CHAPTER 4

ALU-I REPEAT FAMILY AND GENOMIC METHYLATION IN SIX MILLETS WITH A SPECIAL REFERENCE TO GREAT MILLET
SUMMARY

The 0.55 kbp Alu I repeat family of great millet DNA has been characterized with respect to its genomic distribution and homology with the Alu I repeats of five other millet DNAs namely barn yard millet, little millet, fox tail millet, finger millet and pearl millet. This repeat family is arranged in a tandem manner and it reveals a weak homology with Alu I repeats of the other five millets.

Digestion of millet DNAs with Msp-I/Hpa-II suggests a predominance of $^m$CpG methylation in great millet DNA and of $^m$CpC methylation in the other five millet DNAs. Restriction enzyme analyses of millet DNAs with Mbo I, Sau 3A I and Dpn I indicate that some of the 5' GATC 3' sequences are methylated at adenine and/or cytosine residues except in little millet where adenine methylation of the 5' GATC 3' sequences is insignificant and there is a predominance of cytosine methylation in these sequences. Comparison of the Mbo I digestion pattern of great millet shoot DNA with that of embryo DNA indicates methylation of adenine residues at some of the 5' GATC 3' sequences during development from embryonic to the seedling stage.
4.1. INTRODUCTION

A large number of repeat families have been identified and characterized in several plants and their possible biological significance is discussed [2, 9, 15, 19, 29, 51, 52]. However among cereals, such studies have so far been limited only to rye [1], wheat [11, 12, 16-18, 25, 32] and maize [23, 34] and to the best of our knowledge no such information is available on millets. In our laboratory, the DNAs of six millets namely great millet (*Sorghum vulgare*), fox tail millet (*Setaria italica*), little millet (*Panicum miliare*), barn yarn millet (*Echinochloa frumentaceae*), finger millet (*Eleusine coracana*) and pearl millet (*Pennisetum americanum*) have earlier been characterized with respect to their repetitive DNA content and sequence organisation [13, 21, 22, 37, 38, 41-43]. In the present work, Alu I repeat family has been identified and partially characterized in great millet DNA and the methylation status of all the six millet genomes has been assessed by using methylation sensitive restriction enzymes.
4.2. MATERIALS AND METHODS

4.2.1. Seed material and chemicals

Seeds of great millet (var. Maldandi), barnyard millet, foxtail millet, finger millet and pearl millet were obtained as described in Chapter 2. The seeds of great millet varieties namely ICH 153, 296B, 296A, CSH9 and a wild type namely Verticilliflorum Coll 1 (IS 14257) were obtained from ICRISAT, Hyderabad, India.

All chemicals were procured as described in Chapters 2 and 3. Enzymes such as DNA polymerase, DNase I, restriction endonucleases and DNA molecular weight markers (λ DNA digested with HindIII and φ X 174 RF DNA digested with Hae III) were either from Bethesda Research Laboratories (BRL), USA or New England Biolabs (NEB), USA. Radiolabelled α-32P-TTP or CTP were from Radiochemical Centre, Amersham, U.K. Nitrocellulose papers (M.D.I. type SCN, 0.45 microns) for southern hybridization experiments were obtained from Advanced Microdevices, Ambala, India. Black and white ORWO or ILFORD photographic film (35 mm, 125 ASA) and X-ray film AGFA Curix RPI were used for gel photography and autoradiography, respectively.

4.2.2. DNA isolation and criteria of purity

Extraction of DNA from shoots as well as the embryo, and the sizing of DNA were done as detailed in Chapter 2.

4.2.3. Restriction endonuclease digestion and gel electrophoresis

For all restriction enzyme digestion, about 3-4 μgs of DNA was incubated with 10 units of restriction enzyme in a reaction volume
of 20 μl at 37°C, overnight, in appropriate assay buffers which were prepared according to Maniatis et al. [30]. The enzyme to DNA and enzyme volume to reaction volume ratios were carefully adjusted to avoid non-specific (star) activity of restriction enzymes [30]. Following incubation, the enzyme digestion was stopped by addition of 10 X reaction terminating buffer (50% glycerol, 100 mM EDTA, 0.25% bromophenol blue) to a final concentration of 1 X. Control experiments were performed using commercial λ DNA digested with different restriction enzymes to check the reaction conditions.

