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Full Length Research Paper

Methylation sensitive amplified polymorphism (MSAP) reveals that alkali stress triggers more DNA hypomethylation levels in cotton (*Gossypium hirsutum* L.) roots than salt stress

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Cytosine-5 hypomethylation or hypermethylation have been shown to regulate plant development and abiotic stress-responsive genes expression. However, some combinatory stresses in plants like saline and alkaline could have varied responses that could contribute to their adaptive evolution trends. We evaluated root and leaf DNA methylation patterns of cotton in salt (S, 9:1 molar ratio of NaCl to Na_2SO_4, pH 6.96) and alkali (A, 9:1 molar ratio of $NaHCO_3$ to Na_2CO_3, with higher pH 9.21) stresses using methylation sensitive amplified polymorphism (MSAP) method and found out that in general, demethylation events occurred more than methylation events in both leaves and roots samples. In addition, the total methylation variation frequency was only 1.38% in leaves and 2.2% in roots under salt stress. However, the alkali stress triggered more alterations and decreased the DNA methylation level significantly ($P<0.05$) compared to salt stress. This was up to 2.59% and 12.44% in the scored CCGG sites in leaves and roots, respectively. These results suggest that more adaptive and possibly complex gene expression alterations could be occurring in the tolerance of the cotton root in response to salt and alkali stresses coupled with DNA methylation alterations, although exclusion physiological mechanisms cannot be ruled out.

Key words: Salt stress, alkali stress, *Gossypium hirsutum* L., DNA methylation, methylation sensitive amplified polymorphism (MSAP).

INTRODUCTION

DNA methylation is one of the key epigenetic mechanisms among eukaryotes that can modulate gene expression without the changes of DNA sequence. However, in plants, it is the cytosine nucleotide in the genome which is methylated in the symmetrical contexts of CG, CNG (in which N is any nucleotide) and in the asymmetrical sites of CNN (N is either adenine [A], cytosine [C], or thymine [T]). It is reported that numerous cytosine methylation polymorphisms exist between different plants genotypes (Akimoto et al., 2007) and at different developmental stages (Lu et al., 2008). Meanwhile, much evidence indicates that environmental factors have substantial effects on cytosine methylation

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across the plant genome (Burn et al., 1993).

Cytosine-5 methylation, a prominent epigenetic modification, is established and maintained by multiple interacting cellular machineries (Chan et al., 2005; Gehring and Henikoff, 2007). It plays essential roles in plants resistance to various environmental stresses. High human population growth coupled with climate changes, industrial advances and the need to reclaim new agricultural lands have made breeders to encounter new dynamic abiotic and biotic stresses in the bid to have food, fiber and fuel production sufficiency. Salinity and alkalinity are two kinds of Na^+ stresses that have different effects on plant metabolism, especially on root physiology. Hence, there is need to evaluate and address possible influences of salt-alkali combinatory stresses to various key plant developmental processes like DNA methylation (Zhang et al, 2010).

Cotton (*Gossypium hirsutum* L.) has been cultivated widely in many alkaline lands of the world. As an important fiber crop, it has been categorized as a moderately salt tolerant plant with a salinity threshold level of 7.7 dSm-1 (equivalent to 77 mM NaCl). Studies have shown that its growth is severely reduced at high salinity levels, especially at the seedling stage (Ashraf and Ahmad, 2000). However, the responses of cotton to salt and alkali stresses, especially on the gene expression regulation mechanism of DNA methylation, have not been fully understood. The ability of plants to differently regulate gene expression and protein function has been described as a key factor in plant resistance (Lo´pez-Maury et al., 2008). In this regard, epigenetic mechanisms such as DNA methylation are expected to play a crucial role (Angers et al., 2010). Several previous researches have demonstrated that cytosine methylation categorically plays important role in regulating various biotic and abiotic stresses such as low temperature (Steward et al., 2000), water deficient (Labra et al., 2002), bacteria blight (Sha et al., 2005), ion implantation (Yu et al., 2011), hybridization (Hegarty et al., 2011), γ-irradiation (Kravets et al., 2010), heavy metals (Aina et al., 2004), low nutrients (Kou et al., 2011) and tissue culture (Gao et al., 2010; Dann and Wilson, 2011). Cytosine methylation increase in rapeseed (Lu et al., 2007) and decrease in wheat (Zhong et al., 2009) was detected under NaCl stress. However, the coincidence and disparate effects of salt stress (NaCl plus Na_2SO_4) and alkali stress ($NaHCO_3$ plus Na_2CO_3) on DNA cytosine methylation have not been previously investigated.

