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Full Length Article

Effects of DNA Methylation on Growth and Development of *Rehmannia glutinosa*

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Abstract

In this study, 5mC content and methylation level increased along with the growth of *Rehmannia glutinosa*, but degree of DNA methylation was different in leaf and root, and hemi-methylation level was both higher than full-methylation level in leaf or root, which was very significant in root. Furthermore, 5-azaC could inhibit DNA methylation of *R. glutinosa*, compared with the control, although 5mC content was less and showed a decreasing trend along with the increase of 5-azaC concentration, it still increased along with the growth of *R. glutinosa* and reached the peak in Stage IV. In addition, as treated with 5-azaC, DNA methylation of *R. glutinosa* had significant polymorphism, methylation status would change and appear significant demethylation, and degree of DNA demethylation increased along with the increasing concentration of 5-azaC. Further analysis found, under the treatment of 5-azaC, plant height and leaf length were both lower than the control, still increased along with the growth of *R. glutinosa*, however phenotype of *R. glutinosa* could not appear significant change along with the increase of 5-azaC concentration. Thus, under the reduction of DNA methylation, *R. glutinosa* appeared obvious dwarfing phenomenon with smaller leaf, indicating moderate demethylation could inhibit growth and development of *R. glutinosa*, which might be closely related to the status of genomic DNA methylation. © 2018 Friends Science Publishers

Keywords: *Rehmannia glutinosa*; DNA methylation; 5-azaC; Phenotype
Abbreviations: C, Cytosine; CTAB, Cetyl Trimethylammonium Bromide; 5-azaC, 5-azacytidine; 5mC, 5-methycytosine; HPLC, High Performance Liquid Chromatography; PCR, Polymerase Chain Reaction; MSAP, Methylation-sensitive Amplified Polymorphism

Introduction

DNA methylation is one of the most covalent modification in living cells and epigenetic modifications, and can lead to cell differentiation, chromatin inactivation, embryo growth and cancer (Courtier *et al*., 1995; Gonzalgo and Jones, 1997; Yuan *et al*., 2004). DNA methylation is also a vital regulation mechanism of gene expression in many biological processes, such as morphogenesis, development, stress, and alteration (Gaur *et al*., 2012). In the nuclear genome of higher plant, there are about 20–30% cytosine with methylation, and the level of DNA methylation shows large differences in different plants, different tissues, and others (Osabe *et al*., 2014). If the level of DNA methylation is insufficient in growth and development of plant, plant would appear abnormal growth and phenotype (Ronemus *et al*., 1996; Kenneth *et al*., 2006; Marfil *et al*., 2009), for example, plant height becomes shorter, leaf is smaller, and some phenotypic changes can be inherited. Thus, DNA methylation is essential to the normal growth and development of plant.

At the same time, DNA methylation could control the formation of plant tissue morphogenesis and maintain its genetic stability, especially for seeds grown in vitro or cryopreserved seeds (Kaeppler *et al*., 2000; Harding, 2004). At present, study of DNA methylation has been carried out in some plants, such as tissue culture of *Potato* under osmotic stress (Harding, 1994), tissue culture of grape (Harding *et al*., 1996), embryonic development of Siberian ginseng (Chakrabarty *et al*., 2003), flowering and vernalization of chicory (Demeulemeester *et al*., 1999), morphogenesis induction of *Arabidopsis* (Richards *et al*., 1997). DNA methylation inhibitor is base analogue, could reduce the level of DNA methylation, and is composed of 5-azacytidine (5-azaC) and 5-aza-cytosine (5-aza-dC) (Nie and Wang, 2007). The phenotype and development of some plants have been altered by DNA methylation inhibitor 5-azaC, and the altered methylation status could be inherited to offsprings of plants, such as rice (Sano *et al*., 1990), triticale (Heslop-Harrison, 1990), cabbage (Amado *et al*., 1997), tobacco (Vyskot *et al*., 1995), Female lou (Janoušek *et al*., 1996), flax (Fieldes *et al*., 2005), etc.

Rehmannia glutinosa belongs to Scrophulariaceae. *Rehmannia* is a perennial herbaceous plant and pertains to one of the Chinese medicinal materials. Recently, some studies of *R. glutinosa* have been made on physiology, biochemistry, morphology, molecular and other research fields, but research on epigenetics of *R. glutinosa* has not been reported, and the relationship between DNA methylation and growth of *R. glutinosa* has not been explored. In order to study effects of DNA methylation in growth and development of *R. glutinosa*, genomic DNA methylation and phenotype of *R. glutinosa* were analyzed in this research, furthermore, DNA methylation inhibitor 5-azaC was also used to explore the possible relationship between DNA methylation and phenotype of *R. glutinosa*.

