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ORIGINAL PAPER

Extent and pattern of DNA methylation alteration in rice lines derived from introgressive hybridization of rice and *Zizania latifolia* Griseb

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Abstract We have reported previously that introgression by *Zizania latifolia* resulted in extensive DNA methylation changes in the recipient rice genome, as detected by a set of pre-selected DNA segments. In this study, using the methylation-sensitive amplified polymorphism (MSAP) method, we globally assessed the *extent* and *pattern* of cytosine methylation alterations in three typical introgression lines relative to their rice parent at ~2,700 unbiased genomic loci each representing a recognition site cleaved by one or both of the isoschizomers, *Hpa*II/*Msp*I. Based on differential digestion by the isoschizomers, it is estimated that 15.9% of CCGG sites are either fully methylated at the internal Cs and/or hemi-methylated at the external Cs in the rice parental cultivar Matsumae. In comparison, a statistically significant increase in the overall level of both methylation types was detected in all three studied introgression lines (19.2, 18.6, 19.6%, respectively). Based on comparisons of MSAP profiles between the isoschizomers within the rice parent and between parent and the introgression lines, four major groups of MSAP banding patterns are recognized, which can be further divided into various subgroups as a result of inheritance of, or variation in, parental methylation patterns. The altered methylation patterns include hyper- and hypomethylation changes, as well as interconversion of hemi- to full-methylation, or vice versa, at the relevant CCGG site(s). Most alterations revealed by MSAP in low-copy loci can be validated by DNA gel blot analysis. The changed methylation patterns are uniform among randomly selected individuals for a given introgression line within or among selfed generations. Sequencing on 31 isolated fragments that showed different changing patterns in the introgression line(s) allowed their mapping onto variable regions on one or more of the 12 rice chromosomes. These segments include protein-coding genes, transposon/retrotransposons and sequences with no homology. Possible causes for the introgression-induced methylation changes and their implications for genome evolution and crop breeding are discussed.

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Introduction

Whereas cytosine DNA methylation widely exists in diverse eukaryotic organisms—from fungi and plants to mammals, higher plants possess a markedly higher

level of this covalent modification of their DNA, with 20–40% of all cytosine residues in the nuclear DNA being methylated (Gruenbaum et al. 1981; Messeguer et al. 1991). Recent years have seen an increasing interest in the study of various aspects of cytosine DNA methylation owing to realization of its fundamental roles in multiple cellular activities, including control of gene expression, maintenance of genomic integrity, formation and perpetuation of heterochromatin, and control of genomic imprinting (Bourc'his and Bestor 2004; Tariq and Paszkowski 2004; Rangwala and Richards 2004). Consequently, disturbance of intrinsic DNA methylation patterns may have structural and functional consequences to the organisms with this epigenetic code (Wolffe and Matzke 1999; Martienssen and Colot 2001; Tariq and Paszkowski 2004). For example, mutations in any of the three known DNA methyltransferase (DMTase) genes in mouse, leading to genome-wide hypomethylation, are lethal during early embryonic stages (Geiman and Robertson 2002). In *Arabidopsis*, drastic global reduction of cytosine methylation due to loss-of-function mutation of the *Met 1* gene (counterpart of the mammalian *Dnmt1*) or *DDM1* (decrease in DNA methylation1) gene, albeit non-lethal, produces pleiotropically defective phenotypes and developmental abnormality (Finnegan et al. 1996; Kakutani et al. 1996; Ronemus et al. 1996).

Introgressive hybridization, being a frequent phenomenon in natural plant populations, represents a driving force in genome evolution by either direct transfer and/or de novo genesis of adaptive traits (Arnold 2004). Nevertheless, the underlying mechanism for the origin of novel traits (e.g., transgressive segregation) in the derived introgression lines remains obscure (Rieseberg et al. 2003). Similarly, although interspecific crossing and backcrossing (resulting in introgression) are widely used in plant breeding, novel traits that are not explainable by gene transfer or insertional disruption often appear in the derived progenies. Could it be that some of these phenomena have an epigenetic basis? For example, it may be possible that the intrinsic chromatin states and DNA methylation patterns can be disturbed by the integration of chromatin segments from a divergent species, or the introduction of new *trans*-acting inducers/modifiers may direct or regulate the establishment of new epigenetic states that are different from the original one. Indeed, it has been demonstrated repeatedly in mammals that integration of foreign DNA may cause the host genome to undergo extensive and genome-wide alterations in DNA methylation patterns of both cellular genes and transposon-associated DNA repeats (Heller et al. 1995; Remus et al. 1999; Muller et al. 2001).

