

Salt and alkaline stress induced transgenerational alteration in DNA methylation of rice (*Oryza sativa*)

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Abstract

In this study, we sought to explore a possible relationship between salt- and alkali-stressed heritable alterations in DNA methylation and acquisition of stress tolerance in rice. For this purpose, we used four rice genotypes, two salt-tolerant (JL 89-45 and Yangeng 23) and two salt-sensitive (Teyou 12 and Gaochan 106). We analyzed DNA methylation patterns of the salt- or alkaline-stressed rice plants and their selfing progenies growing in non-stressed conditions by methylation-sensitive molecular markers. Results showed that both salt- and alkaline-stresses caused alteration in DNA methylation pattern in the treated plants. Furthermore, the altered patterns persisted in selfed progenies of the stressed plants that grow under non-stressed conditions. Interestingly, the two salt-tolerant genotypes mainly showed hypermethylation while the two salt-sensitive genotypes showed mainly demethylation, indicating that DNA methylation remodeling may play a role in conditioning the salt tolerance. To test this hypothesis, we compared the stress tolerance in a salt-tolerant rice plant (genotype Yangeng 23) and its selfed progenies (S1 and S2 generations) which inherited the methylation alterations of salt-stressed plant (genotype Yangeng 23). Results indicated that both methylation alteration and increased stress tolerance were concomitantly inherited to several S2-generation plants. This is a clear example of epigenetic inheritance, and supports the idea of Lamarckian inheritance of acquired trait.

Keywords: Salt stress, Alkali stress, DNA methylation, Rice, Inheritance of acquired trait.

Abbreviations: ISSR–Inter-simple sequence repeat, S0–immediate plant generation, S1–selfed progeny of S0 plant, S2–selfed progeny of S1 plant, S–salt stress, A–alkaline stress.

Introduction

Cytosine DNA methylation plays important roles in multiple fundamental cellular activities, including control of gene expression, maintenance of genomic integrity, formation and perpetuation of chromatin structure, and control of genomic imprinting (Tariq and Paszkowski, 2005; Rangwala and Richards, 2004; Chan et al., 2005). In mammals, including humans, cytosine methylation is almost exclusively confined to CG dinucleotides. In plants, the degree and spectrum of cytosine methylation are more complex, which occurs not only at CG but also at CHG and asymmetric sites (CHH where, H represents A, T or C). Studies in recent years have revealed that DNA methylation is labile and sometimes responsive to both internal and external perturbations, and biotic and abiotic stresses may cause heritable alterations in cytosine methylation pattern in both plants and animals, which presumably represent adaptive responses (Richards, 2006; Chong and Whitelaw, 2004). Several studies have shown that heritable phenotypic novelty may be related to heritable alterations in DNA methylation (Molinier et al., 2006; Boyko et al., 2007; Chinnusamy and Zhu, 2009). For example, Boyko et al. (2007) reported that the tobacco mosaic virus (TMV) induced heritable DNA hypomethylation of tobacco plants and enhanced tolerance to TMV by their progenies. Abiotic stresses could also induce alteration in DNA methylation, which might bear

relevance to a changed response to the specific stress. For example, Dyachenko et al. (2006) reported that salt stress induced a switch in the photosynthesis mode from C3 to CAM in the facultative halophyte *Mesembryanthemum crystallinum* L., and this metabolic switch was associated with stress-induced specific CHG-hypermethylation of satellite DNA. However, Dyachenko's work only indicated that salt stress is epigenetically mutagenic. It did not explore whether the changed methylations are transgenerationally inheritable. Recently, we showed that chronic starvation of nitrogen induced alterations in DNA methylation of rice and then altered methylation patterns, which associated with enhanced tolerance to the stress in progenies of the stressed plants (Kou et al., 2011). However, this study did not involve different genotypes with contrasting tolerance to the stress. Salt and alkaline (high-pH) stresses are widely occurred at environmental conditions that severely limit crop productivity (Yang et al., 2008; Yang et al., 2009). Both salt and alkaline stresses, but the later in particular, inhibit plant growth and adversely interfere with normal cellular metabolism (Yang et al., 2007; Yang et al., 2009; Charkazi et al., 2010; Ibraheem et al., 2011). This study was aimed to investigate (1) whether there exist differential DNA methylation alteration between two sets of cultivars that were respectively tolerant and sensitive to the stresses; and (2)

whether progenies of the stressed plants would show a “memory” of the stress experienced by their parents, and whether the memory is related to the altered methylation patterns.