DNA digests were analyzed on either 2.4% (for Alu I digests of great millet), or 1% (for all other enzyme digests) neutral agarose horizontal slab gels in TAE 1X at a constant current of 20 mA for 5-8 hours. The gels were stained with ethidium bromide in dark and were visualised on a long wavelength (302 nm) UV transilluminator (UV Products, San Gabriel, California, USA) and photographed with a 35 mm SLR camera (Minolta X 700 with macrophotography and zoom lens system) using a red filter.

4.2.4. Isolation of the 0.55 kbp Alu I fragment of great millet DNA

About 30 μgs of high molecular weight (> 20 kbp) great millet shoot DNA in 10 mM Tris-HCl buffer (pH 7.4) was digested with 100 units of Alu I in a reaction volume of 200 μls according to Maniatis et al.[30]. Following incubation, the enzyme digestion was terminated by the addition of reaction terminating buffer as described in section 4.2.3 and the digest was analysed on a 2.4% neutral agarose gel. After electrophoresis, the gel was stained with ethidium bromide and visualised on the long
wavelength UV transilluminator. The 0.55 kbp Alu I fragments were cut out from the gel and the agarose pieces were then frozen in Tris-saturated phenol at -70°C for minimum 2 hours. Subsequent to this, the frozen gel pieces and phenol were thawed out gradually to 4-10°C and centrifuged at 10,000 rpm for 10 minutes at 4°C using the SIGMA refrigerated microfuge. The top aqueous layer was collected and traces of phenol were removed by treatment with chloroform-isoamyl alcohol (24:1 v/v) mixture. Traces of ethidium bromide were removed by treating the aqueous layer with 1-butanol [30]. The DNA was then precipitated with 2 volumes of chilled ethanol after adjusting the sodium ion concentration of the aqueous layer equivalent to that of 0.25 M sodium acetate, pH 5.5. The DNA precipitate was dried in a vacuum desiccator and dissolved in 10 mM Tris-HCl buffer, pH 7.4.

4.2.5. Southern blotting and hybridization

**Southern blotting**

The transfer of DNA from agarose gels to nitrocellulose membranes was carried out essentially according to Southern [44] and Maniatis et al. [30]. Initially the gels were soaked twice for 15 minutes in 0.25 N HCl at room temperature for depurination of DNA. The DNA was then denatured by soaking the gel in 1.5 M NaCl and 0.5 M NaOH for 1 hour at room temperature with gentle shaking. Neutralisation of the gel was done by soaking the gel in several volumes of 1 M Tris-HCl, pH 8.0 and 1.5 M NaCl for 1 hour at room temperature with constant shaking. The transfer of DNA to nitrocellulose paper was carried out for about 24 hours in 10 X SSC after which the nitrocellulose filters were air dried and baked for 2 hours at 80°C under vacuum.
The filters were stored at room temperature between sheets of Whatman 3 MM paper till further use.

**Preparation of labelled 0.55 kbp Alu I DNA by nick-translation**

The 0.55 kbp Alu I DNA was labelled by the procedure of Rigby et al. [36]. The nick-translation reaction mixture (50 µl) consisted of 1 µg of 0.55 kbp Alu I DNA, 5 mM MgCl₂ and 20 mM each of unlabelled dATP, dGTP and dTTP. Commercial α³²P- CTP (specific activity 3000 Ci/mM) was added to a final concentration of 90-100 picomoles. *E. coli* DNA polymerase I was added to a final concentration of 2-3 U/µg. The reaction was carried out at 15°C for ninety minutes and was terminated by adding freshly distilled phenol saturated with Tris buffer. The aqueous layer was further deproteinized with chloroform-isoamylalcohol mixture and was finally purified by Sephadex G-50 column chromatography. The specific activity of the probe DNA was found to be about 10⁷ cpm/µg. All the counting was done by Cerenkov counting method [3,8], using a Beckman Liquid Scintillation Counter (Model LC 100C).

