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ANALYSIS ON PHENOTYPE, CATALPOL ACCUMULATION AND METHYLATION OF *REHMANNIA GLUTINOSA*

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Abstract

Rehmannia glutinosa is one of traditional Chinese herbal medicines, its phenotype, catalpol accumulation and genomic methylation were studied in this research. During the growth and development of *Rehmannia glutinosa*, leaf length, plant height and catalpol accumulation increased continuously along with the extension of growth stage, and reached peak in the later growth stage. 5-methylcytosine (5mC) content and methylation level in genome also increased along with the growth of *Rehmannia glutinosa*, and reached the highest value in the later growth stage, however genomic methylation in leaf and root was different at the same growth stage and was higher in leaf. As compared with that of *Rehmannia glutinosa* planted in Xinxiang, leaf length, plant height, catalpol accumulation and 5mC content in Wenxian were all higher at the same growth stage, full-methylation was main in genomic methylation of *Rehmannia glutinosa* planted in Wenxian, yet semi-methylation was main for genomic methylation of *Rehmannia glutinosa* plants in Xinxiang. Therefore, Wenxian maybe more suitable to plant *Rehmannia glutinosa*, and this research would help to explore the correlation between ecological environment and growth of *Rehmannia glutinosa*.

Key words: *Rehmannia glutinosa*, Phenotype, Catalpol, DNA methylation.

Introduction

In the covalent modification and epigenetic modification of living cells, DNA methylation is the most common, has a mutagenic effect and could lead to specific gene expression, cell differentiation, chromatin inactivation, embryo growth and cancer (Alghamian *et al*., 2017). DNA methylation occurs in genome of different organisms (Duan *et al*., 2016), the occurrence of DNA methylation in prokaryotes is simultaneous in cytosine and adenine bases, but in most eukaryotes is only limited to cytosine bases. If the level of DNA methylation in plant is insufficient or increased, plant would have abnormal growth and phenotype (Marfil *et al*., 2009; Chen *et al*., 2014; Duan *et al*., 2016), DNA methylation also affects the accumulation of plant secondary metabolites, and has a close and complicated relationship with plant secondary metabolites (Kiselev *et al*., 2015). For example, under treatment of DNA methylation inhibitor 5-azaC, the content of polysaccharide and alkaloid increase considerably in *Dendrobium* (Ni *et al*., 2014), and the synthesis of isoflavone is inhibited in *Maackia* (Xia, 2006).

Some studies have shown that degree and status of genomic methylation may change in different environments (Dowen & Ecker, 2012; Migicovsky & Kovalchuk, 2013; Chen, 2014; Tang *et al*., 2014). For example, water stress leads to hypermethylation in *pea* genome, and methylation degree of second C in CCGG sequence increases by 40% (Labra *et al*., 2002). Low temperature stress makes some of CCGG sequences remethylation and demethylation in *Oryza sativa* genome (Hua *et al*., 2005). Under salt stress, methylation degree of cytosine in CCGG sequence increases by 0.2%-17.6% in *Brassica napus* genome (Lu *et al*., 2007), and methylation level of *Manioc* genome is significantly increased (Mastan *et al*., 2012). It was also found that drought stress could lead to the decrease of methylation level in *ryegrass* genome by up to 10% (Tang *et al*., 2014). Therefore, methylation of plant genome may be affected by environmental factors, and plant would respond quickly to different environments by changes in genomic methylation.

Rehmannia glutinosa belong to Scrophulariaceae, is one of traditional Chinese herbal medicines and has a very high medicinal value (Fei *et al*., 2007; Duan *et al*., 2018). Catalpol is one of main effective components of *R. glutinosa*, its content could vary greatly among different germplasm, different producing area and different source (Yang *et al*., 2012), and better efficacy of *R. glutinosa* is largely related to ecological environment, but geographical environment is very complex, such as soil, water, climate, sunshine, biodistribution etc. (Du & Li, 2013). In order to explore effects of geographical condition on quality of *R. glutinosa*, phenotype, catalpol accumulation and DNA methylation of *R. glutinosa* were studied, and the growth of *R. glutinosa* were also analyzed from the aspects of epigenetics.

Materials and Methods

Materials: *R. glutinosa* cultivar 85-5 was used in this research, and its root tubers were kindly supplied by Agricultural Research Institute of Wenxian County, Henan, China. Primer and adapter were listed in Table S1, and were all synthesized by Invitrogen (Shanghai) Biotechnology Co., Ltd.

