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DNA methylation alterations of rice in response to cold stress

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Abstract

Rice is sensitive to cold stress, and cold tolerance in rice is a complex trait. In the present study, DNA methylation alterations induced by cold stress were examined in two contrasting rice genotypes, Li-Jiang-Xin-Tuan-Hei-Gu (LTH) and IR64, by methylation-sensitive amplified polymorphism analysis. At the seedling stage, a remarkable increase in DNA methylation under cold stress was detected only in the roots of the cold-tolerant genotype of LTH. Demethylation in the panicles of LTH under cold stress and subsequent recovery were determined. An increase in DNA methylation in the leaves and demethylation in the panicles were observed in IR64 at the booting stage under cold stress. These results indicate that cold-induced DNA methylation alterations are specific to genotypes and are dependent on the growth stage and tissue/organ types. Further analysis suggests that a number of cold-induced DNA methylation changes in both genotypes cannot be reverted after recovery, implying that these loci with alterations of DNA methylation may be mitotically heritable and involved in cold stress responsiveness.

Keywords: Rice; Cold stress; DNA methylation; methylation-sensitive amplified polymorphism.
Abbreviations: LTH- Li-Jiang-Xin-Tuan-Hei-Gu; MSAP- Methylation-Sensitive Amplified Polymorphism**;** CT- Cold Tolerance.

Introduction

Low temperature is one of the major abiotic stresses that reduce crop growth and development which is related to seedling establishment, yield and productivity rates in cultivated crops (Badea and Basu, 2009). Rice is a tropical crop that is sensitive to low temperature (the critical low temperature is 12-13°C at seedling establishment stage and 15°C at booting stage); cold stress limits rice growth and reproduction in many rice-growing areas outside the tropics. Rice cultivars exhibit wide contrast in terms of their sensitivity to low temperature; for example, *Japonica* subspecies are generally more cold tolerant than *indica* subspecies (Kaw and Khush, 1986). Cold tolerance (CT) of rice is a genetically complex trait, and numerous quantitative trait loci for CT in rice have been mapped using different mapping populations (Takeuchi et al., 2001; Andaya and Mackill, 2003; Zeng et al., 2009; Suh et al., 2010). A major CT quantitative trait locus called *Ctb1* has been cloned (Saito et al., 2010). However, the genetic and molecular bases of cold tolerance are not clearly understood. In addition, little is known about the role of the epigenetic process involved in the molecular regulation of cold tolerance in rice.

As an important epigenetic modification, DNA methylation plays a key role in regulating gene expression and stress responses (Rassoulzadegan et al., 2006). Cold-induced genome-wide DNA methylation changes were observed in maize, and a gene encoding a retrotransposon was specifically activated under cold stress (Steward et al., 2000, 2002). DNA methylation alterations under environmental stress may be related to the activity of transposon. The activation of Tam3 transposon in *Antirrhinum majus* resulted in the decrease in methylation under low temperature (Hashida et al., 2003, 2006). Demethylation of *NtGPDL* gene in tobacco was followed by leaf-specific up-regulation of this gene under abiotic stresses, such as salt and low temperature (Choi and Sano, 2007; Kamal et al., 2010). Cold-stress-induced DNA methylation changes vary between genotypes. In *Ribes* germplasm, DNA methylation level increased in the tolerant genotype, whereas demethylation was evident in the sensitive genotype (Johnston et al., 2009). In rice, a putative gene encoding an F-box protein was demethylated and up-regulated by cold stress (Hua et al., 2005; Chakrabarti et al., 2011). These results indicate that DNA methylation is involved in the molecular regulation of the cold responsiveness of plants.

In the current study, the temporal-spatial and variety specificities of DNA methylation changes under low-temperature stress and subsequent recovery were determined using the methylation-sensitive amplified polymorphism (MSAP) analysis. The results show the molecular role of DNA methylation in plant response to environmental stimuli.