**Southern hybridization**

This was carried out according to Maniatis et al. [30]. Nitrocellulose filters were prehybridized for 5-6 hrs at 62°C in heat sealed plastic bags containing prehybridization mixture (0.2 ml per square cm of paper). The prehybridization mixture consisted of 6 X SSC, 0.5% SDS, 0.1% Denhardt’s solution (0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.1% BSA) and 250 µgs/ml of denatured calf thymus DNA.
After prehybridization, the solution was removed and hybridization solution (50 uls/cm² of filter) was added to the bag. Hybridization mixture was essentially similar to prehybridization mixture except that it also contained 0.01 M EDTA and ³²P-labelled denatured probe. The hybridization was carried out for 48 hours at 62°C and unhybridized probe was removed by washing the filters as follows:

- 2 x SSC + 0.5% SDS - 5 mins at room temperature
- 2 x SSC + 0.1% SDS - 15 mins at room temperature
- 0.1 x SSC + 0.5% SDS - 2 hours at 62°C with gentle agitation

After 2 hours the buffer was changed and the incubation was continued further for 30 minutes. The filters were then dried at room temperature on a sheet of Whatman 3 MM paper mounted on a thin plastic paper and fixed in X-ray cassette with Agfa Curix X-ray film and intensifying screens. After exposure for about 1-3 days at -70°C, the film was developed with Agfa X-ray developer, washed, dried and photographed with a 35 mm SLR camera (Minolta X 700 with macro-photography and zoom lens system).

4.2.6. HPLC analysis of total DNAs

HPLC analyses was done as described in Chapter 3.
4.3. RESULTS

4.3.1. Identification of repeat families in millets

In order to assess species specific differences and the presence of discrete repeat families, the six millet DNAs were digested with as many as ten different restriction enzymes and the digestion patterns are depicted in Fig. 4.1 A & B. From this figure, it is seen that each of these millet DNAs exhibits differences in the banding pattern as well as the extent of digestion with each restriction enzyme. Some of the important observations from Fig. 4.1 are as follows: Alu I, Bgl II, Eco RI, Hind III and Bgl I families are present in all the six millet DNAs. Pvu I repeat families are present in finger millet and pearl millet. Pvu II repeat families are absent in great millet and barn yard millet. Xho I repeat families are present only in great millet, finger millet and pearl millet. Bcl I repeat families are absent in barn yard millet and fox tail millet while Xba I repeat families are absent in barn yard millet. In some cases, bands are not clearly seen. This may be due to a low copy number of repeat DNA sequences present in them.

4.3.2. Identification and partial characterisation of the Alu I repeat family of great millet

The Alu I/Hind III tandem repeat families have been characterised in a number of plants such as Arabidopsis thaliana [31], Cucumis melo [24], Cucumis sativus [24], Cucurbita pepo [28], Raphanus sativus [19] and Sinapis alba [7]. Comparative data among these plants as
FIG. 4.1A; RESTRICTION ENDONUCLEASE DIGESTION PATTERNS
OF THE MILLET DNAs.

In all the photographs; lane a is great millet, lane b is barn yard millet; lane c is little
millet; lane d is fox tail millet; lane e is finger
millet; lane f is pearl millet; lanes g and h
Hind III digests of λ DNA as the molecular weight marker;
lane i is Hind III digest of λ DNA + Hae III digest of
Φ X 174 RF DNA as the molecular weight markers.

1 % agarose gels were used in all the electrophoresis
experiments.
FIG. 4.1 A
FIG. 4.1B: RESTRICTION ENDONUCLEASE DIGESTION PATTERNS OF THE MILLET DNAs.

