Cytosine methylation changes in rice centromeric and telomeric sequences induced by foreign DNA introgression

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Abstract

Cytosine methylation changes (hyper- or hypomethylation) in centromeric and telomeric sequences were observed in all three studied rice introgression lines containing DNA from wild rice, *Zizania latifolia* Griseb. The changed genomic Southern hybridization patterns were complex and non-concordant between a pair of isoschizomers (*Hpa*II/*Msp*I) digests, indicating methylation modifications at both the inner and outer cytosines of the CCGG sites. The changed patterns were inherited through generations. Possible mechanism for the methylation changes and their potential implications for the phenotypic variation and genome organization are discussed.

Additional key words: chromosome structure, epigenetic changes, introgression lines, *Oryza sativa* L.

Introduction

Cytosine methylation in eukaryotes plays important roles in at least two different cellular processes, control of gene expression and genomic imprinting, and to serve as a host genome defense system, *e.g.*, the repression of mobile elements (Richards 1997, Yoder *et al.* 1997). Many factors are known to cause DNA methylation changes, such as tissue culture, change in chromosome number (polyploidy/aneuploidy), and mutation in DNA methyltransferase genes (Vongs *et al.* 1993, Phillips *et al.* 1994, Scheid *et al.* 1996, Matzke *et al.* 1999). Recently, it was demonstrated that interspecific hybridization in wallaby (*Macropus*) and intraspecific cross in rice all triggered extensive genomic cytosine methylation modifications (O'Neill *et al.* 1998, Xiong *et al.* 1999). We described recently that extensive cytosine methylation changes occurred in rice as a result of foreign DNA introgression from wild rice, *Zizania latifolia* Griseb. (Liu *et al.* 1999b). The affected sequences include both random genomic or cDNA and known-function genes. These epigenetic changes, being non-random and highly heritable, were implicated to be a major cause for the drastically altered phenotypes in the rice introgression lines (Liu *et al.* 1999a,b).

Centromeres and telomeres are the most distinguishable landmarks of eukaryotic chromosomes. Centromeres play a critical role in mitotic and meiotic cell divisions, thus ensuring equal distribution of genetic information to daughter cells and to the next generation. Telomeres are essential to the integrity and stability of chromosomes during replication and division. DNA sequences present in centromeric or telomeric regions are therefore of great interest, as they may play direct/indirect roles for the proper functioning of these two chromosomal regions. Telomeric sequences are highly conserved in all eukaryotes so far studied, having the general structure of $(T/A)_m\ G_n{\cdot}C_a\ (A/T)_m$ (Allshire *et al.* 1988); whereas, centromeric sequences are more complex and are usually conserved only among closely-related species (Jiang *et al.* 1996). Given the extensiveness of the cytosine methylation changes in the rice introgression lines, it is of interest to know whether the changes also affected centromeric and/or telomeric regions. We report in the present paper that cytosine methylation changes occurred in both a centromeric and the telomeric sequences in all three rice introgression lines studied.

Received 6 June 2000, *accepted* 18 September 2000.
Acknowledgements: We thank the National Natural Science Foundation of China (No. 39870425) for support.
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Materials and methods

Plants: Production of a series of rice introgression lines with introgressed genetic material from the donor species *Z. latifolia* was described (Liu *et al.* 1999a). For the present study, three representative lines, designated as line 1, line 2 and line 3, at the 9, 10 and 11 selfed generations were used. These lines were characterized by RFLP analysis and verified to contain *Z. latifolia* species-specific DNA sequences (Liu *et al.* 1999a). Nevertheless, they were identical with their rice parent in more than 99 % of the probe/enzyme combinations (Liu *et al.* 1999a).