Although, salt stress has been studied well from physiological (Suárez and Medina, 2008) and molecular aspects (Yang et al., 2011), and DNA methylation has been proved to be correlated with salt tolerance (Chinnusamy and Zhu, 2009), most of the previous researches considered the individual effects of NaCl (Zhao et al., 2010), $NaHCO_3$ (Gao et al., 2008; Wang et al., 2007a, b, c, 2008; Nishiuchi et al., 2010) and Na_2CO_3 stress (Jin et al., 2006, 2008), with few studies focusing on their combined effect. The different physiological effects of salt stress and alkali stress have been studied in depth in previous researches (Yang et al., 2007, 2009; Chen et al., 2009; Li et al., 2009; Guo et al., 2010; Liu and Shi, 2010; Li et al., 2010), however, little is known about the general pattern of DNA methylation linked with cotton responses to salt stress and alkali stress, and its relationship with salinity and alkalinity tolerance.

Since the root is the primary plant organ that is in contact with saline and alkaline soils and higher-pH regulation procedure such as organic acid secretion, H^+ transportation, ionic flux regulation and CO_2 release through transpiration (Yang et al., 2010), we speculated that cotton roots regulate alkali stress through DNA methylation alteration and that there might be a significant relationship between the extent of methylation alteration and the resistance of salt stress and alkali stress. Therefore, we postulated that the results obtained on such a study would provide valuable breeding and adaptation information about the relationships between DNA methylation and salt/alkali stress responses in cotton for fibre production in marginalized regions.

MATERIALS AND METHODS

Seeds of cotton (*G. hirsutum* L.) were obtained from Xinjiang Province, China and were sown in 17-cm diameter plastic pots that contained 2.5 kg of washed sand. The pots were watered sufficiently with Hoagland nutrient solution every day. All pots were placed in an artificial greenhouse (25.0 ± 1.5°C during the day and 19.0 ± 1.5°C during the night). Plants grew at uniform irradiance in the greenhouse under a photoperiod of 15/9 h (light/dark).

Simulation and treatment of saline and alkaline conditions

Two neutral salts, NaCl and Na_2SO_4, were mixed in a molar ratio of 9:1 for the salt stress treatment (S), while two alkaline salts, $NaHCO_3$ and Na_2CO_3, were also mixed in a molar ratio of 9:1 for the alkali stress treatment (A). The applied stress intensity was 90 mM at pH 6.96 and 9.21 in salt stress and in alkali stress, while the conductivity was 9610 μs/cm in salt stress and 7120 μs/cm in alkali stress, respectively. The seedlings were subjected to stress treatments when they were 4 weeks old. Twelve pots each containing 1 seedling were randomly divided into 3 sets with 4 pots per set whereby each pot was considered a single replicate. One set was used as control (untreated) and the other two sets were treated with salt and alkali, respectively. Stress treatments were imposed daily around 17:00 to 18:00 h with the application of nutrient solutions containing the appropriate salts. All pots were watered thoroughly with treatment solution. Control plants were maintained by watering with nutrient solution.

DNA extraction and MSAP analysis

Genomic DNA was isolated from fresh fibrous leaves and roots of each individual cotton (*G. hirsutum* L.) using the modified cetyltrimethylammonium bromide (CTAB) method and purified by phenol extractions (Xu et al., 2000). The same DNA sample (300 ng) was digested by *EcoR*I plus *Hpa*II and *EcoR*I plus *Msp*I independently at the same time at 37°C for 5 h, and then ligated with *EcoR*I adapter and H/M adapter (Table 1) at 8°C for 4 h. The digested and ligated mixture were used in pre-selective amplification

Table 1. Sequences of adapters and pre-selective and selective primers used for MSAP analysis.

Parameter	*Eco*RI (E)	*Hpa*II/*Msp*I (H/M)
Adapter-1	CTCGTAGACTGCGTACC	GATCATGAGTCCTGCT
Adapter-2	AATTGGTACGCAGTC	CGAGCAGGACTCATGA
Pre-amplification primers	GACTGCGTACCAATTCA	ATCATGAGTCCTGCTGGT
Selective amplification primers	GACTGCGTACCAATTCAAG	ATCATGAGTCCTGCTCGG TCG
	GACTGCGTACCAATTCCC	ATCATGAGTCCTGCTCGG TTC
	GACTGCGTACCAATTCACG	ATCATGAGTCCTGCTCGG TTA
	GACTGCGTACCAATTCAGC	ATCATGAGTCCTGCTCGG TGA
	GACTGCGTACCAATTCAGA	ATCATGAGTCCTGCTCGG TGC
	GACTGCGTACCAATTCATC	ATCATGAGTCCTGCTCGG TAC

as templates with *E* +A and *H*/*M*+T primers (Table 1). The reaction was performed for 30 cycles of 30 s denaturation at 94°C, 45 s annealing at 56°C and 80 s extension at 72°C. The product was diluted 20-fold (v:v) with 1×TE buffer, and then used as the template for the selective amplification reaction. In this step, *Eco*RI and *H*/*M* primers with three additional selective nucleotides were used. The selective PCR was performed in a final volume of 15 µL following the protocol of Vos et al. (1995). The amplicons were fractionated by running through 5% denaturing polyacrylamide gels for 2.5 h at 55 W, 55°C, and visualized by silver staining.

