reno-protective	Doxorubicin-induced nephropathy	Fan et al. (2015)
		5/6 nephrectomized rats	Zhang et al. (2018e)
		Diabetic nephropathy	Hou et al. (2017)
		Minimal change disease rats	Wang et al. (2019)
	Anti-allergic	Ovalbumin-induced allergic asthma mice	Heo and Im (2019)
	Anti-atherosclerosis	Macrophages and ApoE KO mice	Zhang et al. (2014c)
	Anti-diabetic	Alloxan-induced type 1 diabetic mice and streptozotocin-induced type 2 diabetic rats	Qiang et al. (2015)
SAB	Anti-oxidant	Subarachnoid hemorrhage rats	Zhang et al. (2018a)
		HK-2 cells, *Slc7a9* knockout mice	Zhang et al. (2019b)
		γ-irradiation-radiated mice	Zhou et al. (2019)
		HO^-, O_2^-, DPPH and ABTS radicals	Zhao et al. (2008)
	Cardio-protective	Doxorubicin-induced cardiac injury	Chen et al. (2017)
	Neuro-protective	Subarachnoid hemorrhage rat	Zhang et al. (2018a)
		Aβ25-35 peptide-induced Alzheimer's disease mice	Lee et al. (2013)
		Cerebral small vessel disease rat	Wang and Hu (2018)
		Stroke rat	Lv et al. (2015)
		Traumatic brain-injured mice	Chen et al. (2011)
		Parkinson's disease	Zhou et al. (2014)

(continued)

Table 11.1 (continued)

Phenolic acids	Bioactivities	Targets	References
	Anti-platelet	Human platelet	Liu et al. (2014)
		Rat mesentery	Wang et al. (2009)
	Anti-cancer	Colon cancer	Guo et al. (2018), Jing et al. (2016)
		Breast cancer	Sha et al. (2018)
		Hepatocellular cancer	Gong et al. (2016)
	Anti-inflammatory	Rheumatoid arthritis rats	Xia et al. (2018)
		LPS-induced cell injury	Meng et al. (2019)
		Liver injury	Zhao et al. (2019)
	Reno-protective	Ischemic reperfusion-induced renal injury	Ma et al. (2017)
	Anti-atherosclerosis	Macrophages and ApoE KO mice	Bao et al. (2012)
	Anti-diabetic	Streptozotocin-induced diabetic rats	Raoufi et al. (2015)
	Anti-rheumatoid arthritis	Collagen-induced rheumatoid arthritis rat	Xia et al. (2018)
	Anti-infective	*Neisseria meningitidis*	Huttunen et al. (2016)
	Anti-bacterial	*Pseudomonas aeruginosa*	Kong et al. (2017)

prevent cysteine stone formation, protect against oxidative injury (Zhang et al. 2019b). Moreover, SAB and DSU showed potent scavenging activities against HO^{-}, O^{2-}, DPPH, and ABTS radicals than vitamin C (Zhao et al. 2008). SAB also protected the mice from radiation injury through nuclear factor (erythroid-derived 2)-like 2 protein/BTB-mediated anti-oxidant effect (Zhou et al. 2019).

11.2.2 Cardio-protective Activity

Myocardial reperfusion during infarction causes damage in cardiomyocytes. Previous reports indicated that total salvianolic acid injection (TSI) of *S. miltiorrhiza* improved ischemia/reperfusion (I/R)-induced myocardial injury, decreased apoptosis, and reduced infarct size in Sprague-Dawley rats (Huang et al. 2019). Recent studies demonstrated that SAA could attenuate apoptosis and prevent I/R injury in cardiomyocytes through the PI3K/Akt, GSK-3β, JNK, and ERK1/2 pathways, and probably via the JNK-ERK1/2 crosstalk (Qian et al. 2019). Mitochondrial dysfunction contributes to the heart diseases such as coronary heart disease and heart failure. However, pretreatment with SAA could maintain normal mitochondrial function and biogenesis, alleviate the damage, and protect against arsenic trioxide-induced cardiac injury in vivo (Zhang et al. 2018c). Furthermore, treatment with SAB showed protective effect against doxorubicin-induced cardiac injury, which is a common clinical syndrome that causes severe pain to cancer patients (Chen et al. 2017).

11.2.3 Neuro-protective Activity

Previously, the TSI has been approved by Chinese State Food and Drug Administration (SFDA) for the treatment of ischemic stroke (Han et al. 2017). Besides, SAA was confirmed to recover the neurological function, improve the motor ability, and inhibit apoptosis-related proteins in spinal cord injury rats (Yu et al. 2017). In another middle cerebral artery occlusion mice

model, SAA administration ameliorated neuronal damage and decreased infarcted volume via the inhibition of eNOS uncoupling and peroxynitrite formation (Mahmood et al. 2017). Elevated ROS level involves in stroke and other neurodegenerative diseases. However, pretreatment with SAA increased cell survival against ROS-induced neuronal damage (Zhang et al. 2012). Treatment with SAB inhibited oxidative damage, prevented neurologic impairment, and improved cell viability in a rat subarachnoid hemorrhage model and cultured neurons, which was associated with the activation of Nrf2 and SIRT1 signaling pathway (Zhang et al. 2018e). Subchronic SAB administration (10 mg/kg) ameliorated the memory impairment by decreasing the expression of nitric oxide synthase and cyclooxygenase-2 in Aβ25-35 peptide-induced Alzheimer's disease mouse (Lee et al. 2013). In addition, SAB also affected vasculature and cognitive function. Studies revealed that SAB could recover the angiogenesis and cognitive deficits in cerebral small vessel disease rat though modulation of STAT3/VEGF pathway (Wang and Hu 2018). Other studies have indicated that SAB has beneficial effects on stroke, brain injury, and Parkinson's disease (Lv et al. 2015; Chen et al. 2011; Zhou et al. 2014).