Materials and Methods

Plant Materials

In this study, *R. glutinosa* cultivar (85-5) was chosen, and its root tubers were kindly provided by Agricultural Research Institute of Wenxian County, Henan, China. Primers and adapters were synthesized by YingjieJi Trade Co., Ltd., (Shanghai, China) and their sequences are listed in Table S1.

Treatment and Cultivation of *R. glutinosa*

After sterilized for 10 min by 0.1% $HgCl_2$, root tubers of *R. glutinosa* were washed for 3–5 min with sterile water, and were wrapped with gauze, then were respectively treated for 7d (3 times/d) with 0, 15, 30, 50, 100 or 250 μM 5-azaC. Subsequently, root tubers treated with 5-azaC were grown in test field, College of Life Science, Henan Normal University, Xinxiang City, Henan Province, China. In addition, 100 sterilized roots of *R. glutinosa* were treated with 5-azaC in each group, and there were three replicates per group.

Plant height or leaf length of *R. glutinosa* treated with 5-azaC was observed and statistically analyzed in six growth stages of *R. glutinosa*. The growth stages of *R. glutinosa* are usually divided according to the appearance characteristics of root growth (Fig. S1). The details of growth stages are:

Stage I: seedling root is not fleshy
Stage II: plant root is fleshy and cylindrical
Stage III: plant root appears preliminary expansion
Stage IV: plant root appears middle expansion
Stage V: plant root appears late expansion
Stage VI: plant root is spindle-shaped

Tuberous root and leaf of *R. glutinosa* at each growth stage were frozen in liquid nitrogen and stored at -80ºC.

DNA Extraction of *R. glutinosa*

Genomic DNA was extracted from root and leaf of *R. glutinosa* by CTAB method with modifications. About 4 g tuberous root or leaf was put into the pre-cooled mortar and ground into the powder, then immediately transferred to 50 mL centrifuge tube. In the centrifuge tube, 5 mL/g CTAB solution containing two-thousandths of β-mercaptoethanol was added, and put in water bath at 65°C for 2 h accompanied by upside down mixing 1 times/20 min, centrifuged for 10 min at 12000 rpm. Afterwards, the supernatant was transferred, extracted by phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged for 10 min at 12000 rpm. The above process was repeated once, and then the supernatant with isopropyl alcohol was placed for more than 1h and separated by 12000 rpm centrifugation for 15 min. Precipitated DNA was washed twice with 70% ethanol, and dissolved in double-distilled water after air dried for at least 5 min, then 1%RNase was added to DNA solution; put at 37°C for 30 min and extracted with phenol:chloroform:isoamyl alcohol (25:24:1). After further extraction, DNA solution was precipitated by adding 1/3 volume of 3 M NaAc and 2.5 volumes of absolute ethanol, and centrifuged at 12000 rpm for 10 min, then precipitated DNA was washed twice with 70% ethanol and centrifuged at 12000 rpm for 5 min. Subsequently, dried DNA was dissolved in double-distilled water and was stored at -20°C to analyze genomic DNA methylation.

In addition, the integrity of genomic DNA was detected by 0.8% agarose gel electrophoresis, and the yield and purity of genomic DNA were respectively determined by spectrophotometry at 260nm.

HPLC Detection of Genomic Methylation

The content of 5-methyl cytosine (5mC) usually indicates the level of genomic DNA methylation. In this research, level of DNA methylation was detected by HPLC (high performance liquid chromatography). Genomic DNA was hydrolyzed with DNase I, nuclease P1 and alkaline phosphatase, was centrifuged for 5 min at 12000 rpm, then the supernatant was transferred and filtered with 0.45 μm microporous membrane, subsequently was detected by HPLC. The following conditions of HPLC were performed in this experiment; the flow velocity was 0.5 mL/min, the temperature of column was 30°C, the mobile phase was 50 m*M* KH2PO4:8% methanol, the analytical column was Agilent C18 Zorbax XDB column (4.6×150 mm, 5 μM particle size), and the detection wavelength was 285 nm by VWD detector.

The standard substances of 0.0044 g C and 0.0065 g 5mC were respectively dissolved with 10 mL ultrapure water to 40 nM/100 μL (mother liquor), and the mother liquor was diluted to 0.02, 0.05, 0.1, 0.5 and 1.0 nM/100 μL, separately. Each concentration was detected, and the linear equation of standard substance was obtained. The linear equation of standard C was $y = 2172.7x-18.537$, $R^2 = 0.9993$ (y: peak area, x: concentration); the linear equation of standard 5mC was $y = 2276x-7.9806$, $R^2 = 0.9997$

(y: peak area, x: concentration), indicating that C and 5mC both had a good linear relationship in the range of 0.02–1.0 nM/100 μL. Based on the above linear equation of standard substances, the concentration of C and 5mC in genomic DNA of *R. glutinosa* was respectively calculated, then the content of 5mC was obtained according to the following formula: 5mC = [5mC / (5mC + C)] ×100. Inaddition, the 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.