Results

DNA methylation of LTH and IR64 by MSAP analysis

A total of 810 to 846 clear and reproducible DNA bands were detected in at least one sample of LTH and IR64 under different treatments using 32 MSAP primer combinations. All amplified DNA bands were classified into four types (Table S2) according to the presence or absence of the bands as described by Li (2009) and Wang (2011). Type I indicates no methylated cytosine on double strands of DNA or inner methylated cytosine on a single strand (CCGG/GGCC). Type II represents semi-methylation, i.e., outer methylated cytosine on a single

DNA strand (^{5m}CCGG/GGCC). Type III refers to full methylation with inner methylated cytosine on double DNA strands (C^{5m}CGG/GG^{5m}CC). Type IV includes fully methylated cytosines and externally methylated cytosines on double DNA strands (^{5m}C^{5m}CGG/GG^{5m}C^{5m}C and ^{5m}CCGG/GGC^{5m}C). The MSAP results of both lines under control, stressed, and subsequent recovery conditions are shown in Tables 1 and 2. The MSAP% ranged from 21.5% (181 bands) in the roots of LTH at the seedling stage under control conditions to 31.4% (266 bands) in the leaves of IR64 at the booting stage. The various DNA methylation levels were mainly from fully methylated DNA sequences (i.e., MSAP band Types III and IV) in both varieties. The DNA methylation differences between tissues and among tissues at two developmental stages were also detected in both lines with a general pattern of *leaves* > *roots* at the seedling stage and *panicle* > *leaves* at the booting stage, respectively.

DNA methylation level changes in both lines under cold-stress treatment

The DNA methylation levels of LTH and IR64 were dynamic under cold stress treatment when more detailed comparisons were performed. At seedling stage, no DNA methylation level alteration was detected in the leaves of both lines and in the roots of IR64 under cold stress and subsequent recovery. However, an increase in DNA methylation in the roots of LTH under cold stress was observed. This DNA hypermethylation was caused by the cold temperature, and was almost demethylated after recovery (Table 1). At booting stage, DNA methylation slightly decreased in the leaves of LTH under cold stress and subsequent recovery conditions, but obvious demethylation was observed in the panicles of LTH recovered from cold-stress condition. The DNA methylation level in IR64 leaves increased from 26.0% to 31.4% under cold stress, but it decreased from 31.4% to 24.0% after recovery. However, DNA methylation decreased in the panicles of IR64 because of the cold stress, and this demethylation could not be restored after recovery (Table 2).

Alterations of DNA methylation pattern under cold stress and subsequent recovery conditions

To identify the DNA methylation changes (i.e., demethylation or methylation under cold stress and subsequent recovery), we classified all differentially methylated DNA bands into different classes. As indicated in the Table 3, D classes (D1, D2, and D3), which included demethylated DNA fragments under cold stress; M classes (M1, M2, and M3), which comprised methylated DNA fragments induced by cold stress; R classes (RM and RD), which consisted of methylated or demethylated DNA bands detected after recovery; and UN and OT classes, which referred to stable and unclassified bands, respectively. At seedling stage, cold stress caused more cytosine methylation (GGCC content) in the roots of LTH with a total of 35 methylated bands (M1+M2+M3) than in the panicles with 14 demethylated bands (D1+D2+D3). Most of these methylated and demethylated bands were reverted after recovery (26 and 12 out of 35 and 14, respectively).

At booting stage, DNA methylation pattern changes varied based on tissue and genotype. Major parts of cold-induced demethylation and methylation bands were classified under D2 (36 and 43 bands, respectively) and M1 (21 and 26 bands, respectively) classes in the two tissues of LTH (Table 3). This result indicated that most demethylation caused by cold stress cannot be recovered, but most methylation in LTH under cold stress can be reverted at booting stage after release from cold stress. In IR64, more methylation events (76 bands) were detected in the leaves, but more demethylation events (67 bands) were observed in the panicles. Most of the demethylated DNA bands in the leaves and the panicles remained hypomethylated after recovery (D2 classes; 25 and 46 bands, respectively). However, more M1 (41 bands) and M2 classes (13 bands) were found in the leaves and panicles of IR64 at the booting stage, which demonstrate that changes in DNA methylation are unstable in different tissues. Notably, a number of DNA methylation changes (RM and RD class bands) that resulted only after recovery were detected in the two tissues of both genotypes at booting stage. As shown in Table 3, there are more RM and RD bands in IR64 (65 and 43 bands) than in LTH (26 and 33 bands). All these results show that DNA methylation pattern changes under cold stress and subsequent recovery conditions are dependent on genotype, tissue, and developmental stage.