Results

Salt stress-induced alteration in DNA methylation

Methylation-sensitive ISSR fingerprinting and gel-blotting analysis showed that salt stress induced extensive alterations of DNA methylation in the immediately treated rice plants of all four genotypes tested (Figs. 1, 2, 3 and Table 1). However, the methylation alterations induced by the salt stress differed in different genotypes, and both salt-tolerant genotypes showed hypermethylation, while both salt-sensitive genotypes mainly represent demethylation (hypomethylation) (Fig. 2B, D). Further observation indicated that CG hypermethylation was the dominant type of alteration in the salt-tolerant genotype JL 89-45 (Fig. 2B), while for the other salt-tolerant genotype (Yangeng 23) the frequencies of CHG and CG hypermethylation were similar (Fig. 2B). In both salt-sensitive genotypes, Teyou 12 and Gaochan 106, CG hypomethylation was the major type of alteration (Fig. 2D), although for genotype Gaochan 106 some CHG hypermethylation was also detected (Fig. 2B).

Alkaline stress-induced alteration in DNA methylation

In general, effect of alkaline stress on DNA methylation of four rice genotypes was similar to that of salt stress. Methylation-sensitive ISSR fingerprinting and gel-blotting analysis showed that alkaline stress also induced extensive alterations of DNA methylation in the immediately treated rice plants (Figs. 1, 2, 3; Table 1). The methylation alterations induced by alkaline stress also differed in different genotypes, and both salt-tolerant genotypes showed predominantly hypermethylation, while both salt-sensitive genotypes mainly showed demethylation (Fig. 2A, C). In two tolerant genotypes, JL 89-45 mainly showed CG hypermethylation, while Yangeng 23 showed more or less the same extent of CG and CHG hypermethylation. In addition, the total frequency of methylation change in JL 89-45 was much higher than Yangeng 23 (Fig. 2A). Both of sensitive genotypes showed almost the same type and extent of methylation alteration, i.e., CG hypermethylation (Fig. 2C).

Homology analysis of the genomic loci undergoing DNA methylation alteration

From the methylation-sensitive ISSR analysis, 48 loci showing DNA methylation alterations were isolated and sequenced. Homology search of these isolated bands against the publically available databases at NCBI showed that diverse genes participating in cellular basal metabolism such as transcription factors, DNA-binding domains, SHAQKYF class family proteins, and a DNA-directed RNA polymerase β subunit were underlying the methylation alterations as a result of salinity or alkaline stress (Supplementary Table 3).

Transgenerational inheritance of altered DNA methylation patterns

Methylation-sensitive gel-blotting showed that both salt and alkaline stresses could cause alterations of the methylation status in rice, which not only occurred in somatic cells of the immediately treated plants (Fig. 3 and Table 1), but also had

these changes inherited to several untreated selfed progenies (S1) (Fig. 4 and Table 2). Moreover, some selfed progenies showed further changed patterns (Fig. 4 and Table 2). It should be noted that not all selfed progenies of the stressed plant inherited the altered patterns or showed additional alterations. Instead, some progenies completely erased the altered methylation patterns and reverted back to the original patterns of control plants (Fig. 4 and Table 2).

