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Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique

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Abstract DNA methylation is known to play an important role in the regulation of gene expression in eukaryotes. In this study, we assessed the extent and pattern of cytosine methylation in the rice genome, using the technique of methylation-sensitive amplified polymorphism (MSAP), which is a modification of the amplified fragment length polymorphism (AFLP) method that makes use of the differential sensitivity of a pair of isoschizomers to cytosine methylation. The tissues assayed included seedlings and flag leaves of an elite rice hybrid, Shanyou 63, and the parental lines Zhenshan 97 and Minghui 63. In all, 1076 fragments, each representing a recognition site cleaved by either or both of the isoschizomers, were amplified using 16 pairs of selective primers. A total of 195 sites were found to be methylated at cytosines in one or both parents, and the two parents showed approximately the same overall degree of methylation (16.3%), as revealed by the incidence of differential digestion by the isoschizomers. Four classes of patterns were identified in a comparative assay of cytosine methylation in the parents and hybrid; increased methylation was detected in the hybrid compared to the parents at some of the recognition sites, while decreased methylation in the hybrid was detected at other sites. A small proportion of the sites was found to be differentially methylated in seedlings and flag leaves; DNA from young seedlings was methylated to a greater extent than that from flag leaves. Almost all of the methylation patterns detected by MSAP could be confirmed by Southern analysis using the isolated amplified fragments as probes. The results clearly demonstrate that the MSAP technique is highly efficient for large-scale detection of cytosine methylation in the rice genome. We believe that the technique can be adapted for use in other plant species.

Key words DNA methylation · Hybrid rice · Tissue specificity · Isoschizomers · PCR amplification

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Introduction

DNA methylation, especially methylation of cytosine at position 5 (^{5m}C) in eukaryotic organisms, has received considerable attention in recent years. In animals and human, numerous studies suggest that DNA methylation has both epigenetic and mutagenic effects on various cellular activities such as differential gene expression, cell differentiation, chromatin inactivation, genomic imprinting and carcinogenesis (Gonzalgo and Jones 1997a). DNA methylation also plays a role in gene expression in higher plants (Meyer et al. 1994; Ulian et al. 1996; Rossi et al. 1997): actively transcribed sequences are often found to be less methylated than the promoters and certain coding regions of silent genes (Finnegan et al. 1993). Significant differences in the level of cytosine methylation have also been observed among various tissue types in some plant species such as tomato (Messeguer et al. 1991), maize (Lund et al. 1995) and rice (Dhar et al. 1990). In Arabidopsis (Ronemus et al. 1996) and tobacco (Tanaka et al. 1997), demethylation in transgenic plants was observed to be associated with drastic morphological changes.

In parallel with the long-standing interest in the functional role of DNA methylation, there has been a series of developments in the methods used for detecting DNA methylation. Recently developed methods can be classified into two categories according to the principles used in detection. One class of methods is based on bisulfite treatment, in which unmethylated cytosine res-

idues are converted to thymine after bisulfite treatment, whereas methylated cytosine residues remain unchanged (Sadri and Hornsby 1996). Several detection techniques based on bisulfite treatment have been developed to determine DNA methylation levels with quantitative accuracy (Gonzalgo and Jones 1997b; Xiong and Laird 1997).

The other class of methods commonly used involves the application of isoschizomers that differ in their sensitivity to methylation of their recognition sequences. Based on the fact that most ^{5m}C occurs at CG dinucleotides in animals (Doelfler 1983) and at both CG and CNG in plants (Gruenbaum et al. 1981), two restriction enzymes, *Hpa*II and *Msp*I, are frequently used to detect cytosine methylation. Both enzymes recognize the tetranucleotide sequence 5′-CCGG. However, *Hpa*II is inactive if one or both cytosines are fully methylated (both strands methylated) but cleaves the hemimethylated sequence (only one DNA strand methylated), whereas *Msp*I cleaves C^{5m}CGG but not ^{5m}CCGG (McClelland et al. 1994).