11.2.4 Anti-platelet Activity

Salvianolate, the bioactive constituents of *S. miltiorrhiza*, has been approved by Chinese SFDA for the treatment of coronary artery disease since 2005. Clinical research found salvianolate enhanced the anti-platelet activity of standard aspirin plus clopidogrel therapy in acute coronary syndrome patients. Further in vitro studies demonstrated that SAB, the main component of salvianolate (>85%), suppressed thrombin, arachidonic acid, collagen, and U46619-induced platelet aggregation via inhibiting phosphodiesterase and antagonizing P2Y12 receptor (Liu et al. 2014). The beneficial effect of SAB on thrombosis was related to direct peroxide scavenge or indirect adhesion molecules suppression (Wang et al. 2009). SAA inhibited platelet aggregation and attenuates arterial thrombus formation by inhibiting the PI3K expression (Huang et al. 2010). Additionally, intravenous administration of SAA (2.5–10 mg/kg) showed anti-thrombotic activity in vivo. It modulated the hemorheology without influence on the coagulation function and was presumed to be related to the cAMP induction (Fan et al. 2010).

11.2.5 Anti-cancer Activity

SAA exhibited anti-cancer activities against various carcinomas such as acute myeloid leukemia, oral squamous cell, and lung cancer (Pei et al. 2018; Fang et al. 2018; Bi et al. 2013). Moreover, SAA inhibited GRP78 secretion and angiogenesis in tumor microenvironment (Yang et al. 2019). Chemotherapy resistance is a major challenge in cancer treatment. Studies revealed that SAA enhanced the efficacy of cisplatin in lung cancer A549 cells by inhibiting the AKT/mTOR signaling pathway (Tang et al. 2017). Besides, SAA treatment selectively attenuated the growth of multidrug-resistant MCF-7 breast cancer cells, which was correlated with ROS production (Wang et al. 2015b). Overexpression of transgelin 2 increased the resistance of cancer cells to paclitaxel therapy. However, SAA treatment could reverse resistance, induce apoptosis, and inhibit invasion in paclitaxel-resistant breast cancer cells (Cai et al. 2014; Zheng et al. 2015). It was indicated that SAB could reverse the multidrug resistance in colon cancer cells and promote apoptosis in triple-negative breast cancer (Sha et al. 2018; Guo et al. 2018). Autophagy functions as a death executioner that induces autophagic cell death. SAB was demonstrated to be a novel autophagy inducer that mediated colorectal cancer cells autophagy through modulation of the AKT/mTOR signaling pathway (Jing et al. 2016). Evidence also showed that autophagy as well as apoptosis was involved in SAB-induced hepatocellular carcinoma cell death. While pretreatment with autophagy inhibitors attenuates the effects induced by SAB (Gong et al. 2016).

11.2.6 Anti-inflammatory Activity

SAB decreased the levels of inflammatory cytokines (IL-1β, IL-6, and TNF-α) and increased anti-oxidant enzyme (SOD, CAT, and GSH) activities in collagen-induced rheumatoid arthritis rat (Xia et al. 2018). Treatment with SAB markedly ameliorated LPS-induced injury on MH7 A cells via the modulation of NF-κB and JNK pathways, suggesting the potential anti-inflammatory capacity (Meng et al. 2019). Also, SAB decreased the level of pro-inflammatory cytokines in liver injury (Zhao et al. 2019). SAA protected blood-brain barrier by reducing inflammation response and NF-κB inactivation (Zhang et al. 2018d). Liu and colleagues demonstrated that all the phenolic acids isolated from *S. miltiorrhiza* exhibited anti-inflammatory activity in LPS-stimulated THP-1 cells, among which the inhibitory effect of lithospermic acid was the strongest and similar to that of SAB (Liu et al. 2018).

11.2.7 Reno-protective Activity

Nephrotic syndrome is a common nephrology disorder accompanied by heavy proteinuria, hypoalbuminemia, and hyperlipidaemia. Recent studies demonstrated that SAA administration ameliorated histological damages, podocyte injury, and improved hemorheology in doxorubicin-induced nephropathy (Fan et al. 2015). Intraperitoneal with SAA at 10 mg/kg per day relieved urinary proteins and TNF-α level, alleviated pathological lesions in the kidney of 5/6Nx rats (Zhang et al. 2018e). SAA could also protect against early stage diabetic nephropathy, restored glomerular endothelial permeability through suppressing AGE-RAGE pathway (Hou et al. 2017). Furthermore, combination of SAA with prednisone relieved urinary proteins, improved renal function indices including blood urea nitrogen and serum creatinine level in rats (Wang et al. 2019). SAB showed reno-protective activity in a renal I/R rat model by attenuating inflammatory process and oxidative stress through activating the PI3K/Akt signaling pathway (Ma et al. 2017).

11.2.8 Anti-atherosclerosis Activity

SAB acted as a CD36 antagonist that inhibited lipid uptake in macrophages, leading to the inhibition of atherosclerotic lesions formation in ApoE knockout mice (Bao et al. 2012). SAA attenuated TNF-α-induced CC chemokine ligand-20 (CCL-20) secretion, which plays a crucial role in atherogenesis (Zhang et al. 2014c). In addition, DSU prevented atherosclerosis through inhibiting the expression of adhesion molecules in arterial endothelia (Yang et al. 2010).

11.2.9 Anti-diabetic Activity

Diabetic rats treated with SAB (40 mg/kg) for three weeks showed decreased serum glucose and MDA levels as well as increased serum insulin level. Meanwhile, SAB could protect the pancreatic islet cells against cytotoxicity partly through the inhibition of apoptosis and oxidative stress (Raoufi et al. 2015). In alloxan-induced type 1 and high-fat diet with low-dose streptozotocin-induced type 2 diabetic animal models, SAA administration improved mitochondrial function in liver and skeletal muscle, increased ATP production through activating AMPK/CaMKKβ signaling pathway (Qiang et al. 2015).