We have reported recently that, similar to the situation in animals, mentioned above, extensive and heritable changes in DNA methylation patterns of a set of selected DNA sequences occurred in rice lines containing introgressed chromatin segments from *Zizania latifolia* Griseb., a wild grass in tribe *Oryzeae*, and hence, related to cultivated rice (*Oryza sativa* L.) (Liu et al. 2004). It is not clear, however, the *extent* and *pattern* of methylation modifications in the introgression lines from a genome-wide perspective. Here, we have extended the earlier study by analyzing the cytosine methylation patterns of a large number of unbiased loci distributed throughout the rice genome in the same set of introgression lines using a more global method, the methylation-sensitive amplified polymorphism (MSAP) approach (Reyna-Lopez et al. 1997; Xiong et al. 1999; Ashikawa 2001; Cervera et al. 2002). We report that diverse patterns of DNA methylation alterations occurred at loci mapped to variable regions across the rice genome in the introgression lines, that different types of sequences including protein-coding genes and transposons were affected, and that there was a significant increase in the overall relative methylation level in all three introgression lines compared with their original rice parental cultivar.

Materials and methods

Plant material

Three rice introgression lines (RZ1, RZ2, RZ35) derived from introgressive hybridization between rice (cv. Matsumae) and a local accession of *Z. latifolia* Griseb. by a novel sexual hybridization approach (Liu et al. 1999) were used in the present study. The introgression lines are homogeneous in phenotype and exhibit heritable, novel morphological characteristics in multiple traits compared with their rice parent, cv. Matsumae, at the 9th–11th selfed generations (Liu et al. 1999), and hence represent stabilized introgressants. These lines were characterized by genome-wide AFLP fingerprinting as possessing $< 0.1\%$ genomic DNA from *Z. latifolia* (Wang et al. 2005); nonetheless, presence of *Zizania*-specific interspersed DNA repeats was detected, thus verifying their nature as bona fide introgression lines (Shan et al. 2005). All lines are maintained by strict selfing under normal growing conditions.

MSAP analysis

To explore possible alterations in DNA methylation pattern at unbiased, yet specific loci in the introgression lines relative to their rice parent, we used the MSAP analysis method essentially as reported (Reyna-Lopez et al. 1997; Xiong et al. 1999; Ashikawa 2001; Cervera et al. 2002). MSAP is a modified version of the standard Amplified fragment length polymorphism or AFLP technique (Vos et al. 1995), by incorporating a pair of isoschizomers, *Hpa*II/*Msp*I, that possess differential sensitivity to cytosine methylation at the CCGG sites. In total, one pair of pre-selective primers and 44 pairs of selective primers were used for amplifications (Supplementary Table 1). Silver stained sequencing gel was used to resolve and visualize the amplification products. Only clear and reproducible bands that appeared in two independent PCR amplifications (starting from the digestion-ligation step, i.e., the first step of MSAP) were scored.

Recovery and sequencing of MSAP bands

Bands of interest in the silver-stained MSAP 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 AT cloning vector (The Sangong Biotech. Inc., Shanghai, China). The cloned DNA segments were sequenced with vector primers by automatic sequencing. The Advanced BlastN and BlastX programs at the NCBI website (http://www.ncbi.nlm.nih.gov/) were respectively used for mapping and homology analysis of the cloned DNA sequences that gave quality-reads.

DNA gel blot analysis

Genomic DNA was isolated from expanded leaves (precaution was taken to use leaves at the same developmental stage) of individual plants by a modified CTAB method (Kidwell and Osborn 1992) and purified by phenol extractions. Genomic DNA was digested by *Eco*RI together with either of the pair of methylation-sensitive isoschizomers, *Hpa*II or *Msp*I (New England Biolabs Inc.). To ensure complete digestion, an excess of enzymes (10 units enzyme per μg DNA) was used and the incubation time was extended to ~ 48 h. Digested DNA was fractionated by running through 1% agarose gels and transferred onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech) by the alkaline transfer recommended by the supplier. Cloned DNA segments representing different methylation patterns in the MSAP profile were selected as hybridization probes. Hybridization signal was detected by the Gene Images CDP-Star detection module (Amersham Pharmacia Biotech) after washing at a stringency of 0.2 × SSC, 0.1% SDS for 2 × 50 min. The filters were exposed to X-ray film for a few minutes to several hours depending on estimated copy number of the probe sequences.