Planting of *Rehmannia glutinosa*: Root tubers with similar size and number of buds were chosen, and disinfected with 75% ethanol for 30s, and were sterilized with 0.1% mercuric chloride for 10min, then were rinsed for 3-5min with sterile water. Subsequently, disinfected root tubers were planted in test field. Test fields were in Xinxiang (XX) (College of Life Science, Henan Normal University, Xinxiang City) and Wenxian (WX) (Agricultural Research Institute of Wenxian County), Henan, China.

Supplement Materials

Table S1. The sequence of primer and adapter.

Adapter and primer	Sequence
*EcoR*I adapter	5'-CTCGTAGACTGCGTACC CTGACGCATGGTTAA-5'
*Msp*I-*Hpa*II adapter	5'-GACGATGAGTCTAGAA CTACTCAGATCTTGC-5'
*EcoR*I pre-amplification primer	5'-GACTGCCTACCAATTCA-3'
*Msp*I/*Hpa*II pre-amplification primer	5'-GATGAGTCTAGAACGGT-3'
Selective amplification primer	Eo: 5'-GACTGCCTACCAATTC HMo: 5'-GATGAGTCTAGAACGGT
*EcoR*I selective amplification primer	E1: Eo-AAC E2: Eo-AAG E3: Eo-ACA E4: Eo-ACT E5: Eo-AGG E6: Eo-ACC E7: Eo-ACG E8: Eo-AGC
*Msp*I/*Hpa*II selective amplification primer	HM3: ATCATGAGTCCTGCTCGGT HM4: HMo-ACA HM5: HMo-ACC HM6: HMo-CCG HM7: HMo-ACT HM8: HMo-TAA

Table S2. The reaction conditions of pre-amplification.

Cycle name	Temperature (°C)	Time	Number of cycles
Pre-PCR_1	72.0	1m	
Pre-PCR_2	94.0	45s	1
	65.0	30s	
	72.0	1m	
Touch-down PCR	94.0	30s	12
	64.3	30s	12
	72.0	1m	
	72.0	1m	
PCR	94.0	30s	25
	55.9	30s	20
	72.0	1m	
	55.9	30s	
	72.0	1m	
Elongation	72.0	30m	
Store	4.0	-	-

The annealing temperature of Touch-down PCR was 64.3°C, 63.6°C, 62.9°C, 62.2°C, 61.5°C, 60.8°C, 60.1°C, 59.4°C, 58.7°C, 58.0°C, 57.3°C or 56.6°C, respectively

Plant height or leaf length of *R. glutinosa* in XX and WX was statistically analyzed in the following growth stages: I growth stage (seedling root is not fleshy), II growth stage (plant root is fleshy and cylindrical), III growth stage (plant root appears preliminary expansion), IV growth stage (plant root appears middle expansion), V growth stage (plant root appears late expansion) and VI growth stage (plant root is spindle-shaped). In addition, leaf and root tuber of *R.* glutinosa in six growth stage were frozen in liquid nitrogen and stored at -80°C.

Detection of catalpol content: Catalpol content of *R. glutinosa* was detected by high performance liquid chromatography (HPLC). Fresh root and leaf of *R. glutinosa* were put into the oven and dried at 50°C in order to obtain power of root and leaf, 0.8g powder was put into the round-bottom flask with 50ml methylalcohol and heated at 70°C. After heating reflux for 1.5h, the mixture was cooled at room temperature and filtered, then 10ml filtrate was drawn and concentrated to near dry. Subsequently, the residue was dissolved with mobile phase and was filtered, and the filtrate was put into 10ml volumetric flask and was diluted with mobile phase to 10ml. These chromatography analysis conditions were as follows: the mobile phase was mixture of acetonitrile and 0.1% phosphoric acid (1:99), the flow velocity was 1.0ml/min, the column temperature was 25°C, the sensitivity was 0.1, the injection volume was 10μl, the column was Agilcnt C18Zorbax XDB column (150mm x 4.6mm, 5μm particle size) and the detection wave length was 210nm. As shown in HPLC chromatogram of catalpol, the retention time of standard substance was 9.513min, and the retention time of catalpol in *R. glutinosa* was 9.543min.

In addition, 10mg catalpol standard substance was dissolved with mobile phase to 1000μg/ml (mother liquor), and the mother liquor was respectively diluted into 400μg/ml, 500μg/ml, 600μg/ml and 800μg/ml. The injection volume of each concentration was 10μl and was detected, the same method as in reaction liquid was used to detect standard substance. The concentration and peak area was respectively used as abscissa and ordinate, the linear equation of standard substance was y=1.9752*x+6.0855, R^2=0.999 (y: peak area, x: concentration), indicating that catalpol standard substance had a good linear relationship in the scope of 400-1000 μg/ml, and catalpol content of *R. glutinosa* can be calculated according to the linear equation of standard substance. Furthermore, precision, repeatability and stability of HPLC were tested to guarantee the reliability of experiment data, and the detection of catalpol in *R. glutinosa* was repeated three times.