*Hpa*II and *Msp*I, two isoschizomers possessing different sensitivity to cytosine methylation at 5´-CCGG-3´ sites, are employed as "frequent-cutter" enzymes in MSAP analysis. *Hpa*II will not cut if either of the cytosine is fully (double-strand) methylated, but will cut if the external C is hemi-methylated (single strand); in contrast, *Msp*I will not cut only if the external cytosine is fully- or hemi-methylated. Full methylation of the external or both cytosine prevent both enzymes cutting, which make these two methylation patterns indistinguishable by the MSAP technique. Therefore, the real methylation level in plant is a little higher than the results tabulated. For clarity, we hereby defined these two major types of cytosine methylation modifications as CG methylation (digested by *Eco*RI/*Msp*I but not by *Eco*RI/*Hpa*II) and CNG methylation (digested by *Eco*RI/*Hpa*II but not by *Eco*RI/*Msp*I).

Isolation and characterization of amplified fragments

Typical differentially methylated fragments representing different methylation patterns in cotton under salt stress (S) and alkali stress (A) in comparison with control (untreated) plants were excised from the gel with a razor blade, hydrated in 20 to 30 µL of 1×TE buffer and boiled for 5 min. The eluted DNA was amplified with the pre-selective amplification primers. Sequence information was obtained by cloning the fragments in the pMD18-T easy vector and sequencing individual clones. The sequences obtained were compared with nucleotide sequences in the publicly available databases using BlastN and BlastX (http://www.ncbi.nlm.nih.gov/).

Statistical treatments for the molecular data

Only clear and reproducible bands were scored. The scored MSAP bands were transformed into a binary character matrix, 1 for presence and 0 for absence of a band at a particular position in the MSAP profiles. In order to rule out confounding effect of nucleotide sequence change at the CCGG sites in the computed MSAP data, the method described by Cervera et al. (2002) was used. The levels of CG, CNG and the total methylation level for a given plant sample were calculated.

All data were represented by an average of the four replicates (independent plant individuals) and the standard errors (SE). The mean values were compared by post hoc least significant difference (LSD) test at p 0.05. Statistical analysis of the data was performed using the statistical software SPSS 14.0 (SPSS, Chicago, IL, USA).

RESULTS

Alkali stress decreased DNA methylation level significantly in cotton

In the treatment, one pair of pre-selective primers and 19 selective primes were used in MSAP analysis (Table 1), 909 and 1086 bands were detected in leaves and roots, respectively. After tabulating the number of bands representing CG and CNG methylation, we found that CG or CNG methylation level was higher in leaves than roots, and CG methylation occurred more frequently than CNG methylation whether in leaves or roots (Table 2). Furthermore, most of CCGG were shown to be largely unmethylated sites with the values being 63.67% for leaves and 78.87% for roots. As shown in Figure 1, the salt stress had no effect on DNA methylation level, whether in leaves or roots, but alkali stress decreased total methylation level in leaves ($P<0.05$) (Figure 1A) and both CG and CNG methylation level in roots ($P<0.05$) (Figure 1B).

DNA methylation alterations occurred predominantly in cotton root especially under alkali stress

To evaluate the different impacts of salt and alkali stresses on DNA cytosine methylation, only the bands present in all the four replicates were considered. Among the 1086 bands amplified in roots, 17 bands were induced only by salt stress, 114 bands were induced only by alkali stress and 12 bands were induced by both salt and alkali stresses (Figure 2). And among 909 bands in leaves, the

Table 2. Patterns of cytosine methylation in leaf and root of cotton seedlings.

Organ	Total amplified bands	Non-methylated CCGG sites		Methylated CCGG sites				Total methylated bands[c]	MSAP (%)[d]
		Sites	Ratios /%	Hemimethyl[a] CG sites	Ratio/%	Full methyl[b] CHG sites	Ratio/%		
Leaf	909	577	63.48	134	14.74	198	21.78	332	36.52
Root	1086	860	79.12	94	8.65	132	12.14	226	20.79

[a]Hemimethyl (ation) represents those digested by *Eco*RI/*Hpa*II but not by *Eco*RI/*Msp*I; [b]full methyl (ation) represents those digested by *Eco*RI/*Msp*I but not by *Eco*RI/*Hpa*II; [c]total methylated bands = a + b; [d]MSAP (%) = c / Total amplified bands * 100.