Results

Increase in the overall relative cytosine methylation level in the introgression lines versus their rice parent

*Hpa*II and *Msp*I are a pair of isoschizomers that recognize the same restriction site (5′-CCGG) but have different sensitivity to the methylation states of the cytosines: *Hpa*II will not cut if either of the cytosines is fully (double-strand) methylated, whereas, *Msp*I will not cut if the external cytosine is fully- or hemi- (single-strand) methylated (McClelland et al. 1994). Thus, for a given DNA sample, the full methylation of the internal cytosine, or hemi-methylation of the external cytosine, at the assayed CCGG sites, can be revealed as absence in *Hpa*II-digest versus presence in *Msp*I-digest and absence in *Msp*I-digest versus presence in *Hpa*II-digest, of the specific band, respectively, in the MSAP profiles. It should be noted however that because *Hpa*II and *Msp*I cannot differentiate among several other states of the CCGG sites, including unmethylated CCGG, fully methylated $^{m}C^{m}CGG$ or himimethylated $C^{m}CGG$, the methylation percentages calculated by MSAP should be lower than the total *absolute* values (Ashikawa 2001; Cervera et al. 2002). In addition, if the scored bands contain internal methylated CCGG site(s), the percentages will be further underestimated; in fact, this is the case in our analysis, as will be detailed later. Notwithstanding these potential underestimates on the total absolute values of CCGG methylation, capability of the MSAP method for comparison of total *relative* methylation percentages of two major methylation states at the CCGG sites, i.e., full-methylation of the internal Cs and hemi-methylation of the external Cs, is technically reliable and efficient.

By using 44 pairs of *Eco*RI + *Hpa*II/*Msp*I primer combinations, we amplified 836, 925, 990 and 882 clear and reproducible bands respectively for the rice parental line Matsumae, and introgression lines RZ1, RZ2 and RZ35 (Table 1). In Matsumae, of the CCGG

Table 1 Number of bands amplified by MSAP in three introgression lines (RZ1, RZ2, RZ35) and their rice parent (Matsuame)

Rice line	Total bands	None-methylated CCGG sites	Methylated CCGG sites		
			Fully methylated sites (internal C)	Hemi-methylated sites (external C)	Total
Matsumae	836	703 (84.1%)	70 (8.4%)	63 (7.5%)	133 (15.9%)
RZ1	925	747 (80.6%)	90 (9.7%)	88 (9.5%)	178 (19.2%)
RZ2	990	806 (81.4%)	129 (13.0%)	55 (5.6%)	184 (18.6%)
RZ35	882	709 (80.4%)	118 (13.4%)	55 (6.2%)	173 (19.6%)

sites that are not fully methylated at the external Cs, 8.4% are fully methylated at the internal Cs only, and another 7.5% are hemi-methylated at the external Cs only. These values are very close to those calculated for other rice cultivars (~ 16% overall) using the same method (Xiong et al. 1999; Ashikawa 2001). Compared with parental cultivar Matsumae, all three introgression lines (RZ1, RZ2, RZ35) showed alterations in both types of detectable cytosine methylation levels, i.e., full methylation of the internal cytosine and hemi-methylation of the external cytosine, at the CCGG sites. Specifically, in RZ1, levels of both types of methylation are increased, with full methylation of the internal cytosine and hemi-methylation of the external cytosine being 9.7 and 9.5%, respectively (Table 1). In RZ2 and RZ35, the increment for methylation of the internal cytosine is even greater, reaching 13 and 13.4%, respectively; in contrast, methylation of the external cytosine in these two lines is decreased to 5.6 and 6.2%, respectively (Table 1). Taken together, the overall cytosine methylation level of the two states (defined above) in all three introgression lines is significantly elevated compared with parent Matsumae ($a = 0.01$, $t = 10.79$, $t_{2,\ 0.01} = 6.97$).

Inheritance and alteration of locus-specific cytosine methylation patterns from parent to introgression lines

The MSAP profile allows comparison of the cytosine methylation patterns between each introgression line and its rice parent in a locus-specific manner. We found that extensive changes (both hypermethylation and demethylation) in MSAP patterns occurred in the introgression lines (Fig. 1). In addition, some loci showed inter-conversion of the two detectable methylation types, i.e., from full merthylation of the internal cytosine to hemi-methylation of the external cytosine, or vice versa, at the relevant CCGG sites (Fig. 1). We also observed that bands appeared or disappeared in the introgression lines compared with the rice parent, simultaneously in *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I amplifications; we interpret at least some of this type of pattern change as due to nucleotide sequence change at the CCGG site(s).