Methylation-Sensitive Amplified Polymorphism (MSAP) Amplification

The MSAP experiment was performed with modifications according to the method of Vos *et al*. (1995). Under the condition of without changing methylation status, HpaII and MspI have different sensitivity to DNA methylation. HpaII is not sensitive to full methylation (double-stranded-methylation) and could cleave hemi-methylation (single-stranded-methylation), MspI is sensitive to the internal cytosine (CmCGG sequence) and not to the external cytosine (mCCGG sequence) in full methylation. Thus, HpaII and MspI were selected to produce different cleavage fragments which can reveal status and extent of genomic DNA methylation. Genomic DNA of *R. glutinosa* was digested with endonuclease EcoRI/MspI, EcoRI/HpaII, in turn, then was ligated with EcoRI adapter and MspI-HpaII adapter by T4 DNA ligase at 16ºC for 15 h, subsequently Enzyme-Ligation product was stored at -20ºC.

After the pre-amplification system of MSAP was established, Enzyme-ligation product was diluted 10-fold and amplified as a pre-amplification template. The reaction system of MSAP pre-amplification was 50 μL and consisted of 2.5 μM EcoRI pre-amplification primer, 2.5 μM MspI-HpaII pre-amplification primer, 25 μL 2×Taq Mix, 0.5 μL Enzyme-Ligation product, and cycling conditions of PCR pre-amplification were listed in Table S2. After amplification, results of pre-amplification were detected by 1% agarose gel electrophoresis, the pre-amplification product was diluted 10-fold and amplified as template in MSAP selective amplification. The reaction system of MSAP selective amplification was 20 μL and composed of 5 μM EcoRI selective amplification primer, 5 μM MspI/HpaII selective amplification primer, 10 μL 2×Taq Mix and 0.5 μL pre-amplification product. Furthermore, the reaction condition of MSAP selective amplification was the same as pre-amplification except without Pre-PCR_1, subsequently amplification products were detected by 6.0% polyacrylamide gel electrophoresis.

MSAP Data Analysis

In this research, DNA methylation level was quantified by MSAP binary data, the presence or absence of one band was respectively scored as "1" and "0", only clear and reproducible bands were scored after silver staining. For MSAP analysis, bands were scored on the basis of presence or absence in EcoRI/HpaII (H) and EcoRI/MspI (M), and the banding patterns 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 II), and the presence of band only in M was considered DNA full-methylation (class III). In addition, DNA methylation level was calculated with the following formulate: DNA methylation level (%) = (bands of class II+ bands of class III)/(bands of class I+ bands of class II+ bands of class III) ×100, DNA hemi-methylation level (%) = bands of class II/(bands of class I+ bands of class II+ bands of class III) ×100, DNA full-methylation level (%) = bands of class III/(bands of class I+ bands of class II+ bands of class III) ×100.

Compared with the control, DNA methylation patterns of *R. glutinosa* treated with 5-azaC were classified into methylation polymorphism and methylation monomorphism. DNA methylation monomorphism was regarded Type A, DNA methylation polymorphisms included type B (DNA methylation) and type C (DNA demethylation).

Statistical Analysis

In this research, plant height, leaf length, 5mC content and methylation level of *R. glutinosa* were tested for significance level, by using analysis of variance and multiple comparisons of Duncan's multiple range. Content of 5mC and level of DNA methyltion were calculated and analyzed by Excel and DPS7.5, and all histograms were drawn by Origin.

Results

Content of 5mC in Genomic DNA of *R. glutinosa*

As shown in Fig. 1, compared with that in Stage I of *R. glutinosa*, 5mC content in genome of leaf and root both increased along with the growth of *R. glutinosa*, and was the highest in Stage IV, in which 5mC content in genome of leaf and root was respectively 52.0 or 49.5% or so. Subsequently, 5mC content decreased in the later growth stage (Stage V and Stage VI) of *R. glutinosa*, but was still higher than that in the first three stages (Stage I, Stage II and Stage III). For example, 5mC content in genome of leaf and root was about 37.8 and 35.0%, respectively in Stage VI, but was about 33.7or 29.5% in Stage III of *R. glutinosa*.

