African Journal of Biotechnology Vol. 8 (22), pp. 6201-6207, 16 November, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

DNA-methylation changes induced by salt stress in wheat *Triticum aestivum*

Lan Zhong, Yan-hao Xu and Jian-bo Wang*

Key Laboratory of the MOE for Plant Development Biology, College of Life Sciences, Wuhan University, Wuhan, 430072, P. R., China

Accepted 28 September, 2009

The present study was to assess DNA methylation alteration induced by salt stress in two wheat *Triticum aestivum* cultivars differing in salt tolerance (salt-tolerant Dekang-961 and sensitive Lumai-15), comparatively. The changes in the status of methylation of the CCGG sequence of the nuclear genome of the root DNA of plants exposed to different concentrations of NaCl compared with that of untreated plants were determined by methylation-sensitive amplified polymorphism (MSAP) approach. The result showed that CCGG sequences of Dekang-961 control plants were more methylated than that of Lumai-15. NaCl treatment induced some CCGG sites demethylation and some hypermethylation both in Dekang-961 and Lumai-15, with the net result being genome-wide hypomethylation. These results showed a clear alteration of DNA methylation in plants as a response to salt stress and the effect was dose-dependent. These changes may suggest a mechanism for plants adaptation under salt stress.

Key words: DNA methylation, MSAP, salt stress, wheat.

INTRODUCTION

Salt stress is one of the most important constraints to crop production. Plants are immobile organisms and in saline soil roots are the primary point of contact with ionic toxicity and osmotic stress. One of the biochemical changes possibly occurring when plants are subjected to harmful stress conditions is the production of reactive oxygen species (ROS) (Wang et al., 2003). These cytotoxic reactive oxygen species can seriously disrupt normal metabolism through oxidative damage of lipids, proteins and nucleic acids (Apel and Hirt, 2004). While many studies with salt stress have focused on its physiological mechanism of the effect of salinity on plant metabolism, considerably little interest exists on the epigenetic impacts of salt stress.

The ability of plants to differently regulate gene expression and protein function is the base of plant resistance. Epigenetic modifications, which are changes in gene expression occurring without change in DNA sequence, should be one of the molecular mechanism by which plants could silence or superactivate selected DNA templates (Habu et al., 2001). DNA methylation is the most important epigenetic mechanism. Plants contain relatively high levels of 5-methylcytosine (5mC), ranging from 6 to 25% of total cytosines, depending on the species (Steward et al., 2002). Thus DNA methylation is likely to be a regulatory mechanism. In the plant genome, 5mC is found in three nucleotide-sequence contexts: symmetrical CG, symmetrical CNG and asymmetric CNN sites (where N is A, T or C). Similarly to other biochemical modifications such as protein phosphorylation and acetylation, DNA methylation is also reversible (Kress et al., 2001). Methylated DNA is well known to inhibit gene expression, while a reduction in the level of methylation leads to an increase in gene expression (Finnegan et al., 1998). Demethylation may take place as a passive process because of lack of maintenance methylation during several cycles of DNA replication or as an active mechanism in the absence of replication (Kress et al., 2001). In *Arabidopsis thaliana*, DNA demethylation is carried out by bifunctional helix-hairpinhelix DNA glycosylases of the DEMETER (DME) family (Agius et al., 2006; Gehring et al., 2006; Morales-Ruiz et al., 2006; Penterman et al., 2007). The DME family consists of DME, DEMETER-LIKE 2 (DML2), DEMETER-LIKE 3 (DML3), REPRESSOR OF SILENCING 1 (ROS1) and REPRESSOR OF SILENCING

*Corresponding author. E-mail: jbwang@whu.edu.cn. Tel: +86-27-68752213. Fax: +86-27-68752213.

Table 1. Summary of methylation sensitivities of restriction endonucleases *Hpa*II and *Msp*I.