11.2.10 Other Activities

SAA was potential anti-allergic therapy that reduced the number of eosinophils and secretion of inflammatory IL-4 and IL-13 in the lung tissue of ovalbumin-induced allergic asthma mice (Heo and Im 2019). SAB showed anti-rheumatoid arthritis activity on collagen-induced rat model (Xia et al. 2018). In addition, SAB exhibited anti-infective property against human pathogen *Neisseria meningitidis* by inhibiting

meningococcal binding (Huttunen et al. 2016). Protocatechualdehyde and SAB were major components in *S. miltiorrhiza* that possess anti-bacterial properties (Kong et al. 2017). Besides, the combination of salvianolic acid V, a new salvianolic acid, with Colistin sulfate or Levofloxacin showed effects on MRSA or *Acinetobacter baumannii* (Zhang et al. 2018b). Protocatechuic aldehyde markedly inhibited hepatitis B virus replication in vitro and reduced viremia in virus-infected ducks (Zhou et al. 2007).

11.3 Biosynthesis of Phenolic Acids in *S. miltiorrhiza*

11.3.1 Biosynthetic Pathway of Phenolic Acids

Phenolic acids are biosynthesized via two pathways including the phenylpropanoid pathway and the tyrosine-derived pathway in *S. miltiorrhiza* (Xiong et al. 2010; Ma et al. 2015). In the phenylpropanoid pathway, L-phenylalanine produces 4-coumaroyl-CoA under the catalysis of phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), hydroxycinnamate coenzyme A ligase (4CL) (Di et al. 2013; Shi et al. 2019). In tyrosine-derived pathway, L-tyrosine produces 4-hydroxyphenylpyruvic acid under oxidative deamination of tyrosine aminotransferase (TAT), following 4-hydroxyphenylpyruvate reductase (HPPR) and forming 3,4-dihydroxy phenyllactic acid at last (Di et al. 2013; Shi et al. 2019).

Finally, 3,4-dihydroxyphenyllactic acid and 4-coumaroyl-CoA are catalyzed by the RAS to form the precursor substance 4-coumaroyl-3′,4′-dihydroxyphenyllactic acid (4C-DHPL), and finally, the CYP98A14 enzyme synthesizes salvianolic acid (Di et al. 2013). In *S. miltiorrhiza*, 4C-DHPL is regarded as the major intermediate for RA biosynthesis involved in this pathway (Fig. 11.3). Meanwhile, RA is also considered to be a precursor of biosynthesis of SAB which is a structural dimer of RA, but the enzyme involved in catalysis is still unclear (Zhang et al. 2014a, b; Ma et al. 2015).

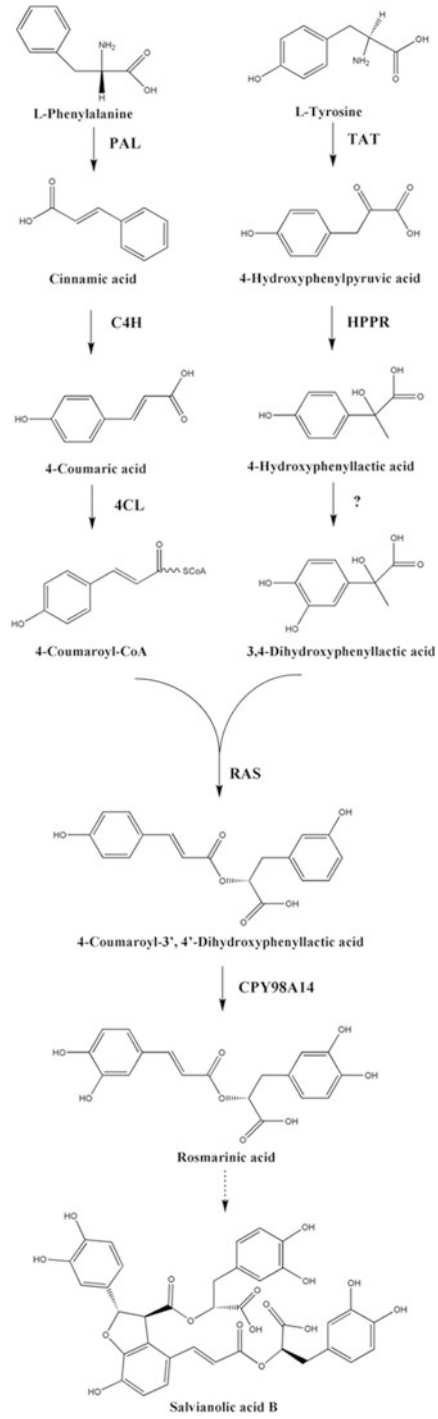

Fig. 11.3 Putative phenolic acid synthesis pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; TAT, tyrosine aminotransferase; HPPR, 4-hydroxyphenylpyruvate reductase; RAS, rosmarinic acid synthase; CYP98A14, a cytochrome P450-dependent monooxygenase

11.3.2 Related Genes Involved in Phenolic Acid Biosynthesis

With the extensive study of secondary metabolic pathways in *S. miltiorrhiza*, several key enzyme genes involved in these two pathways of phenolic acids biosynthesis have been identified, including *SmPAL*, *SmC4H*, *Sm4CL*, *SmTAT*, *SmHPPR*, *SmRAS,* and *CYP98A14* (Table 11.2; Fig. 11.4) (Ma et al. 2015; Shi et al. 2019).