Concordance between altered DNA methylation pattern and inherited stress tolerance

Under salt stress, leaves of the immediately treated plants (S0 generation) of genotype Yangeng 23 were wilted, while S1 and S2 plants were both well-growing, indicating that the stress tolerance of the S1 and S2 plants were stronger than that of the S0 plant (Fig. 5). In addition, under non-stress condition, heights of S1 and S2 plants were taller than that of their S0 generation plant (Fig. 5, $P < 0.05$). Accordingly, as shown in Fig 5 and Tables 1-2, both methylation alteration and increased stress tolerance were stably inherited, whereas both salt-tolerant genotypes showed hypermethylation (Fig. 2). These data suggested that rice salt-stress tolerance was likely interlaced with altered methylation patterns.

Discussion

Biotic and abiotic stresses may cause alterations in DNA methylation pattern in both plants and animals (Richards, 2006; Chong and Whitelaw, 2004). Most of these stress-induced epigenetic modifications are reset to the original patterns once the stress is relieved, while some of the modifications may be persistent. This could be called as “stress memory” and be inherited across mitotic or even meiotic cell divisions (Chinnusamy and Zhu, 2009). Transgenerational changes in DNA methylation patterns are an effective means to modify the expression of a number of genes (Boyko et al., 2007; Boyko and Kovalchuk, 2007; Boyko et al., 2010). Thus, alterations in methylation pattern could be considered as part of the plant protection mechanism in response to environment change. Epigenetic stress memory can help plants more effectively cope with subsequent stresses in an adaptive manner. Recently, Kou et al. (2011) found that in rice the altered DNA methylation patterns are associated with enhanced tolerance to nitrogen-deficiency by progenies of the stressed plants in one rice genotype, suggesting that DNA methylation adjustment may play a role in nitrogen-deficiency tolerance. However, to our knowledge, no report has demonstrated whether heritable DNA methylation alterations contribute to acquisition of transgenerational stress tolerance in plants. Thus, understanding of epigenetic response in different genotypes with different stress tolerance and its relationship with stress tolerance formation represent an important first step towards deciphering this issue. Our results showed that both salt and alkaline stresses not only can change DNA methylation patterns in somatic cells of immediately treated rice plants but also these changes may be persisted in untreated selfed progenies (Table 1 and 2, Fig. 3 and 4). In addition, we observed that the methylation alterations induced by the stresses differed in different genotypes, and two salt-tolerant genotypes both represented hypermethylation (Fig. 2), while both salt-sensitive genotypes represented demethylation, suggesting that DNA methylation adjustment may play an important role in the acquisition of salt tolerance. To test this hypothesis further, we compared the stress tolerance in a tolerant rice plant (genotype Yangeng 23, S0) and its selfed progenies (S1 and S2 generations) that inherited the methylation alteration of the

Table 1. DNA methylation alterations in the S0 generation plants induced by the salt and alkali stresses. Salt-tolerant genotypes, JL 89-45 and Yangeng 23; salt-sensitive genotypes, Teyou 12 and Gaochan 106^a.

Probe	Genbank acc. ^b	Alkaline stress				Salt stress			
		JL 89-45	Yangeng 23	Teyou 12	Gaochan 106	JL 89-45	Yangeng 23	Teyou 12	Gaochan 106
<i>P1</i>	AC111016	n/-	n/n	n/n	n/n	n/n	n/n	n/-	n/n
<i>P2</i>	AK066495	n/-	n/n	n/n	n/n	n/n	n/n	+/n	n/n
<i>P3</i>	AY328087	-/n	n/n	n/-	n/-	-/n	n/n	n/-	n/-
<i>P4</i>	AP004790	n/n	-/n	n/n	n/n	n/n	-/-	+/n	n/n
<i>P5</i>	AL663000	n/-	-/-	n/n	n/n	n/n	-/-	n/n	n/n
<i>P10</i>	AP004139	n/n	n/-	n/n	n/-	n/n	n/-	n/n	n/-
<i>P11</i>	AP004026	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
<i>P14</i>	AC151537	n/-	n/n	n/-	n/-	n/n	n/n	n/-	n/-
<i>P15</i>	AC125411	n/n	-/-	n/n	n/-	n/n	-/-	n/n	n/-
<i>P16</i>	AP006048	n/-	-/-	n/n	n/n	n/-	-/-	n/n	n/n
<i>P17</i>	AP003683	n/n	n/n	n/-	n/-	n/n	n/n	n/-	n/-
Alteration	CG (%)	9.1	36.4	0	0	9.1	36.4	18.2	0
Frequency ^d	CNG (%)	45.5	36.4	27.3	45.5	9.1	45.5	36.4	45.5
	Total (%)	54.6	45.5	27.3	45.5	18.2	45.5	54.6	45.5

