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Predominance and tissue specificity of adenine methylation in rice

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Summary. Using 'A' and 'C' methylation-specific restriction enzymes, namely, MboI, Sau3AI, DpnI, MspI, and HpaII, total rice cv Basmati 370 DNA, repetitive DNAs, and a specific repeat sequence indicated an abundance of adenine methylation. Although cytosine methylation in 5′-CCGG-3′ sequences suggested more CpC methylation than CpG, the 'C' methylation in sequence 5′-GATC-3′ was comparatively less than 'A' methylation. Furthermore, the presence of adenine methylation was tissue specific; it was predominant in rice shoot DNA as compared to embryo DNA. This pattern was also observed in two other cultivars of rice, i.e., R-24 and Sona, and was again confirmed using a cloned probe of a specific repeat sequence. Besides the changes in adenine methylation, there was also a qualitative change in 5mC from CpG to CpC dinucleotides in these two tissue systems.

Key words: Rice – Adenine methylation – Tissue specificity – Genomic rearrangement

Introduction

The role of 5-methyl cytosine, 5mC, has been well examined in animal genomes as compared to plants. It has been shown that the presence of 5mC is negatively correlated with gene activity. Organ- or tissue-specific variations in methylation patterns have also been observed in many animal genes, such as rabbit B-globin and chicken ovalbumin (Mandel and Chambon 1979; Compere and Palmiter 1981). In plants, methylation patterns of rDNA have been thoroughly studied. In general, the ribosomal genes seem to be heavily methylated in a few plant species investigated so far (Goldsbrough and Ellis 1981; Uchimiya et al. 1982; Ellis et al. 1983; Segel and Kolacz 1983; Olmedilla et al. 1984), except the rice rDNA unit. In rice, there is no change in rDNA methylation from seedlings grown in aerobic and anaerobic conditions (Aspart et al. 1983), although there is a change in the synthesis of rDNA genes. Tissue- or stage-specific variations are observed in radish rDNA (Delseny et al. 1984) and zein-light chain genes (Spena et al. 1983). Recently it has been shown in onion, broad bean, and rice that repeat DNA sequences undergo a change during differentiation and dedifferentiation events in cell culture conditions, and such variations are referred to as 'modulation' (Durante et al. 1977; Cremonini et al. 1981; Bassi et al. 1984; Zheng et al. 1985; Kikuchi et al. 1987).

In the present work, we describe the restriction enzyme analysis of total as well as repetitive DNA fractions of rice cv Basmati 370 using methylation-specific enzymes, i.e., MboI, Sau3AI, DpnI, MspI, and HpaII. To assess whether the methylation patterns are tissue/stage-specific, studies were also carried out in two tissue systems, i.e., embryo and shoot. Finally, to examine if changes in the methylation status of rice DNA during development are a general feature of the rice genome or if they are variety specific, similar experiments were carried out using the DNAs of two more rice varieties, namely, R-24 and Sona.

Materials and methods

Isolation of total DNA and repetitive DNA

Native, high-molecular-weight DNAs were prepared from embryos and shoots of three different varieties of rice cultivars, Basmati 370, R-24, and Sona, according to the procedures of Marmur (1961) and Ranjekar et al. (1976).

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Long (9–20 kbp) and short (0.2–0.3 kbp) repeat DNA sequences reassociating by Cot 0.1 and Cot 50 were isolated according to Dhar et al. (1988).

Restriction enzyme analysis, gel electrophoresis, and Southern hybridization

Restriction enzyme digestions of all the DNAs with a few methylation-specific enzymes, such as MboI, Sau3AI, DpnI, MspI, and HpaII, were carried out according to Maniatis et al. (1982). The DNA digests were analyzed on 1% and 1.4% agarose gels or 10% polyacrylamide gels, using both the high- and low-molecular-weight DNA markers. The total pRL7 DNA (3.6 kbp long rice repetitive DNA cloned in pBR 325) and the Sona 2.6 kbp repeat family were labelled by nick-translation as described by Rigby et al. (1977). Southern hybridization experiments were essentially according to Maniatis et al. (1982).