11.3.2.1 Genes in the Phenylpropanoid Pathway

Phenylalanine ammonia-lyase (PAL), the first point enzyme to catalyze in the phenylpropanoid pathway, plays a significant role in primary metabolism and secondary metabolism regulation. Three members in *SmPALs* have been revealed from *S. miltiorrhiza*, including *SmPAL1*, *SmPAL2*, and *SmPAL3*. Gene expression pattern shows that *SmPAL1* and *SmPAL3* highly express in both roots and leaves of

Table 11.2 Key enzymes of phenolic acids biosynthesis pathway

Key enzymes	Accession no.	Functions	Highest expressed tissues	References
SmPAL1	EF462460	Catalyzing the L-phenylalanine to produce trans-cinnamic acid	Root and leaf	Wang et al. (2009, 2015a), Hou et al. (2013)
SmPAL2	GQ249111		Stem and flower	Hou et al. (2013), Wang et al. (2015a), Ma et al. (2015)
SmPAL3	KF220569		Root and leaf	Hou et al. (2013), Wang et al. (2015a), Ma et al. (2015)
SmC4H1	EF377337 DQ355979	Catalyzing the hydroxylation of cinnamate to 4-coumarate	Root and stem	Wang et al. (2015a), Ma et al. (2015)
SmC4H2	KF220564		Root and stem	Wang et al. (2015a), Ma et al. (2015)
Sm4CL1	AY237163	Catalyzing a series of aromatic substrates to form their corresponding hydroxycinnamoyl-CoA esters	Leaf	Wang et al. (2015a), Ma et al. (2015)
Sm4CL2	AY237164		Root	Wang et al. (2015a), Ma et al. (2015)
Sm4CL3	KF220556		Root	Wang et al. (2015a), Ma et al. (2015)
Sm4CL-like1	KF220557		Root	Wang et al. (2015a), Ma et al. (2015)
Sm4CL-like2	KF220558			Wang et al. (2015a), Ma et al. (2015)
Sm4CL-like3	KF220559			Wang et al. (2015a), Ma et al. 2015
Sm4CL-like4	KF220560		Root	Wang et al. (2015a), Ma et al. 2015
Sm4CL-like5	KF220561			Wang et al. (2015a), Ma et al. (2015)
Sm4CL-like6	KF220562			Wang et al. (2015a), Ma et al. (2015)
Sm4CL-like7	KF220563			Wang et al. (2015a), Ma et al. (2015)

(continued)

Table 11.2 (continued)

Key enzymes	Accession no.	Functions	Highest expressed tissues	References
SmTAT1	DQ334606 EF192320	Catalyzing the transamination from L-tyrosine to 4-hydroxyphenylpyruvate	Stem	Wang et al. (2015a), Ma et al. (2015)
SmTAT2	KF220575		Flower	Wang et al. (2015a), Ma et al. (2015)
SmTAT3	KF220555		Root and stem	Wang et al. (2015a), Ma et al. (2015)
SmHPPR1	DQ099741 DQ266514 EF458148	Participate in the synthesis of 4-hydroxyphenyl lactic acid	Stem and flower	Wang et al. (2015a), Ma et al. (2015)
SmHPPR2	KF220565		Stem and leaf	Wang et al. (2015a), Ma et al. (2015)
SmHPPR3	KF220566		Stem	Wang et al. (2015a), Ma et al. (2015)
SmHPPR4	KF220567			Wang et al. (2015a), Ma et al. (2015)
SmRAS1	FJ906696	Catalyzing 4-coumaroyl-CoA and 3, 4-hydroxyphenyllactate to form an ester (4-coumaroyl-3′,4′-dihydroxyphenyllactic acid)	Root	Wang et al. (2015a), Ma et al. (2015), Di et al. (2013)
SmRAS1-like	GU647199		Root	Wang et al. (2015a), Ma et al. (2015), Song (2010)
SmHCT1	KF220570		Root	Wang et al. (2015a), Ma et al. 2015
SmHCT2	KF220571		Stem	Wang et al. (2015a), Ma et al. (2015)
SmHCT3	KF220572		Stem	Wang et al. (2015a), Ma et al. (2015)
SmHCT4	KF220573		Stem	Wang et al. (2015a), Ma et al. (2015)
SmHCT5	KF220574		Stem	Wang et al. (2015a), Ma et al. (2015)
SmCPY98A14	HQ316179	Catalyzing rosmarinic acid synthesis	Root	Wang et al. (2015a), Ma et al. (2015), Di et al. (2013)

S. miltiorrhiza, while *SmPAL2* is mainly expressed in stems and flowers. The full-length ORF of *SmPAL1* is 2827 bp, encoding a 711-amino-acid peptide. Meanwhile, SmPAL1 has been revealed to be induced by abscisic acid (ABA), wounding, polyethylene glycol (PEG), methyl jasmonate (MJ), salicylic acid (SA), Ca^{2+}, gibberellin (GA), and ethylene (Ma et al. 2015; Hou et al. 2013). *SmPAL2* contains 2127 bp ORF encoding a 683-amino-acid peptide, and *SmPAL3* has 2283 bp ORF encoding 760 amino acid, which are induced by PEG and MJ (Ma et al. 2015; Song and Wang 2009). Interestingly, all three *SmPALs* are regulated by MeJA, and the expression of *SmPAL*1 and *SmPAL3* is drastically raised at 6 h after MeJA treatment.

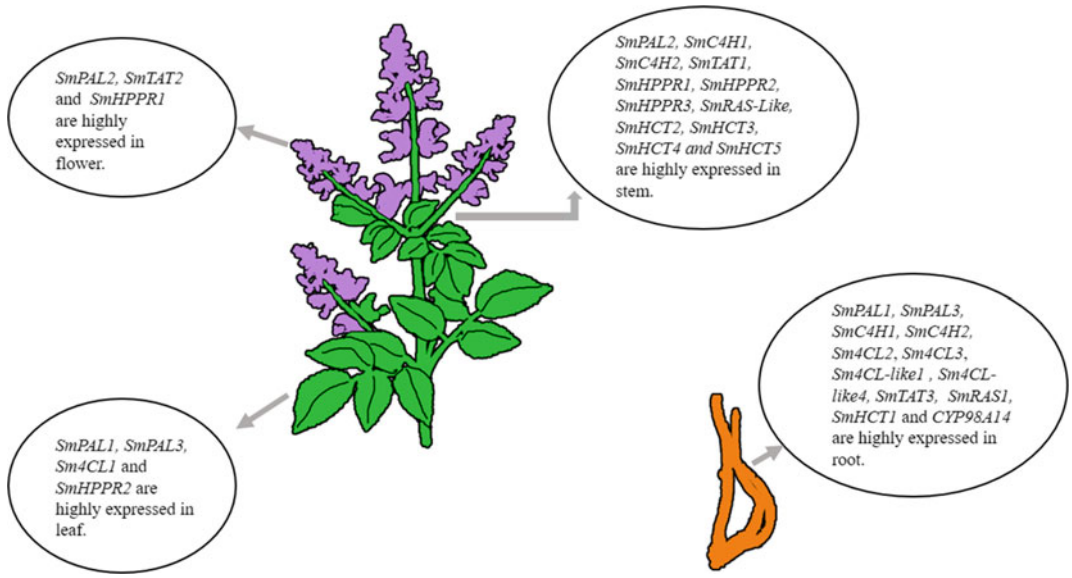

Fig. 11.4 Schematic diagram of key enzyme genes highly expressed in different tissues

