

Changes in DNA Methylation Pattern at Two Seedling Stages in Water Saving and Drought-Resistant Rice Variety after Drought Stress Domestication

ZHENG Xiao-guo^{1,2,#}, CHEN Liang^{1,#}, LOU Qiao-jun¹, XIA Hui¹, LI Ming-shou¹, LUO Li-jun^{1,2}

(¹Shanghai Agrobiological Gene Center, Shanghai 201106, China; ²College of Plant Sciences & Technology, Huazhong Agricultural University, Wuhan 430070, China; [#]These authors contribute equally to this paper)

Abstract: Recent studies revealed that DNA methylation plays an important role in plant growth and development. In this study, a water-saving and drought-resistant rice variety Huhan 3 was subjected to drought stress from tillering to grain-filling stages in six successive growth cycles. The variations in DNA methylation pattern between the original generation (G_0) and the sixth generation (G_6) were analyzed by using methylation sensitive amplification polymorphism method. The results revealed that the methylated loci accounted for 34.3% to 34.8% of the total loci. Among these methylated loci, 83.1% to 84.8% were full- and hyper-methylated and 15.2% to 16.9% were hemi-methylated. The DNA methylation level decreased from the three-leaf to four-leaf stages in Huhan 3. Differentially methylated loci (DML) between generations or/and between different developmental stages accounted for 4.0% of the total loci, most of which were only related to plant development (57.9%). Compared to G_0 , the DNA methylation pattern of G_6 changed after drought domestication, at the three-leaf stage, de-methylation accounting for 59.1%, while at the four-leaf stage, re-methylation for 47.9%. Genome-wide alternations of DNA methylation were observed between the two seedling stages, and DML mainly occurred on the gene's promoter and exon region. The genes related to DML involved in a wide range of functional biology and participated in many important biological processes.

Key words: drought stress; water-saving and drought-resistant rice; DNA methylation; seedling stage; methylation sensitive amplification polymorphism

DNA methylation, the addition of a methyl group to the position 5' of a cytosine base (5mC), is a conserved epigenetic marker which widely exists in varieties of organisms. In mammals, DNA methylation occurs almost exclusively in the symmetric CG context and is estimated occurring at 70%–80% of CG dinucleotides throughout the genome. In plants, DNA methylation commonly occurs at cytosine bases in all sequence contexts: the symmetric CG and CHG contexts (in which H = A, T or C) and the asymmetric CHH contexts (Bird, 2002; Jackson et al, 2002; Goll and Bestor, 2005; Henderson and Jacobsen, 2007). DNA methylation in all the three contexts are established by domains rearranged methyltransferase 2 (DRM2), but maintained by three different DNA methyltransferases. For example, CG methylation is

maintained by DNA methyltransferase 1 (MET1, also known as DMT1), CHG methylation is maintained by chromomethylase 3 (CMT3, a plant-specific DNA methyltransferases), and CHH methylation is maintained by DRM2 (Law and Jacobsen, 2010). DNA methylation is associated with many important biological processes, including heterochromatin formation, defense against transposon proliferation, genomic imprinting, regulation of endogenous gene expression, silencing of transgenes and so on (Paszowski and Whitham, 2001; Bender, 2004; Zhang et al, 2006; Li et al, 2008; Tsukahara et al, 2009).

DNA methylation plays an important role in plant growth and development, and abnormal DNA methylation may cause morphological variations. Recent studies in plants revealed that DNA methylation participates in the regulation of plant height, floral symmetry, flowering time, disease resistance, and the response to environmental stress (Cubas et al, 1999;

Received: 8 February 2014; Accepted: 15 April 2014

Corresponding author: LUO Li-jun (lijun@sagc.org.cn)

Santos et al, 2002; Steimer et al, 2004; Manning et al, 2006; Boyko and Kovalchuk, 2008). In rice, a spontaneous mutant *Epi-d1* was identified (Miura et al, 2009). It is often chimeric, producing both dwarf and normal tillers on a single plant, and *Epi-d1* plants show a wide variation of dwarf and normal features, from completely dwarf to completely normal. The phenotype is mitotically and meiotically inheritable and related to the metastable epigenetic silencing of the *DWARF1* (*D1*) gene. *D1* gene silencing is correlated with repressive DNA methylation marks in the *D1* promoter region (Miura et al, 2009). Another epi-mutant identified in rice is *epi-df*, which is a gain-of-function epi-allele of *FIE1*, and the allele causes a dwarf stature and various floral defects that are inherited in a dominant fashion and caused by ectopically expressed *FIE1* which is hypomethylated in the 5' region in *epi-df* (Zhang et al, 2012). Sha et al (2005) reported that a rice cultivar, Wase Aikoku 3, becomes resistant to the blight pathogen *Xanthomonas oryzae* pv. *oryzae* at the adult stage. Analysis of the DNA methylation patterns revealed that overall levels of methylation are higher in adult plants than in seedlings, and differentially methylated sites between adult plants and seedlings account for 2.3% of the total identified sites. Hyper- or hypo-methylation at specific loci may involve in disease-resistance in adult rice plants (Sha et al, 2005). In addition, abiotic stress, such as drought, salinity and heavy metal, and biotic stress, such as pathogen infection, can induce DNA methylation variations in plants (Aina et al, 2004; Chinnusamy et al, 2008; Pan et al, 2009; Tan, 2010; Verhoeven et al, 2010; Wang et al, 2011; Downen et al, 2012; Karan et al, 2012; Ou et al, 2012). Furthermore, a considerable proportion of these variations can be faithfully inherited to progenies and significantly enhance stress resistance of the progenies (Kou et al, 2011; Ou et al, 2012).