Results

Methylation status of rice shoot DNA

Figure 1 A shows the restriction enzyme digestion patterns of total rice cv Basmati 370 shoot DNA with MboI, Sau3AI, and DpnI. From this figure, it is clear that MboI (lane b) digestion of rice DNA is less than that of Sau3AI (lane c). Since it is known that the restriction enzyme Sau3AI does not cut the sequence 5′-GATC-3′ if C is methylated, whereas MboI does not cut the sequence 5′-GATC-3′ if A is methylated, the difference in the extent of digestion of rice shoot DNA with these two enzymes indicates the abundance of methylated adenine as compared to methylated cytosine in the sequence 5′-GATC-3′. This is further confirmed by the occurrence of extensive digestion of shoot DNA with DpnI (lane d), which digests the DNA only if A is methylated in the sequence 5′-GATC-3′.

Restriction enzyme digestion of total rice shoot DNA with MspI and HpaII is shown in Fig. 1 B. These two restriction enzymes are known to be sensitive towards C methylation in the sequence 5′-CCGG-3′. MspI does not cut the 5′-^{m}CCGG-3′ sequence, whereas it is insensitive to internal C methylation. On the other hand, HpaII is sensitive to internal C methylation and does not cut the sequence, 5′-C^{m}CGG-3′. It can be seen from Fig. 1 B that HpaII (lane c) shows comparatively more digestion of DNA than that with MspI (lane b), suggesting the abundance of 5′-^{m}CCGG-3′ type of sequences rather than 5′-C^{m}CGG or 5′-CCGG-3′ type in rice genome. Thus, this suggested a high frequency of ^{m}CpC dinucleotide as compared to ^{m}CpG dinucleotide in the 5′-CCGG-3′ sequence in rice DNA.

Presence of methylated adenine in rice-repetitive DNA

Since the total rice genomic DNA showed peculiar patterns of 'A' and 'C' methylation, we were interested in studying methylation patterns of its repetitive DNA sequences. For this purpose, long (9–20 kbp) and short (0.2–0.3 kbp) repeats of Cot 0.1 and Cot 50 fractions were isolated and digested with the above two sets of restriction enzymes and the results are shown in Fig. 2 A and B. From Fig. 2 A, it is evident that the digestion of the long repeats of Cot 0.1 DNA is less with MboI (lane b) than that with Sau3AI (lane c), thus revealing the presence of methylated adenine in 5′-GATC-3′ sequences in rice highly repetitive DNA fractions. Though not as distinct as Cot 0.1 DNA, a similar methylation pattern is also observed in the case of Cot 50 DNA (Fig. 2 A). This is further confirmed by the reasonable digestion of the DNAs with DpnI (lane d) compared to MboI (lane b) in both cases. Comparatively more digestion of these repeat sequences by Sau3AI (lane c) also suggests the absence of 'C' methylation in the sequence 5′-GATC-3′. Figure 2 B shows the digestion patterns of long repeats of Cot 0.1 and 50 DNA with MspI (lane b) and HpaII (lane c). Here, HpaII digests the DNAs more than that with MspI, suggesting again the abundance of 5′-^{m}CCGG-3′/5′-CCGG-3′ type of sequences in these repeat fractions. When the digests of short repeat sequences of both Cot 0.1 and Cot 50 with the methylation-specific enzymes were examined by polyacrylamide gel electrophoresis, it was observed that the short repeats were