^aDNA methylation alteration relative to the control plant (C0) were detected by methylation-sensitive Southern blot analysis.

^bDetermined by BlastN at NCBI. ^cn/n: alteration in neither *HpaII* or *MspI*; n/+ : increased methylation in *MspI* but no alteration in *HpaII*; n/- : decreased methylation in *MspI* but no alteration in *HpaII*; +/n: increased methylation in *HpaII* but no alteration in *MspI*; -/n: decreased methylation in *HpaII* but no alteration in *MspI*; -/-: decreased methylation in both *HpaII* and *MspI*; ^d Defined as number of probes showing alterations/total probe number in a given genotype.

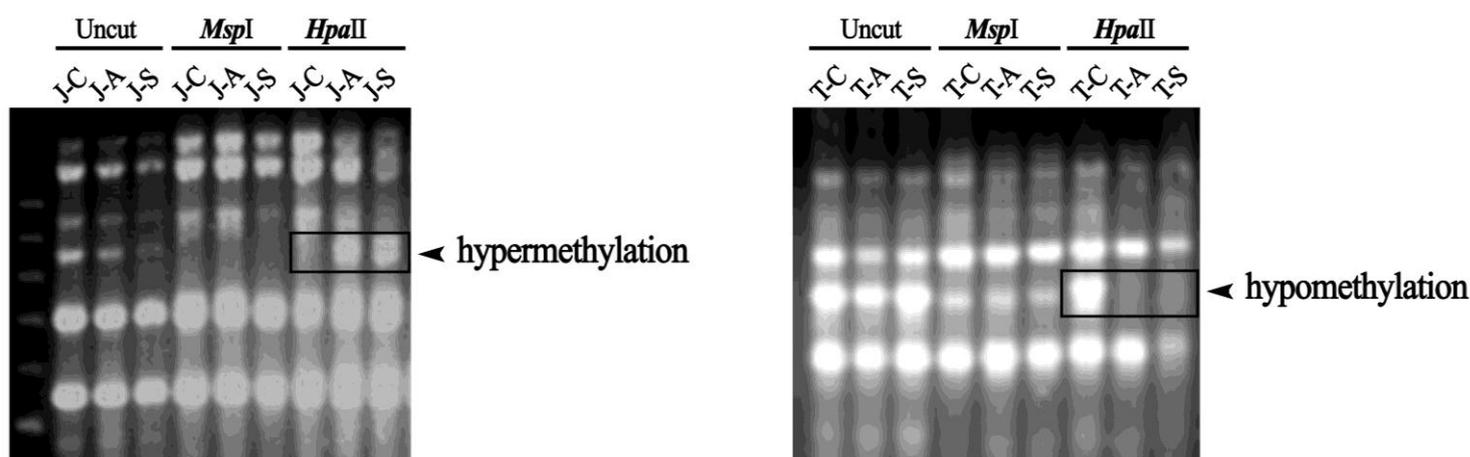


Fig 1. Examples of alterations in DNA methylation induced by salt (S) and alkaline (A) stresses relative to unstressed control (C), revealed by methylation-sensitive ISSR analysis. The DNA was digested by a pair of methylation-sensitive isoschizomers, *HpaII* and *MspI*. The digested DNA was analyzed by ISSR. The JL 89-45 (marked as J), salt-tolerant genotype; Teyou 12 (marked as T), salt-sensitive genotype. Appearance or loss of a band due to alteration in DNA methylation was indicated by rectangles.

