Industrial Crops & Products 124 (2018) 402–411

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

DNA methylation: A new regulator of phenolic acids biosynthesis in *Salvia miltiorrhiza*

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ARTICLE INFO

Keywords:
DNA methylation
Secondary metabolism
Phenolic acid
Biosynthesis
Salvia miltiorrhiza
Regulation

ABSTRACT

Both DNA methylation and secondary metabolism in plants are involved in interactions between plants and their environments. It is speculated that DNA methylation plays an important role in the regulation of plant secondary metabolism. Using *Salvia miltiorrhiza* as a research model, effects of an inhibitor (5-azacytosine, 5-AzaC) and a donor (S-adenosyl methionine, SAM) for DNA methylation on phenolic acid accumulation and expressions of key genes involved in phenolic acid biosynthesis were analyzed. 5-AzaC significantly enhanced phenolic acid accumulation and expressions of key genes involved in phenolic acid biosynthesis, while SAM dramatically inhibited phenolic acid accumulation and the expression of key genes. Both 5-AzaC and SAM can alter methylation patterns of rosmarinic acid synthase gene(*RAS*) promoter. 5-AzaC could significantly reduced methylation levels of CG and CHG sites and increased CHH methylation in *RAS* promoter. S-adenosyl methionine had no effects on CG and CHG methylation and could dramatically reduced CHH methylation level. The gene expression of DNA methyltransferases was reduced by 5-AzaC and increased by SAM, whereas that of DNA demethylases was increased by 5-AzaC and reduced by SAM. It is first proved that DNA methylation regulates the expression of key genes involved in phenolic acids biosynthesis to affect phenolic acids production in *S. miltiorrhiza*. Roles of DNA methylation on phenolic acids accumulation will provide us new insights on the regulation of secondary metabolism in plants.

1. Introduction

Secondary metabolites in plants are a group of active compounds of low molecular weight produced in a specific development period or tissues of plants (Dixon and Strack, 2003). More than 20,000 secondary metabolites are produced in plants, and have important roles in interactions between plants and their environments (Dixon, 2001; Verpoorte and Memelink, 2002). Biosynthesis of secondary metabolites in plants is influenced by environmental factors. For example, herbivore damage results in high concentrations of flavonoid phloridzin in apple plants (Gutbrodt et al., 2011), and microbial attack results in increases in terpenoids, alkaloid and phenolics accumulations (Dixon, 2001). Abiotic factors such as light, water, temperature, mineral elements and phytohormones can also affect the accumulation of secondary metabolites in plants (Falcone Ferreyra et al., 2012). Plants produce various secondary metabolites to reduce injury from stresses in the process (Mouradov and Spangenberg, 2014). Secondary metabolism reflects plant environments more closely than primary metabolism. Knowledge of the interactions between plants and their environments can provide insights on these inner relationships.

DNA methylation is a widespread epigenetic mechanism in organisms. In this modification process, a methyl group is transferred from S-adenosyl methionine (SAM) to cytosine by DNA methyltransferases. Sequences of CG, CHG and CHH are usually the targets for cytosine methylation in higher plant genomes. The level of cytosine methylation is 6–30% in plants and changes with the development of specific plant tissues (Becker et al., 2011). Cytosine methylation plays important roles in development and stress resistance in plants. The levels of genomic DNA methylation in plants are coordinately regulated by both methylation and demethylation (Deleris et al., 2016; Chinnusamy and Zhu, 2009). DNA methylation mainly depends on two pathways: maintenance and *de novo* methylation. DNA methyltransferase (MET) and chromomethylase (CMT) are two key enzymes for maintenance methylation (Zhang et al., 2018). DNA methyltransferase is required for maintenance methylation of CG sites. Chromomethylase is a plant-specific enzyme and is required for maintenance methylation of CHG

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https://doi.org/10.1016/j.indcrop.2018.07.046
Received 24 November 2017; Received in revised form 15 July 2018; Accepted 18 July 2018

sites. Domains rearranged methylase (DRM) is mainly responsible for *de novo* methylation of cytosine. The mechanism of DNA methylation in plants is widely reported and clearly established. However, our knowledge of DNA demethylation is far from complete. DNA glycosylases and methylated DNA binding proteins (MDB) play important roles in the DNA demethylation process. Methylated DNA binding proteins is a critical regulator of DNA demethylation that recruits chromatin remodelers, histone deacetylases, and histone methyltransferases to repress transcription. Arabidopsis MBD7 can bind to highly methylated, CG-dense regions to accelerate DNA demethylation to limit DNA hypermethylation and transcriptional gene silencing (Lang et al., 2015).