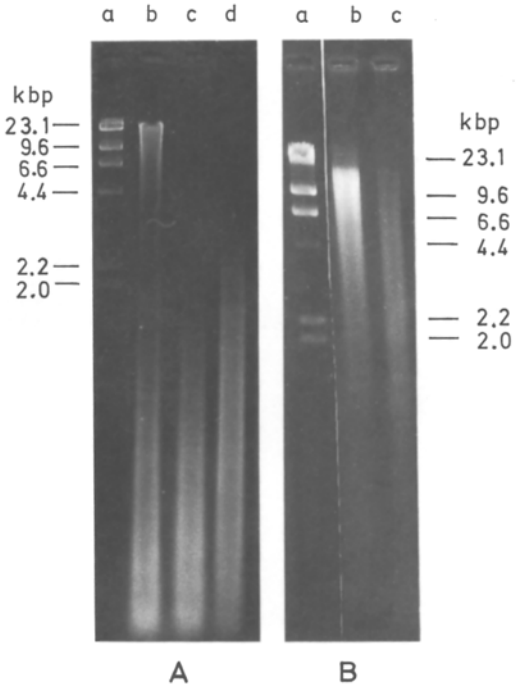

Fig. 1 A and B. Digestion of total rice cv Basmati 370 shoot DNA with MboI, Sau3AI, DpnI (**A**) and MspI, HpaII (**B**). **A** Lambda HindIII digest lane *a*; MboI digest lane *b*; Sau3AI digest lane *c*; DpnI digest lane *d*. **B** Lambda HindIII digest lane *a*; MspI digest lane *b*; HpaII digest lane *c*. Electrophoresis was carried out on 1.4% (**A**) and 1% (**B**) neutral, agarose slab gels in TAE buffer, pH 8.1, at a constant current of 30 mA

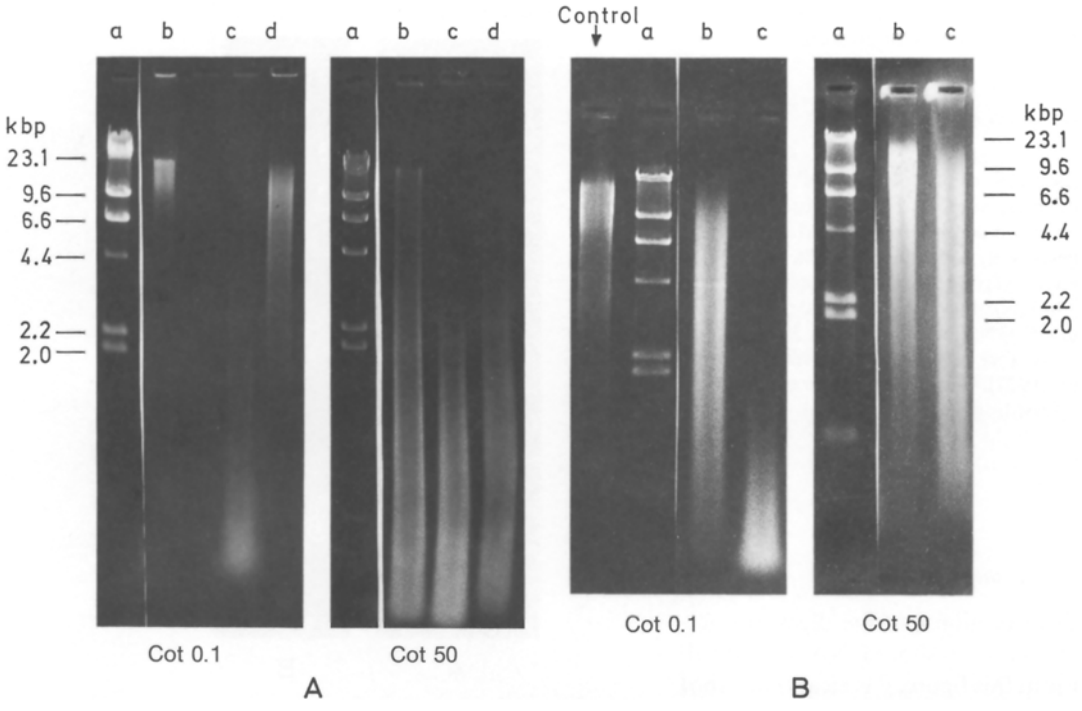

Fig. 2 A and B. Digestion of long repeats of Cot 0.1 and Cot 50 of rice cv Basmati 370 with MboI. Sau3AI, DpnI (**A**) and MspI, HpaII (**B**). **A** Lambda HindIII digest lane *a*; MboI digest lane *b*; Sau3AI digest lane *c*; DpnI digest lane *d*. **B** Lambda HindIII digest lane *a*; MspI digest lane *b*; HpaII digest lane *c*. Electrophoresis was carried out on 1.4% (**A**) and 1% (**B**) neutral, agarose slab gels in TAE buffer, pH 8.1, at a constant current of 30 mA

too small to reveal any distinct differences (data not shown).