Furthermore, during the same growth stage of *R. glutinosa*, 5mC content in genome of leaf and root was different, and was higher in leaf genome of *R. glutinosa*, thus the degree of DNA methylation in *R. glutinosa* had specificity of tissue and growth stage.

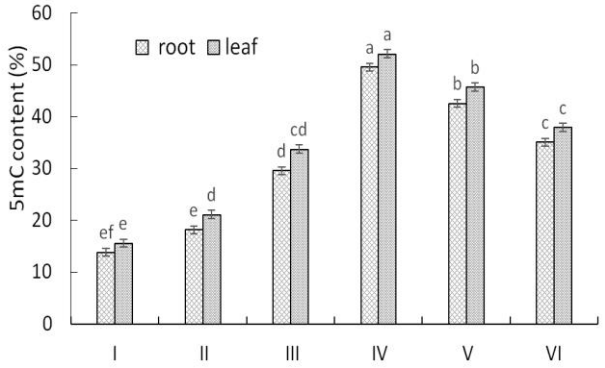

Fig. 1: The content of 5mC in leaf and root of *R. glutinosa*
I, II, III, IV, V and VIrepresented different growth stage of *R. glutinosa*, respectively. The error bar was standard error of mean, and these different lower letters above bar respectively indicated the significant difference in leaf or root among different growth stages of *R. glutinosa* (P < 0.05)

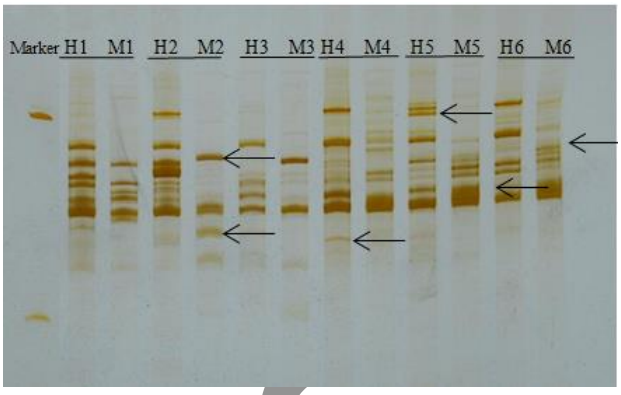

Fig. 2: MSAP amplification bands in root of *R. glutinosa* by E3 /HM4 primer combination
H and M represented bands from genome digested by *Eco*RI/*Hpa*II(H) and *Eco*RI/*Msp*I (M), M: Marker, 2000bp ladder; The arrows only indicated some polymorphic methylation bands between H1M1, H2M2, H3M3, H4M4, H5M5 and H6M6

Level of Genomic Methylation in *R. glutinosa*

In this study, HpaII and MspI with different sensitivity to DNA methylation were selected to produce different cleavage fragments, 6 primer combinations were screened from 48 primer combinations, and 39 DNA polymorphic locis were found. Some selective amplification results are shown in Fig. 2, the polymorphism of bands represented change in DNA methylation.

As can be seen from Fig. 3, methylation level in leaf or root was different during the growth of *R. glutinosa*, and increased along with the extension of growth time. For example, methylation level in leaf or root was about 48.35 and 45.63% in Stage I, and was significantly lower than that in other growth stages (P<0.05), especially was higher in Stage IV and Stage V of *R. glutinosa*. Thus, methylation level was lower in the first growth stages, but was higher in other growth stages of *R. glutinosa*, and was different in root and leaf during the same growth stage of *R. glutinosa*.

In addition, full-methylation level in leaf was also lower in Stage I (18.68% or so), and was about 25.60–27.40% in other growth stages, however hemi-methylation level in leaf was relatively stable and was about 28.0–32.5% except Stage V (Fig. 3a). Full-methylation level in root was about 27.26% in Stage IV, and was also higher than that in other growth stages (*P*<0.05). Compared to that in Stage I, hemi-methylation level in root was higher during the growth of *R. glutinosa*, especially was the highest (42.11%) in Stage V (Fig. 3b).

Further analysis showed that hemi-methylation level was higher than full-methylation level in leaf or root, which was very significant in root (P<0.05). This difference was highly significant in some growth stages of *R. glutinosa* (P<0.01), for example, the level of hemi-methylation or full-methylation in leaf was 29.67 and 18.68%, respectively in Stage I, and was 41.03or 25.64%, respectively in Stage V (Fig. 3a).