Methods for displaying the DNA fragments resulting from digestions with these isoschizomers have also been developed further, and now include restriction landmark genomic scanning (Hatada et al. 1992), methylation-sensitive arbitrary PCR (Gonzalgo et al. 1997) and methylation-sensitive representational difference analysis (Ushijima et al. 1997). More recently, the technique of amplified fragment length polymorphism (AFLP) (Vos et al. 1995) was modified for the detection of 5′-methylcytosine in DNA from dimorphic fungi (Reyna-López et al. 1997), and this approach has proved to be useful for determining DNA methylation status in fungi.

Rice, one of the most important world crops, with a relatively small genome size (430 Mb), has now become a model system for genome research in monocot plants. Although there has been tremendous progress in genome mapping and molecular genetic studies of rice, very little research has been done on the methylation status of the rice genome.

In this study, we investigated the cytosine methylation status of the rice genome in tissues from two developmental stages using a method – methylation-sensitive amplified polymorphism (MSAP) – adapted from the technique of Reyna-López et al. (1997). We also assayed methylation patterns in an elite rice hybrid rice, Shanyou 63, and compared them with those in its parental lines, in the hope of developing a viable approach to assessing the molecular basis of heterosis.

Materials and methods

Plant materials and DNA extraction

The materials used in this study were two indica rice (*Oryza sativa* ssp. *indica*) varieties (Zhenshan 97 and Minghui 63) and their F_1 progeny, the highly heterotic hybrid Shanyou 63, the hybrid most widely grown in China. Tissues assayed included young seedlings and fully expanded flag leaves at the day of heading. DNA was extracted from fresh tissue using the method described by Murray and Thompson (1981).

Methylation-sensitive amplification polymorphism (MSAP) assay

The method was adapted from Reyna-López et al. (1997), who modified the protocol for AFLP described by Vos et al. (1995) to incorporate the use of methylation-sensitive restriction enzymes. The modified protocol involved the use of the isoschizomers *Hpa*II and *Msp*I in place of *Mse*I as the frequent cutter, while the rare cutter *Eco*RI was unchanged. The adapter and the basic primer sequences for the *Eco*RI end were the same as those used in the original protocol (Vos et al. 1995). A new double stranded fragment, referred to as the *Hpa*II-*Msp*I adapter, was designed for the isoschizomer digestions by annealing the oligonucleotides 5′-*GA*TCATGAGTCCTGCT-3′ and 3′-AGTACTCAGGACGA*GC*-5 (the overhanging nucleotides are indicated in italics).

The basic primer sequence for the *Hpa*II/*Msp*I digests (HM + 0) was designed accordingly as 5′-ATCATGAGTCCTGCTCGG-3′. Primers with three selective nucleotides for the *Eco*RI ends (E + 3) and one to four selective nucleotides for the *Hpa*II-*Msp*I ends (HM + 1-HM + 4) were designed and tested. All the adapter and primer sequences were synthesized by BioSynthesis.

To detect MSAP, two digestion reactions were set up at the same time. In the first reaction, 2 μg of genomic DNA was digested with 15 U of *Eco*RI plus 15 U of *Hpa*II (GIBCO-BRL) in a final volume of 50 μl containing 1× R-L buffer (GIBCO-BRL) for 6 h at 37° C. The second digestion reaction was carried out in exactly the same way, except that *Msp*I was used in place of *Hpa*II. The digested fragments were then ligated to the adapter by adding 10 μl of ligation mixture, containing 1× One-Phor-All buffer (Pharmacia), 5 mM DTT, 0.5 pmol *Eco*RI adapter, 5 pmol *Hpa*II-*Msp*I adapter, 1 mM ATP and 10 U T_4 DNA ligase (New England Biolabs), and incubated at 37° C for 4 h. The reactions were stopped by incubating at 65° C for 8 min and diluted to 120 μl for PCR amplification.

Preamplification was conducted by using 3 μl of the above ligation product with E + 0/HM + 0 primers in a volume of 50 μl containing 1× PCR buffer, 0.1 mM each dNTP, 50 ng of each primer and 1 U Taq polymerase. The reaction involved 30 cycles of 94° C for 30 s, 60° C for 30 s, 72° C for 1 min, with a final extension at 72° C for 5 min. The preamplified products were then diluted to 200 μl and stored at −20° C before use.