In all the photographs, lane a is great millet; lane b is barn yard millet; lane c is little millet; lane d is fox tail millet; lane e is finger millet; lane f is pearl millet; lanes g and h are Hind III digest of λ DNA as the molecular weight marker; lane i is Hind III digest of λ DNA + Hae III digest of ϕ X 174 RF DNA as the molecular weight markers.

1% agarose gels were used in all the electrophoresis experiments.
FIG. 4-1 B
well as a few mammals have shown that they show up to 50% homology and probably arise from an ancestral tRNA gene [2,9,15,51]. In addition, the significance of such tandem families with respect to several cellular functions such as determination of chromatin structure has also been discussed [19,29,52]. From the ladder-like banding pattern of the Alu I digests of millet DNAs (Fig. 4.1), it appears that these repeats are tandemly arranged. To confirm the tandem character of the Alu I repeats in great millet, a series of digestions using increasing amounts of enzyme were set up and the digestion products were separated by electrophoresis on a 2.4% agarose gel for better resolution (Fig. 4.2A). From this figure it is seen that the 275 bp band appears to be the basic repeat unit and the successive bands differ by about 275 bps. In order to have a better understanding about the organisation of these Alu I repeats, the 550 bp band was eluted from the gel, nick translated and allowed to probe the southern blot of the above mentioned 2.4% gel (Fig. 4.2B). The 550 bp band was selected because it was more intense than the band of 275 bps. The significant observations from the autoradiogram are as follows:

i) When increasing amount of enzyme is used for digestion, the bands of higher molecular weight decrease in intensity while there is a simultaneous increase in the intensity of the lower molecular weight bands. This is expected for a repeat family that is arranged in a tandem manner.

ii) The basic Alu I repeat unit is 275 bps. The bands of molecular weight 550, 1100 and 1650 bps are more intense than the other bands.
FIG. 4.2. (A) ELECTROPHORESIS ON 2.4% AGAROSE OF GREAT MILLET DNA DIGESTED WITH DIFFERENT AMOUNTS OF

\texttt{Alu I}

0.1 \text{U/\mu g (lane a)};

1 \text{U/\mu g (lane b)};

10 \text{U/\mu g (lane c)};

20 \text{U/\mu g (lane d)};

\texttt{Hae III} digest of \texttt{\phi X 174 RF DNA} as the molecular weight marker (lane e)

(B) SOUTHERN HYBRIDIZATION OF THE 0.55 kbp \texttt{Alu I} FRAGMENT OF GREAT MILLET DNA WITH THE \texttt{Alu I} DIGESTS OF GREAT MILLET DNA

0.1 \text{U/\mu g (lane a)};

1 \text{U/\mu g (lane b)};

10 \text{U/\mu g (lane c)};

20 \text{U/\mu g (lane d)}. 
and represent the dimer, tetramer and hexamer units respectively. The greater intensity of these bands as compared to that of the monomeric 275 bp unit could be explained by considering that there is a loss of the in-between restriction sites due to which not many copies of the basic 275 bp fragment are released on enzyme digestion.

In order to determine if any differences exist in the Alu I digestion patterns in varieties related to the variety Maldandi of great millet, the DNAs of a few varieties namely ICH 153, 296 B, CSH 9, 296 A and a wild variety [verticilliflorum Coll I (IS 142 57)] were restricted with Alu I and the digestion patterns were examined. From Fig. 4.3A. It is seen that no significant differences are seen in the banding patterns of the various digests and that the 550 bp band appears to be prominent in all the varieties.