Enzyme	Sites cut	Sites not cut
*Hpa*II	CCGG ^{m}CCGG GGCC GGCC	^{m}C^{m}CGG ^{m}CCGG C^{m}CGG GG^{m}C^{m}C GGC^{m}C GG^{m}CC
*Msp*I	CCGG C^{m}CGG GGCC GG^{m}CC	^{m}C^{m}CGG ^{m}CCGG ^{m}CCGG GG^{m}C^{m}C GGC^{m}C GGCC

3 (ROS3). DNA demethylation by DME occurs during reproductive development and is required for genomic imprinting and seed viability (Choi et al., 2002; Gehring et al., 2006; Huh et al., 2008; Makarevich et al., 2008; Tiwaria et al., 2008; Hsieh et al., 2009). DNA demethylation by ROS1, DML2, DML3 and ROS3 protects genes from potentially deleterious methylation (Penterman et al., 2007; Zhu et al., 2007; Zheng et al., 2008; Ponferrada-Marín et al., 2009).

Recent studies provided evidence for changes in cytosine methylation under environmental stresses. In rape, cadmium stress stimulated demethylation at specific loci, according to the methylation-sensitive amplification polymorphism (MSAP) approach (Filek et al., 2008). Aluminum (Choi and Sano, 2007), heavy metals (Aina et al., 2004) and water stress (Labra et al., 2002) can cause an increase or decrease in cytosine methylation through out the genome and at specific loci. The treatment with 5-azacytidine (5-azaC), a demethylating agent, could replace low-temperature treatment in several vernalization requiring plant species (Burn et al., 1993; Brock and Davidson, 1994; Demeulemeester et al., 1999; Yong et al., 2003). These facts implied the involvement of epigenetic mechanisms in the regulation of environmental stresses.

The aim of this study was to examine the effect of salinity on the pattern and extent of cytosine methylation in two wheat cultivars having different salt tolerance: Dekang-961 and Lumai-15, as detected by methylation-sensitive amplified polymorphism (MSAP) (Xiong et al., 1999). Using this technique, we asked the following specific questions: (i) Whether epigenetic changes are triggered by salt stress in wheat? (ii) Whether the epigenetic changes in Dekang-961 and Lumai-15 cultivars are similar?

MATERIALS AND METHODS

Plant materials and treatment conditions

Two cultivars of wheat (*Triticum aestivum* L.) were used in this study: Dekang-961 (salt tolerant) and Lumai-15 (salt sensitive), obtained from Shandong Academy of Agricultural Sciences and Shandong Agricultural University, China. Seeds were surface sterilized with 0.1% $HgCl_2$ for 3 min and then washed thoroughly with distilled water. The seeds (200 seeds of each cultivar; 25 seeds per Petri dish) were germinated for 3 days in the dark at 25℃ on filter paper soaked with distilled water. 8-Day-old seedlings were grown in Hoagland solution containing 0, 100 or 150 mM NaCl for 5 days. Seedlings were grown under temperature of 25/15℃ (day/night) and relative humidity of 65 – 75%.

DNA extraction

The entire root was excised from each plant (both control and treated plants). DNA was isolated following a modification of the cetyl trimethyl ammonium bromide (CTAB) extraction method by Murray and Thompson (1980). Root (2 g) was powdered under liquid nitrogen with mortar and pestle. Then 10 ml of extraction buffer (100 mM Tris pH 8.0/1400 mM NaCl/20 mM EDTA/1% 2-mercaptoethanol/2% CTAB) were added and incubated at 65℃ for 60 min in a 50 ml-polypropylene Beckman tube, which was mixed by inversion every 10 min. The tubes were allowed to cool at room temperature and the crude DNA extract was mixed with 10 ml of chloroform: isoamyl alcohol (24:1) followed by centrifugation (4000 rpm, 30 min, 20℃). The supernatant was added by an equal volume of ice-cold isopropanol to precipitate DNA (10 to 20 s). DNA was hooked onto a bent Pasteur pipet and rinsed in a microcentrifuge tube with 70% ethanol. The DNA pellet was dried in the air and dissolved in 600 µl TE. It was digested with 5 µl 10 mg/ml RNase A for 2 h at 37℃ and extracted three times with equal volume of chloroform : isoamyl alcohol (24:1) followed by centrifugation (10000 rpm, 15 min, 4℃). Nucleic acids were precipitated with 1/10 volume 3000 mM NaAc (pH 5.4) and 1 volume of isopropanol for 2.5 h at –20℃ and collected by centrifugation (10000 rpm, 20 min, 4℃). The precipitate was washed with 70% ethanol, air-dried and resuspended in TE (pH 8.0). Concentration and purity of DNA were determined spectrophotometrically (A260 for DNA, A230 for polysaccharides and A280 for proteins).