Methylation status of rice embryo DNA

To assess if the methylation patterns of rice shoot DNA are tissue specific, rice embryo DNA from cv Basmati 370 was digested with MboI, Sau3AI, DpnI, MspI, and HpaII. It is clearly seen from Fig. 3A and B that the trend of 'A' and 'C' methylation is reversed in embryo DNA. For example, the restriction enzyme DpnI, which is specific for A methylation in the sequence 5′-GATC-3′, cuts the rice shoot DNA extensively (Fig. 1A, lane d). However, in rice embryo DNA, the digestion by DpnI (Fig. 3A, lane d) is very small, suggesting low frequency of A methylation in the sequence 5′-GATC-3′. Furthermore, Fig. 3B shows MspI (lane b) digestion to be greater than HpaII (lane c), suggesting a predominance of 5′-C^{m}CGG-3′ type of sequences. Thus 5^{m}C occurs as a part of CpG dinucleotides rather than of CpC dinucleotides in the embryo DNA. Apart from differences in DNA methylation, tissue-specific repeat families are also seen. The Basmati 370 embryo DNA shows a number of bands with both MspI and HpaII in the range of 0.6 to 4.0 kbp, suggesting the presence of these tissue-specific repeat families that are absent in the corresponding shoot DNA digests.

Distribution of pRL7 sequence in rice embryo and shoot DNA

The presence or distribution of a specific repeat sequence was next studied in the embryo and shoot system of cv Basmati 370 using a cloned pRL 7 sequence as a probe. For this purpose, the high-molecular-weight rice embryo and shoot DNAs were digested with methylation-specific restriction enzymes MboI and Sau3AI and a few enzymes like AluI and TaqI (Fig. 4A and B), and were hybridized to ^{32}P-labelled plasmid pRL7 DNA. It can be seen from Fig. 5A that a single strong band is present per lane with each enzyme, with the absence of any background smear in the case of embryonic DNA. The molecular weight of this band varies in a very small range of 2.3 to 2.5 kbp. In the case of shoot DNA, the hybridization pattern is observed to be different with each enzyme (Fig. 5B). With MboI (lane a), for example, two bands of molecular weights of about 15 and 5.5 kbp are prominent. With Sau3AI (lane b), four bands in the region 0.6–1.36 kbp and one band at 0.31 kbp are observed. In the case of AluI and TaqI (lanes c and d), however, many bands are seen in the range of 0.5 to 6.6 kbp. From these results, therefore, it is clear that there is some reorganization in this specific repeat unit in embryo and shoot systems.