Plant methylation pattern varies among species, genotypes or eco-types, and even individual plants may show a unique methylation pattern at different developmental stages or responding to environmental changes (Cervera et al, 2002; Paun et al, 2010). Xiong et al (1999) analyzed the DNA methylation patterns in an elite hybrid rice variety, Shanyou 63, and its parental lines, Zhenshan 97 and Minghui 63. The results revealed that hybrid has different DNA methylation pattern compared with its parental lines (Xiong et al, 1999). Lu et al (2008) analyzed the specific DNA methylation patterns in three different

maize tissues, tassel, bracteal leaf and ear leaf. The results revealed that the three tissues have different DNA methylation patterns and levels (Lu et al, 2008). Sakthivel et al (2010) investigated the pattern and level of cytosine methylation in the leaf tissue of an elite Indian hybrid rice variety KRH2 and its parents at three stages (15 day-old and 35 day-old seedlings, flag leaf). They observed that the methylation levels are high at the initial growth stages and decrease at the later stage and that a considerable portion of cytosine methylation variations are novel in hybrid. Furthermore, demethylation occurs more predominantly during early stages than hyper-methylation, and the frequency of demethylation decreases in flag leaf while that of hyper-methylation remarkably increases in the hybrid (Sakthivel et al, 2010). Pan et al (2009) characterized DNA methylation patterns in leaves and roots at seedling and tillering stages under drought stress for DK106 (drought-resistant introgression line) and IR64 (recurrent parent, drought sensitive line). The results showed that about 20% cytosine of CCGG sequences in rice genome is methylated, and the methylation frequency is similar in coding and non-coding regions on the genome (Pan et al, 2009). The pattern and level of DNA methylation are temporal-spatial specific and variety specific.

Water-saving and drought-resistant rice (WDR) is a new type of modern rice, which is developed through introgressing the water-saving and drought-resistance from the traditional upland rice into the commercialized paddy rice cultivars (Luo, 2010). In this study, a WDR variety, Huhan 3, was subjected to drought stress from tillering to grain-filling stages in six successive growth cycles. The DNA methylation patterns of the original generation (G_0) and the sixth generation (G_6) at three-leaf stage (21 day-old) and four-leaf stage (28 day-old) seedlings were assessed by using the methylation sensitive amplification polymorphism (MSAP) method. We aimed to study the impact of drought entrainment on DNA methylation patterns of WDR variety, study the DNA methylation variations between different developmental stages, and investigate the features of DNA methylation variations on gene level.

MATERIALS AND METHODS

Rice materials and drought treatments

A WDR variety, Huhan 3 (*Oryza sativa* L. ssp. *japonica*) (bred by Shanghai Agrobiological Gene Center,

China), was used in the study (Luo, 2010). The seeds from a single plant of Huhan 3 were designated as the original generation (G_0). Some of these seeds were grown under natural environment. Then, drought stress was imposed on plants from the tillering to grain-filling stages until leaf rolling reaching the highest level according to the grading standard (O'Toole and Cruz, 1980), and the relative water content of leaves was lower than 70%. Seeds from treated plants were harvested, and the same procedure was performed for the other five generations to obtain the G_6 seeds.

To avoid the effects of exogenous environmental factors and obtain more uniform phenotype, the seedlings of both G_0 and G_6 were grown in a growth chamber (CONVIRON CMP6050) after germination and cultivated by hydroponic method. The temperature was 21 °C to 29 °C, light [200 mmol/(m²·s)] was set from 7:00 am to 7:00 pm (12 h), and the relative humidity was 75% to 80%. The seedlings were irrigated with water during the first week and with Yoshida nutrient solution from the second to the forth weeks. Leaves from the three-leaf stage seedlings (21 day-old) were collected as sample 1, and leaves from the four-leaf stage seedlings (28 day-old) were collected as sample 2. Each sample had four replicates and each replicate contained 12 seedlings. Among the 12 seedlings, leaves from 8 seedlings with similar phenotype were collected and mixed to extract genomic DNA with a modified CTAB method (Attitalla, 2011).

MSAP analysis

The MSAP approach is developed from the standard amplified fragment length polymorphism method (Vos et al, 1995). Two isoschizomers with different methylation sensitivity, *Msp* I and *Hpa* II, were used as frequent cutters, and were combined with the same rare cutter (*EcoR* I) in parallel batches, respectively. With some modifications to increase the number of amplified fragments and improve fingerprint readability, the MSAP was performed following the general steps described by Xiong et al (1999). Pre-experiment with 16 primer-pairs showed that there was no difference between four replicates. Then, the genomic DNA of four replicates was evenly mixed for the MSAP analysis.