MSAP analysis

MSAP analysis was performed similarly to AFLP to incorporate the use of methylation-sensitive restriction enzymes (Table 1). The modified protocol involved the use of the isoschizomers *Hpa*II and *Msp*I in place of *Mse*I as the frequent cutter, while the rare cutter *Eco*RI was not changed. The adapters and primers were shown in Table 2.

To detect MSAP, two digestion restrictions were done concurrently. In the first reaction, 500 ng of genomic DNA was digested with 10 units *Eco*RI (New England Biolabs, USA) and 20 units *Hpa*II (New England Biolabs, USA) in a final volume of 20 µl for 6 h at 37℃. The second digestion reaction was carried out in the same way, except that *Msp*I (New England Biolabs, USA) was used in place of *Hpa*II.

The digested fragments were then ligated to the adapters by adding 10 µl of ligation mixture, containing 5 pmol *Eco*RI adapter, 50 pmol *Hpa*II/*Msp*I adapter, 0.2 mM ATP and 2 units T4 DNA ligase (Promega, USA), and incubated at 37℃ for 6 h. The reactions were stopped by incubating at 65℃ for 20 min.

We used two consecutive PCRs to selectively amplify the *Eco*RI–*Hpa*II and *Eco*RI–*Msp*I DNA fragments. The pre-amplification

Table 2. Sequences of adapters and primers used for MSAP analysis.

Primers/adapters	Sequence (5′–3′)
*Eco*RI adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTCTAC
E + 1 primer E + 3 primers	GACTGCGTACCAATTC+A GACTGCGTACCAATTC+ AAC (E1) GACTGCGTACCAATTC+ ACG (E2) GACTGCGTACCAATTC+ ACT (E3) GACTGCGTACCAATTC+ AGT (E4)
*Hpa*II/*Msp*I adapter	GATCATGAGTCCTGCT CGAGCAGGACTCATGA
HM + 1 primer HM + 3 primers	ATCATGAGTCCTGCTCGG+T ATCATGAGTCCTGCTCGG+ TAA (HM1) ATCATGAGTCCTGCTCGG+ TCC (HM2) ATCATGAGTCCTGCTCGG+ TTC (HM3)

was performed by using 2 µl of the above ligation product with E+1/HM+1 primers in a volume of 15 µl containing 1 × PCR buffer, 1.5 mM $MgCL_2$, 0.2 mM each dNTP, 40 ng of each primer and 1 unit of Taq polymerase (Promega, USA). PCR reactions were performed with the following profile: 94℃ for 60 s, 25 cycles of 30 s denaturing at 94℃, 30 s annealing at 56℃ and 60 s extension at 72℃, ending with 10 min at 72℃ to complete extension. After checking for the presence of a smear of fragments (100-1000 bp in length) by agarose electrophoresis, the amplification product was diluted 10 times in 0.1 × TE.

Selective amplification was carried out using a total of 12 primer combinations obtained with four *Eco*RI primers in combination with three *Hpa*II/*Msp*I primers with three selective bases each (E+3, HM+3) listed in Table 2. Selective PCR reactions were performed in volumes of 15 µl, containing 5 µl of the pre-amplification product, 30 ng of the *Eco*RI primer and 40 ng of *Hpa*II/*Msp*I primer; the remaining components of the reaction were the same as in the pre-amplification reaction. The PCR reactions were performed with the following profile: 94℃ for 60 s, 36 cycles of 30 s denaturing at 94℃, 30 s annealing and 60 s extension at 72℃, ending with 10 min at 72℃ to complete extension. Annealing was initiated at a temperature of 65℃, which was then reduced by 0.7℃ for the next 12 cycles and maintained at 56℃ for the subsequent 23 cycles.