Preparation of probes: A rice centromeric sequence (*RCS*) was obtained by the procedure of Aragon-Alcaide *et al.* (1996). PCR amplification was done on rice genomic DNA with a pair of primers, *i.e.*, primer-1: 5'-TGCATCTATTCTTGCTTGTT and primer-2: 5'-CGTCGCTCTAAAATGTACAGC, designed according to a cereal centromeric sequence (Aragon-Alcaide *et al.* 1996). The amplification product (~260 bp) was gel-purified (*Weizard PCR Preps Purification System, Promega*, Madison, USA) and labeled with α-^{32}P-dCTP by the random primer method (Feinberg and Vogelstein 1983). The telomeric sequence (*TS*) was obtained by the method of Cox *et al.* (1993). PCR reaction was performed in the absence of template DNA with a pair of oligonucleotide primers T_1 (5'-TTTAGGG)$_5$ and T_2 (5'-CCCTAAA)$_5$. The crude PCR product was then directly labeled with α-^{32}P-dCTP (Cox *et al.* 1993).

Southern blot hybridization: At each generation, genomic DNA was isolated from young leaves of an individual plant of each of the three introgression lines, as well as from their rice parental cultivar (cv. Matsumae) and the donor species *Z. latifolia*. Care was taken to select leaves at the same stage. The purified DNAs (*ca.* 3 µg each sample) were then digested with a pair of isoschizomers, *i.e.*, *Hpa*II/*Msp*I. To ensure complete digestion, a 3-fold excess of enzymes was used (3 U per 1 µg DNA), and the digestion was performed overnight twice. Digested DNAs were fractionized by electrophoresis in 0.8 % agarose gel and transferred onto Hybond N$^+$ membrane by the alkaline method (*Amersham Pharmacia Biotech*, Little Chalfont, England). The labeled *RCS1* and *TS* were used as probes. A sequence (*Zg12*) that was known to be unmethylated in both rice and *Z. latifolia* as well as in all the introgression lines (Liu *et al.* 1999b), was also used as a probe to confirm complete digestion of the genomic DNAs. Pre-hybridization, hybridization and post-hybridization washing conditions were as described (Liu *et al.* 1997). The filters were then exposed to X-ray films at -80 °C for a few hours to several days.

Results

*Hpa*II and *Msp*I recognize the same tetranucleotide sequence 5'-CCGG (a predominant cytosine methylation site in eukaryotes), but with different sensitivities to cytosine methylation at specific sites: *Hpa*II will not cut if one or both cytosines are fully methylated whereas *Msp*I cleaves C^{5m}CGG but not ^{5m}CCGG (McClelland *et al.* 1994). Apparently, any changes in the Southern blot hybridization patterns in only one of the enzymes, or noncocordant changes in both enzymes are the results of cytosine methylation changes. According to this criterion, it is evident that, in comparison with the rice parent, all three introgression lines underwent cytosine methylation changes in both centromeric and telomeric sequences (Fig. 1*a*, *b*). For the centromeric sequence (Fig. 1*a*), with *Hpa*II-digest, novel lower molecular mass fragments appeared in introgression line 1 and 3, thus likely denoting demethylated elements of *RCS*, while with *Msp*I-digest, all three lines produced novel fragments. In line 1, absence of a higher molecular mass parental fragment (~12 kb) was also observed. All these changes in the hybridization patterns could be interpreted as cytosine methylation changes at both the inner and the outer Cs of multiple CCGG sites within or adjacent to the *RCS* elements. Another common feature in all three introgression lines is an apparent increased hybridization signal compared to the rice parent, indicating possible sequence amplification. Alternatively, the increased signal may be due to introgression of the *Z. latifolia* homologs of *RCS*, as *Z. latifolia* exhibited a much stronger hybridization signal than rice (Fig. 1*A*). For the telomeric sequence (Fig. 1*B*, line 1 changed in *Hap*II-digest, *i.e.*, it gave a smaller-sized smear, but did not change in *Msp*I, indicating demethylation at only the inner cytosines of the CCGG sites. Line 2 did not change in *Hpa*II-digest, but gave a higher molecular mass smear in *Msp*I, indicating hypermethylation at only the outer cytosines of the CCGG sites. Line 3 generated a significantly lower molecular mass smear in *Hpa*II-digest but gave a higher molecular mass smear in *Msp*I, thus denoting extensive demethylation at the inner cytosines and hypermethylation at the outer cytosines of the CCGG sites. To rule out the possibility that the changed patterns in the introgression lines were caused by incomplete (differential) digestion of the genomic DNA, precaution was taken by using both excessive enzymes and two-round overnight digestions. In addition, a single-copy