The digestion and ligation reactions were separately performed. In the digestion reaction, DNA samples were separately digested with double enzyme combinations, *EcoR* I/*Msp* I and *EcoR* I/*Hpa* II. The reaction solution contained 250 ng genomic DNA, 2 μL of 10 × T4 DNA ligase buffer (Promega, Madison,

WI, USA), 10 U *EcoR* I, 10 U *Msp* I (or *Hpa* II) (New England Biolabs, USA), and ddH₂O with a final volume of 20 μL, subsequently incubated at 37 °C for 2 h. Approximately 5 μL of digestion products were checked with 0.5% agarose gels to confirm the DNA template was completely digested. Then, 15 μL of digestion products were mixed with 5 pmol *EcoR* I adapter, 50 pmol *Hpa* II/*Msp* I adapter (Table 1), 1.5 U T4 ligase and 1.5 μL of 10 × T4 ligation buffer. ddH₂O was added to a final volume of 30 μL and the solution was incubated at 16 °C for 8 to 16 h for ligation reaction. Enzymes were afterwards denatured at 65 °C for 10 min. Negative control samples were included at all steps to prevent contamination. The resultant products were diluted 20-fold and used as templates in the following pre-amplification.

Pre-amplification was conducted in a 20 μL reaction system containing 2 μL of 10 × PCR reaction buffer (Tiagen, Beijing, China), 1 μL of dNTPs (2.5 mmol/L), 1.5 U *Taq* polymerase, 5 μL of diluted product (as DNA template), 5 μmol/L pre-amplification primers (*EcoR* I and *Hpa* II/*Msp* I, Table 1), and ddH₂O. The reaction was catalyzed for 29 cycles in a thermocycler of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min with a final extension at 72 °C for 10 min. A total of 20 μL volume for the selective amplification contained 2 μL of 10 × PCR reaction buffer, 1 μL of dNTPs (2.5 mmol/L), 1.5 U *Taq* polymerase, 1 μL of pre-amplification product (as

Table 1. Sequences of adapter and primer used in methylation sensitive amplification polymorphism (MASP) assay.

Adapter and primer	<i>EcoR</i> I	<i>Hpa</i> II/ <i>Msp</i> I
Adapter 1	5'-CTCGTAGACTGCG TACC-3'	5'-GACGATGAGTCTA GAA-3'
Adapter 2	5'-AATTGGTACGCAG TCTAC-3'	5'-CGTTCTAGACTCA TC-3'
Pre-amp primer	5'-GACTGCGTACCAA TITCA-3' (E1)	5'-GATGAGTCTAGAA CGGT-3' (HM1)
Sel-amp primer	E1 + TA (E01) E1 + TG (E02) E1 + TC (E03) E1 + GA (E04) E1 + GC (E05) E1 + GT (E06) E1 + CA (E07) E1 + CG (E08) E1 + CT (E09) E1 + CC (E10) E1 + TT (E11) E1 + GG (E12) E1 + AG (E13) E1 + AC (E14) E1 + AT (E15) E1 + AA (E16)	HM1 + AA (HM31) HM1 + AG (HM32) HM1 + AC (HM33) HM1 + AT (HM34) HM1 + GA (HM35) HM1 + GT (HM36) HM1 + GG (HM37) HM1 + GC (HM38) HM1 + CA (HM39) HM1 + CT (HM310) HM1 + CG (HM311) HM1 + CC (HM312) HM1 + TA (HM313) HM1 + TG (HM314) HM1 + TC (HM315) HM1 + TT (HM316)

Table 2. Activity of enzymes and classification of methylation statuses in CpG islands.

Enzyme	Free-methylated [I (1, 1)]	Full-methylated [II (1, 0)]	Hemi-methylated [III (0, 1)]	Hyper-methylated [IV (0, 0)]
Sequence	CCGG GGCC	CCGG GGCC	CCGG GGCC	CCGGCCGG GGCCGGCC
<i>Msp</i> I	CCGG GGCC	CCGG GGCC	Non	Non
<i>Hpa</i> II	CCGG GGCC	Non	CCGG GGCC	Non

Classification of methylation was signified in the brackets.

DNA template) and 1 μ L of *EcoR* I selective amplification primer (10 μ mol/L), 1 μ L of *Hpa* II/*Msp* I selective amplification primer (10 μ mol/L) and 13.5 μ L of ddH₂O. The selective amplification was performed with a touchdown program of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min, decreasing the annealing temperature by 0.7 °C per cycle during 12 cycles and then 24 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min with a final extension at 72 °C for 10 min. The primers used in selective amplification are listed in Table 1. The final products were separated using 6% polyacrylamide gels and visualized via silver staining (Xu et al, 2002).

Band scoring and data analysis

The two isoschizomers (*Msp* I and *Hpa* II) recognize the same sequence (5'-CCGG-3') but differ in their sensitivities to DNA methylation. The scoring of differential methylation status on a specific site is based on the presence (scored as 1) or absence (scored as 0) of bands in the *EcoR* I/*Msp* I and *EcoR* I/*Hpa* II lanes. Comparing the two profiles of these two lanes allows for the assessment of the methylation status of the restriction sites. In total, there are four types of band combinations in the two lanes that represent four types of DNA methylation statuses of the restriction sites (5'-CCGG-3') (Table 2). The sites that are free from methylation are recognized by both isoschizomers with the band type represented by type I (1, 1). The full methylation sites (methylation at the internal C residue of both strands, MeCpG) are only recognized by *Msp* I with the band type represented by type II (1, 0). Moreover, plant-specific hemi-MeCpCpG sites (methylation at the external C residue in one DNA strand but not in its complement strand) are only recognized by *Hpa* II with the band type represented by type III (0, 1). Sites that are hyper-methylated at both the internal and external Cs and those are fully methylated at the external Cs on both strands are cut by neither two enzymes, and the band type is represented by type IV (0, 0).