Selective PCR products were mixed with 15 µl of formamide dye (98% formamide, 10 mM EDTA, 0.01% w/v bromophenol blue and 0.01% w/v xylene cyanol), denatured at 95℃ for 4 min and separated by electrophoresis on 5% denaturing polyacrylamide sequencing gels (5% acrylamide 19:1, 7 M Urea) in 1×TBE buffer. The gels were pre-run at 110 W for about 30 min before 4.5 µl of the mix was loaded. Gels were run at 80 W for about 2.5 h and silver stained as described by Bassam et al. (1991).

The gel was fixed in 10% acetic acid for 30 min, washed three times with a large quantity of ultrapure water for 6 min, transferred to a silver impregnation solution (1 g/l $AgNO_3$, 0.056% formaldehyde) for 30 min and then rinsed with ultrapure water for 5 to 10 s. All the above steps were performed with slow agitation on a shaker. Image development was carried out with manual agitation for 1 to 3 min in developer (30 g/l Na_2CO_3, 0.056% formaldehyde, 400 g/l sodium thiosulfate). To stop development and fix the gel, 10% acetic acid was added directly to the developing solution and incubated with shaking for 2 to 3 min. The gel was then rinsed briefly in ultrapure water and dried at room temperature.

*Hpa*II and *Msp*I are a pair of isoschizomers and they possess differential sensitivity to cytosine methylation at CCGG sites. The specificities of *Hpa*II and *Msp*I are described in the REBASE database of restriction enzymes (Roberts et al., 2007). In summary, *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. Methylation of the external cytosines (on both strands) or a full methylation of both cytosines prevents cutting, which makes these two methylation patterns indistinguishable by the MSAP technique. To follow changes in methylation, MSAP analysis of each treatment was performed in duplicate with the *Hpa*II–*Eco*RI and *Msp*I–*Eco*RI products (in the *Hpa*II and *Msp*I lanes, respectively). A product appearing in both lanes indicates that the corresponding CCGG site is unmethylated. A product appearing in *Msp*I lanes is caused by hypomethylation of the outer C relative to the internal C. A product appearing in *Hpa*II lanes reflects hemi-methylation of the outer C. The absence of a product in both *Eco*RI + *Hpa*II digestions and *Eco*RI + *Msp*I digestions is attributed to methylation of the external cytosines (on both strands) or a full methylation of both cytosines.

Scoring of MSAP bands

For the MSAP analysis, the replicate gel was run from the same DNA sample but from a different digestion–ligation–amplification reaction, only the reactions showing full reproducible results between replicas were used for data analysis. Moreover, with MSAP gels, the upper part and the lower part of the gel, where resolution is not satisfactory, was not used for band scoring. The scored MSAP bands were transformed into a binary character matrix, using “1” and “0” to indicate the presence and absence, respectively, of a band at a particular position.

RESULT

To explore changes in DNA methylation induced by salinity in wheat, we employed MSAP analysis to assess cytosine methylation at specific restriction sites through out the genome. An example of methylation pattern detected in cultivar Dekang-961 through MSAP analysis was reported in Figure 1. Table 3 summarized the methylation status in the NaCl treated plantlets and the control. Differences in banding patterns obtained from digestion