Recent research indicates that various environmental stresses can lead to changes in DNA methylation levels in plants, including pathogens, heavy metals, cool, drought and high salt. Moreover, many genes regulated by DNA methylation are related to stress resistance (Dowen et al., 2012; Wada et al., 2014). In tobacco, infection of tobacco mosaic virus can drastically increase levels of genomic methylation (Boyko et al., 2007), whereas the levels of methylation of stress-responsive genes are decreased, and the expression levels of these genes are increased to improve plant disease resistance (Wada et al., 2004). Cold stress can also change levels of DNA methylation in maize seedlings, and genome-wide demethylation mainly occurs (Steward et al., 2002). Drought-induced DNA methylation changes show a significant level of developmental and tissue specificity in rice, and 70% of the changed sites are reversed to their original status after recovery. These observations demonstrate that alteration of DNA methylation in plants is related to drought resistance. DNA methylation can be considered a very important regulatory mechanism for rice plants to adapt to drought (Wang et al., 2011). Under environmental stresses, increases in genomic DNA methylation benefit genomic stability. Simultaneously, demethylation of stress resistance-related genes can improve expression levels to enhance stress resistance and reduce the injury induced by stresses. Therefore, DNA methylation is considered as important regulatory mechanism for plants to adapt to environmental stresses.

In summary, it is found that both secondary metabolism and DNA methylation are critical responsive and regulatory mechanisms for plants to adapt to environmental stresses. Therefore, it is speculated that DNA methylation plays an important role in environmental stress-induced secondary metabolism in plants. There are few reports about the relationship between DNA methylation and plant secondary metabolism. In *Taxus*, paclitaxel accumulation is probably related to DNA methylation alteration (Li et al., 2013). In *Vitis amurensis*, the DNA demethylation agent 5-azacytosine (5-AzaC) significantly increased stilbene synthase 10 gene (*VaSTS10*) expression and resveratrol content and reduced DNA methylation levels in the promoter and coding regions of the *VaSTS10* gene. DNA methylation may be involved in the control of resveratrol biosynthesis (Kiselev et al., 2013). However, no additional evidence about the relationship between DNA methylation and secondary metabolism has been reported, and the regulatory mechanism remains unclear.