sequence (*Zg12*) known to be unmethylated in both rice and *Z. latilolia* (Liu *et al.* 1999b), was used to probe the same set of blots. The sequence produced a monomorphic hybridization fragment in all three introgression lines and in the rice and *Z. latifolia* parental lines in both *Hap*II/*Msp*I digests, thus verifying the observed changes in both centromeric and telomeric sequences in the introgression lines are due to cytosine methylation changes.

To study the heritability of the methylation changes, individual plants of each of the three introgression lines at respectively the 10 and 11 selfed generations (S_{10} and S_{11}) were analyzed. It was found that the hybridization pattern for a given line at both generations was invariably identical to that of the 9-generation (data not shown). This underscores the stable inheritance of the cytosine methylation changes in both the centromeric and telomeric sequences of the introgression lines. This observation is also consistent with the results revealed by other types of DNA sequences (Liu *et al.*1999b and unpublished data). Together, these results suggest that cytosine methylation changes in the rice introgression lines occurred extremely early (maybe instantaneously upon introgression) and/or non-randomly, and stably inherited thereafter.

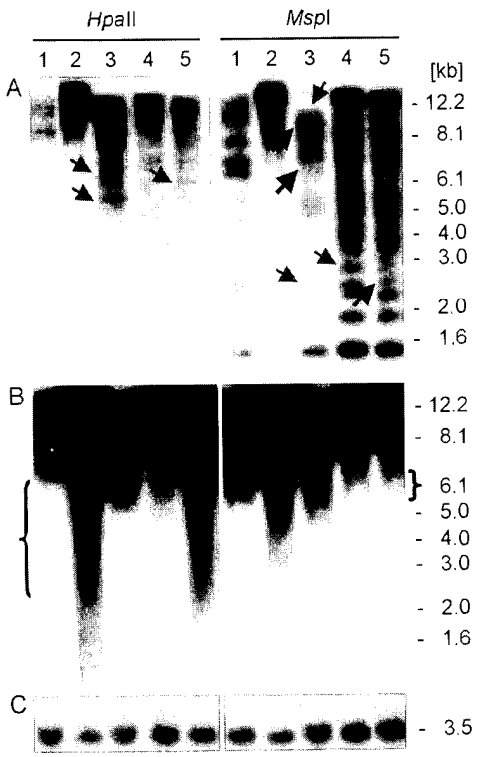

Fig. 1. Cytosine methylation changes in centromeric and telomeric sequences in 9-generation-old rice introgression lines. *A* - Southern blot hybridization of a rice centromeric sequence (*RCS*) to *Hpa*II- or *Msp*I-digested genomic DNAs of three introgression lines (*lanes 3 to 5*) and their rice parent, cv. Matsumae (*lane 1*), as well as the wild donor species *Z. latifolia* (*lane 2*). Appearance/absence of hybridization fragments in the introgression lines relative to their rice parent are due to methylation modifications at specific cytosines of the CCGG sites, and marked by *arrows*. Apparently, all three introgression lines exhibit changes in the banding patterns in at least one of the enzyme digests. Autoradiography was on X-ray film at -80 °C for 2 d.
B - Hybridization of the same set of blots as in *A* to a telomeric sequence (*TS*). Appearance or absence of the smear regions (or portion thereof) (marked by parentheses) denotes hypomethylation at the inner cytosine and/or hypermethylation at the outer cytosine of the CCGG sites. Apparently, all three introgression lines exhibited changes in at least one of the enzyme digests. Autoradiography was on X-ray film at -80 °C for 12 h.
C - Hybridization of the same set of blots as in *a* and *b* to a single-copy sequence (*Zg12*) known to be unmethylated in both rice, *Z. latifolia* and the three introgression lines. A monomorphic hybridization fragment of all plant materials in both *Hpa*II and *Msp*I digests verifies the complete digestion of the genomic DNAs. Autoradiography was on X-ray film at -80 °C for 7 d.