In Matsumae, the MSAP loci can be divided into four major groups (Table 2, Fig. 1): group A refers to loci that are characterized by monomorphic band(s) present in both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I amplifications; group B refers to loci that are present in *Eco*RI/*Hpa*II amplification but absent in the corresponding *Eco*RI/*Msp*I amplification; group C refers to

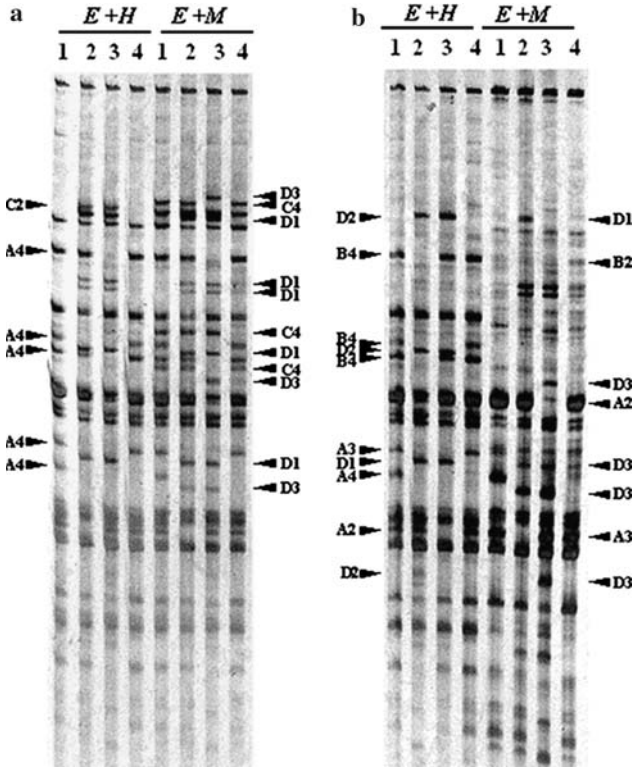

Fig. 1 Examples of changing MSAP patterns detected in the three introgression lines (RZ1 *lane 2*, RZ2 *lane 3*, RZ35 *lane 4*) as compared with their rice parent (Matsumae—lane 1) using primer combinations E + AAG/HM + TTC (**a**) and E + ACA/HM + TTC (**b**). E + H and E + M refer to digestion with *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I, respectively. Typical changing methylation patterns, as detailed in the text and Table 2, are marked by *arrowheads*

Table 2 Cytosine methylation patterns in the rice introgression lines (RZ1, RZ2, RZ35) and their rice parent (Matusumae)

Pattern	Matsumae		Introgression line					
	H	M	H	M	Number and frequency of sites			
					RZ1	RZ2	RZ35	Average
A1	+	+	+	+	646 (91.9%)	613 (87.2%)	658 (93.6%)	639 (90.9%)
A2	+	+	+	−	19 (2.7%)	14 (2.0%)	12 (1.7%)	15 (2.1%)
A3	+	+	−	+	11 (1.6%)	29 (4.1%)	16 (2.3%)	18.7 (2.7%)
A4	+	+	−	−	27 (3.8%)	47 (6.7%)	17 (2.4%)	30.3 (4.3%)
Total					703 (100%)			
B1	+	−	+	−	30 (47.6%)	15 (23.8%)	20 (31.7%)	21.7 (34.4%)
B2	+	−	+	+	27 (42.9%)	34 (54.0%)	10 (15.9%)	23.7 (37.6%)
B3	+	−	−	+	2 (3.2%)	7 (11.1%)	8 (12.7%)	5.7 (9.0%)
B4	+	−	−	−	4 (6.3%)	7 (11.1%)	25 (39.7%)	12 (19.0%)
Total					63 (100%)			
C1	−	+	−	+	51 (72.9%)	40 (57.1%)	48 (68.6%)	46.3 (66.1%)
C2	−	+	+	+	9 (12.9%)	13 (18.6%)	13 (18.6%)	11.7 (16.7%)
C3	−	+	+	−	0 (0%)	4 (5.7%)	3 (4.3%)	2.3 (3.3%)
C4	−	+	−	−	10 (14.2%)	13 (18.6%)	6 (8.5%)	9.7 (13.9%)
Total					70 (100%)			
D1	−	−	+	+	65 (50%)	146 (66.1%)	28 (29.8%)	79.7 (53.7%)
D2	−	−	+	−	39 (30%)	22 (10.0%)	20 (21.3%)	27 (18.2%)
D3	−	−	−	+	26 (20%)	53 (23.9%)	46 (48.9%)	41.7 (28.1%)
Total					130 (100%)	221 (100%)	94 (100%)	148.4 (100%)

loci that are present in *Eco*RI/*Msp*I amplification but absent in corresponding *Eco*RI/*Hpa*II amplification; and group D refer to loci that are not present in parent but appeared de novo in introgression lines.