The general DNA methylation level was analyzed based on two strands of DNA and calculated by the following formula: $(II \times 2 + III + IV \times 2) / [(I + II + III + IV) \times 2] \times 100\%$, in which II, III and IV were methylated loci. Any variations in DNA methylation between generations or treatments could be detected by comparing the methylation status of the corresponding samples.

RESULTS

Pattern and level of DNA methylation

The DNA methylation of a total of 4 744 CCGG loci were assessed by using the 256 MSAP primer-pairs. The data revealed that more than 65.2% of the total identified loci were unmethylated, while methylated loci accounted only for 34.3%–34.8% (Table 3). Among these methylated loci, full- and hyper-methylated loci accounted for 83.1%–84.8%, and hemi-methylated loci accounted for 15.2%–16.9%. DNA methylation level was 31.4%–32.1%.

Comparison of DNA methylation pattern between the two developmental stages revealed that the

Table 3. DNA methylation patterns and levels of Huhan 3 in different generations and developmental stages.

Index of methylation	G ₀		G ₆	
	21 d	28 d	21 d	28 d
Type I	3 094	3 119	3 099	3 112
Type II	1 280	1 316	1 284	1 317
Type III	251	274	262	272
Type IV	119	35	99	43
Total amplified bands	4 744	4 744	4 744	4 744
DNA methylation level (%)	32.1	31.4	31.9	31.5
Methylated loci ^a	1 650	1 625	1 645	1 632
MSAP proportion (%) ^b	34.8	34.3	34.7	34.4
Full- and hyper-methylated loci ^c	1 399	1 351	1 383	1 360
Type II and IV proportion (%)	84.8	83.1	84.1	83.3
Hemi-methylated loci	251	274	262	272
Type III proportion (%) ^d	15.2	16.9	15.9	16.7

^a Methylated loci = II + III + IV; ^b MSAP (%) = (II + III + IV) / (I + II + III + IV) \times 100; ^c Full- and hyper-methylated loci = II + IV; ^d Type III proportion (%) = III / (I + II + III + IV) \times 100.

MSAP, Methylation sensitive amplification polymorphism.

proportion of methylated loci and the DNA methylation level decreased from the three-leaf to four-leaf stages in both G_0 and G_6 . The proportion of methylated loci declined by 0.5% and 0.3%, while the DNA methylation level declined by 0.7% and 0.4%, in G_0 and G_6 , respectively (Table 3). It is suggested that G_0 had more variations of DNA methylation than G_6 at the two developmental stages. Comparison of DNA methylation pattern between the two generations uncovered that G_0 had a higher DNA methylation level than G_6 at the three-leaf stage. However, at the four-leaf stage, the DNA methylation level of G_0 was lower than that of G_6 . Hence, it is likely that different developmental stages, as well as different generations, had different DNA methylation levels. The results indicated that the DNA methylation pattern of G_6 changed after growth under drought stress for six successive generations, and G_6 had less variation than G_0 at the two seedling stages.

Analysis of differentially methylated loci (DML)

One hundred and ninety loci, 4.0% of the total 4 744 identified, showed variations in DNA methylation between different generations or/and between different developmental stages. Among these 190 loci, 44 and 48 loci had variations in DNA methylation between generations at three-leaf and four-leaf stages, respectively. DNA methylation patterns of these loci were different between G_0 and G_6 , which implied that the variations in these loci can be attributed to drought stress domestication. In addition, 132 and 171 loci of 190 loci had variations in DNA methylation between different developmental stages in G_0 and G_6 , respectively. DNA methylation patterns of these loci were different between 21 d (three-leaf stage) and 28 d (four leaf- stage), which implied that the variations in these loci were related to rice growth and development (Table 4).

DNA methylation refers to de-methylation (types II and IV changed to types I and III, and type III changed to type I), re-methylation (type I changed to types II, III and IV, and type III changed to types II and IV) or interchange event (changes between types II and IV) (Table 4). Compared to G_0 , G_6 de-methylation occurred more predominantly at three-leaf stage than re-methylation while the frequency of de-methylation decreased largely at the four-leaf stage with a remarkable increase in re-methylation. At the three-leaf stage, 26 de-methylated loci accounted for 59.1% of the total 44 loci while re-methylated loci accounted

Table 4. Variations in DNA methylation between generations and between developmental stages.

Patterns of variation		Developmental stage		Generation	
Original band type	New band type	21 d	28 d	G_0	G_6
I (1, 1)	II (1, 0)	5	11	5	9
	III (0, 1)	5	3	4	1
	IV (0, 0)	2	4	9	23
II (1, 0)	I (1, 1)	2	3	3	5
	III (0, 1)	1	1	0	2
	IV (0, 0)	2	7	2	7
III (0, 1)	I (1, 1)	1	6	14	16
	II (1, 0)	0	0	0	0
	IV (0, 0)	1	5	0	11
IV (0, 0)	I (1, 1)	15	3	25	25
	II (1, 0)	3	0	37	38
	III (0, 1)	7	5	33	34
Total band		44	48	132	171
Proportion (%)		23.2	25.3	69.5	90.0
Re-methylated band ^a		13	23	18	44
Proportion of re-methylation events (%)		29.5	47.9	13.6	25.7
De-methylated band ^b		26	18	75	82
Proportion of de-methylation events (%)		59.1	37.5	56.8	48.0

^a Re-methylated bands = Band number of types II, III and IV changed from type I + band number of types II and IV changed from type III; ^b De-methylated bands = Band number of types I and III changed from types II and IV + band number of type I changed from type III.

only for 29.5%. However, the proportion changed at four-leaf stage, de-methylated loci accounted for 37.5% of the total 48 loci, less than re-methylated loci (47.9%) (Table 4). In addition, both G_0 and G_6 had more de-methylation events than re-methylation events along with the development of rice plants, while G_0 had more de-methylation events (56.8%) and less re-methylation events (13.6%) than G_6 (48.0% and 25.7%) (Table 4).