Meanwhile, RNAi of *SmPAL1* has been found to cause a significant decrease in the content of RA and SAB in *S. miltiorrhiza*, indicating that *SmPAL1* plays a more significant role in phenylpropanoid pathway (Wang et al. 2015a; Song and Wang 2011).

Cinnamate 4-hydroxylase (C4H) is a cytochrome P450 enzyme involved in the catalysis of the production of *p*-coumaric acid by trans-cinnamic acid produced by PAL (Huang et al. 2008a). The full length of *SmC4H1* is 1512 bp encoding a 504-amino-acid protein. *SmC4H1* is highly expressed in roots and stems; meanwhile, it is induced by elicitor such as MJ, ABA, Ag^+, and UV-B radiation. *SmC4H2* encoding a 397-amino-acid protein is absent ER-targeting peptide, probably causing by N-terminal deletion of gene (Shi et al. 2019; Wang et al. 2015a, b). The tissue expression of *SmC4H2* is the same to *SmC4H1*, expressed highly in stem and root (Ma et al. 2015). However, *SmC4H2* does not seem to be involved in MJ regulation because of their insensitive to MJ, but its promoter contains some elements that respond to other stresses including fungal attack and salicylic acid (Wang et al. 2015a, b).

4-Coumarate: CoA ligase (4CL) catalyzing 4-coumaroyl acid to form 4-coumaroyl-CoA in phenylpropanoid pathway has ten members such as *Sm4CL1*, *Sm4CL2*, *Sm4CL3*, *Sm4CL-like 1*, *Sm4CL-like 2*, *Sm4CL-like 3*, *Sm4CL-like 4*, *Sm4CL-like 5*, *Sm4CL-like 6*, and *Sm4CL-like 7*. *Sm4CL1* has been found highly expressed in stem, low in leaves and rear in roots, while the expression levels of *Sm4CL2*, *Sm4CL3*, *Sm4CL-like1*, and *Sm4CL-like4* are high in roots. Furthermore, the expression of *Sm4CL1* is affected by elicitors like MJ and YE, and *Sm4CL2* is induced by MJ, YE and Ag^+, implying that *Sm4CL1* may be involved in the biosynthesis of phenolic acids in the stems and leaves, while *Sm4CL2* may participate in the biosynthesis of phenolic acids in roots of *S. miltiorrhiza* (Jin et al. 2012; Shi et al. 2019; Wang et al. 2015a; Zhao et al. 2006).

11.3.2.2 Genes in the Tyrosine-Derived Pathway

Tyrosine aminotransferase (TAT) and 4-hydroxyphenylpyruvate reductase (HPPR) are two main key enzymes which are involved in forming 3,4-dihydroxyphenyllactic acid (DHPL) in tyrosine-derived pathway. Tyrosine aminotransferase (TAT) is the first enzyme in the tyrosine-derived pathway of phenolic acids

biosynthesis. *SmTAT1* contains an ORF of 1233 bp encoding 411 amino acid (Huang et al. 2008b). The expression patterns show that *SmTAT1* is expressed higher in stem compared to root and leaf. Meanwhile, *SmTAT1* responds to MJ, ABA, SA, UV-B, GA, ethylene, Ag^+, YE (Xing et al. 2015; Yan et al. 2006; Liang et al. 2013). *SmTAT2* and *SmTAT3* are found to express in flower, stem, and root, respectively, also have been cloned from *S. miltiorrhiza*. *SmTAT1* and *SmTAT3* are grouped in the same branch in the phylogenetic tree analysis, but *SmTAT2* is divided into another branch, indicating that *SmTAT1* and *SmTAT3* may play a similar role in the phenolic acid synthesis pathway (Wang et al. 2015a).

4-Hydroxyphenylpyruvate reductase (HPPR) is the second enzyme in tyrosine-derived pathway, catalyzing 4-hydroxyphenylpyruvic acid to form 4-hydroxyphenyllactic acid. Three HPPRs are designed as *SmHPPR1*, *SmHPPR2*, and *SmHPPR3*. *SmHPPR1* and *SmHPPR2* encode 313 amino acid, and *SmHPPR3* encodes a 319-amino-acid protein. Promoter region of *SmHPPR1* expressed highest in stem which possesses many stress-responsive elements. At the same time, *SmHPPR1* has been found that it can be induced by MJ, SA, GA3, ABA, Ag^+, and UV-B radiation (Xing et al. 2015; Wang et al. 2015a; Xiao et al. 2011).

11.3.2.3 Genes Involved in Rosmarinic Acid (RA) Biosynthesis

Rosmarinic acid synthase (RAS) is regard as a rate-limiting enzyme catalyzing 4-hydroxyphenyllactic acid to form precursor 4-coumaroyl-3′,4′-dihydroxyphenyllactic acid (4C-DHPL). Seven *SmRASs* including *SmRAS1*, *SmRAS1-like,* and five *SmHCTs* have been revealed so far (Ma et al. 2015; Shi et al. 2019). *SmRAS1* containing 1284 bp ORF and encoding a 426-amino-acid polypeptide has been proved expressed predominantly in roots and stems, and induced by MJ, Ag^+ (Di et al. 2013; Ma et al. 2015). *SmRAS-like* is expressed higher in stem than other tissues and sensitive to *Pseudomonas lachrymans*, MJ, light, and SA (Song. 2010; Ma et al. 2015). *SmHCTs* include *SmHCT1*, *SmHCT2*, *SmHCT3*, *SmHCT4,* and *SmHCT5*, encoding 341, 425, 426, 439, 427 amino acid, respectively. *SmHCT2*, *SmHCT3*, *SmHCT4*, and *SmHCT5* are highly expressed in stem, while *SmHCT1* is highly expressed in root. Meanwhile, *SmHCT3*, *SmHCT4*, and *SmHCT5* were responsive to MJ elicitation (Wang et al. 2015a; Ma et al. 2015).