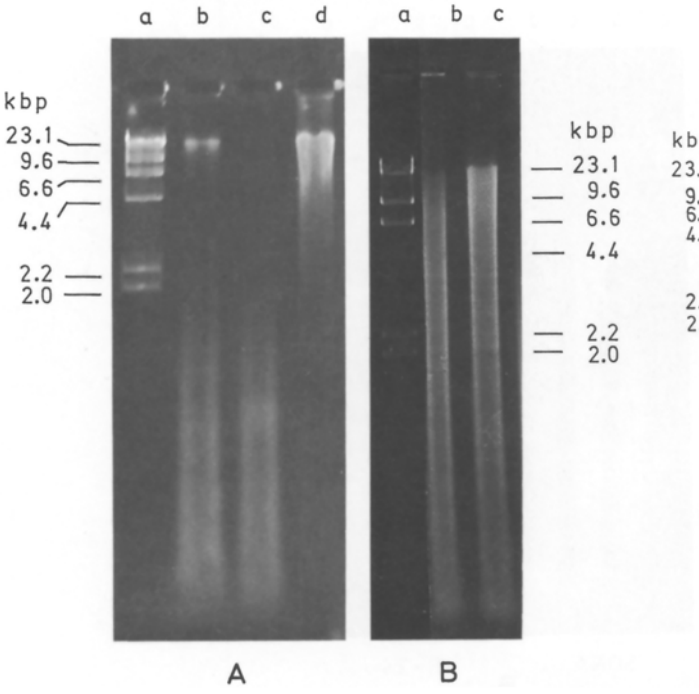

Fig. 3 A and B. Digestion of rice cv Basmati 370 embryo DNA with MboI, Sau3AI, DpnI (**A**) and MspI, HpaII (**B**). **A** Lambda HindIII digest lane *a*; MboI digest lane *b*; Sau3AI digest lane *c*; DpnI digest lane *d*. **B** Lambda HindIII digest lane *a*; MspI digest lane *b*; HpaII digest lane *c*. Electrophoresis was carried out on 1.4% (**A**) and 1.0% (**B**) neutral, agarose slab gels in TAE buffer, pH 8.1, at a constant current of 30 mA

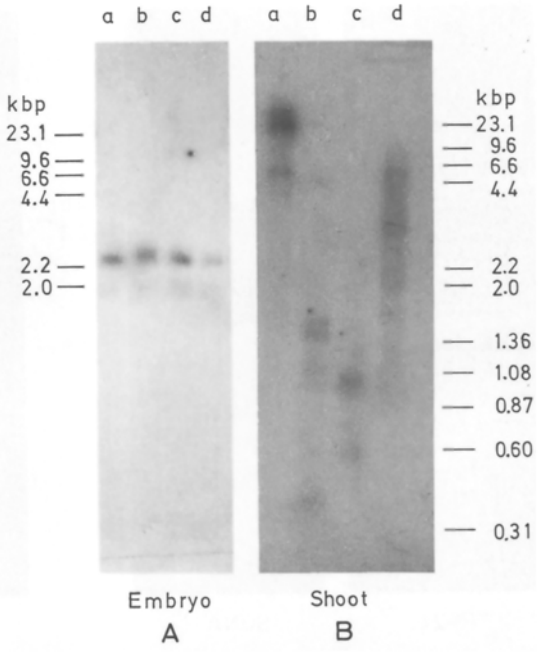

Fig. 5 A and B. Autoradiograms showing the hybridization of pRL7 cloned DNA with rice cv Basmati 370 embryo (**A**) and shoot DNAs (**B**) digested with different restriction enzymes. **A and B** MboI digest lane *a*; Sau3AI digest lane *b*; AluI digest lane *c*; TaqI digest lane *d*.

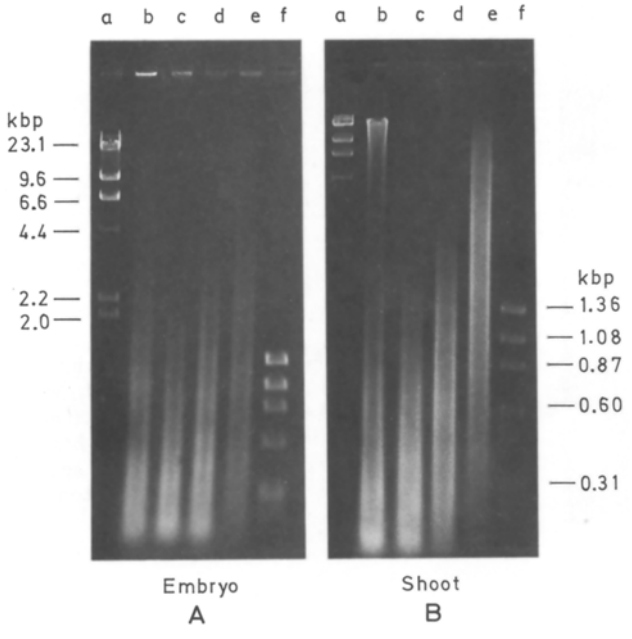

Fig. 4 A and B. Digestion of total rice embryo (**A**) and shoot DNA (**B**) with different restriction enzymes. **A and B** Lambda HindIII digest lane *a*; MboI digest lane *b*; Sau3AI digest lane *c*; AluI digest lane *d*; TaqI digest lane *e*; $\varnothing \times 174$ HaeIII digest lane *f*. Electrophoresis was carried out on 1.4% agarose gels in TAE buffer, pH 8.1, at a constant current of 30 mA