Genomic DNA extraction: Genomic DNA in leaf and root of *R. glutinosa* was extracted by modified CTAB method. About 4g fresh leaf or root was put into the pre-cooled mortar and ground into powder, was immediately transferred to 50ml centrifuge tube in which 5ml/g CTAB solution with two-thousandths of β-mercaptoethanol was added, centrifuge tube was put in water bath at 65°C for 2h accompanied by upside down mixing 1time/20min. After centrifuging for 10min at 12000rpm, the supernatant was transferred, extracted with phenol: chloroform: isoamyl alcohol (25: 24: 1) and was centrifuged for 10min at 12000rpm, and was repeated twice. Subsequently, the supernatant with isopropyl alcohol was placed for more than 1h and was separated by 12000rpm centrifugation for 15min. DNA precipitation was washed twice with 70% ethanol, and was dissolved in double-distilled water after air-drying for 5min. In addition, DNA solution added with one percent RNase was put at 37℃ for 30min, and was extracted with phenol: chloroform: isoamyl alcohol (25: 24: 1). After further extraction, DNA solution was precipitated with 1/3 volume of 3M NaAc and 2.5 volume of absolute ethanol, and was centrifuged at 12000rpm for 10min, then DNA precipitation was washed twice with 70% ethanol and centrifuged at 12000rpm for 5min, subsequently, dried DNA was dissolved in double-distilled water and was stored at -20°C.

Furthermore, the yield and purity of genomic DNA were determined by micro-spectrophotometer at 260nm, and OD value of 260nm/280nm was between 1.8 and 2.0. The integrity of genomic DNA was detected by 0.8% agarose gel electrophoresis, it was found that DNA band were clear, bright and no trailing, and spot hole was not bright, thus genomic DNA extracted from *R. glutinosa* can be used for analysis of DNA methylation.

HPLC detection of genomic methylation: It is well known, the content of 5-methylcytosine (5mC) in genome generally represents level of genomic methylation, in this research, 5mC content was detected by HPLC (Duan *et al*., 2018). After genomic DNA of *R. glutinosa* was orderly hydrolyzed with DNase I, nuclease P1 and alkaline phosphatase, was centrifuged at 12000rpm for 5min, then the supernatant was transferred and was filtered with 0.45μm organic microfiltration membrane, subsequently was detected by HPLC. These chromatography conditions performed in this study were as follows: the mobile phase was composed of 50mM KH_2PO_4 and 8% methanol (92: 8), the flow velocity was 0.5ml/min, the column temperature was 30°C, the sensitivity was 0.1, the injection volume was 20μl, the analytical column was Agilent C18 Zorbax XDB column (4.6×150mm, 5μm particle size), and the detection wavelength was 285nm.

The standard substance of 0.0044g C and 0.0065g 5mC was separately dissolved with 10ml ultra pure water to 40nM/100μL (mother liquor), and the mother liquor was diluted to 0.02nM/100μl, 0.05nM/100μl, 0.1nM/100μl, 0.5nM/100μl, 1nM/100μl, then each concentration of standard substance (20μl) was detected. As shown in HPLC chromatogram, the standard substance of C and 5mC both had a good linear relationship in the range of 0.02-1.0nM/100μl, and the linear equation of C or 5mC as follows: y = 2172.7x-18.537 (R^2 = 0.9993), y = 2276x-7.9806 (R^2 = 0.9997), in which x or y represented concentration and peak area of standard substance respectively. According to the above linear equation of standard substance C and 5mC, the concentration of C and 5mC could be respectively calculated, then the content of 5mC was obtained according to the following formula: 5mC = [5mC / (5mC + C)] ×100. In addition, precision, repeatability and stability of HPLC were tested to guarantee the reliability of experiment data, and the detection of C and 5mC in genomic DNA was repeated three times.

MSAP amplification of genomic DNA: Under the condition of without changing methylation status, *Hpa*II and *Msp*I have different sensitivity to DNA methylation, *Hpa*II is not sensitive to full methylation (double-stranded methylation) and could cleave hemi-methylation (single-stranded methylation), *Msp*I is sensitive to internal cytosine (CmCGG sequence) and not to the external cytosine (mCCGG sequence) in full methylation. Thus, *Hpa*II and *Msp*I were selected to produce different cleavage fragments which could reveal the status of genomic methylation, reaction system and amplification condition of methylation-sensitive amplified polymorphism (MSAP) was performed according to method of Duan (2018). Genomic DNA of *R. glutinosa* was orderly digested by *EcoR*I / *Msp*I and *EcoR*I/ *Hpa*Π, then was ligated with *EcoR* I adapter and *Msp* I -*Hpa* II adapter by T4 DNA ligase at 16°C for 12h. Subsequently, the Enzyme-Ligation product was stored at -20°C in order to perform MSAP which consisted of pre-amplification and selective amplification.