BLAST result of the differentially methylated DNA sequences induced by cold stress

A set of 15 cold-induced, differentially methylated DNA bands were cloned and sequenced. As shown in Table 4, most of these cloned DNA fragments can be mapped on the gene-body regions, except for the sequence of W5, which is from a non-coding region. Among them, two fragments (W28 and W45) are homologous to gene encoding retrotransposon proteins. W14 is located in the promoter region of a gene (Loc_Os10g04860) encoding putative aldehyde oxidase, which is responsible for the conversion of ABA-aldehyde to ABA. Aldehyde oxidase plays a role in ABA accumulation and regulation of plant response to abiotic stresses, such as cold stress (Zdunek-Zastocka et al., 2004; Sun et al., 2009). W24 is homologous to the coding region of a gene encoding the gibberellin 2-beta-dioxygenase involved in GA inactivation (Santes and Garcia-Martinez, 1995; Sponsel and Hedden, 2004). W35 is highly homologous to the gene encoding bromodomain-associated family protein (Loc_Os12g38620); this gene is related to histone modification and transcription regulation (Wu and Chiang, 2007; Morinière et al., 2009). The remaining sequences are homologous to gene encoding ATPase (W4), aquaporin (W15), phosphoribulokinase/Uridine kinase (W23), F-box containing protein (W37), and transporter (W29), indicating that a number of genes with different functions are affected by cold stress through DNA methylation changes.

Discussion

DNA methylation is one of epigenetic mechanisms in plants that respond to environmental stimuli by regulating gene expression. In the current study, the results indicate that DNA methylation changes induced by cold stress are tissue-specific and dependent on growth stage. Most importantly, the patterns of DNA methylation alteration under cold stress and subsequent recovery vary based on tissues and genotypes. There are common and heritable DNA methylation differences among ecotypes (Cervera et al., 2002; Vaughan et al., 2007). A low correlation between the frequency of DNA methylation difference and genetic distance was also found in different genotypes of rice (Takata et al., 2005). In the present study, a few DNA methylation differences in response to cold stress were detected between the two genotypes with contrasting phenotypes. These differences are dependent on the tissue/organ and the developmental stage (more evident at seedling stage). However, the relation of the difference in genotype-dependent DNA methylation to cold tolerance in rice needs to be further elucidated. Environmental conditions can

Table 1. DNA methylation levels in leaves and roots of LTH and IR64 at seedling stage under cold stress

MSAP Band Types [a)]	LTH						IR64					
	Leaves			Roots			Leaves			Roots		
	Control	Cold	Recovery	Control	Cold	Recovery	Control	Cold	Recovery	Control	Cold	Recovery
I	582	582	584	662	641	660	608	609	605	631	633	628
II	6	6	6	6	6	6	4	4	4	3	3	3
III	118	118	118	115	115	114	118	118	118	100	100	100
IV	137	137	135	60	81	62	80	79	83	76	74	79
Total amplified bands	843	843	843	843	843	842	810	810	810	810	810	810
Total methylated bands[b)]	261	261	259	181	202	182	202	201	205	179	177	182
MSAP (%)	31.0	31.0	30.7	21.5	24.0	21.6	24.9	24.8	25.3	22.1	21.9	22.5
Full methylated bands[c)]	255	255	253	175	196	176	198	197	201	176	174	179
Full methylation ratio (%)	30.2	30.2	30.0	20.8	23.3	20.9	24.4	24.3	24.8	21.7	21.5	22.1

a) Type I: no methylated cytosine on double strands of DNA or inner methylated cytosine on a single strand; Type II: semi-methylation, i.e., outer methylated cytosine on a single DNA strand; Type III: full methylation with inner methylated cytosine on double DNA strands. Type IV: fully methylated cytosines and externally methylated cytosines on double DNA strands. b) Type II+III+IV. c) Type III+IV.