Further analysis of 190 DML revealed that most of them (57.9%) related only to plant development, showing no variations between generations (Fig. 1-A). Their DNA methylation pattern was $G_0 = G_6$ and 21 d \neq 28 d. Among the remained 80 DML (42.1%), whose DNA methylation pattern was $G_0 \neq G_6$, most occurred only at the three-leaf stage (40.0%) or the four-leaf stage (45.0%), and a small proportion occurred both at the three-leaf and four-leaf stages (15.0%). In addition, 123 DML which had DNA methylation variations between two developmental stages, occurred in both G_0 and G_6 . However, for the remained DML, more occurred in G_6 only than in G_0 only (Fig. 1-B).

Distributions of DML at chromosome and gene levels

To characterize the distributions of DML at

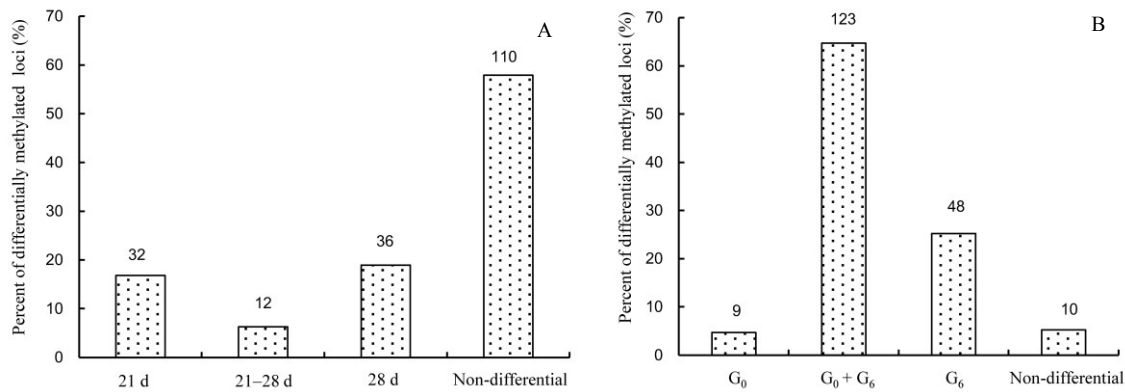


Fig. 1. Analysis of DNA methylation variations between generations and between developmental stages.

A, Comparison between developmental stages (three-leaf and four-leaf stages). Only 21 d means that DML occurred between G₀ and G₆ only in three-leaf stage, but not in four-leaf stage; 21-28 d means that DML occurred between G₀ and G₆ both in three-leaf and four-leaf stages. Only 28 d means that DML occurred between G₀ and G₆ only in four-leaf stage. Non means no DML occurred between G₀ and G₆ in both three-leaf and four-leaf stages, but two developmental stages had different methylation pattern, i.e. G₀ = G₆, but 21 d ≠ 28 d. B, Comparison between generations (G₀ and G₆). Non means no DML occurred between the two stages in both G₀ and G₆, but two generations had different methylation pattern, i.e. 21 d = 28 d, but G₀ ≠ G₆.

Data above a column is number of differentially methylated loci (DML).

chromosome and gene levels, 89 of 190 differential methylated fragments were isolated and re-amplified with the corresponding selective primer combinations. The sequences of the differentially methylated fragments were used as query searches against the nucleotide databases of Gramene (<http://www.gramene.org/>) for homology and function annotation. As shown in Fig. 2-A, the DML were widely distributed on all the 12 chromosomes. Further analysis revealed that 89 sequences were involved in 125 loci, among which 43 loci distributed in the promoter region (34.4%), 55 loci distributed in the gene body (35 in exon and 20 in intron, accounting for 44.0%), 11 loci distributed in the 3'-UTR (8.8%), and 16 loci distributed in intergenic region (12.8%) (Fig. 2-B).

Functional analysis of genes relating to DML

To analyze the functions of genes relating to DML, 190 DML were assorted into three classes. Class I included 110 loci that involved in rice development only, having the DNA methylation pattern of G₀ = G₆ & 21 d ≠ 28 d, i.e. these loci had DNA methylation variations between different developmental stages, but had no DNA methylation variations between different generations (Fig. 1-A). Thirty-four loci of this class were randomly sequenced, relating to 37 genes. Class II included 10 loci related only to drought entrainment, and the DNA methylation pattern of these DML was G₀ ≠ G₆ & 21 d = 28 d, i.e. these loci had DNA methylation variations between different generations,