After pre-amplification, the system of MSAP was established, the Enzyme-Ligation product was diluted 10-fold and amplified as the pre-amplification template. The reaction system of MSAP pre-amplification (50μl) was composed of 2.5μm *EcoR* I pre-amplification primer, 2.5μm *Msp* I -*Hpa*Π pre-amplification primer, 25μl 2×Taq Mix, 0.5μl Enzyme-Ligation product, and reaction conditions of PCR pre-amplification were listed in Table S2, then the pre-amplification products were detected by 1% agarose gel electrophoresis, which were clear and uniform. Subsequently, the pre-amplification products were diluted 10-fold and amplified as the selective amplification template. The reaction system of MSAP selective amplification (20μl) was composed of 5μm *EcoR* I selective amplification primer, 5μm *Msp* I */Hpa*Π selective amplification primer, 10μl 2×Taq Mix and 0.5μl pre-amplification product, and the reaction condition of MSAP selective amplification was the same as pre-amplification except without Pre-PCR_1. After MSAP selective amplification, the amplification products were detected by 6.0% polyacrylamide gel electrophoresis for 5.5h or so, and the polyacrylamide gel was performed as the following, such as fixing with glacial acetic acid, rinsing with double-distilled water, staining with silver nitrate, coloration, and finally only clear and reproducible bands in the polyacrylamide gel were calculated and analyzed.

MSAP analysis: DNA methylation level was quantified by MSAP binary data, the presence or absence of one band was scored as "1" or "0" respectively. The band was scored according to presence or absence in *EcoR* I */Hpa*Π (H) and *EcoR* I */Msp* I (M), and band pattern of MSAP amplification could be divided into three classes: the presence of band in H and M was considered no methylation (class Ⅰ), the presence of band only in H was considered

DNA hemi-methylation (class Π), and the presence of band only in M was considered DNA full-methylation (class Ⅲ). DNA methylation level was calculated by the following formulate: DNA methylation level (%) = (bands of class Π+ bands of class Ⅲ)/(bands of class Ⅰ + bands of class Π+ bands of class Ⅲ) ×100, DNA hemi-methylation level (%) = bands of class Π/(bands of class Ⅰ + bands of class Π+ bands of class Ⅲ) ×100, DNA full-methylation level (%) = bands of class Ⅲ/(bands of class Ⅰ + bands of class Π+ bands of class Ⅲ) ×100. In addition, some band patterns with indeterminable change of genomic methylation were also found in this study, but not shown in these results.

Statistic analysis of data: Significance level, ANOVA analysis and multiple comparison of Duncan's multiple range test were performed by DPS7.5 software, such as experiment data of plant height, leaf length, catalpol content, 5mC content and methylation level, etc.

Results

The growth and development of *Rehmannia glutinosa*: As shown in Fig. 1(a), compared with that in Ⅰ growth stage of *R. glutinosa,* plant height increased along with the growth of *R. glutinosa,* and reached the maximum value in Ⅵ growth stage (p<0.05). Compared with that in XX, plant height of *R.glutinosa* in WX was higher at the same growth stage (p<0.05), for example in Ⅵ growth stage, plant height of *R. glutinosa* in XX was about 16cm, which was significantly lower than that in WX (20cm or so) (p<0.05).

It can be seen from Fig. 1(b) that leaf length of *R. glutinosa* was also increased along with the extension of growth stage, and reached the maximum value in Ⅵ growth stage (p<0.05). Compared with that in XX, leaf length of *R. glutinosa* in WX was significantly longer at III-Ⅵ growth stage (p<0.05). In Ⅵ growth stage, leaf length of *R. glutinosa* in XX was about 24cm, and was significantly shorter than that in WX (31cm or so) (p<0.05).

The accumulation of catalpol in *Rehmannia glutinosa*: It can be seen from Fig. 2(a), the accumulation of catalpol in root increased continuously along with the growth of *R. glutinosa* (p<0.05), and reached peak at Ⅴ and Ⅵ growth stage. Compared with that in XX, catalpol content of root in WX was significantly higher during the same growth stage (p<0.05), for example, at Ⅴ growth stage, catalpol content in XX was 4.797%, and was lower than that in WX (5.551%).