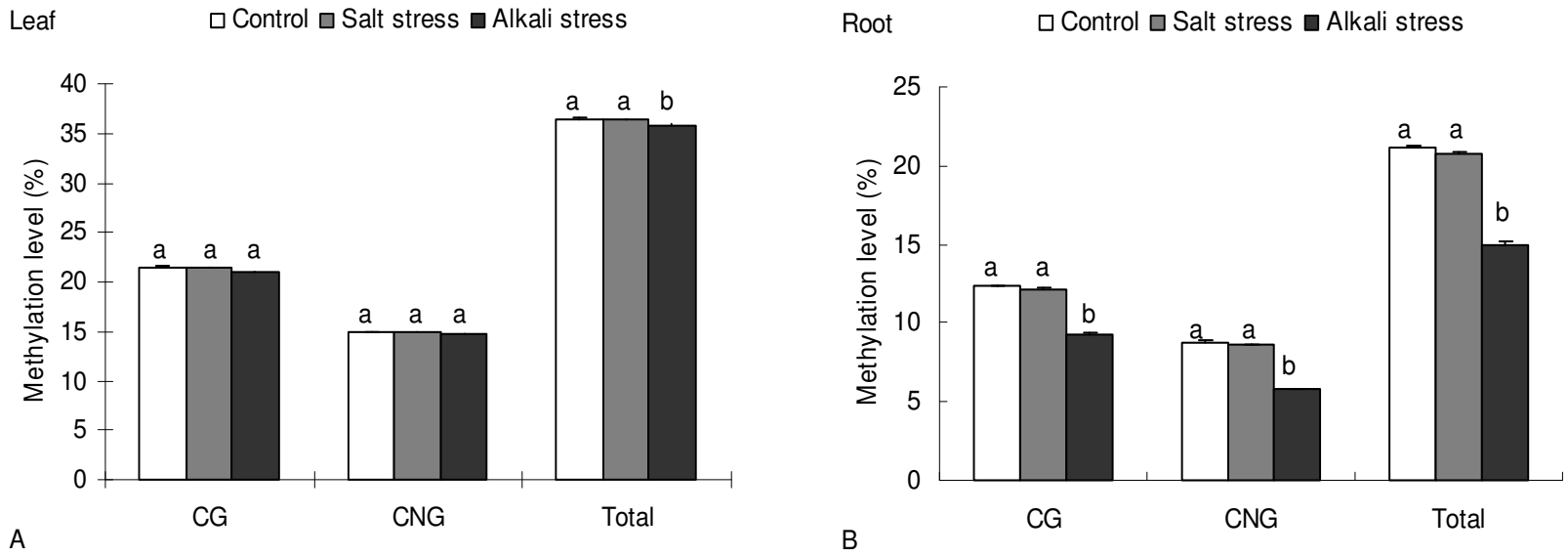

Figure 1. Effects of salt stress (9:1 molar ratio of NaCl to Na_2SO_4) and alkali stress (9:1 molar ratio of $NaHCO_3$ to Na_2CO_3) on DNA methylation level in cotton leaf (A) and root (B). The shown total methylation level is equivalent to CG+CNG methylation. Data represent means ± S.E. of four replicates, while the different letters represent significant difference among treatments at the 5% level according to the least significant difference (LSD) test.

corresponding numbers were 3, 9 and 15. These results show that alkali stress induced much more novel or caused the disappearance of bands. This meant that hyper/hypomethylation events mainly occurred in cotton subsequent to alkali stress in which the DNA methylation alterations were predominantly focused on the cotton roots (Table 3).

The amplified bands in cotton under salt and alkali stresses were classified into three categories, in contrast with untreated plant; no polymorphic bands (A-C), demethylation events (D-H) and methylation events (I-M) (Table 3). According to different cytosine sites methylated, methylation alteration could be classified into CG, CNG and