Restriction enzyme analysis of two other cultivars of rice

Restriction enzyme analysis of shoot and embryo DNAs from two more rice cultivars, viz., R-24 and Sona, was next undertaken. The aim of this experiment was to see whether the tissue/stage-specific methylation changes observed in Basmati 370 were variety specific or could also be found in other varieties. Figures 6 and 7 A, B show the digestion patterns of embryo and shoot DNAs of the two rice varieties R-24 and Sona with MboI, Sau3AI, DpnI, and MspI, HpaII. The digestion patterns with MboI, Sau3AI, and DpnI clearly indicate the predominance of 'A' methylation in the shoot DNAs (Fig. 6 B) as compared to the embryo DNAs (Fig. 6 A) in both varieties. Also, the MspI and HpaII digestion patterns (Fig. 7) in these DNAs are consistent with that of Basmati 370, wherein a quantitative change in $5^{m}C$ from CpG to CpC dinucleotides is observed during the transition from embryo to shoot.

Another finding from Figs. 6 and 7 is that the methylation-specific restriction enzymes also show variety as well as tissue-specific band patterns. In Sona shoot DNA, for instance, the MboI (Fig. 6 B, lane b) digest shows two bands of 2.6 kbp and 0.9 kbp, while Sau3AI (Fig. 6 B, lane c) reveals a single band of 2.6 kbp. This indicates the presence of regularly arranged, unmethylat-