According to model of inheritance/alteration in the introgression lines, each of the four groups can be further divided into several subgroups or altering patterns (e.g., Fig. 1), as is detailed in Table 2 and briefly summarized as follows: (1) of the 703 group A loci, the great majority [639 on averaged (90.9%)] showed stable Mendelian inheritance in the introgression lines, the rest showed either variable inheritance between *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I amplifications (patterns A2 and A3), or non-inheritance in both amplifications (pattern A4); (2) of the 63 group B loci, only 21.7 on average (34.4%) showed stable inheritance in the introgression lines, with the rest showing variable inheritance between *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I amplifications (patterns B2, B3 and B4); (3) of the 70 group C loci, 46.3 on average (66.1%) showed stable inheritance to the introgression lines, with the rest showing variable inheritance between *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I amplifications (patterns C2, C3 and C4); (4) on average, 148.4 group D loci appeared de novo in the introgression lines in either or both of *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I amplifications.

Of these diverse changing patterns in the introgression lines versus their rice parental cultivar, apart from the fourth category in each group, namely, A4, B4 and C4, which can be caused by nucleotide sequence changes leading to loss or gain of one or more CCGG sites, all rest should be due to methylation alteration at the internal or external cytosine of the assayed CCGG site(s).

Validation of the cytosine methylation changes and their meiotic transmission in the introgression lines by DNA gel blot analysis

Because the MSAP technique involves two rounds of PCR amplifications, it is necessary to rule out possible PCR artifacts as a cause for the observed differential methylation patterns in an introgression line versus parent. Thus, to validate the methylation changes revealed by MSAP in the introgression lines, we selected 15 isolated MSAP bands (MSAP2, MSAP6, MSAP10, MSAP11, MSAP13, MSAP16, MSAP17, MSAP18, MSAP19, MSAP21, MSAP22, MSAP26, MSAP27, MSAP28, MSAP84) as probes for Southern blot analysis using *Eco*RI plus *Hpa*II or *Msp*I double enzyme-digestions, which represented several different MSAP patterns (Fig. 1, Table 2). Southern blot hybridization revealed that, 7 of these 15 probes gave a smearing hybridization signal denoting their repetitive nature (data not shown), and hindered further analysis; eight probes (MSAP2, MSAP6, MSAP10, MSAP13, MSAP16, MSAP22, MSAP28, MSAP84) produced discrete bands, and in all but one case (probe MSAP6) alteration in banding patterns in one or more introgression lines versus their rice parent, which can be