stressed S0 plant (Fig. 5). We observed an interesting result, in which the S1 and S2 plants both represented much stronger stress tolerance than the S0 plant. This means that both methylation change and the stress tolerance trait were concomitantly inherited. This is a clear example of epigenetic inheritance, and supports the idea of Lamarckian inheritance which suggested acquired traits to be heritable. We noted that, even under non-stress condition, the S1 and S2 plants also exhibited a higher growth rate than the S0 plant (Fig. 5), suggesting that the changed methylation patterns might have caused a change in the intracellular metabolism via differential gene expression, as supported by the gene homology analysis (Supplementary Table 3). Therefore, it is possible that the combined use of salt stress treatment and some

epigenetically mutagenic treatments such as 5-azadeoxycytidine represents a useful means for stress tolerance improvement, and should be investigated future.

Materials and methods

Plant growth condition and stress treatment

Four rice (*Oryza sativa* L.) ssp. *japonica* genotypes, including two salt-tolerant (JL 89-45 and Yangeng 23) and two salt-sensitive (Teyou 12 and Gaochan 106), were chosen as plant material for this study (Yang et al., 2010). Evidence of

Table 2. Inheritance of altered DNA methylation patterns in selfed progenies (S1) of two individual stressed rice plants. Alkali stress treatment, genotype JL 89-45; salt stress treatment (genotype Yangeng 23). DNA methylation alterations of 1-16 individual plants of selfed progenies (S1) were detected by methylation-sensitive Southern blot analysis. The selfed progenies plants grown in unstressed test field.

Probe	Stressed plants Stress	Selfed progeny (S1) of the stressed plants																Frequency (%)			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Inheri.	Rev.	New	
		H/M	H/M	H/M	H/M	H/M	H/M	H/M	H/M	H/M	H/M	H/M	H/M	H/M	H/M	H/M	H/M				
<i>P2</i>	Alkali stress (genotype JL 89-45)	n/-	n/r	n/r	n/i	n/i	n/i	n/r	n/r	n/r	n/i	n/r	n/r	n/r					33.3	66.7	0
<i>P17</i>	Alkali stress (genotype JL 89-45)	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n					na	na	na
<i>P5</i>	Salt stress (genotype Yangeng 23)	n/-	n/--	n/--	n/r	n/i	n/--	n/i	n/i	n/i	n/--	68.8	6.2	25							
<i>P17</i>	Salt stress (genotype Yangeng 23)	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	na	na	na

H: *HpaII*; M: *MspI*, *Inheri.* or *i*: inheritance of altered methylation patterns; Rev. or r: reversion to the original pattern of control plants; New or n: *de novo* further alterations in the S1 generation; -: demethylation; --: further demethylation; na: not applicable