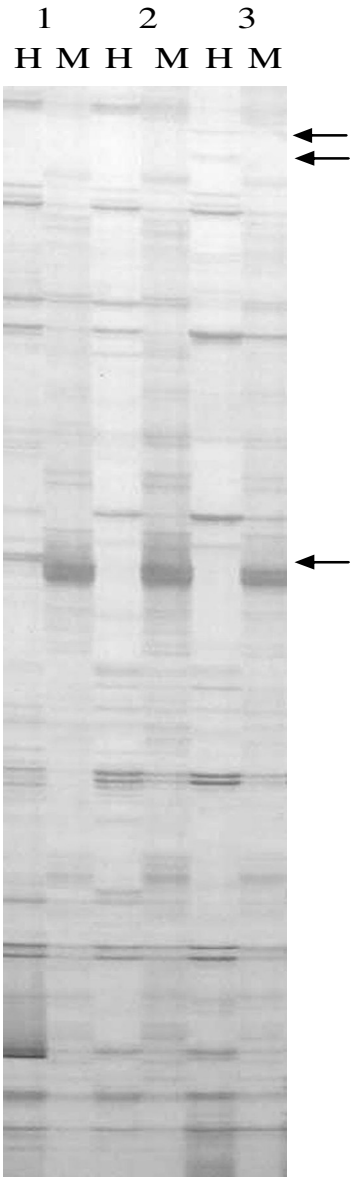

Figure 1. Example of methylation patterns detected in Dekang-961 using the primer combination E4/HM1. Lanes 1 - 3 showed patterns from the DNA of the root of control, seedlings treated with 100 mM NaCl and 150 mM NaCl. H and M refered to digestion with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I, respectively. The arrows indicated polymorphism bands.

with *Msp*I or *Hpa*II made it possible to determine the targeting sites of methylation.

MSAP analysis using 12 pairs of primers, performed on three groups, a control and two salt-treated groups, produced a total of 1025 and 1035 bands for Dekang-961 and Lumai-15, respectively. In control plants, the analysis of the CCGG sequences showed that most of them were unmethylated sites, equal to 71.5% of 1025 for Dekang-961 and 73.9% of 1035 for Lumai-15. The remaining percentages were ascribed to methylation sites. In detail, the methylation of internal cytosines (resulting in cleavage by *Msp*I but not *Hpa*II) was accounted for 10.1% (104 out of 1025) of the methylated sites in Dekang-961 and for 9.7% (100 out of 1035) in Lumai-15. The bands obtained only from *Eco*RI-*Hpa*II digestion that indicated a hemimethylation or a methylation of external cytosine, represented 14.7% (151 out of 1025) in Dekang-961 and 14.9% (154 out of 1035) in Lumai-15. The methylation occurred in the external cytosines or both the cytosines was 37 (3.6%) and 16 (1.5%) in Dekang-961 and Lumai-15, respectively.

In plants grown on a low concentration (100 mM) of NaCl, 45 of 1025 fragments (4.4%) in Dekang-961 and 20 of 1035 (1.9%) in Lumai-15 showed hypomethylation (Table 3, Class D-H); 17 cases (1.7%) in Dekang-961 and 12 (1.2%) in Lumai-15 were hypermethylation (Table 3, Class I-M). After treatment with a high concentration (150 mM) of NaCl, 6.0% (61 out of 1025, Table 3, Class D-H) was hypomethylation and 2.0% (20 out of 1025, Table 3, Class I-M) was hypermethylation events in Dekang-961 and 33 of 1035 bands (3.2%, Table 3, Class D-H) showed hypomethylation and 14 of 1035 bands (1.4%, Table 3, Class I-M) showed hypermethylation in Lumai-15. As shown in Table 3, the salt stress induced DNA methylation changes comprised both hypomethylation and hypermethylation events and the former were more frequently observed than the later, leading to a net hypomethylation in the genome.

It should be noted that there exists three cases of comparison of the methylation status of the CCGG sequence between control and treated plants showing unambiguous information. As shown in Table 3, the patterns of methylation are different in class N and O, but the levels of methylation do not always change between the control and the salt treated plantlets. This approach does not allow us to distinguish methylation of the external cytosines from a full methylation of both cytosines (Table 3, Class P).

DISCUSSION

In this study, MSAP approach was used to assess whether salt stress caused DNA methylation changes in salt-tolerant wheat, Dekang-961 and salt-sensitive wheat, Lumai-15. Based on our results, both wheat cultivars showed a significant, genome-wide dose-dependent hypomethylation in the CCGG sequence. On the contrary, hypermethylation occurred in heterochromatin of tobacco cell culture (Kovarik et al., 1997) and in the CCWGG sequences of *Mesembryanthemum crystallinumin* (Dyachenko et al., 2006) in response to salt stress. That was because the level of CCGG-methylation of the whole genome was determined here, but changes in the DNA methylation status of two heterochromatic loci of tobacco

Table 3. Results of the MSAP analysis. + Band present, - band absent.