Selective amplifications were conducted in volumes of 20 μl, containing 5 μl of the preamplification product, 30 ng of *Eco*RI primer, and 40 ng of *Hpa*II-*Msp*I primer end-labeled with [γ-^{32}P]ATP; the remaining components of the reaction were the same as in the preamplification reaction. The PCR amplification reactions were performed using the touch-down cycles as described in the original AFLP protocol (Vos et al. 1995). The denatured PCR products were separated on a 6% denaturing polyarylamide gel at 65 V for 3 h (Maughan et al. 1996). The gel was then dried and exposed to an X-ray film for 2–8 h.

Cloning and Southern analysis of differentially methylated fragments

Fragments containing methylated cytosine at 5′-CCGG sites were excised from the sequencing gel and reamplified using the same PCR conditions as used in the selective amplification. The fresh reamplified products (less than 24 h old) were ligated into the Vector pCR 2.1 using the Original TA Cloning Kit (Invitrogen) and transformed into *E. coli* strain DH5α. Plasmids were extracted using the alkaline lysis method (Sambrook et al. 1989). The insert DNA fragments were amplified and purified for use as probes in Southern analysis.

The procedure for Southern analysis essentially followed the method described by Liu et al. (1997) for RFLP assay, except that, for each sample, the total DNA was digested with either *Hpa*II or *Msp*I. The two digestions were loaded side by side on an agarose gel to facilitate comparison.

Results

Application of the methylation-sensitive amplified polymorphism (MSAP) technique

A preliminary experiment was conducted using the preamplification product from Minghui 63 in order to define conditions that would yield distinct amplified fragments on the sequencing gel. In this preliminary experiment, we tested a large number of combinations of selective primers, with three selective nucleotides at the *Eco*RI end (E + 3) and one to four selective nucleotides at the *Hpa*II-*Msp*I end (HM + 1 to HM + 4). Only E + 3/HM + 4 produced scorable bands; other combinations either resulted in a smear or yielded too many amplified fragments to allow accurate scoring.

Since *Hpa*II is sensitive to methylation of either cytosine residue at the recognition site and *Msp*I is sensitive only to methylation at the external cytosine, methylation of the internal cytosine would lead to the appearance of a fragment in the sequencing gel loaded with the amplification product from the *Eco*RI + *Msp*I digest but not the *Eco*RI + *Hpa*II digest. Indeed, we found a large number of fragments that were present among the selective amplification products of the *Eco*RI + *Msp*I digest but not in the other (Fig. 1). However, we also detected a few fragments in *Eco*RI + *Hpa*II digestion that were absent in the *Eco*RI + *Msp*I digest (Fig. 1). McClelland et al. (1994) attributed this sort of difference to hemimethylation of the recognition site 5′-CCGG, in which the external cytosine is methylated only in one strand. We also tested the reproducibility of the technique and found that almost all the bands could be repeatedly detected in different reactions, indicating that this method is robust for efficient detection of DNA methylation.

Cytosine methylation status in the parental lines

We used 16 pairs of primers to detect cytosine methylation at 5′-CCGG in flag leaf tissue and 11 pairs of primers in seedling tissue (Table 1). In flag leaf tissue, a total of 1076 fragments were amplified by 16 pairs of selective primers; each of the fragments represented a recognition site cleaved by one or both of the isoschizomers. Of the 1076 fragments, 195 were differentially amplified from the two digests for at least one of the parental lines, due either to full methylation of the internal cytosine – resulting in cleavage by *Msp*I but not *Hpa*II – or to hemimethylation of the external cytosine – resulting in cleavage by *Hpa*II but not *Msp*I. The two parents showed essentiallly the same degree of methylation; 174 differentially amplified fragments were detected in Zhenshan 97 and 176 were observed in Minghui 63. Thus, approximately 16.3% (175/1076) of 5′-CCGG sites in the rice genome were cytosine-methylated in this tissue, as detected by the differential recognition of the two isoschizomers. Full methylation of internal cytosines accounted for 87.6% of the methylated sites, and the remaining 12.4% were due to hemimethylation (Table 1). The situation was very similar in the seedling tissue (Table 1).