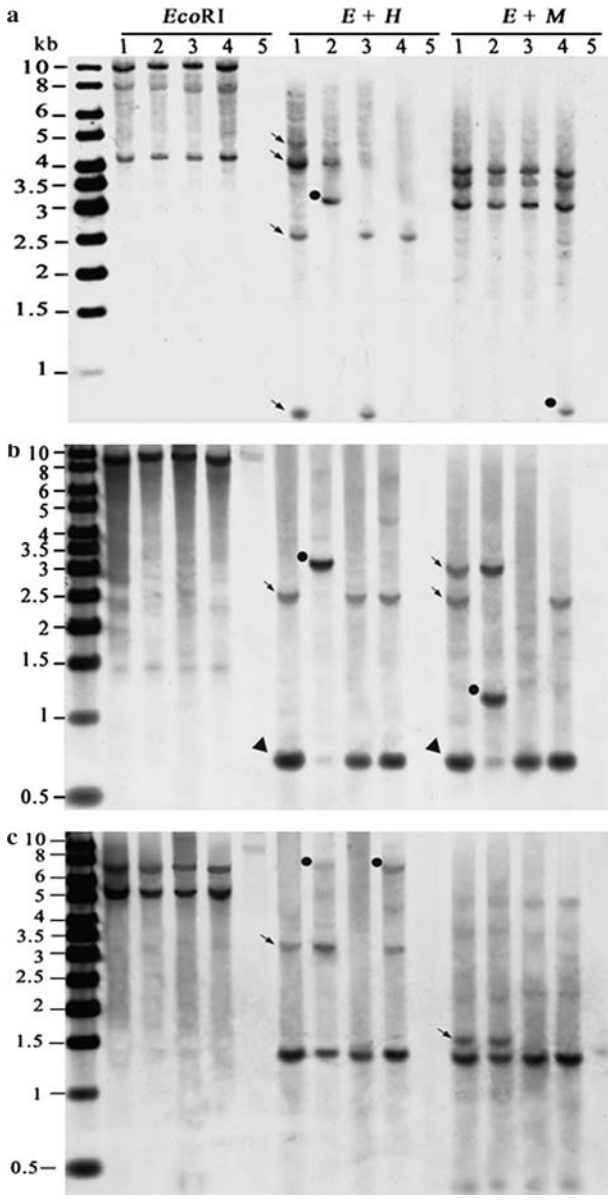

Fig. 2 Examples of validation by methylation-sensitive DNA gel blot analysis on alterations in DNA methylation pattern in the introgression lines relative to their rice parent. Hybridization of probes MSAP16 (**a**), MSAP22 (**b**) and MSAP28 (**c**) to *Eco*RI + *Hpa*II- or *Eco*RI + *Msp*I-digested genomic DNA of the rice parent Matsumae (*lane 1*), *Zizania latifolia* (*lane 5*) and the three introgression lines RZ1, RZ2 and RZ35 (*lanes 2–4*). For all three probes, the banding patterns in *Eco*RI alone digest are mornomorphic among the rice lines, indicating lack of sequence change at the *Eco*RI restriction sites. Loss of parental bands (marked by *arrows*) and gain of novel bands (marked by *solid circles*) in *Eco*RI + *Hpa*II- or *Eco*RI + *Msp*I-digest but not in both should unequivocally denote cytosine methylation alterations at the relevant CCGG sites. A single band (marked by *arrowheads*) disappeared in both enzyme combinations of probe MSAP22 (**b**) is probably due to sequence change at the CCGG site(s). The 1 kb DNA ladder (Fermentas Inc., Maryland, USA) was used as molecular size marker

unequivocally ascribed as alteration in cytosine methylation, was observed (Fig. 2 and data not shown). Probe MSAP6 produced a complex pattern that can also be caused by sequence changes at the CCGG sites (data not shown). It is thus appeared that the majority of MSAP pattern alterations at low-copy genomic regions in the introgression lines can be validated by gel blot analysis as bona fide methylation alterations at the CCGG sites.

To investigate if the altered cytosine methylation patterns in the introgression lines were mitotically stable and meiotically heritable, genomic DNA from leaves of three randomly selected individual plants taken from each of the three successive selfed generations (9th–11th) were digested with *Hpa*II or MspI and hybridized against the seven selected MSAP bands (see above) that showed clear methylation alterations compared with the parent. Results showed that complete uniformity in the newly acquired methylation patterns among the individuals for a given introgression line was detected both within and between the selfed generations (Fig. 3 and data not shown), indicating stable mitotic perpetuation and meiotic inheritance of the altered methylation patterns in these lines.

Cytosine methylation changes in the introgression lines are genome-wide in scope and affecting both cellular genes and transposons

Based on BlastN analysis at the Gramenae (Ware et al. 2002) website (http://www.gramene.org), the 31 clones that produced quality sequencing reads are mapped to one or more of all 12 rice chromosomes, indicating that

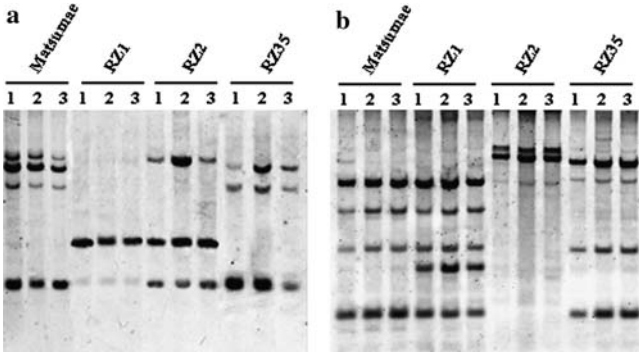

Fig. 3 Examples of meiotic inheritance of altered methylation patterns at both the internal and external cytosines in the introgression lines, as evidenced by homogeneity of the newly acquired patterns in Southern blot analysis among three random individual plants within an introgression line. **a**, **b** being probe MSAP10 with *Msp*I digest and probe MSAP84 with *Hpa*II digest, respectively

the alterations in DNA methylation pattern in the introgression lines are likely genome-wide in scope.