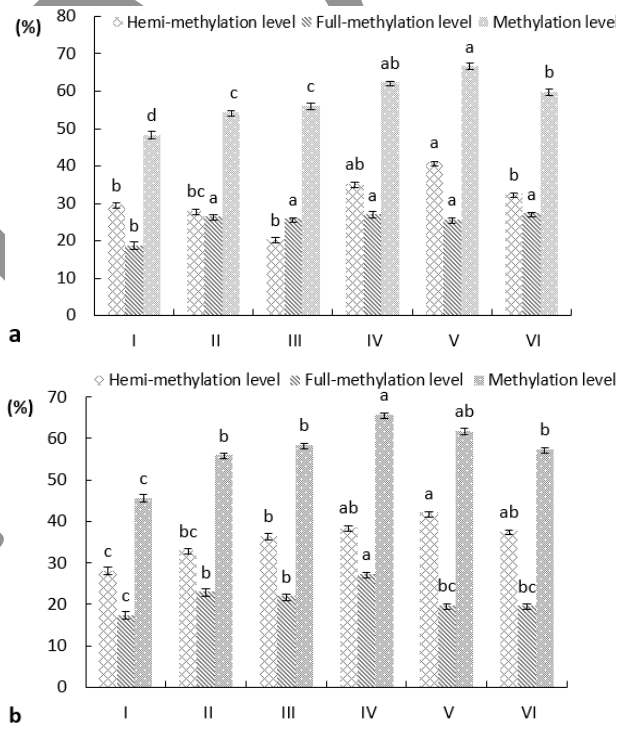

Fig. 3: The level of genomic methylation in *R. glutinosa*
a. The level of DNA methylation in leaf of *R. glutinosa*, b. The level of DNA methylation in root of *R. glutinosa*. I, II, III, IV, V and VI represented different growth stage of *R. glutinosa*, respectively. The error bar was standard error of mean, and these different lower letters above bar respectively indicated significant difference of methylation among different growth stages of *R. glutinosa* (P < 0.05)

In root of *R. glutinosa*, the level of hemi-methylation or full-methylation was 42.11 and 19.74%, respectively in Stage V, and was 37.70 or 19.67% in Stage VI (Fig. 3b). Thus, the hemi-methylation maybe dominant in genome of *R. glutinosa*.

Effects of 5-azaC on Phenotype of *R. glutinosa*

Compared with the control, plant height of *R. glutinosa* was lower as treated with 5-azaC, but still increased along with the growth of *R. glutinosa*. Plant height of *R. glutinosa* in Stage IV was shown in Fig. 4a, under the treatment of 5-azaC, plant height of *R. glutinosa* was 20 cm or so, and was significantly lower than the control (29.0 cm) ($P<0.05$), especially was only 18.2 cm as treated with 15 μM 5-azaC. However, plant height of *R. glutinosa* could not appear significant change along with the increase of 5-azaC concentration, so 15–250 μM 5-azaC had analogous and significant effect on plant height of *R. glutinosa*.

Along with the growth of *R. glutinosa*, leaf was longer and longer, and the growth trend of leaf was similar to that of plant height, but was shorter than the control. As shown in Fig. 4b, leaf length of *R. glutinosa* in Stage IV was shorter under the treatment of 5-azaC, compared with the control, leaf length was shorter, and was significantly influenced except 15 μM 5-azaC ($P<0.05$). For example, as treated with 30–250 μM 5-azaC, leaf length of *R. glutinosa* was only 14.0 cm or so, was significantly lower than the control ($P<0.05$), but would not show significant change along with the increase of 5-azaC concentration, so 30–250 μM 5-azaC had analogous and significant effect on leaf growth of *R. glutinosa*.

Thus, under the treatment of 30–250 μM 5-azaC, *R. glutinosa* appeared obvious dwarfing phenomenon with smaller leaf, indicating that *R. glutinosa* would be more sensitive to this concentration of 5-azaC which had significant inhibitory effect on growth and development of *R. glutinosa*.