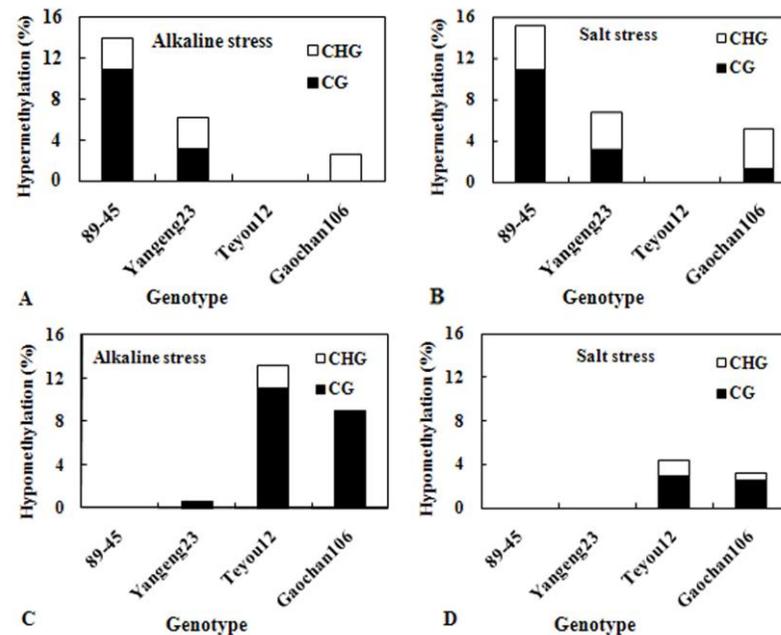


Fig 2. Tabulated data on alterations in DNA methylation of different rice genotypes under salt- and alkaline- stresses revealed by methylation-sensitive ISSR analysis. The DNA was digested by a pair of methylation-sensitive isoschizomers, *HpaII* and *MspI*. The digested DNA was analyzed by ISSR. Salt-tolerant genotypes are JL 89-45 and Yangeng 23, and salt-sensitive genotypes are Teyou 12 and Gaochan 106.

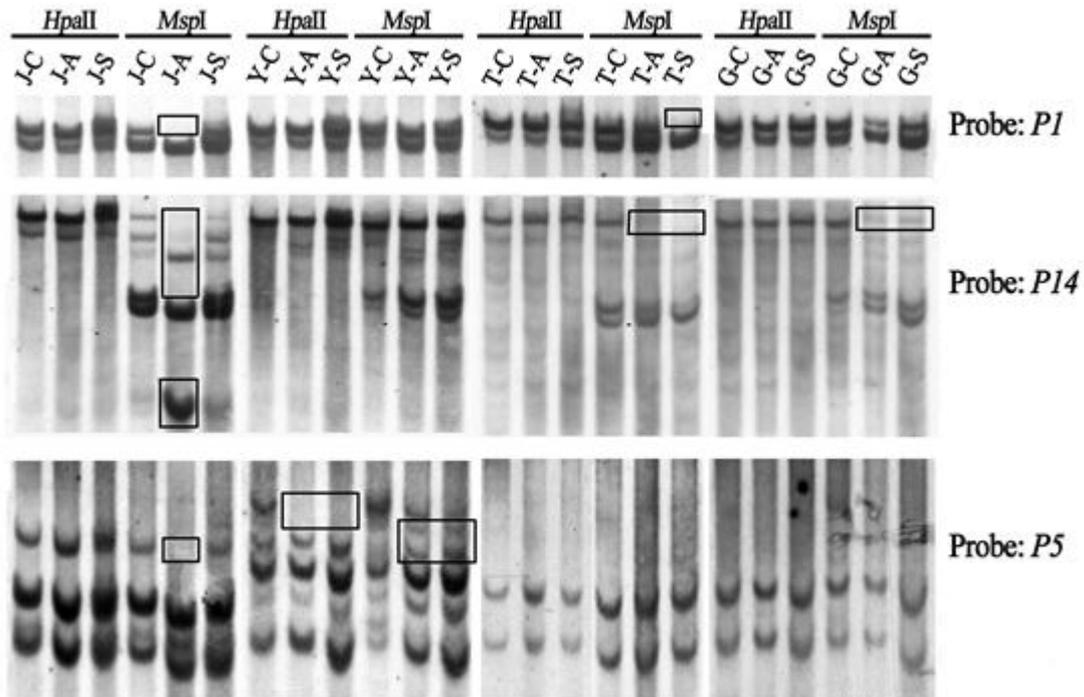


Fig 3. DNA methylation alterations in the S0 generation plants subsequent to salt (S) and alkaline (A) stresses compared with the unstressed control (C), revealed by methylation-sensitive DNA gel-blotting. Salt-tolerant genotypes are JL 89-45 (marked as J) and Yangeng 23 (marked as Y) and salt-sensitive genotypes are Teyou 12 (marked as T) and Gaochan 106 (marked as G). Gel-blotting patterns were generated by hybridizing the probes to DNA samples digested with a pair of methylation-sensitive isoschizomers, *HpaII* (H) and *MspI* (M). Rectangles denote the bands undergoing methylation alterations.