Based on BlastX analysis, of the 31 clones, 17 showed significant similarities to known-function genes, sequences encoding for hypothetical proteins or sequences related to transposons/retrotransposons, whereas 14 identified no meaningful match (Table 3 and Supplementary Table 2). This result indicates that both cellular genes and transposable elements have been targets for methylation alterations in the introgression lines.

Sequence characterization revealed that 11 out of the 31 MSAP fragments (35%) resulted from *Hpa*II-*Msp*I/*Hpa*II-*Msp*I restriction (Supplementary Table 2), which is incongruent with previous studies (Reyna-Lopez et al. 1997; Xiong et al. 1999; Ashikawa 2001; Cervera et al. 2002) wherein usually only *Hpa*II-*Msp*I/*Eco*RI hetrofragments were obtained. We believe that the major reason for this discrepancy is because all previous studies used ^{33} P-labeled *Eco*RI primers, such that only fragments with the *Eco*RI adaptor were visible; in contrast, we used silvers staining where all amplified fragments can be visualized (method).

Discussion

Several previous studies have demonstrated that the MSAP technique is highly efficient and reliable for large-scale detection of cytosine methylation at unbiased loci in plant genomes (Xiong et al. 1999; Ashikawa 2001; Cervera et al. 2002; Portis et al. 2003). The fact that the overall relative cytosine methylation level at the 5′-CCGG sites in rice cultivar Matsumae estimated in this study virtually gave the same value as the two previous independent studies (Xiong et al. 1999; Ashikawa 2001) strongly suggests consistency and reproducibility of the MSAP technique. This was further bolstered by the result that most MSAP-detected alterations in the cytosine methylation patterns at low-copy genomic regions in the introgression lines could be validated by methylation-sensitive gel blot analysis using the isolated DNA segments as probes.

We recently reported that rice lines introgressed by *Z. latifolia* exhibited extensive and heritable alteration in DNA methylation patterns (detected by DNA gel blot analysis) in a set of pre-selected loci including both protein-coding genes and transposon-related sequences, yet, the *extent* and *pattern* of the methylation alteration in these lines on a genome-wide scale was not known (Liu et al. 2004). By the MSAP technique, we have showed in this paper that (1) extensive cytosine methylation alterations including hyper- and hypomethylation as well as inter-conversion of methylation types (from hemi- to full-methylation or vice visa) occurred at multiple genomic loci in the introgression lines; (2) according to their nature of occurrence in the parent and their inheritance to, or variation in the introgression lines, the altered patterns can be divided into distinct groups and subgroups; (3) most alterations at low-copy genomic regions in the introgression lines can be confirmed by DNA gel-blot analysis, and the newly-acquired patterns are stably inherited; (4) the estimated relative overall cytosine methylation levels at the 5′-CCGG sites in all three studied introgression lines are significantly higher than that of the parental cultivar; and (5) based on sequence homology, the loci underwent methylation alterations in the introgression lines are diverse, including protein-coding genes, transposon/retrotransposon-related sequences and sequences with unknown functions.

A salient observation in this study is the unexpected high instability of group B loci (hemi-methylation of external Cs), and the moderate stability of group C loci (full methylation of the internal Cs), at the assayed CCGG sites (Table 2), as a result of *Zizania* introgression. The underlying mechanism is unknown, nonetheless, it underscores the emerging view that in plants different categories of methylation patterns are specified and maintained by divergent enzymatic machineries (Chan et al. 2005).

Possible causes for these dramatic methylation alterations in the rice lines derived from introgressive hybridization remains enigmatic. Given the scope (genome-wide) of the methylation alterations versus the minute amount (< 0.1%) of introgressed DNA, as well as occurrence of alterations in sequences that do not have homologues in the donor species *Zizania* (e.g., Fig. 2; Liu et al. 2004), it is unlikely that the alterations are caused by homology-dependent mechanisms (Bender 1998; Matzke et al. 2002), as has been proposed responsible for the remodeling of

Table 3 Classification of cloned DNA segments showing alteration in DNA methylation pattern in the introgression lines based on the MSAP profile

Category	Number of DNA segment	Percentage
Known-function gene	11	35.5
Putative protein-coding gene	4	12.9
Transposon and retrotransposon	2	6.5
No similarity	14	45.1
Total	31	100