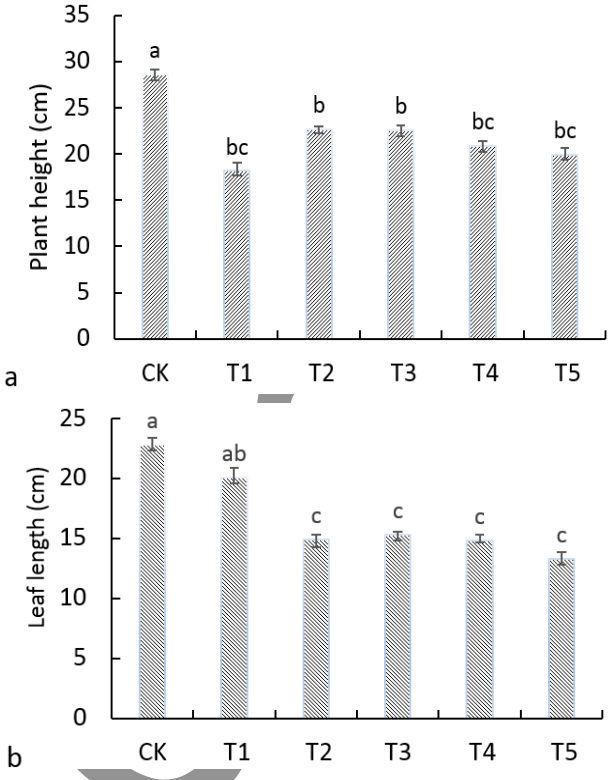

Fig. 4: Effect of 5-azaC on phenotype of *R. glutinosa*
a. The plant height of *R.glutinosa*, b. The leaf length of *R.glutinosa*. CK, T1, T2, T3, T4 and T5 respectively represented *R.glutinosa* treated with 0, 15, 30, 50, 100 or 250μM 5-azaC. The error bar was standard error of mean, and these different lower letters above bar respectively indicated the significant difference among *R.glutinosa* treated with different concentration of 5-azaC ($P < 0.05$)

Effect of 5-azaC on the Degree of Genomic Methylation

As shown in Fig. 5a, compared with the control, 5mC content was less in root genome of *R. glutinosa* treated with 5-azaC, but increased along with the growth of *R. glutinosa*, and reached the peak in Stage IV. Similarly, under the treatment of 5-azaC, although 5mC content was less in leaf genome, still increased along with the growth of *R. glutinosa*, and also reached the peak in Stage IV (Fig. 5b), indicating that 5-azaC could inhibit DNA methylation of *R. glutinosa*, and 5mC content in root and leaf was higher in the maturity stage of *R. glutinosa*.

Further analysis showed that during the same growth stage of *R. glutinosa*, 5mC content in genome of leaf and root both showed a decreasing trend along with the increase of 5-azaC concentration, especially was significantly less as treated with 50–250 μM 5-azaC ($P<0.05$). For example, when *R. glutinosa* was treated with 50, 100 or 250 μM 5-azaC, 5mC content in leaf genome was 40.75, 35.96 or 29.83%, respectively in Stage IV, and significantly lower than the control (52%) (Fig. 5c). As treated with the same concentration of 5-azaC, 5mC content in genome of root and leaf was also different, and was higher in leaf genome, thus effects of 5-azaC on DNA methylation degree in root and leaf were different.

Pattern of Genomic Methylation in *R. glutinosa* Treated with 5-azaC

The change of genomic DNA methylation status of *R. glutinosa* in Stage IV was analyzed by MSAP, compared with the control, DNA methylation status of *R. glutinosa* treated with 5-azaC was mainly divided into three major types with 12 kinds of band type (Table 1). Type A was monomorphic band, indicating that methylation status at CCGG site would keep constant, methylation pattern of *R. glutinosa* were the same between the control and treatment group, in which A1, A2 and A3 was regarded to be non-methylation model, hemi-methylation model or full-methylation model, respectively.

Type B and C were polymorphic bands, and Type B was remethylation band pattern. Compared to the control, methylation pattern of B1-B2, B3-B5 possibly indicated that *de novo* methylation or hyper-methylation could occur under the treatment of DNA methylation inhibitor 5-azaC, respectively.

Table 1: DNA methylation pattern of *R. glutinosa* treated with 5-azaC

Type	Digestion				Changes of methylation patterns		Types of methylation pattern	
	H1	M1	Hx	Mx	CK	Treatment		
1	1	1	1	1	CCGG GGCC	CCGG GGCC	A1	A
2	1	0	1	0	CCGG CCGG GGCC GGCC	CCGG CCGG GGCC GGCC	A2	
3	0	1	0	1	CCGG GGCC	CCGG GGCC	A3	
4	1	1	1	0	CCGG GGCC	CCGG CCGG GGCC GGCC	B1	B
5	1	1	0	1	CCGG GGCC	CCGG GGCC	B2	
6	0	1	0	0	CCGG GGCC	CCGG GGCC	B3	
7	1	0	0	0	CCGG CCGG GGCC GGCC	CCGG GGCC	B4	
8	1	1	0	0	CCGG GGCC	CCGG GGCC	B5	
9	0	1	1	1	CCGG GGCC	CCGG GGCC	C1	C
10	1	0	1	1	CCGG CCGG GGCC GGCC	CCGG GGCC	C2	
11	0	0	0	1	CCGG GGCC	CCGG GGCC	C3	
12	0	0	1	1	CCGG GGCC	CCGG GGCC	C4	