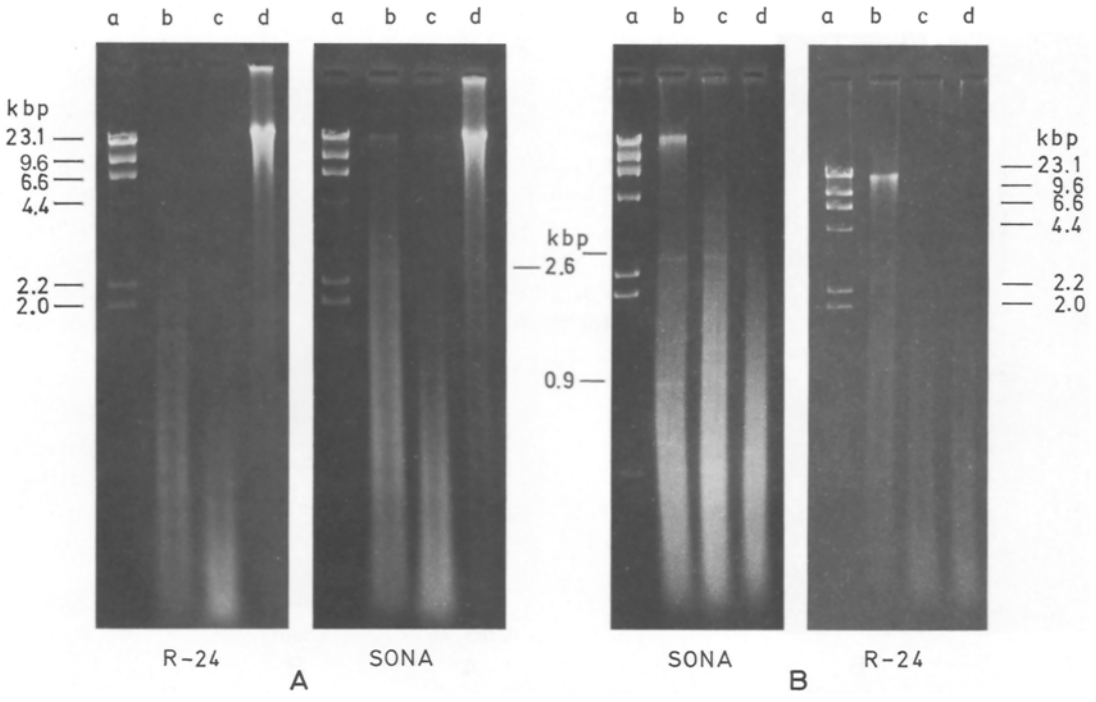

Fig. 6 A and B. Digestion of rice cv R-24 and sona embryo (**A**) and shoot DNAs (**B**) with MboI, Sau3AI, and DpnI. **A and B** Lambda HindIII digest lane *a*; MboI digest lane *b*; Sau3AI digest lane *c*; DpnI digest lane *d*. Electrophoresis was carried out on 1.4% neutral, agarose slab gels in TAE buffer, pH 8.1, at a constant current of 30 mA

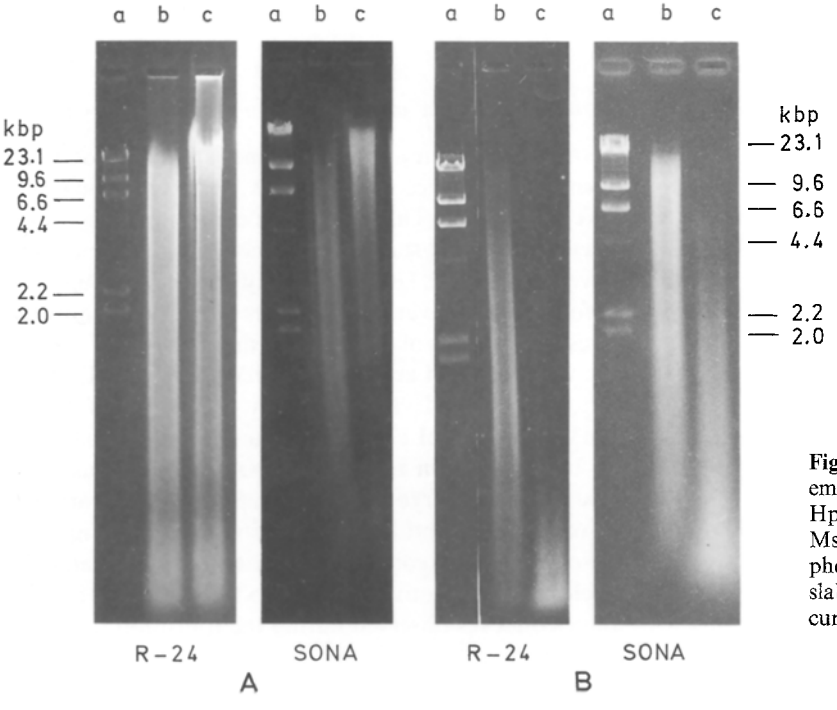

Fig. 7 A and B. Digestion of rice cv R-24 and sona embryo (**A**) and shoot DNAs (**B**) with MspI, HpaII. **A and B** Lambda HindIII digest lane *a*; MspI digest lane *b*; HpaII digest lane *c*; Electrophoresis was carried out on 1% neutral, agarose slab gels in TAE buffer, pH 8.1, at a constant current of 30 mA

ed 5′-GATC-3′ sequences in the Sona shoot DNA. Also, it shows that there are DNA sequences that include methylated 5′-GATC-3′ sequences arranged at a regular interval of 2.6 kbp. These methylated as well as unmethylated sequences may or may not belong to same/similar type of repeat families. In Sona embryo DNA (Fig. 6A), the 2.6-kbp Sau3AI and 0.9-kbp MboI repeat elements are absent, whereas the 2.6-kbp MboI repeat family is present. The R-24 embryo DNA, when digested with MboI, shows many bands with varying molecular