Differential methylation patterns among parental lines and hybrid

The two parental lines and their F_1 hybrid were compared for the patterns of differential amplification (Table 2, Fig. 1) using tissues from flag leaves and seedlings. Four major classes of banding patterns were identified among the differentially amplified fragments (Table 2). In the first class (class A), the same methylation sites were detected in both parents and in the hybrid; these are referred to as monomorphic with respect to cytosine methylation, within the resolving power of this technique. In the flag leaf tissue, 125 fragments detected by 16 primer pairs reflected full methylation of the internal

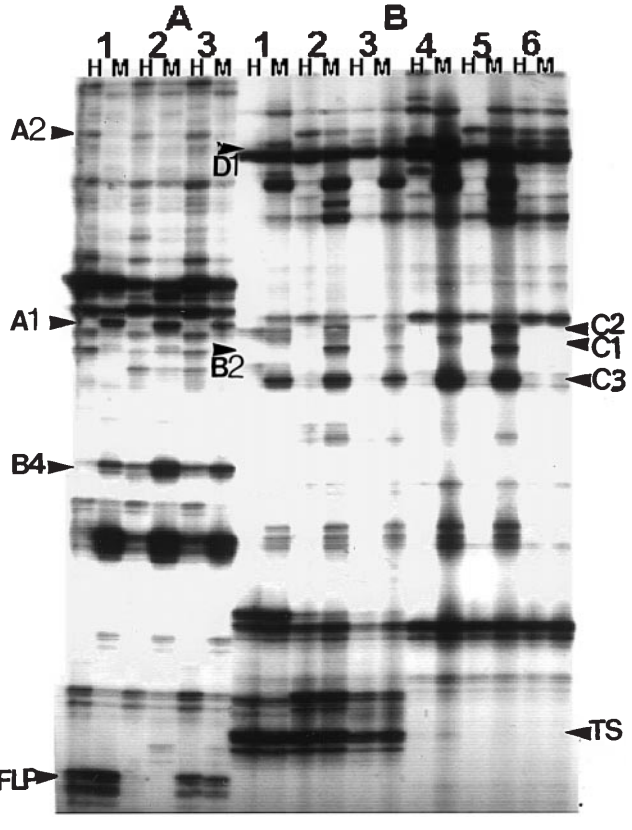

Fig. 1 Examples of methylation patterns detected in Zhenshan 97 (lanes 1 and 4), Minghui 63 (lanes 2 and 5), and F_1 (lanes 3 and 6) using the primer combinations HM + TCAA/E + AGA (A) and HM + TCAA/E + AAG (B). Lanes 1–3 show patterns from flag leaves, and those in lanes 4–6 are from seedlings. H and M refer to digestion with *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I, respectively. Designations of the methylation patterns (*arrowheads*) follow those in Table 2 except for TS, tissue specificity (pattern E in Table 3); the polymorphism marked AFLP resulted from conventional AFLP

Table 1 Numbers of bands amplified using various primer combinations in the cultivars Zhenshan 97 and Minghui 63 and their F1 hybrid