We were next interested in knowing if the Alu I repeats of great millet were species specific or they exhibited some homology with Alu I repeats of the other five millets. For this purpose the 550 bps Alu I band of great millet DNA was nick translated and allowed to probe the Alu I digests of the other five millet DNAs (Fig. 4.3B) at a hybridisation temperature of 60°C. Under such conditions, a weak hybridisation signal is observed with the Alu I repeats of the other five millets co-migrating with the great millet 550 bp Alu I fragment. In case of pearl millet the probe also hybridises very weakly to two additional bands of molecular weights 1.7 kbp and 2.0 kbp.
FIG. 4.3. A: ELECTROPHORESIS ON 1% AGAROSE OF THE DNAs OF GREAT MILLET VARIETIES DIGESTED WITH 

Alu I:

Hind III digest of λ DNA (lane a), Maldandi (lane b), MR 750 (lane c); ICH 153 (lane d); 296 B (lane e); CSH 9 (lane f); wild verticilliflorum Coll I (ISI 14257) (lane g).

FIG. 4.3. B: SOUTHERN HYBRIDIZATION OF THE 0.55 kbp Alu I FRAGMENT OF GREAT MILLET DNA WITH THE Alu I DIGESTS OF THE DNAs OF OTHER FIVE MILLETS; great millet (lane a); barn yard millet (lane b); little millet (lane c); fox tail millet (lane d); finger millet (lane e); pearl millet (lane f); Hind III digest of λ DNA as the molecular weight marker (lane g, h, i).
Alu-1 digest of the DNAs of great millet varieties

FIG. 4.3
4.3.3. Methylation status of millet genomes

4.3.3.1. Content of 5 methyl cytosine

Prior to digestion with methylation specific enzymes, the amount of 5 methyl cytosine in the total nuclear DNA of the six millets was determined by HPLC [Fig.4.4 A & B and Table 4.1]. From Table 4.1, it is seen that the 5 methyl cytosine content ranges from 3% in barn yard millet to 9.6% in great millet. Great millet embryo DNA shows a slightly greater amount of 5 methyl cytosine than that of shoot DNA. The fraction of the cytosine residues methylated was also determined and the values vary between 14% in little millet to 31% in pearl millet. These values are higher than the corresponding values of animal cells and thus show a trend complying with that observed in plants [20]. As the presence of 5 methyl cytosine in the DNA is known to increase its Tm [10, 14], the relatively high Tm of 89.9°C of pearl millet DNA could be explained by the fact that almost 31% of its cytosine residues are methylated.

4.3.3.2. Methylation of the 5'CCGG3' sequences

The methylation status of the 5'CCGG3' sequences in the six millet DNAs was assessed by using the isoschizomer pair of restriction enzymes namely Msp I and Hpa II. Both the enzymes recognise the sequence 5'CCGG3', but show a differential sensitivity to cytosine methylation. Msp I cleaves the sequence when the internal cytosine is methylated, while Hpa II cleaves when the external cytosine is methylated. Fig. 4.5 depicts the digestion patterns of the millet DNAs with Msp I and Hpa II. From this figure, it is seen that great millet DNA is digested to a slightly greater extent with Msp I than
FIG. 4.4. A: HPLC PROFILES OF THE TOTAL NUCLEAR DNAs of millets.

a - standard mixture of bases;
b - calf thymus DNA;
c - barn yard millet;
d - fox tail millet
e - little millet

C, G, T and A indicate peaks of cytosine, guanine, thymine and adenine respectively. The arrow indicates 5-methyl cytosine.
FIG. 4.4 (B); HPLC PROFILES OF THE TOTAL NUCLEAR DNAs OF MILLETS.

f - pearl millet;
g - finger millet;
h - great millet;
i - great millet embryo.

C, G, T and A indicate peaks of cytosine, guanine, thymine and adenine respectively. The arrow indicates 5-methyl cytosine.
<table>
<thead>
<tr>
<th></th>
<th>% of 5-methyl cytosine</th>
<th>% of the total cytosines methylated</th>
</tr>
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<tbody>
<tr>
<td>Great millet</td>
<td>9.62 ± 1.05</td>
<td>19.76 ± 0.75</td>
</tr>
<tr>
<td>Barn yard millet</td>
<td>3.02 ± 0.55</td>
<td>15.66 ± 1.07</td>
</tr>
<tr>
<td>Little millet</td>
<td>4.19 ± 0.76</td>
<td>14.52 ± 0.63</td>
</tr>
<tr>
<td>Fox tail millet</td>
<td>7.51 ± 0.82</td>
<td>25.48 ± 0.79</td>
</tr>
<tr>
<td>Finger millet</td>
<td>3.59 ± 0.87</td>
<td>23.72 ± 1.1</td>
</tr>
<tr>
<td>Pearl millet</td>
<td>7.28 ± 0.61</td>
<td>30.92 ± 2.12</td>
</tr>
<tr>
<td>Great millet embryo</td>
<td>11.75 ± 0.99</td>
<td>16.92 ± 0.92</td>
</tr>
</tbody>
</table>