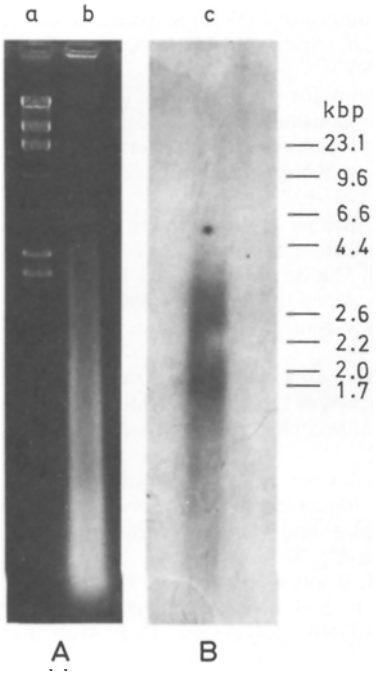

Fig. 8 A and B. Digestion pattern and autoradiogram of sona embryo DNA digested with Sau3AI :and hybridized to Sona shoot Sau3AI repeat family (2.6 kbp) as a probe. Lambda HindIII digest lane *a*; Sona embryo Sau3AI digest lane b; hybridization of 2.6 kbp Sona shoot Sau3AI repeat family to the above digest lane *c*

weights in the range of 0.5 to 2.2 kbp, while these are absent in the corresponding shoot DNA digest.

When the 2.6-kbp band that is present in the Sau3AI digest of Sona shoot DNA (Fig. 6B, lane c) and absent in the embryo DNA (Fig. 6A, lane c) is eluted, labelled, and hybridized to the gel blot of the embryo Sau3AI digest, two bands of about 2.6–2.7 kbp and 1.7 kbp are observed (Fig. 8). This suggests that there is a change in the Sau3AI repeat element with respect to either its copy number or methylation of its 5′-GATC-3′ sequences during the transition from embryo to shoot stage in Sona DNA.

Discussion

An important feature of the present studies is that rice shoot DNA and repetitive DNA have an abundance of 'A' methylation. This is a novel finding in view of the very few reports on adenine methylation in higher plants (Pintor-Toro 1987). It is also observed that this pattern of methylation is tissue specific, i.e., there is a predominance of 'A' methylation in rice shoot DNA as compared to embryo DNA. Since tissue-specific methylation is observed in three varieties of rice, this information may be of structural or functional significance in rice development and may help to assign a role to the repeat sequences in this process. The occurrence and role of adenine methylation is still unclear in higher plants. Recently, methylated adenine has been observed in zein genes, but the experimental evidence suggests that it is unrelated to their regulation of expression (Pintor-Toro 1987). In the case of animals and microorganisms, however, methylated adenine has defined roles (Sterglanz and Bugg 1973; Engel and Von Hippel 1978; Hattman 1982; Plasterk et al. 1983; Pukkila et al. 1983; Cheng et al. 1985; Hare and Taylor 1985). For instance, in *E. coli* the rate of adenine methylation within 5′-GATC-3′ sequences modulates the activity of certain promoters (Hattman 1982; Plasterk et al. 1983). Engel and Von Hippel (1978) have shown that the presence of 6-methyladenine results in a destabilization of the DNA helix, presumably because the N1-*cis* orientation of the methyl group is favored. Such effects may be significant in protein – DNA interactions (Sterglanz and Bugg 1973; Cheng et al. 1985).

Another important feature of the present work is the differential methylation of repeat DNA that corresponds to a specific, cloned repeat element pRL 7 during the transition from embryo to shoot stage. This repeat unit is present in a well-organized manner in the embryo DNA, whereas in the case of shoot DNA, many copies of this sequence are present in a varied environment with random distribution, while some are well organized to give a particular band pattern. From this data it appears that there is some form of genomic rearrangement of a specific, repetitive sequence and it may partly be due to the changes in methylation of this repeat unit. In the literature, there are a few reports on genomic alterations in tissue culture conditions. In rice, for example, Zheng et al. (1985) have reported the amplification of a specific, highly repeated sequence in cultured cells of variety Roncarlo during the differentiation process. Kikuchi et al. (1987) have given preliminary results in rice (cultivar Nipponbare) showing variations in the copy number of two repeated DNA sequences, one being amplified and the other being reduced in copy number in the differentiation process under cultured conditions.

Ours is probably the first report that describes the differential methylation status of specific repeat sequences in rice (cv Basmati 370) during the transition from embryo to shoot without any external inducing factor.

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