As shown in Fig. 2(b), the accumulation of catalpol in leaf increased continuously along with the growth of *R. glutinosa* (p<0.05), and reached peak at Ⅳ and Ⅴ growth stage. Compared with that in XX, catalpol content of leaf in WX was higher during the same growth stage, for example, at Ⅳ growth stage, catalpol content of leaf in XX was 5.369%, and was lower than that in WX.

The methylation degree of *Rehmannia glutinosa* genome: As listed in Fig. 3(a), 5mC content in root genome increased along with the growth of *R. glutinosa*, and reached peak at Ⅳ growth stage. Compared with that in XX, 5mC content of root genome in WX was significantly higher during the same growth stage (p<0.05), for example, at Ⅳ growth stage, 5mC content in XX was 52.01%, and was significantly lower than that in WX (57.81%).

It can be seen from Fig. 3(b), the variation trend of 5mC content in leaf genome was also inverted bell during the growth of *R. glutinosa*, and reached peak at Ⅳ growth stage. Compared with that in XX, 5mC content of leaf genome in WX was significantly higher during the same growth stage of *R. glutinosa* (p<0.05), for example, at Ⅳ growth stage, 5mC content in XX was 49.48%, and was significantly lower than that in WX (55.28%).

Genomic methylation pattern of *Rehmannia glutinosa*: The level of genomic methylation in leaf of *R. glutinosa* was shown in Fig. 4. It can be seen from Fig. 4(a) that methylation level of leaf genome in WX increased along with the growth of *R. glutinosa*, reached the highest value at Ⅵ growth stage, and was about 60.29% which was significantly higher than that at other growth stages (p<0.05). Compared with semi-methylation level, full-methylation level of leaf genome was always higher, especially was significantly higher at Ⅵ growth stage (35.29% or so) (p<0.05). Methylation level of leaf genome in XX also increased, and reached the highest value (66.67% or so) at Ⅴ growth stage, which was significantly higher than that at other growth stages (p<0.05) (Fig. 4b). However, during the growth of *R. glutinosa* in XX, semi-methylation level was higher as compared with full-methylation level, especially at Ⅴ growth stage, semi-methylation level was about 41.03% and was significantly higher than full-methylation level with 25.64% (Fig. 4b).

The level of genomic methylation in root of *R. glutinosa* can be seen in Fig. 5. As shown in Fig. 5 (a), along with the growth of *R. glutinosa* in WX, methylation level of root genome increased, and reached the highest value at Ⅵ growth stage (63.79% or so) which was significantly higher than other growth stages (p<0.05). Furthermore, full-methylation level of root genome in WX was always higher as compared with semi-methylation level, especially at Ⅴ growth stage, full-methylation level was about 36.21% and was significantly higher than semi-methylation level (21.99% or so) (Fig. 5a). Methylation level of root genome in XX also increased, and reached the highest value at Ⅲ growth stage (65.79% or so), which was significantly higher than that at other growth stages (p<0.05) (Fig. 5b). Compared with full-methylation level, semi-methylation level of root genome in XX was higher, especially at Ⅴ growth stage, semi-methylation level was about 34.71% and was significantly higher (p<0.05) (Fig. 5b).

As compared with that in WX, methylation level of leaf genome in XX was higher during Ⅰ-Ⅴ growth stages, full-methylation level was also higher during Ⅰ-Ⅳ growth stages, and semi-methylation level was always higher. Furthermore, methylation level of root genome in XX was higher during Ⅰ-Ⅳ growth stages compared with that in WX, semi-methylation level was always higher during the growth of *Rehmannia glutinosa*, yet full-methylation level was lower. In general, as compared with that in WX, at the early growth stage of *R. glutinosa*, genomic methylation level in XX was higher, but was lower at the later growth stage.