The percentage of 5-methyl cytosine as well as the percentage of total cytosines methylated were determined from the peak height and peak area. The values in this table are an average of three DNA samples each done in duplicate.
with Hpa II while the reverse is observed in case of all the other five millet DNAs. These data indicate a predominance of \(^{m}\text{CpG} \) methylation in great millet DNA and of \(^{m}\text{CpC} \) methylation in the \(5'\text{CCGG}3'\) sequences of the other five millet DNAs.

4.3.3.3. Methylation of the \(5'\text{GATC}3'\) sequences

The millet DNAs were next restricted with the restriction enzymes Mbo I, Sau 3AI and Dpn I which recognise the sequence \(5'\text{GATC}3'\) but show a differential sensitivity to cytosine and adenine methylation. Dpn I cleaves the sequence only if adenine is methylated while Mbo I will not cleave the sequence if adenine is methylated and is insensitive to cytosine methylation. Sau 3A I will not cleave the sequence if the cytosine is methylated and is insensitive to adenine methylation. Fig. 4.4 depicts the digestion of all the six millet DNAs with Mbo I, Sau 3A I and Dpn I. Based on the extent of digestions with these enzymes, the following conclusions can be arrived at.

The DNAs of great millet, barn yard millet and fox tail millet show a small extent of digestion with Dpn I (as compared to the respective control DNAs) indicating that a few of the \(5'\text{GATC}3'\) sequences are methylated at the adenine residues. Among the six millets, finger millet shows relatively more digestion with Dpn I indicating the presence of more number of methylated adenine residues as compared to great millet, barn yard millet and fox tail millet. In case of pearl millet and little millet, no significant difference is observed between the Dpn I digest and the control DNA suggesting that adenine methylation of the \(5'\text{GATC}3'\) sequences is probably absent.
b) While comparing the digestion of the millet DNAs with Mbo I and Sau 3AI, it is seen that in each case, except pearl millet and little millet, Mbo I and Sau 3AI digest the DNAs extensively and to a similar extent indicating that many of the 5'GATC 3' sequences are not methylated at cytosine and adenine residues. In case of little millet and pearl millet, Mbo I digests the DNA to a greater extent that Sau 3AI, suggesting that most of the 5'GATC 3' sequences are methylated only at the cytosine residues. This difference is especially very significant in little millet. In each plant DNA, several bands of similar molecular weight are shared by the Mbo I and Sau 3AI digests. These bands represent repeat families. It is observed that some of these repeat families differ in intensity in the Mbo I and Sau 3AI digests. This difference is because some members of these repeat families are methylated at adenine or cytosine residues.

c) A band of approximately 23 kbp is seen in the Mbo I and Sau 3AI digests of barn yard millet, fox tail millet and pearl millet. This could be either due to lack of 5'GATC 3' sequences or due to methylation at both C and A residues. However, in case of great millet and finger millet, such a band is seen only in the Mbo I digest and is absent or insignificant in the Sau 3AI digest indicating thereby that this 23 kbp DNA fragment in these two millets contains 5'GATC 3' sequences methylated at adenine residues.