Table 2. DNA methylation levels in leaves and panicles of LTH and IR64 at booting stage under cold stress

MSAP Band Types [a)]	LTH						IR64					
	Leaves			Panicles			Leaves			Panicles		
	Control	Cold	Recovery	Control	Cold	Recovery	Control	Cold	Recovery	Control	Cold	Recovery
I	633	638	640	617	625	654	626	580	643	620	662	657
II	14	16	27	18	30	34	24	40	35	33	23	29
III	108	101	104	137	99	89	95	140	90	116	91	101
IV	79	79	63	62	80	57	101	86	78	77	70	59
Total amplified bands	834	834	834	834	834	834	846	846	846	846	846	846
Total methylated bands[b)]	201	196	194	217	209	180	220	266	203	226	184	189
MSAP (%)	24.1	23.5	23.3	26.0	25.1	21.6	26.0	31.4	24.0	26.7	21.7	22.3
Full methylated bands[c)]	187	180	167	199	179	146	196	226	168	193	161	160
Full methylation ratio (%)	22.4	21.6	20.0	23.9	21.5	17.5	23.2	26.7	19.9	22.8	19.0	18.9

a) Type I: no methylated cytosine on double strands of DNA or inner methylated cytosine on a single strand; Type II: semi-methylation, i.e., outer methylated cytosine on a single DNA strand; Type III: full methylation with inner methylated cytosine on double DNA strands. Type IV: fully methylated cytosines and externally methylated cytosines on double DNA strands. b) Type II+III+IV. c) Type III+IV.

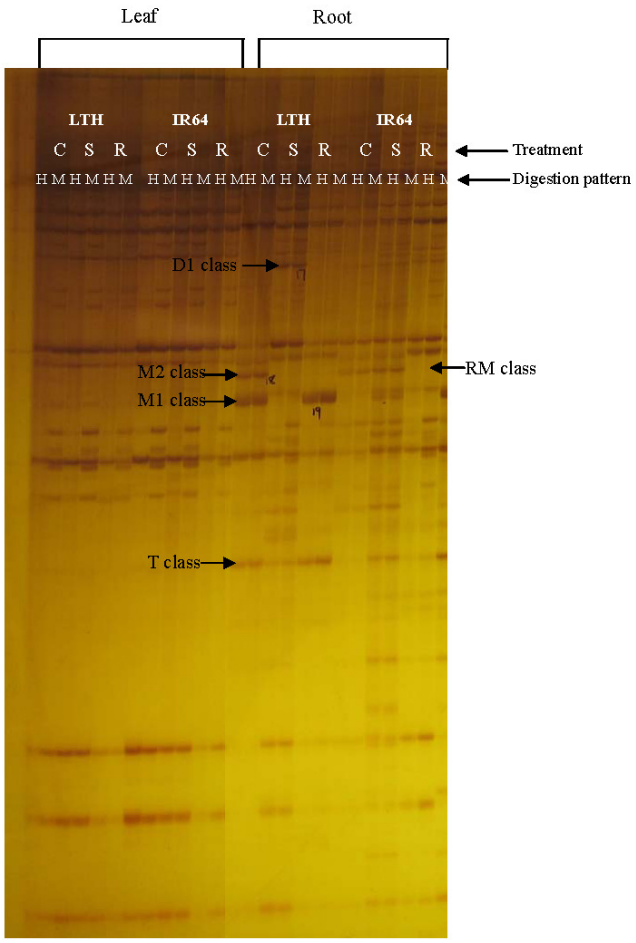

Fig 1. DNA MSAP patterns in leaf (L) and root (R) under three treatments at seedling stage. H: digested by *Eco*R I/*Hpa* II; M: digested by *Eco*R I/*Msp* I; C: Control condition; S: Cold stress condition; R: Recovery; D1 class: demethylated by stress, but remethylated after recovery; M1 class: Methylated by stress, but demethylated after recovery; M2 class: methylated by stress, and remaining hypymethylated after recovery; RM class: only demethylated by recovery, T class: Tissue-specific methylation.

