CHAPTER V

RESTRICTION ENDONUCLEASE CLEAVAGE PATTERNS OF
THE DNAs OF FOUR VIGNA SPECIES AND CAJANUS CAJAN
INTRODUCTION

Restriction endonucleases have revolutionised the approaches to genome analyses, and have served as extremely powerful tools in (i) identification of repeated sequence families; (ii) detection of sequence specific methylation patterns; (iii) localization of specific gene fragments (1); and (iv) analysing the overall distribution of base composition in total DNAs as well as in the vicinities of specific DNA sequences.

Analyses of restriction endonuclease cleavage patterns of eukaryotic DNAs have resulted in the emergence of concepts like 'molecular drive' or 'concerted evolution' (2,3) and restriction fragment length polymorphism (RFLP) of defined DNA segments (4-8). In fact, RFLP has been proposed as a parameter for deciding genome donors in plant breeding (8). The above concepts and studies have also been used to establish phylogenetic relationships amongst groups of related organisms.

In gross terms, two major classes of reiterated DNA sequences namely tandem repeats and dispersed repeats can be identified by the restriction enzyme digestions. Tandem repeats are seen as a ladder of bands in partial digests of the DNAs, while dispersed repeats may or may not be multiples of a basic repeat unit, but invariably occur in clusters of different reiteration frequencies. Using restriction endonuclease analyses of DNAs, extensive work has been reported with respect to repeat families as well as the extent and nature
of methylation in animal genomes (9-22). In plants, distinctly dispersed repeats have been shown in lily (23) and *Vicia faba* (24), while clusters of different tandem repeat families have been shown in garlic (25), rye (26) and a few other plant species (27-30).

This chapter describes the results of our studies on the restriction endonuclease cleavage analyses of DNAs of four *Vigna* species and *Cajanus cajan*.

**MATERIALS AND METHODS**

**Chemicals and materials**

Formamide was obtained from SD*, Bombay (India, AR grade) and was distilled and deionised prior to use. Ficoll was from Pharmacia Fine Chemicals, Uppsala (Sweden) while BSA (Bovine serum albumin), PVP (polyvinyl pyrrolidone) and PIPES (1,4-piperazine diethane sulfonic acid) were from Sigma Chemical Company, Missouri (USA).

Nitrocellulose membrane filters (0.45 μm type SCN) were procured from Advanced Microdevices Pvt. Ltd., Ambala (India) while the X-ray films (Agfa Curix RPl) were from Agfa Gevaert India Limited, Bombay (India). The X-ray cassettes were from Umasons, Aurangabad (India) while the intensifying screens were purchased from Kiran Screens, Bombay (India).
Enzymes

Restriction endonucleases BamHI, EcoRI, HaeIII, HindIII, Mbol, MspI, and PvuI and the enzymes like E. coli DNA polymerase I and T4 DNA ligase were procured from New England Biolabs, Massachusetts (USA) while restriction endonucleases AluI, HhaI, HpaII, Sau3AI and SmaI and the enzyme SI Nuclease were purchased from Bethesda Research Laboratories, Maryland (USA).

Elution of high molecular weight DNA

The isolated nuclear DNAs were mostly heterogeneous in sizes (chapter III, Fig.III.1). When the size of the isolated plant DNA was examined by agarose gel electrophoresis, the DNA showed a band at a size of ~20 kbp along with a trail in the size range 0.5 to 20 kbp. This makes the isolation of high molecular weight DNA for restriction analyses mandatory. The large number of protocols are available, wherein, the high molecular weight DNA may be recovered by ultracentrifugation, electroelution from agarose gels, thermal elution from low melting agarose gels, extraction from solubilized agarose and freeze-squeeze elution from agarose. Most methods of eluting DNA from agarose gels, for various reasons, gave low recoveries of DNA. I, therefore, tried the freeze thaw method of Dharmalingam (40) and found it to be convenient and rapid. The actual method, however, required to be modified and to be scaled up to preparative proportions. Briefly, 11 cm x 10 cm x 1 cm slabs of 0.7% agarose in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 7.8 - 8.1) were prepared.
The edges of the glass plates were lined with PVC tape to form a trough. While allowing the gel to set, four preparative (1.2 x 0.15 x 0.7 cm) slots were formed using an acrylic slot former. In the preparative slots, 100 - 120 µl of DNA was loaded while 20 - 40 µl of DNA was used in the analytical slot. After electrophoresis, the analytical track was cut off from the slab, stained in ethidium bromide at 1 µg/ml concentration for 30 min and visualized on a transilluminator. The rest of the preparative slab was aligned with the analytical slab and the tracks were cut across the region corresponding to the high molecular weight DNA in the analytical track. These agarose pieces were transferred to a Sorvall SE 12 tube, submerged in phenol equilibrated with Tris buffer and frozen at -70°C for 2 hours. Subsequent to this, the frozen gel piece and phenol were thawed out gradually to 4 - 10°C and centrifuged at 10000 rpm for 20 min at 4°C using Sorvall SE 12 rotor. The aqueous layer was collected, freed of traces of phenol with chloroform - isoamylalcohol (24:1, v/v) mixture and the DNA precipitated with 2 volumes of chilled ethanol after adjusting the sodium ion concentration of aqueous layer equivalent to that of 0.25 M sodium acetate, pH 5.5. The DNA was allowed to precipitate either overnight at -20°C or for 20 min at -70°C. The DNA precipitate was collected by centri­fugation, washed once with 70% ethanol before drying and dissolved in a suitable volume of T10 buffer (10 mM Tris HCl, pH 7.5).
Restriction endonuclease digestions and agarose gel electrophoresis