Roots of *Salvia miltiorrhiza* is one of the most popular Traditional Chinese Medicine (TCM). It is usually used in combination with *Panax notoginseng* (Burk.) F. H. Chen in the treatment of cardiotonic disease. Phenolic acids and tanshinones are the most active ingredients in *S. miltiorrhiza*. Phenolic acids mainly including salvianolic acid B, rosmarinic acid and caffeic acid are water-soluble. Tanshinones mainly including tanshinone IIA, cryptotanshinone, tanshinone I and dihydrotanshinone I are lipophilic. In recent years, more and more attention has been paid to phenolic acids because of their strong activities. Salvianolic acid B and rosmarinic acid have various strong activities including antioxidant, anti-inflammatory, anticancer effects and cardiotonic protection (Lam et al., 2006; Lin et al., 2006; Petersen, 2013). Besides antioxidants, salvianolic acids also act as effective inhibitors of leukocyte-endothelial adherence, inflammation and metalloproteinases expression from aortic smooth muscle cells. (Ho and Hong, 2011). Polyphenol injection is a new dosage form of *S. miltiorrhiza* and have been widely used in treatment of coronary heart disease. However, our understanding of the regulation of phenolic acid biosynthesis in *S. miltiorrhiza* is far from complete. Phenolic acids are biosynthesized *via* the phenylpropanoid pathway and the tyrosine-derived pathway in *S. miltiorrhiza*. Many enzymes are involved in their biosynthesis, including phenylalanine ammonia lyase (PAL), 4-coumarate-CoA ligase (4CL), tyrosine aminotransferase (TAT), cinnamic acid 4-hydroxylase (C4H), 4-hydroxyphenylpyruvate reductase (HPPR), rosmarinic acid synthase (RAS) and CYP98A14. Phenolic acids accumulation in *S. miltiorrhiza* is dramatically influenced by environmental stresses including drought, Ag+, MeJA, YE, SA and ABA (Dong et al., 2010; Li et al., 2016; Liang et al., 2013; Liu et al., 2011; Yan et al., 2014; Yang et al., 2012; Zhang et al., 2015, 2014). However, the relationship and molecular mechanism between phenolic biosynthesis and DNA methylation have not been reported. In this study, an inhibitor (5-AzaC) and a donor (SAM) of DNA methylation were used to treat *S. miltiorrhiza* hairy roots. The effects of 5-AzaC and SAM on the accumulation of phenolic acids in *S. miltiorrhiza* hairy roots were investigated to reveal the relationship between DNA methylation and phenolic biosynthesis. The expression levels of genes involved in phenolic biosynthesis and DNA methylation were analyzed to uncover the regulatory mechanism.

2. Materials and methods

2.1. S. miltiorrhiza hairy roots culture and treatment

S. miltiorrhiza hairy roots were obtained after infection of aseptic leaves with *Agrobacterium rhizogenes* (ATCC15834) (Yang et al., 2012). Fresh hairy roots (0.2 g) were subcultured in a 150-ml flask with 50 ml of 6,7-V liquid medium in an orbital shaker at 110 rpm at 25 °C in the dark.

SAM and 5-AzaC were dissolved in distilled water, filter-sterilized through 0.22-μm filters and stored at 4 °C prior to use. On the day 18 of hairy roots subculture, different concentrations of 5-AzaC (10, 100, 250 and 500 μM) and SAM (1, 10, 100 and 1000 μM) were added. The hairy roots were collected on day 6 after treatment. Treatment with 10 μM 5-AzaC and 1000 μM SAM had most significant influence on phenolic acid accumulations in *S. miltiorrhiza* hairy roots. Therefore, 10 μM 5-AzaC and 1000 μM SAM were added to hairy root cultures on day 18 after subculture. The hairy roots were then collected on the day 1, 2, 3, 6 and 9 after treatment. All treatments were performed in quadruplicate. The collected hairy roots were blotted dry to obtain the fresh weight (FW) and then dried at 50 °C in an oven until constant weight to obtain the dry weight (DW).

2.2. Extraction and analysis of phenolic acids in S. miltiorrhiza hairy roots

The dried hairy roots were ground to powders in a mortar and sieved through a 0.45-mm screen. The powders (0.500 g) were added to a tube with 5 mL of methanol-water solution (7:3) and extracted for 45 min ultrasonically. Then, the extraction solutions were centrifuged at 12,000 rpm for 2 min and filtered through 0.45-μm Millipore filters.

Standards of salvianolic acid B, caffeic acid, rosmarinci acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China. Stock solutions of the standards were prepared in methanol-water solution (7:3) and diluted to the desired concentrations. A series of the standard solutions were used to determine the linear range of the analytes. The detector response was linearly correlated with concentration of the tested compounds with a range of 0.672–13.44 μg for salvianolic acid B, 0.2233–2.233 μg for caffeic acid, 0.275–5.5 μg for rosmarinci acid. The linearity of each standard curve was confirmed by plotting the peak area (y, μV S) against the corresponding concentration (x, μg mL^{-1}) of the analytes. The regression equations are y = 1,047,231.08x-476816.0101 (r^2 = 0.999) for salvianolic acid B, y = 1,788,562.991x-484549.6257 (r^2 = 0.999) for rosmarinci acid, y = 4,653,441.752x-564398.2227