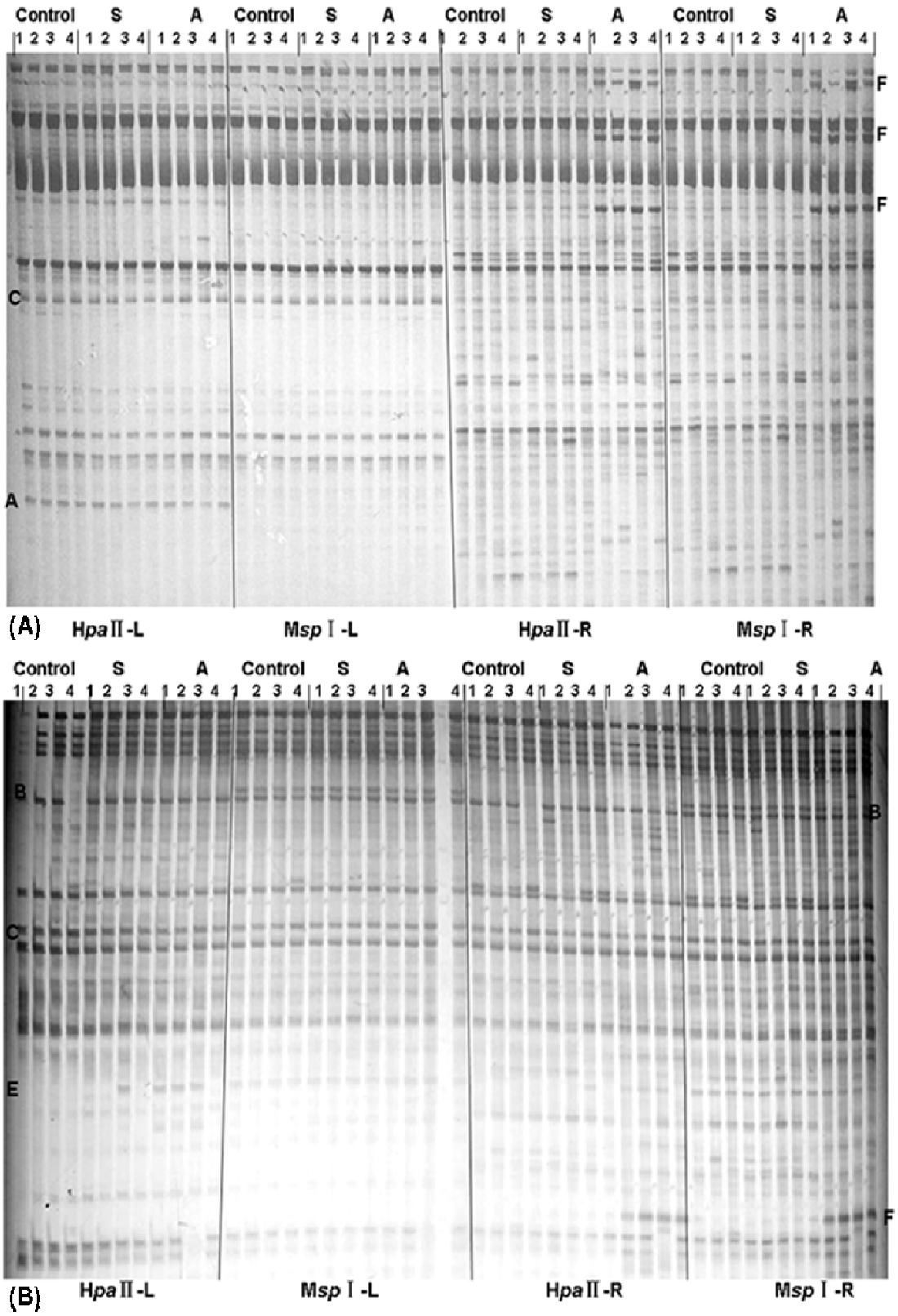

Figure 2. Examples of MSAP profiles showing the various types of variation in cytosine methylation patterns at 5'-CCGG-3' sites in leaf (L) and root (R) of cotton experiencing salt stress (S, 9:1 molar ratio of NaCl to Na_2SO_4) and alkali stress (A, 9:1 molar ratio of $NaHCO_3$ to Na_2CO_3) in comparison with control (untreated) plants, using primer combinations E-AAG/HM-TTC (A) and E-ATC/HM-TGA (B). The capital letters, A-F, represents nonpolymorphic and polymorphic bands as described in the text.

Table 3. Analysis of the demethylation events (Hypo) and methylation events (Hyper) detected in cotton experiencing salt stress (S, 9: 1 molar ratio of NaCl to Na_2SO_4, pH 6.96) and alkali stress (A, 9: 1 molar ratio of $NaHCO_3$ to Na_2CO_3, with higher pH 9.21) with comparison to control (untreated) cotton.

Parameter	Class	Effect	Band displayed in MSAP gel				Frequency of sites (%)[a]			
			Control		Treated		Salt stress		Alkali stress	
			H	M	H	M	Leaf	Root	Leaf	Root
No polymorphic bands	A	No change	+	−	+	−	1.20 ± 0.02	1.20 ± 0.04	1.11 ± 0.04	0.52
	B	No change	−	+	−	+	6.22 ± 0.08	4.18 ± 0.04	5.70 ± 0.11	3.37 ± 0.07
	C	No change	+	+	+	+	91.24 ± 0.06	92.60 ± 0.13	90.57 ± 0.15	84.68 ± 0.22
Demethylation events	D	CNG Hypo	+	−	+	+	0.02 ± 0.02	0.09 ± 0.04	0.07 ± 0.02	0.70
	E	CG Hypo	−	+	+	+	0.39 ± 0.08	0.50 ± 0.04	0.85 ± 0.07	1.26 ± 0.06
	F	CG/CNG Hypo	−	−	+	+	0.07 ± 0.02	0.26 ± 0.06	0.26 ± 0.04	6.11 ± 0.09
	G	CNG Hypo	−	−	−	+	0.09	0.17 ± 0.04	0.22 ± 0.03	0.33 ± 0.07
	H	CG Hypo	−	−	+	−	0	0.09 ± 0.06	0	0.13 ± 0.03
Methylation events	I	CNG Hyper	+	+	+	−	0.22 ± 0.03	0	0.24 ± 0.02	0.20 ± 0.07
	J	CG Hyper	+	+	−	+	0.17 ± 0.04	0.26 ± 0.04	0.35 ± 0.04	0.44 ± 0.04
	K	CG/CNG Hyper	+	+	−	−	0.24 ± 0.04	0.50 ± 0.05	0.33 ± 0.05	1.96 ± 0.08
	L	CNG Hyper	−	+	−	−	0	0.02 ± 0.02	0.07 ± 0.04	0.07 ± 0.02
	M	CG Hyper	+	−	−	−	0.17	0.01 ± 0.02	0.22 ± 0.03	0.17