Cytochrome P450-dependent monooxygenase (CYP98A14) in *S. miltiorrhiza* participates the synthesis of RA by catalyzing 4C-DHPL. *SmCYP98A14* has 1525 bp ORF encoding a 508-amino-acid protein, and its expression is higher in roots than that in stems and leaves; moreover, *SmCYP98A14* is sensitive to MJ and Ag^+ (Di et al. 2013; Wang et al. 2015a; Ma et al. 2015).

11.4 Biotechnological Approaches to Improve the Production of Phenolic Acids

Due to the serious degradation of the quality of traditionally cultivated *S. miltiorrhiza*, slow growth cycle and low yield of active ingredients, the yield and quality of *S. miltiorrhiza* cannot meet the growing market demand. Phenolic acids are active ingredients with important economic and medicinal properties; its synthetic route has been basically clear, how to improve the amount of phenolic acid compounds in *S. miltiorrhiza* has become a research hotspot and difficulty. The application of plant biotechnology in improving biological activity and the required ingredients is more attractive and efficient than traditional methods. Here, we will introduce several methods which were reported to enhance the production of PAs including elicitors, metabolic engineering, and transcriptional regulation.

11.4.1 Elicitation Treatment to Increase the Production of Phenolic Acids

Elicitors are usually an agent that stimulates a plant defense response. Simulating biotic and abiotic stresses, elicitors classified into biotic elicitor and abiotic elicitor can stimulate plants and plant cultures to respond to them and lead to the accumulation of secondary metabolites in plants and plant cultures, which is important to produce valuable pharmaceutical ingredients (Wang and Wu 2013). Some elicitors have been utilized to enhance the production of secondary metabolites such as phenolic acids in the hairy roots or cell culture of *S. miltiorrhiza*, including methyl jasmonate (MeJA), yeast extract (YE), abscisic acid (ABA), silver ions (Ag^+), gibberellic acid (GA), salicylic acid (SA), polyamines, and ethylene. For example, treating with MeJA (0.1 mM), RA and lithospermic acid B accumulation were significantly increased. Meanwhile, several RA biosynthesis genes were induced by 0.1 mM MeJA, including phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase, tyrosine aminotransferase, 4-hydroxyphenylpyruvate reductase, and 4-hydroxyphenylpyruvate dioxygenase (Xiao et al. 2009). SA was utilized to treat suspension cultures of *S. miltiorrhiza*, which lead to a significant increase in the RA content. Furthermore, increase of the PAL activity was measured with SA treatment (Jiao et al. 2012). As the most studied heavy metal ion elicitor, low concentration of Ag^+ can stimulate 3-*O*-glucosylresveratrol production, which do not affect cell growth at the same time (Cai et al. 2013). With Ag^+ treatment, the total phenolic acids of *S. miltiorrhiza* hairy roots were increased and the tyrosine aminotransferase (TAT) activity showed a remarkably rise (Yan et al. 2006). It was found that accumulation of RA on day 6 after treatment of *S. miltiorrhiza* hairy roots with Ag^+ treatment was significantly increased to 1.3 times than that of the control and productions of caffeic acid and ferulic acid were also increased with Ag^+ treatment. However, productions of DSU, cinnamic acid, and lithospermic acid B (LAB) were significantly dropped by Ag^+ treatment. The results of qRT-PCR showed that expression of five key enzymes genes in RA biosynthesis pathways was significantly up-regulated by Ag^+ (Xing et al. 2015). YE was reported much more effective than Ag^+ to enhance the content of RA and accumulation of total phenolic acids. The activity of PAL showed a notable repression with YE treatment, while the activity of TAT was enhanced by YE (Yan et al. 2006). It was reported that exogenous ABA and polyamines increased the production of salvianolic acids in hairy root cultures of *S. miltiorrhiza*. The results of HPLC showed that contents of SAB and SAA enhanced 2.0-fold and 3.3-fold, respectively, after 80 μmol L^{-1} ABA treatment in *S. miltiorrhiza* hairy roots. Meanwhile, PAL activity also was detected, which increased 1.8-fold after ABA treatment (Hao et al. 2012). Liang et al. (2013) found that three significant phytohormones including abscisic acid (ABA), gibberellin (GA), and ethylene (Eth) could enhance the production of phenolic acids and activities of PAL and TAT in *S. miltiorrhiza* hairy roots.

11.4.2 Metabolic Engineering for Production of Phenolic Acids in *S. miltiorrhiza*

The use of modern biotechnology and molecular biology methods regulates the content of phenolic acids in *S. miltiorrhiza*. The first is the regulation of key enzyme genes in its synthetic pathway. Hairy roots have the advantages of high genetic stability, rapid growth, etc., which is considered as a promising system to generate pharmacological active ingredient of traditional Chinese medicine plant (Guillon et al. 2006; Kai et al. 2012; Shi et al. 2019, 2014). In order to improve the synthesis of pharmacologically active ingredients such as phenolic acids in *S. miltiorrhiza*, researchers attempted to overexpress one or more key enzymes of the phenolic acid synthesis pathway in hairy roots of *S. miltiorrhiza*. Previous research revealed that the content of rosmarinic

acid (RA) was ~3.6-fold more than control in *SmC4H* transgenic lines. Furthermore, the concentration of LAB enhanced 11.1-fold of that in control (EV) when overexpressed *SmC4H* in hairy roots. Overexpression of *SmTAT* and *SmHPPR* in hairy root also showed an increase in RA and LAB levels. It is worth noting that the *SmTAT-SmHPPR* co-transformed lines had the highest level of metabolites that the content more than 16.1 and 18.8 times that in EV line (Xiao et al. 2011). Overexpressed genes such as *Sm4CL*, *SmRAS,* and Sm*CYP98A14* in the biosynthesis pathway of phenolic acids are also a strategy to be used for enhancing the yields of phenolic acids in the future. Not only endogenous genes, but also foreign genes can be involved in the regulation of phenolic acid synthesis. It was reported that the accumulation of phenolic acids in *S. miltiorrhiza*, especially SAB, was affected by overexpressing the foreign gene *AtPAP1* (Zhang et al. 2010).