Primer (HM + 4/E + 3)[a]	Total number of bands	Fully methylated sites[c, d]	Hemimethylated sites	Methylation on polymorphic sites	Sites hyper- or demethylated in hybrid[d]	Tissue-specific sites
/E + ACA	63, 63	13, 13	2, 2	2, 2	0, 1	1
/E + AGA	72, -	14, -	3, -	5, -	3, -	–
/E + ACC	74, 74	11, 11	3, 3	4, 5	1, 1	1
/E + AAA	79, -	15, -	2, -	2, -	0, -	–
/E + AAC	64, 64	10, 10	0, 0	2, 1	1, 0	1
/E + AAG	65, 63	9, 11	2, 2	4, 5	1, 3	5
/E + AAT	61, 61	8, 8	1, 1	2, 2	0, 2	2
/E + AGG	73, 73	12, 12	2, 2	5, 5	0, 1	1
/E + AGC	54, 54	8, 8	1, 1	2, 2	1, 1	0
/E + AGT	66, 66	7, 7	0, 0	0, 0	0, 0	0
/E + CAA	72, -	11, -	0, 0	4, -	2, -	–
/E + CAT	68, 67	13, 14	1, 1	4, 5	2, 2	2
/E + CGC	67, -	12, -	2, 0	3, -	2, -	–
/E + CTG	62, -	6, -	0, -	1, -	1, -	–
/E + CAC	75, 75	12, 12	2, 2	4, 3	2, 1	1
/E + CGT	61, 61	10, 10	3, 3	2, 3	0, 1	2
Total	1076, 721	171, 116	24, 17	46, 33	16, 13	16

[a] The selective primer HM + 4 is 5′-CATGAGTCCTGCTCGG*T-CAA* (HM + TCCA) and was used in combination with each of the 16 E + 3 primers listed. The core sequence of the *Eco*RI primer is exactly the same as that used in the AFLP protocol by Vos et al. (1995)

[b] Each of the bands represents a recognition site cleaved by one or both of the isoschizomers. The patterns of methylation were deduced on the basis of polymorphisms in the pattern of the amplified fragments between the two digestions of each sample. The numbers separated by a comma represent data based on flag leaf (*left*) and seedling (*right*) tissues, respectively. A minus sign indicates that the corresponding primer was not used in the analysis. Four primer combinations were not used for seedlings

[c] Internal cytosine fully methylated

[d] The data in these columns are based on analysis of the two parental lines only

cytosine, and 24 fragments were the result of hemimethylation. In the seedling tissue, the numbers of fully methylated and hemimethylated sites were 83 and 17, respectively.

Methylation polymorphism was detected among parents and hybrid at 46 sites resolved by the 16 primer pairs in flag leaves, and 33 sites resolved by 11 primer pairs in seedlings (Table 1). These sites fell into a number of patterns that could be grouped into three major classes (classes B, C and D in Table 2). In class B, consisting of four patterns, 30 sites in the flag leaf tissue and 20 sites in seedlings showed differential cytosine methylation between the parental lines. The banding patterns in each case indicated that one of the parents was methylated at the internal cytosine and the other parent was methylated at the external cytosine or both. A common feature of the patterns in class B was that the bands detectable in the parents displayed simple Mendelian inheritance: irrespective of the enzyme digest; a band that was detected in either or both parents was also detected in the hybrid.

In class C, consisting of three patterns, the fragments resulting from the *Eco*RI-*Msp*I digests that were detected in one or both parents were not observed in the

Table 2 Patterns of cytosine methylation in the cultivars Zhenshan 97, Minghui 63 and their F_1 hybrid

Pattern[a]	Zhenshan 97		Mingshui 63		F_1		Number of sites[b]
	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	
A1	–	+	–	+	–	+	125, 83
A2	+	–	+	–	+	–	24, 17
B1	–	+	–	–	–	+	15, 10
B2	–	–	–	+	–	+	12, 9
B3	+	+	–	+	+	+	1, 1
B4	–	+	+	+	+	+	2, 0
C1	–	+	–	–	–	–	3, 1
C2	–	–	–	+	–	–	4, 4
C3	–	+	–	+	–	–	2, 4
D1	–	+	–	–	+	+	4, 2
D2	–	–	–	+	+	+	3, 2

[a] Class A patterns are monomorphic (i.e., the same methylation sites are found in both parental lines and in the hybrid), B patterns are characterized by differences in levels of methylation between the two parental lines; C-type patterns reveal hypermethylation in the F_1, and D-type indicate demethylation in the F_1. +, band present; –, band absent

[b] The numbers separated by comma in each cell of this column are based on flag leaf (*left*) and seedling (*right*) tissues, respectively

F_1, indicating increased methylation (or hypermethylation) in the hybrid (Table 1). The two patterns in class D represented a decrease in the level of methylation in the hybrid compared to the parents; fragments detected only in the *Eco*RI-*Msp*I digest in one of the parents were detected in both digests in the hybrid. This indicates that demethylation had occurred at those sites in the hybrid (Table 1).