Column H, Pattern after digestion with *Eco*RI and *Hpa*II; column M, pattern after digestion with *Eco*RI and *Msp*I. The symbol (+) indicates presence and the symbol (–) absence of a fragment. According to the methylation status of the 5'-CCGG sequence in leaves and roots of whole seedling treated or not treated with salt or alkali stress, three categories of bands were identified (classes A to C represent no changes; classes D to H represent demethylation events; classes I to M represents methylation events). [a]Variation rates represent the corresponding ration of events that occurred in cotton leaves and roots under salt stress and alkali stress.

CG/CNG hyper/hypo-methylation. This showed that most of CCGG methylated status in leaves (97.38 to 98.60%) and roots (88.57 to 97.98%) were not significantly changed (Table 3). In general, demethylation events were more than methylation events whether in leaves or roots (Figure 3A to C). In addition, the total methylation variation frequency was only 1.38% in leaves and 2.2% in roots under salt stress; in contrast, it was 2.59% in leaves and 12.44% in roots under alkali stress (Figure 3). Furthermore, CG/CNG sites were the dominant sites for both methylation and demethylation events especially in roots (Figure 4). The results therefore pointed out that most methylation variations occurred in cotton roots under alkali stress, and could be correlated with the root resistance to salt and alkali stresses.

Analysis of polymorphic fragment sequences

To obtain more information about the sequences amplified in cotton roots that might be correlated with the resistance of salt and alkali stresses, 16