11.4.3 Transcriptional Regulation of Phenolic Acids Biosynthesis in *S. miltiorrhiza*

The effects of overexpressing one or more critical enzyme genes of synthetic pathway to enhance the target product are limited, and the increase in the yield of the target product is often limited. The regulatory functions and mechanisms of transcription factors for plant secondary metabolism have become research hotspots. As research continues, it has been found that the use of upstream transcription factors to activate the entire metabolic regulatory network is often more efficient than simply transferring to one or several rate-limiting enzyme genes. JA is involved in plant growth and development regulation as a plant hormone, as well as response to stress and leads to accumulation of secondary metabolites (Wasternack and Hause 2013; Namdeo. 2007). With the continuous advancement of sequencing technology, transcription factors mainly including ERF, WRKY, bHLH, MYB, and other transcription factors in responsive to JAs have been discovered and cloned for secondary metabolic regulation in *S. miltiorrhiza* (Zhou and Memelink 2016; Yu et al. 2018; Cao et al. 2018; Du et al. 2018; Ding et al. 2017).

Members of the AP2/ERF TF family responding to JA are significant biosynthesis of secondary metabolites in plant (Zhou and Memelink 2016). *SmERF115*, most sensitive to MeJA, has been isolated and characterized. The phenolic acids production of hairy roots in *S. miltiorrhiza* is enhanced when *SmERF115* overexpressed, while silencing of *SmERF115* leads to the decreased of phenolic acids. Meanwhile, *SmERF115* is binding directly the promoter of *SmRAS* and up-regulating the expression of *SmRAS* to mediate the yield of phenolic acids (Sun et al. 2019).

Basic helix-loop-helix (bHLH) transcription factor, one of the largest families of transcription factors in plants, plays an important role in plant growth and development and secondary metabolism. SmbHLH37, SmbHLH51, and SmbHLH148 in *S. miltiorrhiza* were revealed to participate the regulation of phenolic acids. SmbHLH37 was reported binding the promoter regions of *SmTAT* and *SmPAL* to repress their expression, inhibiting the SAB synthesis pathway and resulting in decreased SAB production. In contrast, SmbHLH51 and SmbHLH148 played a positive role in mediating the pathway of phenolic acid biosynthesis. The content of *SmbHLH51*-OE lines increased 2.19-fold and 1.59-fold, respectively, and overexpressed SmbHLH148 significantly improved three salvianolic acid levels including caffeic acid, rosmarinic acid, and salvianolic acid B (Du et al. 2018; Wu et al. 2018; Xing et al. 2018a, b). As a special member of the bHLH family, the MYC2 transcription factor has been widely studied. MYC2 is not only the core of the response MJ in plants, but also plays a significant role in secondary metabolism and various growth and development processes (Kazan and Manners 2013; Gangappa et al. 2010). For example, it was reported that SmMYC2 regulated the generation of phenolic acids by activating both primary and secondary metabolic pathways in *S. miltiorrhiza*. Overexpressed *SmMYC2* transgenic plants

showed higher SAB content which was 1.88-fold higher than in control lines (Yang et al. 2017).

As the largest transcription factor family, MYB TFs are widely found in plant. The MYB transcription factors are divided into four subfamilies, called 1R-MYB, 2R-MYB, 3R-MYB, and 4R-MYB, respectively, depending on the number of incomplete repeats (one, two, three, or four) in the DNA-binding domain (Katiyar et al. 2012; Zhang et al. 2013; Li and Lu 2014). MYB transcription factors have been shown to be involved in primary and secondary metabolism, cell fate and traits, developmental processes, and responses to biotic and abiotic stresses (Dubos et al. 2010; Katiyar et al. 2012). As a R2R3-MYB transcription factor, SmMYB36 was in involved in tanshinones and phenolic acids biosynthesis regulation. In the overexpressing SmMYB36 hairy roots, the content of SAB, RA, and total phenolic acid decreased significantly compared with the control lines, while the content of tanshinone increased in overexpressing lines (Ding et al. 2017). It also was reported that SmMYB111 promoted accumulation of SAB and RA. The concentrations of RA in *SmMYB111*-OE lines were 3.05 and 3.10 times higher than that in control lines. Meanwhile, the content of SAB in *SmMYB111*-OE lines was about 3.54- and 2.50-fold higher than control (Li et al. 2018).

JASMONATE ZIM-DOMAIN (JAZ) transcriptional repressor plays an important role in the JA signaling pathway. Meanwhile, JAZs can interact with MYC2 transcription factor and repress it function when MJ absent (Thines et al. 2007). SmJAZ8 was found involved in biosynthesis of phenolic acids and tanshinones in *S. miltiorrhiza* hairy roots. The production of phenolic acids was decreased when overexpressed *SmJAZ8*, but increased in RNAi transgenic lines. Meanwhile, the content of tanshinones also declined or enhanced in *SmJAZ8*-OE lines or RNAi lines, respectively (Pei et al. 2017).