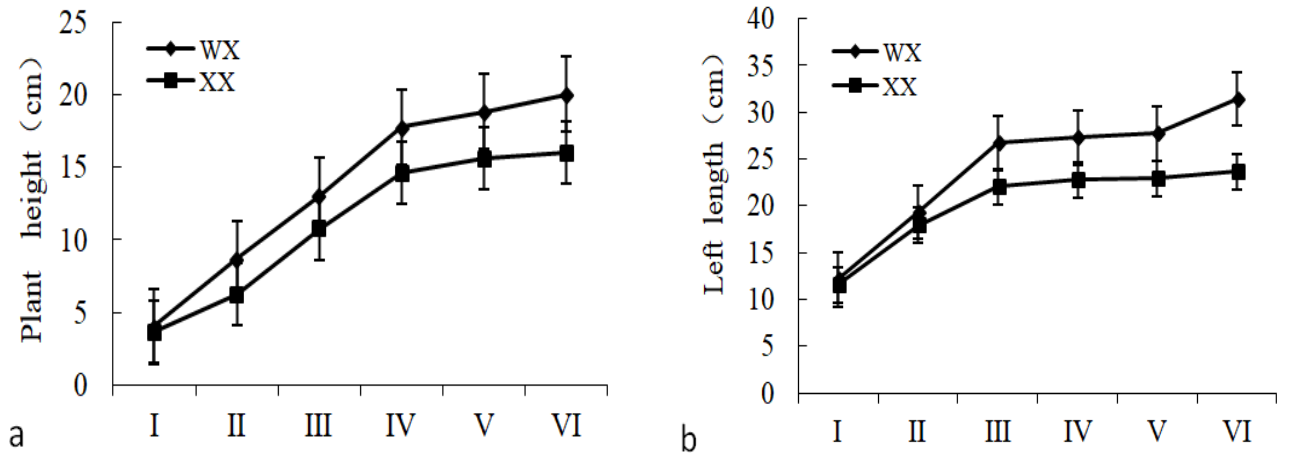

Fig. 1. The phenotype of *Rehmannia glutinosa*
(a) Plant height of *R. glutinosa* at different growth stages, (b) Leaf length of *R. glutinosa* at different growth stages. WX and XX represented *R. glutinosa* planted in Wenxian or Xinxiang, respectively. Ⅰ, Ⅱ, Ⅲ, Ⅳ, Ⅴ and Ⅵ respectively represented the different growth stage of *R. glutinosa*, and the error bar was the standard error of mean.

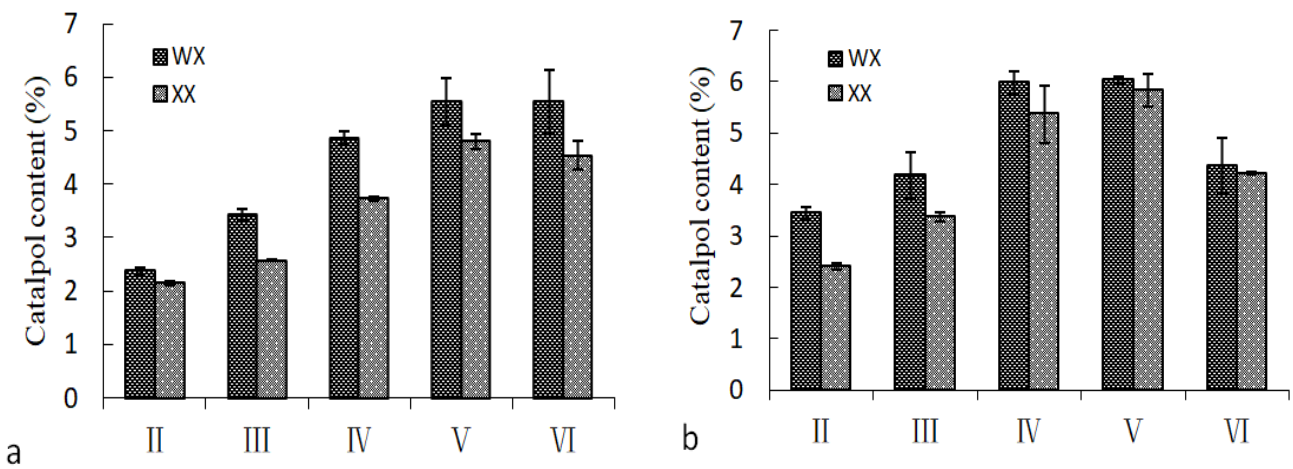

Fig. 2. Catalpol content of *Rehmannia glutinosa*
(a) Catalpol content in root of *R. glutinosa* at different growth stages, (b) Catalpol content in leaf of *R. glutinosa* at different growth stages. WX and XX represented *R. glutinosa* planted in Wenxian or Xinxiang, respectively. Ⅱ, Ⅲ, Ⅳ, Ⅴ and Ⅵ respectively represented the different growth stage of *R. glutinosa*, and the error bar was the standard error of mean.