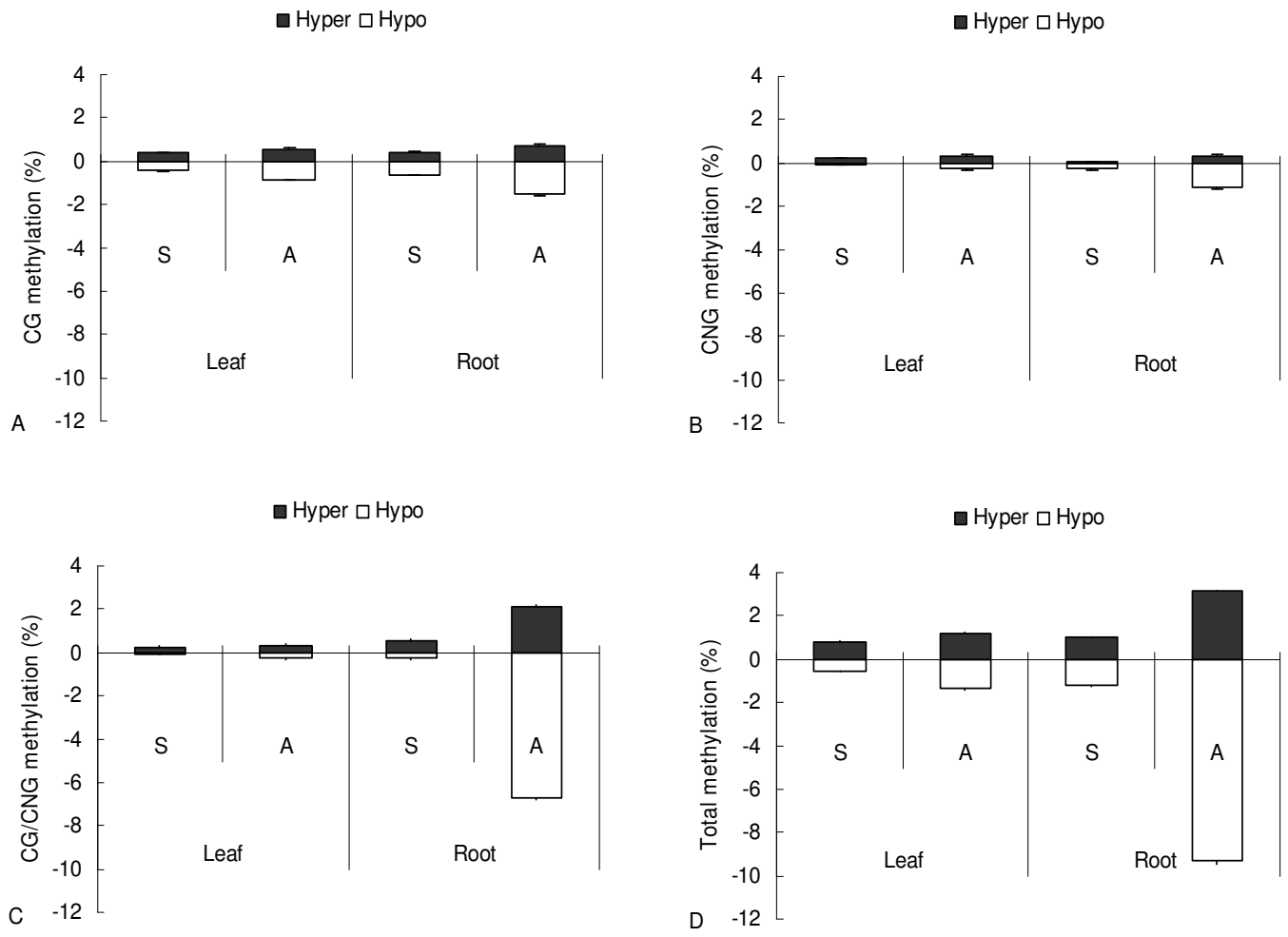

Figure 3. Effects of salt stress (S, 9:1 molar ratio of NaCl to Na_2SO_4) and alkali stress (A, 9:1 molar ratio of $NaHCO_3$ to Na_2CO_3) on CG (A), CNG (B), CG/CNG (C), and total methylation(D) alterations (hypermethylation and hypomethylation) occurring in cotton leaf and root. Data represent means ±S.E. of four replicates.

fragments were isolated and sequenced using pMD 18-T vector in Shenggong Company, China. The sequences obtained were analyzed by NCBI BLAST (http://www.ncbi.nlm.nih.gov/). Homology was detected in GenBank for two fragments in this study, one had homology to *Populus trichocarpa* predicted mRNA and the other had homology to Ty3- gypsy reverse transcriptase. This was inline with genome-wide analysis of DNA methylation in plants by McrBC digestion, methylcytosine immunoprecipitation (MeDIP), or sequencing of bisulfate-stressed DNA, which indicated that transposons were heavily methylated at both CG and non-CG sites (Lister et al., 2008).

DISCUSSION

Shi and Sheng (2005) demonstrated that soil salinization and alkalization always co-occurred, and salt stress is indeed the stress of neutral salts (NaCl and Na_2SO_4), while alkali stress is the stress of alkaline salts ($NaHCO_3$ and Na_2CO_3). High concentrations of salt cause physiological drought (Yang et al., 2008), ion injury (Li et al., 2009) and nutrient imbalance (Hasegawa et al., 2000), and the elevated pH surrounding the roots under alkali stress not only affects the roots vitality, but also causes inorganic ions to precipitate (Shi and Zhao, 1997), thereby resulting in mineral nutrition stress that further disrupts the ionic homeostasis, charge balance and pH stability (Yang et al., 2007). Previous studies had shown that cytosine methylation alterations play a crucial role in regulating gene expression in plant responses to environmental stresses. For example, drought and salinity can cause demethylation at coding regions of certain genes encoding glycerophosphodiesterase-like proteins and subsequently activate their expression (Choi and Sano, 2007).

Herein, our studies showed that although cytosine methylation levels remained unchanged or nearly so in cotton leaves and roots under salt stress (Figure 1), there