4.3.3.4. Methylation of the 5'GCGC 3' and 5'GGCC 3' sequences

In addition to the above enzymes, the enzymes Hha I and HaeIII which show sensitivity to cytosine methylation were used. Hha I recognises the sequence 5'GCGC 3' and will not cleave if either of the cytosine
FIG. 4.5: RESTRICTION ENDONUCLEASE CLEAVAGE ANALYSIS ON 1% AGAROSE GELS OF THE SIX MILLET DNAs digested with Msp I (lane b), Hpa II (lane c), Hae III (lane d), Mbo I (lane f), Sau 3AI (lane g) and Dpn I (lane h). Control DNA is in lane a; Hind III digest of \( \lambda \) DNA (lane i) and Hind III digest of \( \varphi \) X 174 RF DNA (lane e).
GREAT MILLET
GREAT MILLET
BARN YARD MILLET
BARN YARD MILLET
LITTLE MILLET
LITTLE MILLET
FOX TAIL MILLET
FOX TAIL MILLET
FINGER MILLET
FINGER MILLET
PEARL MILLET
PEARL MILLET
FIG. 45
is methylated. Relatively less digestion is observed with the DNAs of great millet, fox tail millet and pearl millet while more digestion is seen in case of the DNAs of barn yard millet and little millet. Among these millets, the DNA of finger millet is digested extensively. A greater extent of digestion with Hha I indicates a less predominance of CpG methylation while a low extent of digestion is indicative either of a low frequency of occurrence of 5' GCGC 3' sequences or some CpG methylation (Fig. 4.6A). Hae III recognises the sequence 5' GGCC 3' but will not cut it if the internal cytosine is methylated. Since all the six millet DNAs show extensive digestion with Hae III, it indicates that mCpC methylation is not common in the 5' GGCC 3' sequences.

4.3.3.5. Methylation of the 5' CCGG 3' and 5' GATC 3' sequences in great millet embryo DNA

A few reports are available wherein specific plant genes have been analysed with respect to their methylation status during development [4,26,33,45,48]. In the present work, we have only analysed the methylation status of the total millet genomes. Preliminary HPLC analyses of the 5-methyl cytosine content showed that great millet embryo DNA contained slightly more 5 methyl cytosine than the shoot DNA. We were therefore, interested to know if such a difference could be reflected by comparing the digestion patterns of the embryo and shoot DNAs with the methylation sensitive restriction enzymes namely Msp I/Hpa II and Mbo I/ Sau 3 Al. From Fig. 4.6B, it is seen that the extent of digestion of great millet embryo DNA with Msp I and Hpa II is the same. This behaviour is slightly different from that of shoot DNA where the digestion
with Msp I is slightly more than that with Hpa II. In the Mbo I digest of these two DNAs, however a 23 kbp band is present in the shoot DNA while no such band is seen in the embryo DNA. Moreover, the presence of this 23 kbp band in the Mbo I digest of shoot DNA and its absence in the Sau 3AI digest indicates the presence of clusters of 5'GATC 3' sequences methylated at adenine residues in the 23 kbp fragment. The above observations indicate methylation of adenine residue in some 5'GATC 3' sequences during the transition from the embryonic to the seedling stage.
FIG. 4.6 A: Restriction endonuclease cleavage analysis on 1% agarose gels of the DNAs of great millet (lane b), barn yard millet (lane c), little millet (lane d), fox tail millet (lane f), finger millet (lane g) and pearl millet (lane h) with Hha I. Lanes a and l are Hind III digest of λ DNA; lane e is Hind III digest of λ DNA + Hae III digest of φ X 174 RF DNA.

FIG. 4.6 B: Restriction endonuclease cleavage analysis on 1% agarose gel of great millet embryo DNA digested with Msp I (lane b), Hpa II (lane c), Mbo I (lane d) and Sau 3AI (lane e). Lane a is control DNA and lane f is Hind III digest of λ DNA + Hae III digest of φ X 174 RF DNA.
FIG. 4-6 A