Discussion

Compared with the rice parent, all three introgression lines used in the present study exhibited drastically altered phenotypes in numerous traits, although RFLP analyses showed that they were identical with the rice parent in more than 99 % of the probe/enzyme combinations, when methylation-insensitive enzymes were used (Liu *et al.* 1999a). When methylation-sensitive isoschizomers were used, however, nearly half of the sequences detected polymorphism between introgression lines and the rice parent, indicating genome-wide methylation changes (Liu *et al.* 1999b). Given that methylation modifications, both hyper- and hypo-methylations, could affect expression of the affected gene and/or its nearby genes (Richards 1997), it was assumed that DNA methylation changes could be the underlying cause for at least some of the phenotypic variations of the introgression lines (Liu *et al.* 1999a). Although centromeres and telomeres are usually composed of mainly heterochromatins and hence non-genic sequences, extensive cytosine methylation modifications could conceivably affect DNA replication and therefore chromatin structure and/or organization (Phillips *et al.* 1994). Consequently, the changes could be pertinent to epigenetic or even structural genomic changes, and hence modification on gene expressions. The present study shows that complex patterns of cytosine methylation changes have occurred in both centromeric and telomeric sequences in all three introgression lines studied. Non-

concordant appearance/absence of hybridization fragments in both *Hpa*II and *Msp*I digests indicated hyper- and/or hypomethylation changes at both the inner and outer cytosines of the CCGG sites in the centromeric and telomeric sequences. These epigenetic changes could possibly lead to intensive reorganization of the centromeric and/or telomeric regions. This is in accordance with a recent finding in mammals: interspecific hybridization in two wallaby species induced genome-wide methylation changes, extensive amplification of retroelements and *de novo* structural changes at centromeric regions in the hybrid genome (O'Neill *et al.* 1998). These rapid changes were proposed to play a major role in hybrid genome evolution and species speciation by facilitating reproductive isolation.

The mechanism for cytosine methylation changes as a result of foreign DNA introgression is not clear. It is possible that a surveillance mechanism similar to, but probably more general than, the RIP (repeat-induced point mutation) system in *Neurospora* (Selker 1997) also exists in the plant genome. This system could be triggered, presumably by certain sequence repeats (DNA introgression from related species apparently provides sequence redundancy) and cause genome-wide (instead of sequence-specific as in *Neurospora*) methylation changes. In fact, this possibility has been proposed to explain tissue culture-induced genome-wide methylation changes, including both hyper- and hypomethylations (Phillips *et al.* 1994). A feature of RIP system in *Neurospora* is the indiscrimination of methylation on all cytosines of the targeted sequence, *i.e.*, methylation is not confined to CpG dinucleotides and CpNpG trinucleiotides (Selker 1997). The mobilization of a similar mechanism in the rice genome would largely explain the complex hybridization patterns in both *Hpa*II and *Msp*I digests in the centromeric and telomeric sequences of the introgression lines (Fig. 1*a*, *b*). Alternatively, foreign DNA introgression may activate cryptic mobile elements, whose amplification/ transposition, in turn, would cause DNA methylation modifications. This should be highly probable if the primary function of DNA methylation is to serve as a host genome defense system (Yoder *et al.* 1997). The fact that both centromeric and telomeric sequences are often associated with mobile elements seems to further corroborate this possibility.

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