DNA methylation patterns in newly formed plant wide-hybrids and allopolyploids (Matzke et al. 1999; Wendel 2000; Shaked et al. 2001; Madlung et al. 2002; Pikaard 2001; Comai et al. 2003; Levy and Feldman 2004; Comai 2005). Instead, it is more plausible that the cytosine methylation alterations in the introgression lines are caused by one or more of the following not-necessarily-mutually exclusive mechanisms: (1) A general disturbance of the intrinsic epigenetic chromatin states constituted by, or interrelated to, cytosine DNA methylation patterns as a result of alien chromatin insertion, as was proposed to be responsible for the occurrence of similar phenomena in animals with integrated foreign DNA (Heller et al. 1995; Remus et al. 1999; Muller et al. 2001). (2) The introduction of an exogenous *trans*-acting ''methylation-modifying'' factor(s) from the donor species *Zizania* or activation of otherwise cryptic endogenous factor(s) in rice, as a result of alien introgression, that may have caused alteration on the original parental cytosine methylation patterns following cell divisions. This later scenario is consistent with findings by a recent study on possible controlling mechanisms of naturally occurring ecotype-specific cytosine methylation patterns in *Arabidopsis* (Riddle and Richards 2002). In the study, two QTLs corresponding to *trans*-acting methylation modifiers were identified, which are believed to contribute to both establishment and maintenance of ecotype-specific cytosine methylation patterns (Riddle and Richards 2002). Moreover, such genotype-specific methylation pattern modifiers were also shown to present in mouse (Schumacher et al. 2000). (3) The altered cytosine methylation patterns are directed by genomic rearrangements that lead to the formation of ''aberrant structures'' in the introgression lines. It has been demonstrated in *Arabidopsis* that some naturally occurring aberrant genomic structures like direct or inverted DNA repeats may induce de novo DNA methylation of the underlying sequence and to sequences homologous to it (Luff et al. 1999; Melquist et al. 1999). This, coupled with the often faithful inheritance of newly acquired methylation patterns across organismal generations in plants (Scheid et al. 2003; Riddle and Richards 2002; Chan et al. 2005), may lead to methylation diversification from the original patterns. In this regard, it is notable that the rice introgression lines used in this study indeed contained a large amount of structural anomalies that are not accounted for by direct transfer from the donor species (Wang et al. 2005).

Despite our current ignorance of the mechanisms, the extensive and genome-wide occurrence of methylation alterations as a result of introgressive hybridization may have conspicuous structural and functional bearing to the introgression lines. For example, arrays of heritable transgressive phenotypic traits, including the overall plant form, disease-resistance, flowering time, yield-component traits etc., appeared de novo in the introgression lines (Liu et al. 1999; unpublished data). Given the wide-occurrence of DNA methylation alterations detected in the MSAP profiles, it might be possible that some of the novel traits have an epigenetic basis, i.e., they are caused by DNA methylation-mediated changes in gene expression. In addition, two types of transposable elements, the *copia*-like LTR retrotransposon *Tos17* (Hirochika et al. 1996) and a MITE transposon *mPing* (Jiang et al. 2003; Kikuchi et al. 2003; Nakazaki et al. 2003), are found as transiently mobilized in some of the introgression lines whereas both elements remain static in the parent cultivar (Liu and Wendel 2000; Shan et al. 2005). Furthermore, at least for *Tos17*, the element activity is correlated with its cytosine methylation status, an observation consistent with the genome defense role by cytosine DNA methylation on repressing activity of transposable elements in eukaryotes (Wessler 1996; Wang et al. 1996; Yoder et al. 1997; Grandbastien 1992; Grandbastien 1998; Wolffe and Matzke 1999; Martienssen and Colot 2001; Ros and Kunze 2001; Kato et al. 2004; Zilberman and Henikoff 2004). Therefore, element activation in the introgression lines is likely the consequence of cytosine methylation alterations detected in this study. Thus, an apparent implication of findings of this paper is on our appreciation of plant breeding materials derived from introgressive hybridization. Based on this study, it appears possible that at least in some cases introgression of genomic DNA from divergent species may impose a broader impact on the recipient cultivar than hitherto recognized, and some of the transgressive traits that are traditionally believed to result from genic interactions (epistasis) may actually have an epigenetic underpinning like altered DNA methylation patterns and its downstream consequences. Similarly, another implication with respect to the present findings is related to genome evolution under natural conditions mediated by introgression. Given prevalence of introgressive hybridization events between differentiated plant populations (Rieseberg 1995; Arnold 1997), it is likely that stochastic epigenetic alleles may have been generated by introgression, which are subjected to natural evaluation and selection, and hence, contribute to adaptive evolution (Finnegan 2001; Kakutani 2002;

Kalisz and Purugganan 2004; Rapp and Wendel 2005). Thus, an added role by introgression on genome evolution is likely due to its potentiality to generate epi-alleles in the form of altered DNA methylation patterns.

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