cause DNA methylation changes, such as decrease in DNA methylation, which is linked to vernalization in *Arabidopsis thaliana* (Finnegan et al., 1998) and winter wheat (Sherman and Talbert, 2002). Moreover, environmental conditions can somatically alter the degree of TAM3 DNA methylation, and are positively correlated with growth temperature (Hashida et al., 2006). In the present study, MSAP analysis detected evident DNA methylation changes in both genotypes at the booting stage. This result may support the idea suggested by Andaya and Mackill (2003) that rice plants are more sensitive to cold stress at the reproductive growth stage. Notably, the results of cold-induced DNA hypermethylation in the panicles of IR64 were different from those of cold-induced DNA demethylation in the panicles of LTH, which indicate that these different DNA methylation pattern changes may be correlated to cold tolerance in rice. DNA methylation is a reversible epigenetic process affected by complex gene by environment interactions (Ramchandani et al., 1999). Our previous study on drought-induced DNA methylation changes also indicated that most demethylation/methylation loci caused by drought can be reversed to their original status after recovery (Wang et al., 2011). However, a large number of cold-induced, differentially methylated DNA loci cannot be reverted after recovery (D2 and M2 classes). This observation indicates that these altered DNA methylation loci may be mitotically heritable, and they can affect stable stress-responsive gene regulation and long-term resistance within-generation stress memory (Chinnusamy and Zhu, 2009).

Materials and methods

Plant materials and stress treatment

Two rice varieties, Li-Jiang-Xin-Tuan-Hei-Gu (LTH) and IR64, were used in this experiment. IR64 is a cold-sensitive *indica* rice variety developed at the International Rice Research Institute. LTH is a cold-tolerant *japonica* rice variety kindly provided by the Yunnan Academy of Agricultural Sciences. All seeds were sown and grown in plastic pots filled with Turface and supplied with half nutrient solution (Yoshida, 1976) in a glasshouse. The rice seedlings were grown under a constant condition of 12 h light/12 dark at 28 °C/25 °C (day/night) and a relative humidity of 60%-80% in a phytotron. The cold-stress treatment was performed as follows: at seedling stage, the rice plants were kept at 4 °C for 2 d in the growth chamber; At booting stage, the plants were kept in a cold room at 10 °C with 12 h light/12 h dark for 2 d. The stressed plants were allowed to recover under normal growth conditions (25 °C) for 2 d. Leaf and root tissues were collected from 10 plants which under stressed, controlled, and recovered conditions at seedling stage. At booting stage, leaf and young panicle tissues were sampled from 3 plants for the three treatments. Three replicates were prepared from each sample to analyze DNA methylation.

Methylation-sensitive amplified polymorphism analysis

DNA extraction was performed using the standard CTAB method. MSAP analyses were conducted in accordance with the method by Xiong (1999) with minor modification. Double enzyme combinations *Eco*R I/*Msp* I and *Eco*R I/*Hpa* II were selected for the analysis. The designed adapters, as well as the primary and secondary PCR primers, are shown in Table S1. First, we used several primer combinations to identify the repeatability of three replicates for each sample, the results showed identical MSAP bands among three replicates (Data not shown). Then we mixed the replicates as pool sample for the following MSAP analysis. A total of 300 ng genomic DNA was double digested and ligated in one step with a 25 µl reaction volume (1×T4 ligase buffer, 1×YANG^{+}/TANGO buffer, 3 U *Eco*R I and 3 U *Hpa* II-*Msp* I, 1.5 U T4 ligase, 50 pmol adapters for *Eco*R I, and 5 pmol for *Hpa* II-*Msp* I), which was kept at 37 °C for 8-10 h. The product was diluted 10-fold and used as template in the subsequent pre-amplification. The volume of pre-amplification was 20 µl, which contains 2 µl of the aforementioned diluted mixture, 1× PCR buffer, 10 mM dNTP 2 µl, 10 µM *Eco*R I and HM primer, and 0.5 U *Taq* DNA polymerase. The PCR reaction was performed for 20 cycles with 30 s denaturation at 94 ºC, 1 min annealing at 56 ºC, and 1 min extension at 72 ºC. The PCR product was diluted 10-fold and used for the second selective amplification with the same primers, but with two selection nucleotides at the 3' end. The final product was denatured, separated on 6% denaturing polyacrylamide gels, and visualized by silver staining. The differentially amplified fragments (differentially methylated DNA fragments) were recovered from the gel, re-amplified, and purified with Wizard SV gel by using a PCR clean-up system (A9280, Promega Corp. USA). The purified DNA fragments were cloned with T-vector (Takara) and *E. coli*