Parameter	Class	Band display pattern in MSAP gel				Number of sites			
		Control		Treated		Dekang-961		Luami-15	
		*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	100 mM	150 mM	100 mM	150 mM
Total bands						**1025**		**1035**	
Not polymorphic bands	A	+	-	+	-	138	136	149	144
	B	-	+	-	+	83	79	93	88
	C	+	+	+	+	721	718	755	754
Demethylation events	D	+	-	+	+	8	10	3	7
	E	-	+	+	+	20	25	7	12
	F	-	-	+	+	7	11	4	6
	G	-	+	+	-	0	0	0	0
	H	-	-	+	-	10	15	6	8
Methylation events	I	+	+	+	-	1	2	0	0
	J	+	+	-	+	9	11	6	7
	K	+	+	-	-	2	2	4	4
	L	+	-	-	+	0	0	0	0
	M	+	-	-	-	5	5	2	3
Not informative	N	-	-	-	+	1	0	0	0
	O	-	+	-	-	1	0	0	0
	P	-	-	-	-	19	11	6	2

cell culture and in the sequence CCWGG of the nuclear genome of *Mesembryanthemum crystallinumin* were investigated (Kovarik et al., 1997; Dyachenko et al., 2006). Demethylation of genomic DNA has occasionally been reported in several plant species with environmental factors, such as cold, heavy metals and aluminium (Finnegan and Kovac, 2000; Lizal and Relichova 2001; Alina et al., 2004; Choi and Sano 2007). According to literature, salt stress causes oxidative stress (Dionisio-Sese and Tobita, 1998; Meneguzzo et al., 1999) that induces DNA hypomethylation, as reviewed by Cerda and Weitzman (1997). ROS attack in the DNA vicinity is known to result in formation of 8-hydroxyguanosine (also denoted 8-oxoguanine) as a major adduct. Wachsman (1997) reported that the presence of 8-oxoguanine in CG sequences strongly inhibits the methylation of adjacent cytosine residues (Cerda and Weitzman, 1997). Indeed, reduction in methylation was found upon exposure to paraquat, an effective reactive oxygen species generator (Choi and Sano, 2007). Since salinity is known to generate reactive oxygen species (Zhu, 2003), the present data suggested that the induction of hypomethylation upon salt treatment might have been mediated through oxygen radicals.

For the loss of DNA methylation, active or passive mechanisms have been proposed (Jost et al., 2001; Zhu et al., 2007). The active mechanism involves enzymes that excise methyl from cytosine and the passive mechanism is considered to be that newly replicated DNA is not methylated (Jost et al., 2001). Demethylation upon environmental stress must involve the former mechanism, since DNA is scarcely replicated in stressed tissues, while demethylation takes place rather rapidly (Steward et al., 2000). The recently identified enzyme ROS1, a 5-methylcytosine DNA glycosylase/lyase, shows clear demethylation activity towards 5mC in DNA and ubiquitously expressed in plant tissues (Agius et al., 2006). Zhu et al. (2007) found that active DNA demethylation caused by ROS1 was important in pruning the methylation patterns of the genome and even the normally "silent" transposons and other sequences were under dynamic control by both methylation and demethylation.

Both hypermethylation and hypomethylation were induced by salinity in Dekang-961 and Lumai-15. Similarly, one hallmark of cancer cells is local hypermethylation and global hypomethylation of chromosomal DNA (Smith, 2000). This uncommon DNA methylation is thought to modulate gene expression by hypermethylation of tumour suppressor genes and/or hypomethylation of oncogenes (Cheah et al., 1984; Counts and Goodman, 1995; Crossen and Morrison, 1999).

What is suggested by the present work is that the DNA methylation changes induced by salt stress were similar in Dekang-961 and Lumai-15 cultivars and there was a larger demethylation in the former, despite Lumai-15 having lower methylation level in control. Changes in DNA methylation can be considered either a simple indirect effect of salt stress or a precise defensive mechanism for regulating the gene expression. These will open new windows for a better understanding of the plant stress adaptation mechanism. Further study on how these changes function in plant stress adaptation would be

worthwhile.

ACKNOWLEDGEMENT

This work was carried out with the financial support from the National Natural Science Foundation of China (No. 30521004).

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