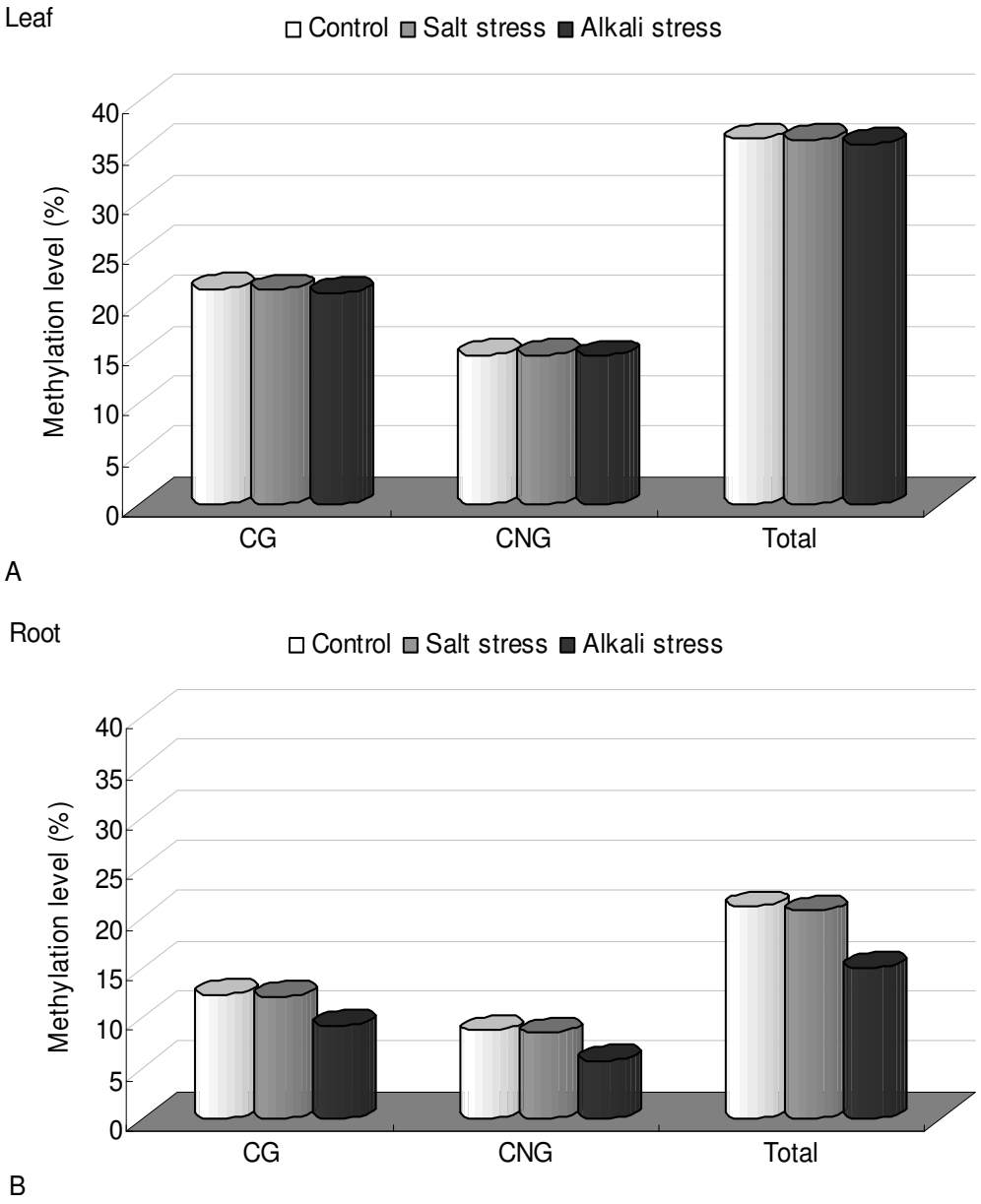

Figure 4. Effects of salt stress (S, 9:1 molar ratio of NaCl to Na_2SO_4) and alkali stress (A, 9:1 molar ratio of $NaHCO_3$ to Na_2CO_3) on DNA hypermethylation (A) and hypomethylation (B) occurring in cotton leaf and root. Data represents means of four replicates.

were some alterations of CG, CNG and CG/CNG methylation patterns that did occur (Figure 3). However, alkali stress particularly tended to reduce the overall DNA methylation levels in leaves and roots ($P<0.05$) (Figure 1) in which the methylation alterations affected up to 2.59% and 12.44% of the scored CCGG sites in leaves and roots, respectively (Figure 3D). Our results are consistent with previous reports that showed that environmental factors such as cold (Choi and Sano, 2007), heavy metals (Aina et al., 2004) and water stress (Wang et al., 2011) tends to cause demethylation of genomic DNA. These results indicated that methylation alterations mainly focused on cotton under alkali stress whether in the case of hypermethylation or in hypomethylation (Figure 4). Also, previous reports showed that alkali stress exerted much more severe impacts on plant growth, photosynthesis, solute accumulation, ion balance than salt stress (Yang et al., 2007, 2008; Liu and Shi, 2010). The present results suggested that such prominent DNA methylation alterations which occurred in cotton may be correlated with the resistance of alkali stress and afforded supportive evidence to the physiological conclusion that higher alkali stress is much more destructive.

Another important phenomenon was that the total

methylation variation frequency was only 1.38% in leaves and 2.2% in roots under salt stress; in contrast, it was 2.59% in leaves and 12.44% in roots under alkali stress (Figure 3D). This suggested that more adaptive and possibly complex and frequent gene expression alterations may occur in the resistance of the cotton root to salt and alkali stresses coupled with DNA methylation alterations. These may be due to the evolution of some special mechanisms to adapt into changes in salt and alkali stresses as it has been also postulated by other studies. However, exclusion mechanisms could not be ruled out as the concentrations have been shown to be much lower in roots than the external solution, and did not increase with time at high salinities (Storey and Wyn, 1978). Yang has also demonstrated that the physiological responses in the shoots were almost similar, while the responses of roots were dramatically different under salt stress and alkali stress (Yang et al., 2007).

Although, we did not find any gene which was directly correlated with alkali stress resistance, the present level of findings indicated that this possibility warrants attention and it deserves to be studied in-depth in the future. This is more so as exploration in understanding the interrelations between environmental, genetic and epigenetic variations in plants is gaining much attention among researchers (Bossdorf and Zhang, 2011). Furthermore, accumulating evidence showed that plants due to their sessile nature seem to have complex epigenetic mechanisms which are influenced by environmental changes. However, the flexibility of the epigenome may enable the plants to adapt and thrive in many dynamic environmental stresses.

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