Hha I

abcdefghi

Great millet embryo DNA methylation

FIG. 4-6 B
4.4. DISCUSSION

It has been suggested that Alu I repeats of both animals and plants are derived from a common monomer unit of about 60 bp which has been implicated in the determination of chromatin structure. The monomer unit in Arabidopsis thaliana, Cucumis melo, Cucumus sativus, Cucurbita pepo, Raphanus sativus and Sinapis alba are 180, 380, 180, 351, 177 and 172 bp respectively and each unit is a multiple of nearly 60 bp unit [2,5,6,19,27,34,40,49,50]. The great millet Alu I monomer is 275 bp long which appears to be a multiple of 55 bp which is close to a 60 bp unit. Sequence analyses of the Alu I repeats from animals and plants have shown them to be derived from a tRNA gene. Attempts are being made to clone and sequence, the 550 bp Alu I repeat DNA in great millet and try to correlate the sequence with the available sequence data of distantly related plants and animals with a view to find out if the concept of the tRNA gene ancestor could be extended to the millets.

A partial homology (upto 50%) has been observed among the Alu I repeats of a few plants as well as distantly related animals [2,9,15,51]. The occurrence of a weak homology among the Alu I repeats of six millets indicates that only a few regions of these repeats might be conserved probably because they have some biological functions such as organisation of interphase nuclei [19,29,52]. The weak homology observed among the Alu I repeats in millets could probably be explained by considering that an ancient Alu I repeat was present in a progenitor genome. Ampli-
fication of these diverged sequences with unequal crossing over at or after the speciation probably resulted in the present day Alu I repeats in the six millet genomes.

The Msp I and Hpa II digestion patterns suggest a predominance of \(^{m}\)CpG methylation in great millet and of \(^{m}\)CpC methylation in the other five millets in the \(^{5'}\)CCGG\(^{3'}\) sequences. The available data on the methylation status of plant DNAs have shown that over 25% of the cytosine residues are methylated in plant cells while only 2-8% of the total cytosines are methylated in animal cells [20]. This difference is due to the fact that though in both plants and animals, the primary site of cytosine methylation is the C-G dinucleotide which is more frequent in plant DNA (3-4%) than in animal DNA (0.5 to 1%). Moreover in plants, 5 methyl cytosine is found at a variety of cytosine containing dinucleotides besides CpG which are a part of the basic tri-nucleotide C-X-G (20), which is not the case in animal DNAs. Thus all the methylatable sites of plant DNA are not full methylated. Approximately 80% of C-G or C-A-G sites are methylated and only 50% of the C-C-G sites are modified at the external cytosine [20]. It has been suggested that it is this incomplete methylation which makes these sites potential factors in cellular regulation.

The restriction enzyme data using Mbo I, Sau 3A I, and Dpn I indicates that some of the \(^{5'}\)GATC\(^{3'}\) sequences are methylated at adenine and/or cytosine residues in these millets except in little millet and pearl millet where adenine methylation is insignificant. The restriction enzyme digestion of great millet embryo DNA further indicates methylation of the adenine residues in a few \(^{5'}\)GATC\(^{3'}\) sequences, during the
change from embryo to seedling stage. The detection of 6-methyladenine in plants may support the idea on the possible new role of cytokinins (N^6 -substituted adenine derivatives) in cell differentiation as modulators of adenine residue methylation in plant DNA [46]. The presence of 6-methyladenine in the DNA of higher plants relates them in a definite degree to algae which are considered to be predecessors of higher plants. As compared with algae, however, the capacity for methylation of adenine in the DNA has decreased considerably in higher plants and the capacity for methylation of cytosine has increased in the course of evolution [47].

A role of 6-methyl adenine in DNA repair in plant cells has been envisaged by Pintor-Toro [26]. This odd base has been associated with the regulation of gene activity as well as of DNA regulation and repair [35,39]. At present, we can only speculate that in plants also, this DNA modification may be engaged in a similar regulatory process. However, previous work on zein genes has shown that adenine methylation is unrelated to the regulation of gene expression [26]. Further characterisation of the 23 kbp fragment in the Mbo I digest of great millet shoot DNA would be required before attributing any specific role of adenine methylation during differentiation.
4.5. REFERENCES

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