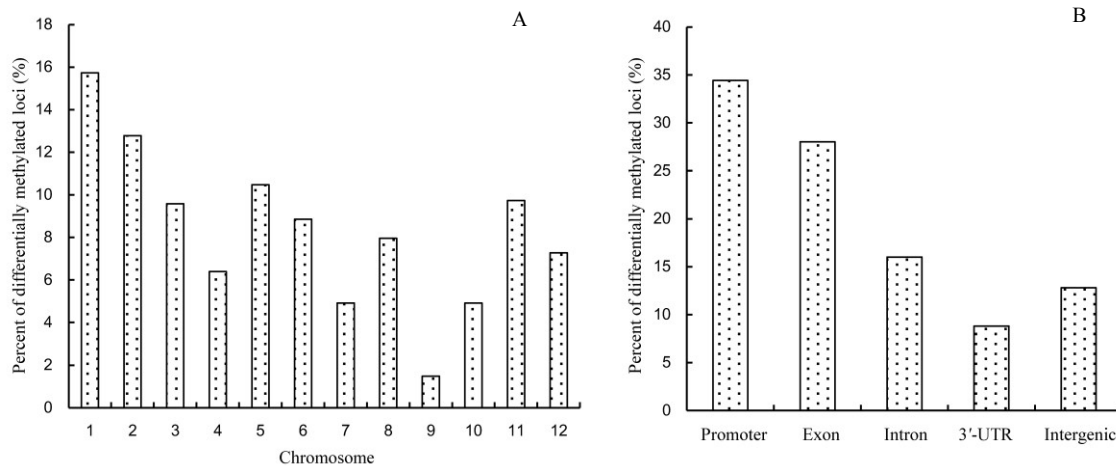


Fig. 2. Distributions of differentially methylated loci (DML) at chromosome level (A) and gene level (B).

but had no variation between different developmental stages (Fig. 1-B). Nine class II loci were randomly sequenced, which were aligned to 27 genes. Class III included 70 loci related to both development and drought entrainment, i.e. these loci had DNA methylation variations between different developmental stages and between different generations. The DNA methylation pattern was $G_0 \neq G_6$ & 21 d \neq 28 d. Forty-six sequenced class III loci were aligned to 61 genes.

GO (gene ontology) analysis was performed for these three classes of DML related genes. The results revealed that, except some transposon and expressed protein with unknown function, most of the genes were functional. Many class I genes had enzyme activity, such as catalytic activity (LOC_Os02g04700, LOC_Os10g33420 and LOC_Os02g44550), hydrolase activity (LOC_Os01g52550, LOC_Os04g26790, LOC_Os06g39875 and LOC_Os10g33420), transferase activity (LOC_Os01g52550, LOC_Os04g26790, LOC_Os06g39875 and LOC_Os10g33420), kinase activity (LOC_Os12g06490, LOC_Os02g29080 and LOC_Os03g61010), and so on. They participated in many important biological processes, such as tRNA synthesis (LOC_Os02g04700), fibre synthesis (LOC_Os02g09930), embryo development (LOC_Os03g35340), post-embryonic development (LOC_Os03g35340), signal transduction (LOC_Os01g52550), and so on. Class II genes not only had similar functions as class I genes, but also had functions in stress responses, such as LOC_Os11g11990 and LOC_Os05g32110. Class III genes involved in embryo and post-embryo developments (LOC_Os05g49770), anatomical structure morphogenesis (LOC_Os02g24190 and LOC_Os11g38240), pollen-pistil interaction (LOC_Os07g36570), responses to abiotic and biotic stimuli (LOC_Os02g24190, LOC_Os03g50210 and LOC_Os10g02070), and so on.

DISCUSSION

In this study, a water-saving and drought-resistant rice variety, Huhan 3, was subjected to drought stress for six successive generations, and the DNA methylation patterns of G_0 and G_6 at the three-leaf stage (21 day-old seedlings) and four-leaf stage (28 day-old seedlings) were assessed by MSAP method. The DNA methylation level of Huhan 3 was 31.4%–32.1%, and the methylated loci accounted for 34.3%–34.8% of the total loci identified (Table 3). This result is consistent

with the results of a previous study in which the methylated loci account for 28%–39% (Sakthivel et al, 2010), but inconsistent with the results of Xiong et al (1999) and Pan et al (2011) in which the methylated loci account for 16.3% and 20.0%, respectively. The methylation differences might result from the different materials and much more amplified loci used in the present study. Among the methylated loci, full- and hyper-methylated loci accounted for 83.1%–84.8%, and hemi-methylated loci accounted for 15.2%–16.9%, which is similar to the results of previous study (87.6% and 12.4%, respectively) (Xiong et al, 1999).

It was reported that the DNA methylation level increases along with rice grain development during the adult stage (Sha et al, 2005). However, in our study, the DNA methylation level of the four-leaf seedlings was lower than that of the three-leaf seedlings. It was speculated that de-methylation induces activation of gene expression along with rice development at seedling stage, as DNA methylation plays an important role in regulation of gene expression (Zhang et al, 2010). While at adult stage, re-methylation induced suppression of gene expression along with rice grain development. Comparison between two generations revealed that the DNA methylation pattern of G_6 was different from G_0 at both seedling stages (Table 3). Compared to G_0 , at the three-leaf stage, de-methylation predominately occurred in G_6 (59.1%), while re-methylation accounted only for 29.5%. However, at the four-leaf stage, more re-methylation (47.9%) than de-methylation occurred in G_6 (37.5%) compared to G_0 (Table 4).

DML accounted for 4.0% of the total identified loci, among which some were re-methylated and some were de-methylated, indicating that DNA methylation pattern was developmental stage specific in rice (Table 4), which is consistent with the previous report that DNA methylation pattern is temporal-spatial specific and variety specific (Pan et al, 2009). Furthermore, most of the DML (57.9%) only occurred between different developmental stages, and none occurred between different generations, indicating that drought domestication induced less DNA methylation variations than that induced by plant (Fig. 1-A). Comparison between two developmental stages revealed that G_6 had more DNA methylation variations than G_0 , indicating that the epi-genome of G_6 was more unstable than that of G_0 .