Tissue specificity of cytosine methylation

Methylation status at 5′-CCGG sites in the parental lines and the hybrid was compared between seedlings and flag leaves. A total of 16 fragments were differentially amplified between the two tissues in at least one of the parents or in the hybrid (Tables 1, 3), indicating that these sites were differentially methylated in the two tissues. Of the 16 differentially methylated sites, seven were detected in the hybrid (patterns A and B of Table 3), four in Zhenshan 97 (pattern C) and two in both Minghui 63 and the F_1 (pattern D). The tissue-specific methylations at the remaining three sites were detected in all three materials (pattern E) (also see Fig. 1).

The banding patterns indicated that tissue-specific methylation occurred in such a way that 11 of the 15 sites (patterns A, B and C of Table 3) were methylated at either the external cytosine or at both cytosines in the seedling tissues but methylated only at the internal cytosine in flag leaf tissues. The reverse was the case for the two sites at which tissue-specific methylation occurred in both F_1 and Minghui 63 (pattern D). Although the banding patterns for the remaining three sites indicated that they were fully methylated in the seedling, these sites were completely demethylated in flag leaf tissues (pattern E and also see Fig. 1). Thus, DNA in the seedling tissue appears to be cytosine-methylated to a greater extent than in flag leaf tissues.

Confirmation of methylation patterns by Southern analysis

Southern analysis was performed to confirm the methylation patterns detected by MSAP. Fifteen amplified fragments representing different methylation patterns were recovered from the gels. These isolated fragments were cloned and used as probes for Southern hybridization. Total DNA from flag leaves or seedlings was digested with either *Hpa*II or *Msp*I. The two digests of each sample were loaded next to each other on the gel, blotted onto a nylon membrane and hybridized with the DNA fragments representing different methylation patterns.

Four of the 15 fragments were recovered from bands displaying methylation pattern A1 in Table 2. Southern hybridization revealed that, for each of the four probes, the detected fragment resulting from *Msp*I digestion was much smaller than that in the *Hpa*II digest (see Fig. 2A). This is in perfect agreement with the expected methylation of the internal cytosine of the restriction site.

Six fragments were recovered from bands displaying methylation patterns B1 and B2 in Table 2. Results of Southern hybridization for four of the probes agreed with the expectation based on the assumption that one of the parents was methylated at the internal cytosine and the other parent was methylated at the external cytosine or both, with the hybrid being heterozygous (Fig. 2B). One of the remaining two probes failed to detect methylation polymorphism among the parents and F_1 (i.e. the same as pattern A1), and the other showed a smear in the autoradiograph.

Two fragments displaying patterns C1 and C2, respectively, and one fragment of pattern D1 were recovered for Southern analysis. The probe for pattern C2 derives from a site at which Minghui 63 differs from Zhenshan 97 and the F_1 (lanes 4–6, Fig. 2C), in agreement with pattern C2 in Table 2. However, the tissue that showed this methylation polymorphism was seedling, while flag leaves were used for the amplification experiment. The other two probes detected multiple sequences.

We also recovered two fragments showing tissue-specific methylation in all three materials (pattern E of Table 3), in which the external cytosine, or both cytosines, presumably methylated in seedlings was demethylated in the flag leaves. Southern hybridization with one of the probes showed differences in banding patterns between flag leaf and seedling in all three materials