their stress tolerances was shown in supplementary Fig. 1. The seeds for each genotype were divided into three groups and packed in petri dishes in a growth cabinet (26 °C during the day and 20°C during the night, 12 h photoperiod at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Salt (0.4% NaCl), alkaline (0.4% NaHCO_3), and distilled water were applied, and marked as salt stress (S), alkaline stress (A) and control (C) groups, respectively. For salt stress, the seeds were consecutively germinated and grown in 0.4% NaCl for 7 days. For alkaline stress treatment, the seeds were germinated in distilled water for 14 d, and then transferred to 0.4% NaHCO_3 for another 7 days. Treatment solutions were changed daily. All seedlings were tagged and later analyzed. Panicles of the tagged plants were bagged, and selfing seeds were collected from each individual plant.

DNA isolation and digestion

Genomic DNA was isolated from expanded leaves by CTAB method (Ou et al., 2009) and purified by phenol extractions. The DNA was digested by a pair of methylation-sensitive isoschizomers, *HpaII* and *MspI*. To ensure complete digestion, an excess of enzymes (10 units enzyme per μg DNA) was used and the incubation time was extended to ~48 hrs. The restriction enzymes were purchased from the New England Biolabs Inc. (Beverly, Massachusetts, USA). Digested DNA were used for Southern blot and ISSR analysis.

Methylation-sensitive ISSR fingerprinting and homology analysis

The above digested DNA was analyzed by ISSR. In total, 34

pairs of selective primers were used for amplifications (Supplementary Table 1) at an annealing temperature of 50-55 °C. Representative ISSR gels were eluted and re-amplified with the appropriate selective primer combinations. Sizes of the PCR products were verified by agarose gel electrophoresis, and then cloned into the PMD-18Tcloning vector (Takara Biotech. Inc., Dalian, China). The cloned DNA segments were sequenced with vector primers by automatic sequencing. The Advanced BlastN and BlastX programs at NCBI (<http://www.ncbi.nlm.nih.gov/>) were used for homology analysis.

Methylation-sensitive DNA Southern-blotting

The digested DNA was run through 1% agarose gels and transferred onto Hybond N⁺ nylon membranes (Amersham Pharmacia Biotech, Piscataway, New Jersey) by the alkaline transfer recommended by the supplier. The probes were chosen according to corresponding ISSR bands undergoing DNA methylation alterations. A set of isolated bands from the methylation-sensitive ISSR profiles were used as probes (Table 1).

These probes were prepared by sequence-specific PCR amplifications using specific primers (Supplementary Table 2). Authenticity of all PCR-amplified probe fragments was verified by sequencing. The fragments were gel-purified and labeled with fluorescein-11-dUTP by the Gene Images Random Prime-labeling Module (Amersham Pharmacia Biotech). A hybridization signal was detected by the Gene Images CDP-Star detection module (Amersham Pharmacia Biotech) after washing at a stringency of 0.2 x SSC, 0.1% SDS for 2 x 50 min. The filters were exposed to x-ray films.

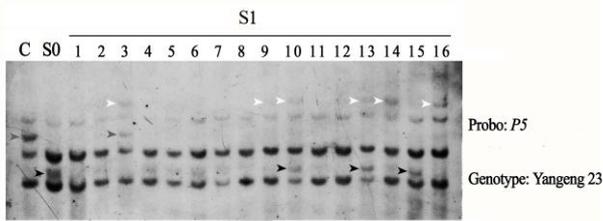


Fig 4. Transgenerational inheritance of altered DNA methylation patterns induced by the salt stress to selfed progenies (S1) of a salt-stressed rice plant (genotype Yangeng 23). C: unstressed control plant; S0: an immediately stressed plant; S1: selfed progenies of the stressed S0 plant. These selfed progenies were grown under unstressed normal conditions. S1 individual plants in Lanes 1-16 were chosen randomly. Yangeng 23, salt-tolerant genotype. Gel-blotting patterns were generated by hybridizing the probes to DNA samples digested with a methylation-sensitive isoschizomers, *MspI* (M). Black arrow showed that the altered methylation patterns were inherited into selfed progenies (S1). Gray arrow showed that methylation changes caused by salt stress were erased and recovered to the original pattern of control plants. The occurrence of *de novo* alterations is marked by white arrow.