Distribution analysis of DML at chromosome and gene levels showed that drought domestication and

plant development induced genome-wide alterations of DNA methylation, and the variations mainly occurred in promoter regions and bodies (including exon and intron) of genes. These regions are crucial to regulate gene expression (Zhao and Han, 2009). This result is consistent with previous report that the DNA methylation variation in coding region is similar to that in non-coding region on the genome (Pan et al, 2009). In addition, it was reported that 5% of genes' promoters are methylated in plants, and these genes are of tissue-specific expression (Zhang et al, 2006). In our study, GO analysis of genes relating to DML showed that they involved in a wide range of functions, including regulation of embryo and post-embryo development, pollen-pistil interaction, response to abiotic and biotic stimuli and other functions relating to plant development. Hence, it is likely that DNA methylation plays crucial roles in regulation of plant development and stress response.

In this study, drought stress was performed from tillering to grain-filling stages, during which drought stress has great impacts on rice development and grain yield (Chen et al, 2013). And simultaneously, 'stress memories' (drought induced DNA methylation variations) were generated in this stage and stored in seeds. In the present study, seedling stage was selected to read 'stress memories' for two reasons. Firstly, at seedling stage, the development status of plants directly reflected differences of the amount of nutrients and genetic information (including epigenetic information) between different seeds obtained from last generation. Secondly, the DNA methylation pattern could be easily affected by environmental factors at adult stage. Further study is required to investigate the tendency and extent of DNA methylation variations in different domestication generations subjected to drought stress also at adult stage. In addition, MSAP method only detects the CCGG context and produces a relative higher rate of false positive identification in DNA methylation survey. Thus, a more precise method is needed to identify the whole-genome DNA methylation status in rice.

ACKNOWLEDGEMENTS

This study was supported by the National High-Technology R&D Program of China (Grant No. 2012AA101102), the Project for High-Level Talents of China (Grant No. 2010C1120), and Shanghai Key Program for Agriculture Science and Technology, China.

REFERENCES

- Aina R, Sgorbati S, Santagostino A, Labra M, Ghiani A, Citterio S. 2004. Specific hypomethylation of DNA is induced by heavy metals in white clover and industrial hemp. *Physiol Plant*, **121**(3): 472–480.
- Attitalla I H. 2011. Modified CTAB method for high quality genomic DNA extraction from medicinal plants. *Pak J Biol Sci*, **14**(21): 998–999.
- Bender J. 2004. DNA methylation and epigenetics. *Annu Rev Plant Biol*, **55**: 41–68.
- Bird A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev*, **16**(1): 6–21.
- Boyko A, Kovalchuk I. 2008. Epigenetic control of plant stress response. *Environ Mol Mutagen*, **49**(1): 61–72.
- Cervera M T, Ruiz-Garcia L, Martinez-Zapater J. 2002. Analysis of DNA methylation in *Arabidopsis thaliana* based on methylation-sensitive AFLP markers. *Mol Genet Genom*, **268**(4): 543–552.
- Chen X R, Liu L Y, Yan C H, Zhong L, Zhu C L, Peng X S, He X P, Fu J R, OuYang L J, He H H. 2013. Effects of rewating after drought at heading date on seed setting and some physiological indexes in early rice varieties with different yields. *Chin J Rice Sci*, **27**(1): 77–83. (in Chinese with English abstract)
- Chinnusamy V, Gong Z Z, Zhu J K. 2008. Absciscic acid-mediated epigenetic processes in plant development and stress responses. *J Integr Plant Biol*, **50**(10): 1187–1195.
- Cubas P, Vincent C, Coen E. 1999. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature*, **401**: 157–161.
- Downen R H, Pelizzola M, Schmitz R J, Lister R, Downen J M, Nery J R, Dixon J E, Ecker J R. 2012. Widespread dynamic DNA methylation in response to biotic stress. *Proc Natl Acad Sci USA*, **109**(32): E2183–E2191.
- Goll M G, Bestor T H. 2005. Eukaryotic cytosine methyltransferases. *Annu Rev Biochem*, **74**: 481–514.
- Henderson I R, Jacobsen S E. 2007. Epigenetic inheritance in plants. *Nature*, **447**: 418–424.
- Jackson J P, Lindroth A M, Cao X F, Jacobsen S E. 2002. Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature*, **416**: 556–560.
- Karan R, Deleon T, Biradar H, Subudhi P K. 2012. Salt stress induced variation in DNA methylation pattern and its influence on gene expression in contrasting rice genotypes. *PLoS One*, **7**(6): e40203.
- Kou H P, Li Y, Song X X, Ou X F, Xing S C, Ma J, von Wettstein D, Liu B. 2011. Heritable alteration in DNA methylation induced by nitrogen-deficiency stress accompanies enhanced tolerance by progenies to the stress in rice (*Oryza sativa* L.). *J Plant Physiol*, **168**(14): 1685–1693.
- Law J A, Jacobsen S E. 2010. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet*, **11**(3): 204–220.
- Li X Y, Wang X F, He K, Ma Y Q, Su N, He H, Stolc V, Tongprasit