Table 3 Tissue-specific cytosine methylation in flag leaves and seedlings

Pattern	Flag leaf						Seedling						Number of sites
	Zhenshan 97		F1		Mingshui 63		Zhenshan 97		cF1		Minghui 63		
	*Hpa*II	*Mse*I	*Hpa*II	*Mse*I	*Hpa*II	*Mse*I	*Hpa*II	*Mse*I	*Hpa*II	*Mse*I	*Hpa*II	*Mse*I	
A	–	+	–	+	–	+	–	+	–	–	–	+	4
B	–	–	–	+	–	+	–	–	–	–	–	+	3
C	–	+	–	+	–	+	–	–	–	+	–	+	4
D	–	+	–	–	–	–	–	–	–	+	–	+	2
E	+	+	+	+	+	+	–	–	–	–	–	–	3

+, band present; –, band absent

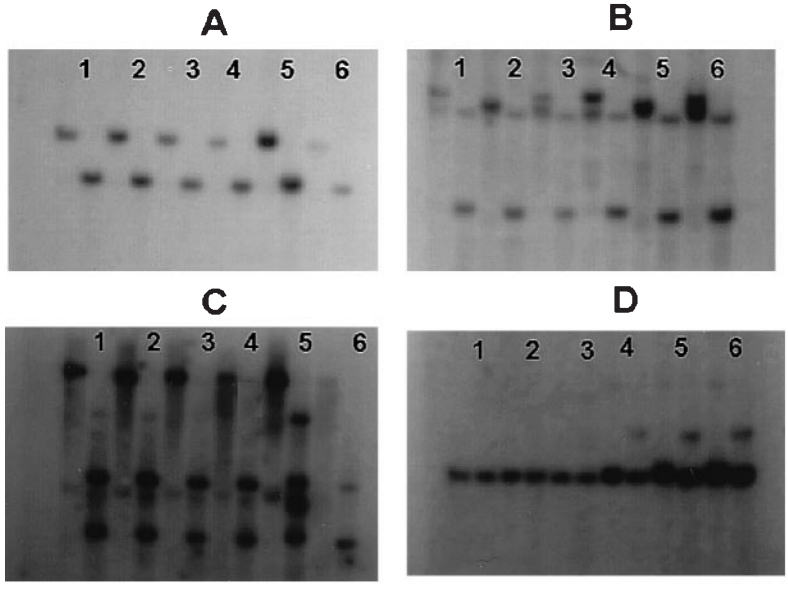

Fig. 2A–D Southern analysis to confirm the methylation patterns. Each DNA sample was separately digested with *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I and the two digests were loaded on the gel side by side (*Hpa*II digest on the left and *Msp*I digest on the right). The samples in each panel are from Zhenshan 97 (numbers 1 and 4) and Minghui 63 (2 and 5), and F_1 (3 and 6). Samples 1–3 in each panel are from flag leaf tissues, and 4–6 are from seedlings. Different hybridization probes were used for each panel, as follows: a fragment displaying methylation pattern A1 (Table 2) amplified using primer combination HM + TCAA/E + AGA (**A**); a fragment displaying methylation pattern B1 (Table 2) amplified using primer combination HM + TCAA/E + AGA (**B**); a fragment displaying methylation pattern C2 (Table 2) amplified using primer combination HM + TCAA/E + AAG (**C**); and a fragment displaying pattern E (Table 3) amplified using primer combination HM + TCAA/E + AAG (**D**). This last fragment revealed differential methylation between seedlings and flag leaves in all the three materials

(Fig. 2D). However, the hybridization pattern for the other probe suggested that demethylation had occurred in Zhenshan 97 and the hybrid, but not in Minghui 63.

Altogether, 11 of 15 fragments that showed various methylation patterns in parents and hybrid detected methylation by Southern hybridization, although the patterns did not fully agree with the expectations based on MSAP analysis.

Discussion

We have adapted a methylation-sensitive amplified polymorphism (MSAP) technique for the detection of cytosine methylation in the rice genome. The results showed that this technique is highly efficient for large-scale detection of cytosine methylation in the rice genome. Confirmation using Southern analysis further substantiated the usefulness of this technique in the detection of methylation. A distinct advantage of this approach compared to other methods is the ease of recovery of the differentially amplified fragments from the gel, which facilitates rapid identification of the methylated sequences in the genome. We also believe that this technique can be adopted for the detection of cytosine methylation in other plant species.