The above results indicate that it is feasible to increase phenolic acid production at the level of transcription factor regulation. Altering the expression of a transcription factor can directly or indirectly result in a change in the expression of one or more key enzyme genes in the phenolic acid biosynthetic pathway to accumulate or reduce phenolic acid. The production of phenolic acids can be increased to meet needs of markets by constructing single plants or hairy roots that overexpress transcription factors that positively regulate phenolic acid synthesis.

11.4.4 Callus Cultures of *S. miltiorrhiza* for Enhancing Production of Phenolic Acids

Hairy roots have been previously introduced for the mass production of valuable secondary metabolites, especially some slow-growing medicinal plants. However, studies on using callus and cell suspension cultures in *S. miltiorrhiza* to generate secondary metabolite, especially important pharmacological components, are limited. In 1996, *Taxus* cell cultures were reported that could be an alternative source of paclitaxel and related taxane production. A significant increase in paclitaxel and baccatin III levels was observed in cultured cells of *Taxus* species after treatment with MeJA (Yukimune et al. 1996). In recent years, callus cultures of *S. miltiorrhiza* were reported to be used for producing RA and SAB. Callus cultures were cultivated in MS medium using stem and leaf explants; subsequently, the active ingredients of the extracts from callus cultures were analyzed by high-performance liquid chromatography coupled to DAD and MS (HPLC-DAD-MS). The results showed that extraction of callus cultures from stem produced higher amounts of RA and SAB than callus leaves. The content of RA in stem was $1.27 \pm 0.38\%$ but $0.28 \pm 0.02\%$ in leaves, meanwhile, the SAB production in stem and leaves was $0.87 \pm 0.20\%$ and $0.07 \pm 0.03\%$, respectively (Wu et al. 2016).

Plant callus and cell suspension cultures possessing high-value secondary metabolites are a promising potential alternative source for industrial production of medicinal ingredients like phenolic acids. Cell cultures are insensitive to the external environment and rapidly produce metabolites with pharmacological active

ingredients (Shi et al. 2019). How to use the suspension cells and callus of *S. miltiorrhiza* to carry out the production of secondary metabolites of pharmaceutical ingredients needs further investigation.

11.4.5 Making Use of Endophytic Fungus in *S. miltiorrhiza* to Produce Phenolic Acids

Making use of endophytic fungus to produce plant secondary metabolites is a novel technology. *Phoma glomerata* D14, an endophytic fungus isolated from *S. miltiorrhiza*, was also found to produce salvianolic acid C (SAC). However, HPLC analysis found that the production of salvianolic acid C in extract of mycelium and broth was very low, 47.67 ± 0.04 μg/g and 0.054 μg/mL, respectively (Li et al. 2016b). Furthermore, *Fusarium proliferatum* SaR-2 and *Alternaria alternata* SaF-2 were found to exhibit higher levels of phenolics than plant roots. Results of total phenol production detection showed that total phenolic content of *F. proliferatum* SaR-2 and *A. alternata* SaF-2 was 21.75 ± 0.11 mg/g and 20.53 ± 0.08 mg/g, respectively (Li et al. 2015). *Trichoderma atroviride* D16, an endophytic fungus isolated from roots of *S. miltiorrhiza*, has been reported to produce tanshinone I and tanshinone IIA, which was a potential source for industrially production of tanshinone I and tanshinone IIA to meet pharmaceutical needs (Lou et al. 2013). *Chaetomium globosum* D38 isolated from *S. miltiorrhiza* roots could enhance the production of tanshinones, especially for dihydrotanshinone I and cryptotanshinone. Furthermore, this endophytic fungus could co-exist with the root of *S. miltiorrhiza* without toxicity. Both live fungus and its mycelia extract were revealed that could increase the production of tanshinones (Zhai et al. 2018). Due to the low variety and low content of endophytic fungus-producing phenolic acids, more endophytic fungus that produce phenolic acid should be explored and culture conditions optimized to increase phenolic acids production in the future.

11.5 Conclusions and Prospects

S. miltiorrhiza is an important traditional Chinese herbal medicine, which has been widely used in the treatment of cardiovascular and cerebrovascular diseases. Phenolic acid is one group of biologically active compounds in *S. miltiorrhiza*. It has great curative effect and pharmacological activity. However, due to the market demand is increasing, the germplasm resources of *S. miltiorrhiza* are degraded, and the content of phenolic acids in traditionally cultured *S. miltiorrhiza* is low. How to improve the production of phenolic acid has become an urgent problem to be solved.

It was a strategy that the use of modern biological means to regulate the synthesis of phenolic acids, through the expression of genes leading to the accumulation of secondary metabolites. However, its mechanism of action and regulation mode needs further exploration due to its complex secondary metabolic network. Combined with molecular biology, transcriptomics, and metabolomics, the expression of secondary metabolite-related genes and their mechanisms are important for understanding the synthesis and regulation mechanisms of phenolic acids. Currently, the upstream pathway of RA synthesis and the key enzymes involved in regulation have been basically understood, including SmPAL, SmC4H, Sm4CL, SmTAT, SmHPPR, SmRAS, and SmCYP98A14. However, the synthetic pathways of other phenolic acids such as SAB downstream of RA are still unclear. Therefore, exploring the synthetic pathways downstream of RA to other phenolic acids, discovering the key enzymes involved in catalysis and further researching the metabolic mechanism of metabolites, will be beneficial to promote the production of phenolic acids and drug development in *S. miltiorrhiza*.

To date, several types of transcription factors such as bHLH, MYB, JAZ, and AP2/EARF have been reported to regulate phenolic acid

biosynthesis. Through the genome and transcriptome data of *S. miltiorrhiza*, it is possible to dig deeper and isolate more genes involved in the regulation of phenolic acids. The strategy of constructing a "transcription factor-biosynthetic pathway critical gene-metabolite" network will contribute to the improved synthesis of phenolic acids in *S. miltiorrhiza*. Meanwhile, comprehensive utilization of synthetic biology, elicitors, hairy roots and transgenic plants, endophytic fungus, genetic engineering and transcriptional regulation to enhance phenolic acid production in *S. miltiorrhiza* is also the direction of future research.

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