H1 and M1 represented bands from genome digested with *Eco*RI/*Hpa*II (H) and *Eco*RI/*Msp*I (M) in *R. glutinosa* under normal condition, Hx and Mx represented bands from genome digested by H and M in *R. glutinosa* treated with 5-azaC. "1 1", "1 0" or "0 1" was considered to be pattern of no DNA methylation, pattern of DNA hemi-methylation and DNA full-methylation, respectively

Table 2: The change of DNA methylation pattern in *R. glutinosa* treated with 5-azaC

No. of change Sites Treatment	Leaf		Root	
	Type B	Type C	Type B	Type C
CK-15μM	4	9	3	7
CK-30μM	8	11	7	12
CK-50μM	9	21	7	20
CK-100μM	5	29	5	25
CK-250μM	2	34	2	26

Type B and Type C respectively indicated DNA remethylation type or DNA demethylation type, which represented the change of methylation pattern in *R. glutinosa* under the treatment of 15, 30, 50, 100 and 250μM 5-aza as compared with the control (CK)

Type C was demethylation band pattern, DNA methylation pattern of C1–C4 showed that demethylation would occur under the treatment of 5-azaC. In addition, some band patterns with indeterminable change of methylation were also found in this research (data not shown).

Change of Methylation Status in *R. glutinosa* Treated with 5-azaC

As shown in Table 2, genomic DNA methylation in leaf of *R. glutinosa* had significant polymorphism as treated with 5-azaC, there were 2–9 re-methylation sites (Type B) and 9–34 de-methylation sites (Type C), so change of DNA methylation might take de-methylation as principle and re-methylation as supplement in leaf of *R. glutinosa*. Furthermore, degree of DNA de-methylation in leaf was different as treated with different concentration of 5-azaC, and increased along with the increasing concentration of 5-azaC, especially was higher under the treatment of 100–250 μM 5-azaC, in which type C was respectively 29 and 34 as treated with 100 or 250 μM 5-azaC.

Similarly, DNA methylation also appeared significant polymorphism in root of *R. glutinosa* as treated with 5-azaC (Table 2), there were 2–7 re-methylation sites (Type B) and 7–26 de-methylation sites (Type C), and change of DNA methylation might also take de-methylation as principle in root of *R. glutinosa*. Further analysis showed that degree of DNA de-methylation also increased with the increasing concentration of 5-azaC, especially was higher with 25–26 de-methylation sites as treated with 100–250 μM 5-azaC. Therefore, under the treatment of 5-azaC, the status of genomic DNA methylation in *R. glutinosa* would change, appeared significant de-methylation, and effect of 5-azaC on methylation status might be almost same in leaf and root of *R. glutinosa*.

Discussion

DNA methylation could influence growth and development of plant mainly by regulating gene expression (Finnegan *et al*., 2000), and cytosine methylation is very important to regulate gene expression in tissue specificity or development stage of plant (Chan *et al*., 2005; Gehring and Henikoff, 2007). However, methylation level of genomic DNA has large differences in different tissues and development stages of plant (Finnegan *et al*., 1998; Chen *et al*., 2007; Solís *et al*., 2012). For example, embryonic development and seed viability of *A. thaliana* is associated with DNA methylation level (Wen *et al*., 2006), the frequent occurrence of DNA methylation in *Navel orange* leads to higher budding frequency (Hong and Deng, 2005). In this study, 5mC content and methylation level increased along with growth and development of *R. glutinosa*, and degree of DNA methylation was different in leaf and root during the same growth stage of *R. glutinosa*, which was higher in leaf of *R. glutinosa*. Thus, genomic DNA methylation of *R. glutinosa* had the specificity of tissue and growth stage,