It should be pointed out, however, that there are two major constraints associated with the resolving power of this technique. First, this method can only investigate a small proportion of the cytosines in the genome. This is because detection is restricted to the recognition sites of the isoschizomers used, e.g., 5′-CCGG in the present study, while the expected frequency of this sequence in the genome is on the order of 1/256. The second constraint results from the basic principle of the method, which relies on differential sensitivity of the two restriction enzymes to cytosine methylation. As mentioned previously, *Hpa*II is inactive if either cytosine is fully methylated but cuts the hemimethylated sequence in which only one DNA strand is methylated, whereas *Msp*I can cleave $C^{5m}CGG$ but not $^{5m}CCGG$. It is clear that although differences between banding patterns obtained following digestion with *Hpa*II and *Msp*I are the result of cytosine methylation in the restriction sites, methylation of cytosine residues in the restriction sites in many cases does not necessarily produce such differences between the two digestions. Consequently, the MSAP technique is unable to detect many of the cytosine methylations in the restriction sites.

It should also be noted that the amplified fragments in the same lanes often differed in intensity, as can be clearly seen in Fig. 1. Such differences in band intensity suggests the possibility of variations in the level of methylation at the recognition site among the cells in the targeted tissue.

The major objective of this study was to estimate the extent of cytosine methylation in the rice genome. The results showed that cytosine methylation occurs at approximately 16.3% of the 5′-CCGG sequences in the rice genome, with full methylation of $C^{m}CGG$ occurring

more often than hemimethylation. This value is certainly an underestimate given the technical limitations in the resolving power of the MSAP technique. The major causes of underestimation include the occurrence of methylation at both of the cytosine residues and methylation at the external cytosine, neither of which can be detected by the MSAP technique.

An interesting feature of cytosine methylation in the rice genome revealed by the MSAP method is the degree of tissue specificity; more cytosine methylation was detected in seedlings than in flag leaves. Tissue specificity of adenine methylation has also been reported in embryos and shoots of rice (Dhar et al. 1990). Such tissue specificity is certainly expected based on current knowledge of the role of methylation as one of the regulatory mechanisms of gene expression during development and differentiation (Finnegan et al. 1993), although how methylation functions to regulate gene expression remains to be elucidated.

Another objective of this study was to develop an approach to investigating the possible role of methylation in the expression of heterosis, as such a possibility has been put forward previously (Tsaftaris et al. 1997). There have been a number of studies in rice addressing the genetic basis of heterosis and the relationship of heterosis to molecular heterozygosity and gene expression (Zhang et al. 1994, 1995, 1996; Xiao et al. 1995; Yu et al. 1997; Xiong et al. 1998). The present study has identified three classes of patterns of cytosine methylation characterized by differences in degree of methylation between the hybrid and the parent al lines: (1) the same level of methylation in the parents and hybrid; (2) an increased level of methylation in the hybrid compared to the parents, and (3) a decreased level of methylation in the hybrid. In the first case, the banding patterns appeared to follow simple Mendelian inheritance, and in both of the latter cases, the banding patterns were not inherited in a Mendelian fashion. Such increased or decreased methylation in the hybrid compared to the parents may provide an explanation for parent-specific and/or hybrid-specific differential gene expression as demonstrated in our previous study (Xiong et al. 1998). Thus, the MSAP technique may provide a very useful tool for comparative assessment of the levels of DNA methylation in parents and hybrids, hence probing the relationship between DNA methylation and heterosis. Moreover, the ability to clone the differentially methylated fragments and confirm their methylation status by Southern analysis may also provide an avenue for direct identification of the sequences (or the genes) that are differentially methylated between parents and hybrid. In conjunction with efforts to map QTLs for heterosis, the MSAP method may provide an additional tool for elucidating the genetic and molecular basis of heterosis.

In summary, we conclude that the MSAP technique represents an efficient tool for detection of DNA methylation that should be useful for studying a number of important biological problems. It may be particularly useful in testing the hypothesis that methylation plays a role in heterosis.

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