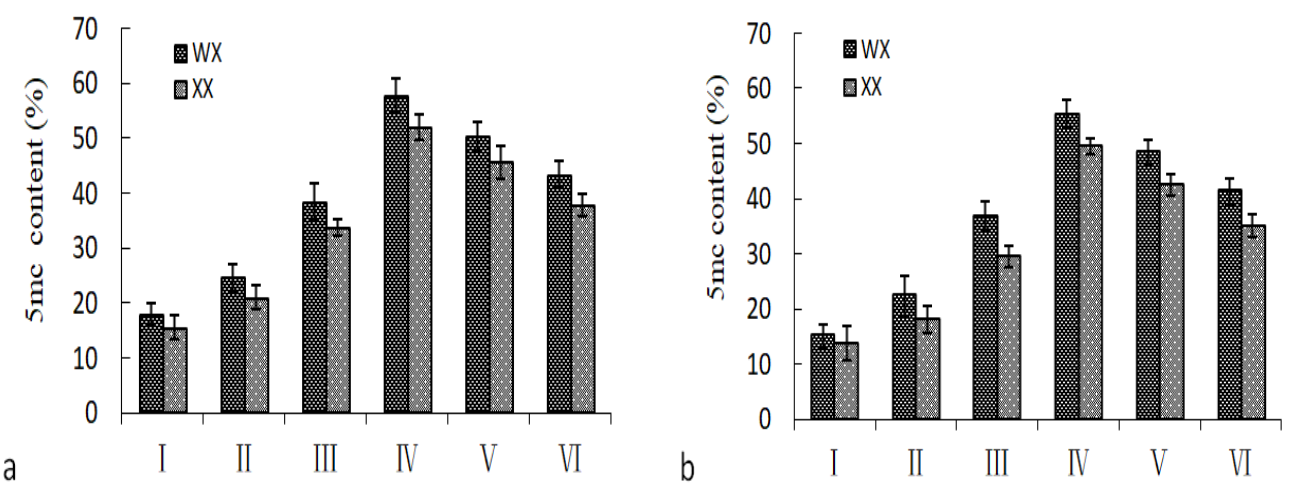

Fig. 3. 5mC content in genome of *Rehmannia glutinosa*
(a) 5mC content in root genome of *R. glutinosa* at different growth stages, (b) 5mC content in leaf genome of *R. glutinosa* at different growth stages.WX and XX represented *R. glutinosa* planted in Wenxian or Xinxiang, respectively. Ⅰ, Ⅱ, Ⅲ, Ⅳ, Ⅴ and Ⅵ respectively represented the different growth stage of *R. glutinosa*, and the error bar was the standard error of mean.

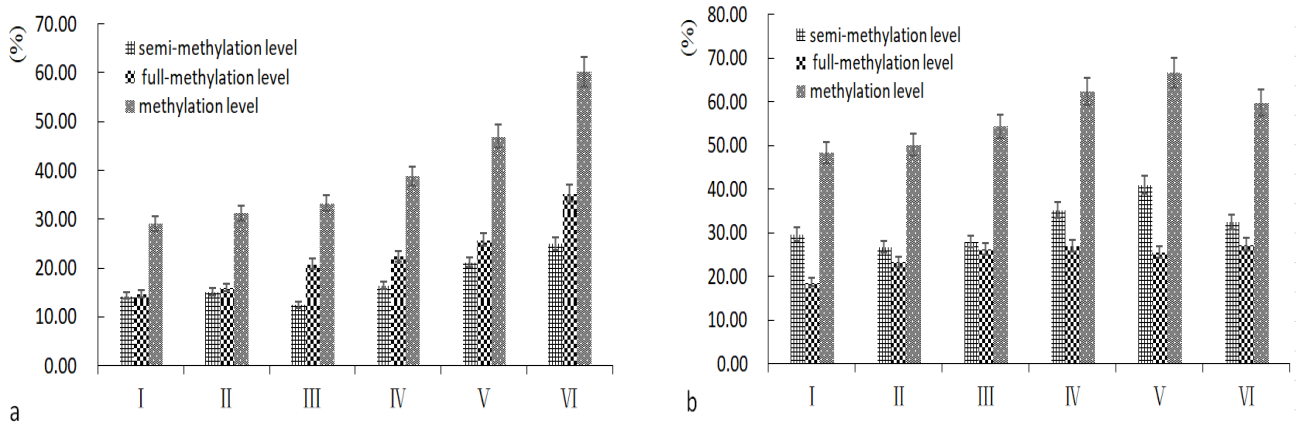

Fig. 4. The level of genomic methylation in leaf of *Rehmannia glutinosa*
(a) Genomic methylation level of *R. glutinosa* leaf in Wenxian, (b) Genomic methylation level of *R. glutinosa* leaf in Xinxiang. Ⅰ, Ⅱ, Ⅲ, Ⅳ, Ⅴ and Ⅵ represented the different growth stage of *R. glutinosa*, respectively. The error bar was the standard error of mean.