Table 3. Summary of DNA methylation pattern changes of LTH and IR64 at booting stage under cold stress.

Band Class [a)]	Band No in LTH				Band No in IR64			
	Seedling Stage		Booting Stage		Seedling Stage		Booting Stage	
	Leaves	Roots	Leaves	Panicles	Leaves	Roots	Leaves	Panicles
D1	1	12	12	3	4	7	12	13
D2	0	2	36	43	0	0	25	46
D3	0	0	3	2	0	0	2	8
(D1+D2+D3)	1	14	51	48	4	7	39	67
M1	0	26	21	26	0	0	41	8
M2	1	9	15	13	3	8	27	13
M3	0	0	4	6	0	0	8	3
(M1+M2+M3)	1	35	40	45	3	8	76	24
RM	0	0	16	15	0	0	32	19
RD	3	5	10	18	0	5	33	24
(RM+RD)	3	5	26	33	0	5	65	43
OT	0	0	4	10	0	0	29	5
UN	838	789	713	698	803	790	637	707
Total changed bands	5	54	121	136	7	20	209	139
Total amplified bands	843	843	834	834	810	810	846	846

a) D1- demethylated by stress, but remethylated after recovery; D2- demethylated by stress, and remaining hypomethylated after recovery; D3- Demethylated by stress, but remethylated in a different pattern after recovery; M1- Methylated by stress, but demethylated after recovery; M2- methylated by stress, and remaining hypymethylated after recovery; M3- methylated by stress, but demethylated in a different pattern after recovery; RM- only methylated by recovery; RD- only demethylated by recovery; UN-not changed; OT-other classes of bands.

Table 4. BLAST result of the DNA methylation polymorphic sequences

Code	Size (bp)	BLAST result
W1	136	Loc_Os04g01250, amidase family protein
W4	168	Loc_Os05g25550, E1-E2 ATPase domain containing protein
W5	136	Non-coding region chr09
W12	132	Loc_Os04g16722, putative uncharacterized protein ycf68
W14	139	Loc_Os10g04860, putative aldehyde oxidase
W15	243	Loc_Os10g35050, putative aquaporin protein
W23	153	Loc_Os04g50880, phosphoribulokinase/Uridine kinase family protein
W24	138	Loc_Os01g22920, putative gibberellin 2-beta-dioxygenase
W28	108	Loc_Os04g34960, putative retrotransposon protein, Ty1-copia subclass
W29	140	Loc_Os11g47320, putative protein transporter
W35	227	Loc_Os12g38620, bromodomain associated family protein
W37	156	Loc_Os10g03780, OsFBX351 - F-box domain containing protein
W39	125	Loc_Os01g58530, putative NB-ARC/LRR disease resistance protein
W45	181	Loc_Os07g34510, putative retrotransposon protein, unclassified
W81	113	Loc_Os03g17780, WD domain, G-beta repeat domain containing protein

DH5*α*, and sequenced in the Key Laboratory of the National Key Facility for Crop Gene Resources and Genetic Improvement in Chinese Academy of Agricultural Sciences. The resulting sequences were analyzed by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Rice Genome Annotation Project BLAST Search (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml).

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Supplementary data

Table S1. Adaptor and primer sequences for MSAP

Table S2. Modified methylation patterns of *Hpa* II and *Msp* I digested genomic DNA

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