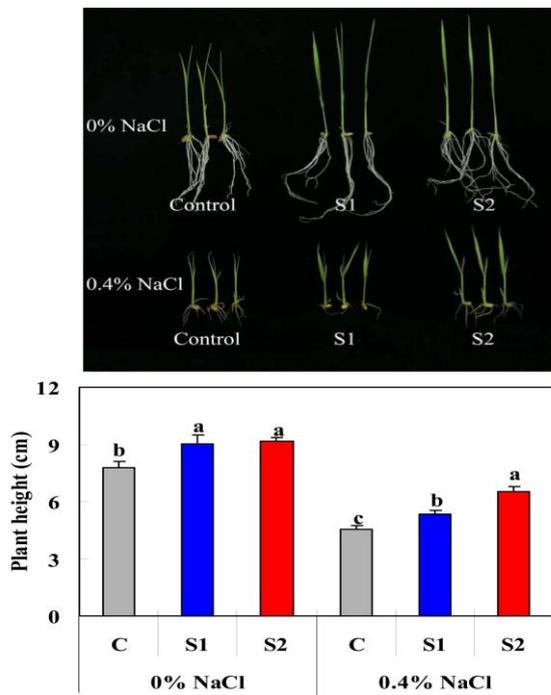


Fig 5. Comparison of salt stress tolerance in a tolerant rice plant (Control plants of genotype Yangeng 23, labeled as C) and the S1 and S2 selfed progenies of stressed Yangeng 23 plant. S2 plant was selfed progenies of S1 plant. These S1 and S2 plants both inherited the altered methylation alteration in the stressed S0 plant. The seeds of S0, S1 and S2 plants were germinated and grown in 0.4% NaCl for 7 days. Control plants (C) were germinated and grown together in distilled water for 7 days. The values of plant height are means and standard errors (\pm SE) of 6-18 individual plants. Means followed by different letters among generations of the same treatment are significantly different according to the least significant difference (LSD) test ($P < 0.05$).

Test for heritability of altered DNA methylation patterns and salt stress tolerance by progenies of stressed plants

A tolerant rice genotype (Yangeng 23) was used for this experiment. DNA methylation patterns in salt- and alkaline-stressed individual plants and their selfed progenies (S1 and S2 generation) were detected by methylation-sensitive Southern blotting as described. The S1 and S2 plants both were grown in unstressed test field. Plant growth condition was the same as described above.

Statistical analysis

Data were analyzed using the statistical software SPSS 14.0 (SPSS Inc., Chicago, USA). Plant height was represented by means and standard errors (S.E.) of 6-18 individual plants. Plant height means among generations of the same treatment were compared by the least significant difference (LSD) test at $P < 0.05$ level.

Conclusions

Our results indicated that rice cultivars with different tolerance response to salinity (tolerant and sensitive) showed contrasting patterns of DNA methylation under both salt and alkaline-stresses. This finding suggests that DNA methylation remodeling upon stress can play a conditioning and adaptive role in response to the stresses. The concomitant inheritance of altered methylation patterns and enhanced stress tolerance suggest a possibility for improving stress tolerance of crops by epigenetic manipulations such as changing in DNA methylation patterns. Further studies are required to explore the causal relationship between an epigenetic alteration and change of phenotypic or physiological traits, as well as their concomitant inheritance.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No. 30970232 and No. 30870178), Research Projects by Jilin Provincial Government (No. 20106023 and No. 20090567), and Science Foundation for Young Teachers of Northeast Normal University (No.09QNJJ021).

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