- W, Jin W W, Jiang J M, Terzaghi W, Li S G, Deng X W. 2008. High-resolution mapping of epigenetic modifications of the rice genome uncovers interplay between DNA methylation, histone methylation, and gene expression. *Plant Cell*, **20**(2): 259–276.
- Lu Y L, Rong T Z, Cao M J. 2008. Analysis of DNA methylation in different maize tissues. *J Genet Genom*, **35**(1): 41–48.
- Luo L J. 2010. Breeding for water-saving and drought-resistance rice (WDR) in China. *J Exp Bot*, **61**(13): 3509–3517.
- Manning K, Tor M, Poole M, Hong Y G, Thompson A J, King G J, Giovannoni J J, Seymour G B. 2006. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nat Genet*, **38**(8): 948–952.
- Miura K, Agetsuma M, Kitano H, Yoshimura A, Matsuoka M, Jacobsen S E, Ashikari M. 2009. A metastable *DWARF1* epigenetic mutant affecting plant stature in rice. *Proc Natl Acad Sci USA*, **106**(27): 11218–11223.
- O'Toole J C, Cruz R T. 1980. Response of leaf water potential, stomatal resistance, and leaf rolling to water stress. *Plant Physiol*, **65**(3): 428–432.
- Ou X F, Zhang Y H, Xu C M, Lin X Y, Zang Q, Zhuang T T, Jiang L L, von Wettstein D, Liu B. 2012. Transgenerational inheritance of modified DNA methylation patterns and enhanced tolerance induced by heavy metal stress in rice (*Oryza sativa* L.). *PLoS One*, **7**(9): e41143.
- Pan Y J, Fu B Y, Wang D, Zhu L H, Li Z K. 2009. Spatial and temporal profiling of DNA methylation induced by drought stress in rice. *Sci Agric Sin*, **42**(9): 3009–3018. (in Chinese with English abstract)
- Paszkowski J, Whitham S A. 2001. Gene silencing and DNA methylation processes. *Curr Opin Plant Biol*, **4**(2): 123–129.
- Paun O, Bateman R M, Fay M F, Hedrén M, Civeyrel L, Chase M W. 2010. Stable epigenetic effects impact adaptation in allopolyploid orchids (Dactylorhiza: Orchidaceae). *Mol Biol Evol*, **27**(11): 2465–2473.
- Sakthivel K, Girishkumar K, Ramkumar G, Shenoy V V, Kajjidoni S T, Salimath P M. 2010. Alterations in inheritance pattern and level of cytosine DNA methylation, and their relationship with heterosis in rice. *Euphytica*, **175**(3): 303–314.
- Santos F, Hendrich B, Reik W, Dean W. 2002. Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev Biol*, **241**(1): 172–182.
- Sha A H, Lin X H, Huang J B, Zhang D P. 2005. Analysis of DNA methylation related to rice adult plant resistance to bacterial blight based on methylation-sensitive AFLP (MSAP) analysis. *Mol Genet Genom*, **273**(6): 484–490.
- Steimer A, Schob H, Grossniklaus U. 2004. Epigenetic control of plant development: New layers of complexity. *Curr Opin Plant Biol*, **7**(1): 11–19.
- Tan M P. 2010. Analysis of DNA methylation of maize in response to osmotic and salt stress based on methylation-sensitive amplified polymorphism. *Plant Physiol Biochem*, **48**(1): 21–26.
- Tsukahara S, Kobayashi A, Kawabe A, Mathieu O, Miura A, Kakutani T. 2009. Bursts of retrotransposition reproduced in *Arabidopsis*. *Nature*, **461**: 423–426.
- Verhoeven K J F, Jansen J J, van Dijk P J, Biere A. 2010. Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytol*, **185**(4): 1108–1118.
- Vos P, Hogers R, Bleeker M, Reijans M, Van De Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucl Acids Res*, **23**(21): 4407–4414.
- Wang W S, Zhao X Q, Pan Y J, Zhu L H, Fu B Y, Li Z K. 2011. DNA methylation changes detected by methylation-sensitive amplified polymorphism in two contrasting rice genotypes under salt stress. *J Genet Genom*, **38**(9): 419–424.
- Xiong L Z, Xu C G, Saghai Maroof M A, Zhang Q F. 1999. Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique. *Mol Gen Genet*, **261**(3): 439–446.
- Xu S B, Tao Y F, Yang Z Q, Chu J Y. 2002. A simple and rapid methods used for silver staining and gel preservation. *Hereditas*, **24**(3): 335–336. (in Chinese with English abstract)
- Zhang L G, Cheng Z J, Qin R Z, Qiu Y, Wang J L, Cui X K, Gu L F, Zhang X, Guo X P, Wang D, Jiang L, Wu C Y, Wang H Y, Cao X F, Wan J M. 2012. Identification and characterization of an epi-allele of *FIE1* reveals a regulatory linkage between two epigenetic marks in rice. *Plant Cell*, **24**(11): 4407–4421.
- Zhang M S, Kimatu J N, Xu K Z, Liu B. 2010. DNA cytosine methylation in plant development. *J Genet Genom*, **37**(1): 1–12.
- Zhang X Y, Yazaki J, Sundaresan A, Cokus S, Chan S W L, Chen H M, Henderson I R, Shinn P, Pellegrini M, Jacobsen S E, Ecker J R. 2006. Genome-wide high-resolution mapping and functional analysis of DNA methylation in *Arabidopsis*. *Cell*, **126**(6): 1189–1201.
- Zhao Z M, Han L. 2009. CpG islands: Algorithms and applications in methylation studies. *Biochem Biophys Res Commun*, **382**(4): 643–645.