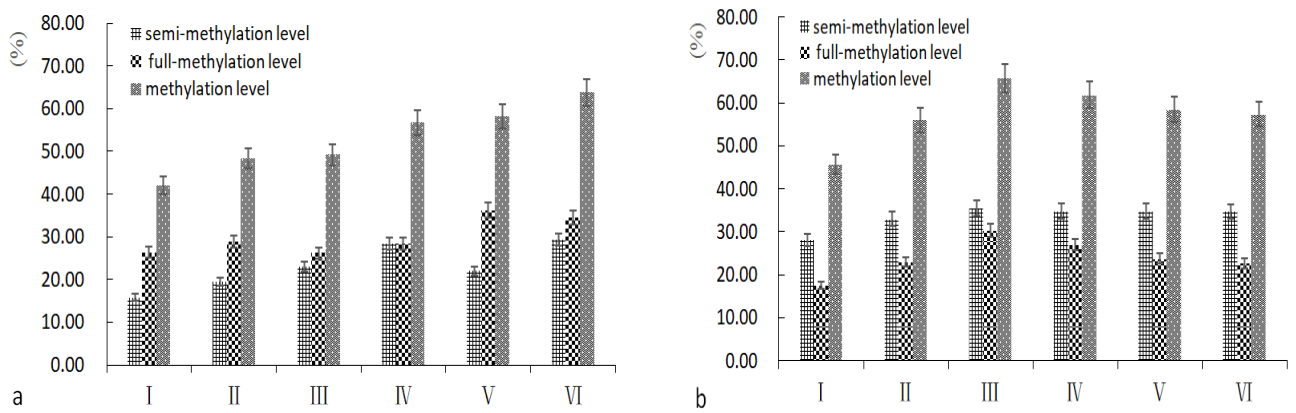

Fig. 5. The level of genomic methylation in root of *Rehmannia glutinosa*
(a) Genomic methylation level of *R. glutinosa* root in Wenxian, (b) Genomic methylation level of *R. glutinosa* root in Xinxiang. Ⅰ, Ⅱ, Ⅲ, Ⅳ, Ⅴ and Ⅵ represented the different growth stage of *R. glutinosa*, respectively. The error bar was the standard error of mean.

Discussion

DNA methylation could influence the growth and development of plant, and is very important to regulate gene expression in tissue specificity or development stage of plant (Finnegan *et al*., 2000; Duan *et al*., 2016). However, methylation level of genomic DNA has large differences in different tissues and development stages of plant (Solís *et al*., 2012), for example, embryonic development and seed viability of *Arabidopsis thaliana* is associated with DNA methylation level (Xiao *et al*., 2006). The frequent occurrence of DNA methylation in *Navel orange* leads to higher budding frequency (Hong & Deng, 2005). In this study, 5mC content and methylation level increased along with the growth and development of *R. glutinosa*, and peaked at Ⅳ growth stage. However, genomic methylation in leaf and root was different during the same growth stage, and was higher in leaf of *R. glutinosa*. Thus, genomic methylation of *R. glutinosa* has the specificity of tissue and growth stages, which is also found in other plants, such as *rice* (Xiong *et al*., 1999), *tomato* (Messeguer *et al*., 1991) and *bamboo* (Guo *et al*., 2011).

In this study, along with the growth of *R. glutinosa*, plant height and leaf length increased and the accumulation of catalpol also increased at the early growth stage of *R. glutinosa* and reached maximum at Ⅴ growth stage, which was consistent with research result of wang (Wang *et al*., 2004). Compared with that in Xinxiang, plant height and leaf length of *R. glutinosa* were higher in Wenxian, the accumulation of catalpol in root and leaf of *R. glutinosa* was always higher in Wenxian. Perhaps Wenxian is Geo-authentic habitats of *R. glutinosa*, similar results were also found by Du & Li (2013). Furthermore, genomic methylation of *R. glutinosa* in Xinxiang and Wenxian was also different, in which 5mC content of *R. glutinosa* planted in Wenxian was significantly higher, genomic methylation level of *R. glutinosa* in Wenxian increased along with the extension of growth stage, and full-methylation was main form of genomic methylation, however genomic methylation level in Xinxiang increased first and then decreased along with the growth of *R. glutinosa*, and semi-methylation was main form of genomic methylation. These results might confirm that methylation status of plant genome is also specific for specific environment, and plant could respond to different environment by changing methylation of genomic DNA, furthermore, genomic methylation of *R. glutinosa* may be related to the formation of authentic medicinal materials, but this relationship between DNA methylation and catalpol of *R. glutinosa* need to be further studied.

Acknowledgments

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