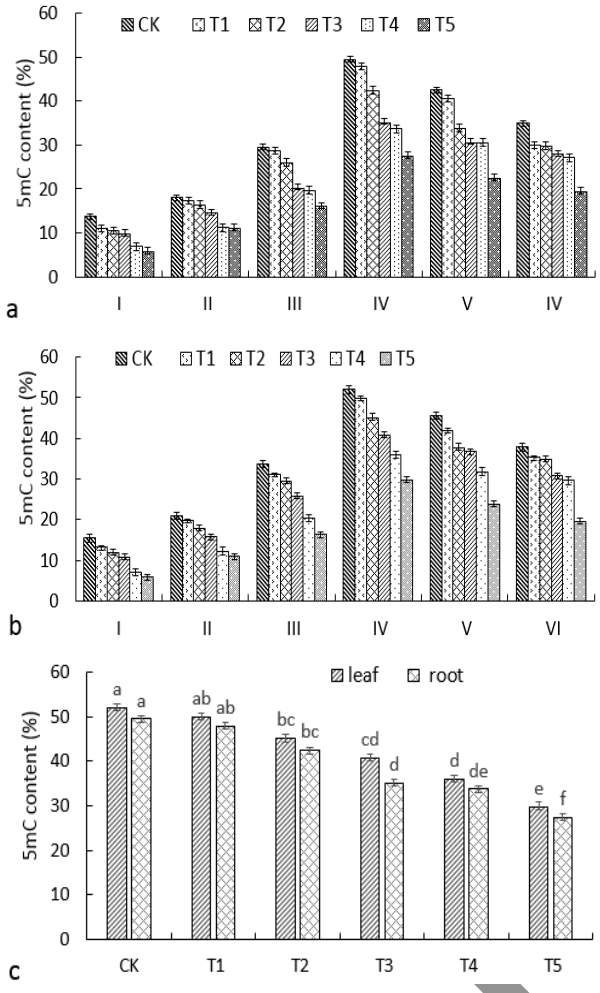

Fig. 5: Effect of 5-azaC on 5mC content of *R. glutinosa*

a.5mC content in root genome of *R. glutinosa* treated with 5-azaC, b. 5mC content in leaf genome of *R. glutinosa* treated with 5-azaC, c. 5mC content in genome of root and leaf during Stage IV of *R. glutinosa* treated with 5-azaC. CK, T1, T2, T3, T4 and T5 respectively represented *R. glutinosa* treated with 0, 15, 30, 50, 100 or 250μM 5-azaC. I, II, III, IV, V and VI represented the different growth stage of *R. glutinosa*, respectively. The error bar was standard error of mean, and these different lower letters above bar respectively indicated the significant difference of 5mC content among leaf or root of *R. glutinosa* ($P < 0.05$)

which was also found in other plants, such as rice (Xiong *et al*., 1999), tomato (Messeguer *et al*., 1991), rape (Lu *et al.*, 2005), bamboo (Guo *et al*., 2011), wheat (Duan *et al*., 2016), etc. Further analysis found that hemi-methylation level was higher than full-methylation level in leaf or root of *R. glutinosa*, which was very significant in root; therefore, hemi-methylation maybe dominant in genome of *R. glutinosa*.

As is well known, DNA methylation is essential for growth and development of plant. If DNA methylation is insufficient or too high, plant would show abnormal growth and shape (Ronemus *et al*., 1996; Kenneth *et al*., 2006; Marfil *et al*., 2009). DNA methylation inhibitor 5-azaC could decrease methylation degree in genomic DNA (Fieldes *et al*., 2005; Akimotoi *et al*., 2007), and has been widely used to regulate DNA methylation of plant, such as *A. thaliana* (Finnegan *et al*., 2000), rice (Akimotoi *et al*., 2007), *Linum usitatissimum* (Fieldes *et al*., 2005), wheat (Duan *et al*., 2016), etc. In this study, 5-azaC could also inhibit DNA methylation of *R. glutinosa*, which was relatively strong along with the increase of 5-azaC concentration, especially was significant as treated with 50–250 μM 5-azaC. Furthermore, under the treatment of 5-azaC, methylation status in *R. glutinosa* would change, appeared significant demethylation, and the degree of DNA demethylation increased along with the increasing concentration of 5-azaC, especially was higher at 100–250 μM 5-azaC. In addition, the reduction of genomic DNA methylation plays pleiotropic role in regulating growth and development of plant (Duan *et al*., 2016), for example, as treated with 5-azaC, *A. thaliana* (Burn *et al*., 1993), radish (Wang *et al*., 2005) and cabbage (Li *et al*., 2003) has early flowering time, the growth and development of cabbage or rice display some abnormalities, such as small leaf, dwarf plant and plexiform plant (Sano *et al*., 1990; King, 1995). In this study, compared with the control, *R. glutinosa* also appeared dwarf with smaller leaf under the treatment of 5-azaC, especially 30–250μM 5-azaC had a significant inhibitory effect on growth and development of *R. glutinosa*, this phenomenon was also found in other studies (Nie and Wang, 2007; Duan *et al*., 2016).

Conclusion

Taken together, the degree of genomic DNA methylation in *R. glutinosa* had specificity for tissues and growth stages, and the hemi-methylation maybe dominant in genome of *R. glutinosa*. Under the treatment of 5-azaC, degree of DNA methylation decreased in *R. glutinosa*, methylation status would appear significant demethylation, and the reduction of genomic DNA methylation would lead to dwarfing and small leaf of *R. glutinosa*. Therefore, DNA methylation should be important to growth and development of *R. glutinosa*, but the correlation between growth and DNA methylation of *R. glutinosa* need to be further studied.

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