Table 1 lists the enzymes used in the present studies, their assay buffer conditions, temperature of incubation, recognition sequence and effect of methylation of this sequence on enzyme activity. For all digestions, 3 - 5 μg of DNA was incubated with 6 - 10 units of the enzyme at 37°C for 15 - 18 hrs in appropriate buffers as listed in Table 1. The enzyme-DNA ratio and enzyme volume to reaction volume ratio were carefully adjusted to avoid non-specific (star) activity of the enzymes (41, 68-73). Following incubation, the enzyme digestion was stopped by addition of 1/10th volume of a 10x reaction stop solution (50% glycerol, 100 mM EDTA and 0.25% bromophenol blue) and the digests were analyzed by agarose gel electrophoresis on 1.0 or 1.4% neutral agarose gels in TAE 1X (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 7.8) at a constant current of 20 mA for 5 - 8 hours. The DNA bands were stained with ethidium bromide and were visualized on a long wavelength (302 nm) UV transilluminator. The gels were photographed using a Minolta X 700 35 mm camera with red filter.

Southern blotting

Transfer of DNA from agarose gels to nitrocellulose membranes was carried out essentially according to Southern (74)
<table>
<thead>
<tr>
<th>ENZYME (SOURCE)</th>
<th>RECOGNITION SEQUENCE</th>
<th>ASSAY BUFFER (b)</th>
<th>TEMPERATURE (°C)</th>
<th>METHYLATED SEQUENCE</th>
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<tr>
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<td>MS</td>
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<td>(BRL)</td>
<td>CCCCGG</td>
<td>SB</td>
<td>37</td>
<td>na</td>
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a: BRL- Bethesda Research Laboratory, Maryland, USA NEB-New England Biolabs, Massachusetts, USA

b: MS- Medium salts buffer - 10 mM Tris HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT (41)
HS- High salts buffer - 50 mM Tris HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT (41)
LS- Low salts buffer - 10 mM Tris HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT (41)
SB- Sma I buffer - 10 mM Tris HCl, pH 8.0, 20 mM KCl, 10 mM MgCl₂, 1 mM DTT (41)
c: m in the sequence refers to methylation of the base following m.

d: The references pertain to effect of methylation on cleavage only.

na: Information not available.
and Maniatis et al. (41) except that neutralization of the gel was done with 3 M NaCl buffered in 0.25 M sodium acetate pH 5.5 instead of NaCl-Tris buffer. After blotting, the nitrocellulose membranes were dried at 62°C overnight and stored at -20°C in moisture free plastic wrappings.

**Isolation and nick translation of Cot 1.0 x 10^{-1} M.s. DNA**

Unsheared pigeonpea DNA in 0.18 M NaCl buffered with 0.006 M PIPES buffer, pH 6.8, was denatured in a boiling water bath for 10 min and then incubated at 62°C to Cot 1.0 x 10^{-1} M.s. The reassociation was stopped by chilling the reaction mixture and adjusting the buffer to 1 x S1 nuclease buffer.

The unreassociated DNA was digested with S1 nuclease (10 units/µg) at 37°C for 30 min. The digestion mixture was then deproteinised with chloroform-isoamylalcohol mixture (24:1, v/v) and the S1 nuclease resistant Cot 1.0 x 10^{-1} M.s. DNA was precipitated from the aqueous layer with 2 volumes of chilled ethanol. The DNA precipitate was dried, dissolved in a suitable volume of T 10 buffer (10 mM Tris HCl, pH 7.5) and was labelled by nick translation procedure of Rigby et al. (75) as described in chapter IV except that the labelled DNA was purified by Sephadex G-50 column chromatography.
Southern Hybridization

The nitrocellulose filters were incubated for 5 hours in prehybridization buffer SSC 3X (SSC 1x = 0.15 M NaCl + 0.15 M trisodium citrate, pH 7.0) containing 1x Denhardt's solution (0.02% polyvinyl pyro idone, 0.02% BSA, 0.02% Ficoll), 50% formamide, 0.02% SDS and sheared heat denatured calf thymus DNA, 250 μg/ml. The prehybridization was carried out in heat sealed polyethylene bags at 62°C, following which the radioactive probe DNA (Cot 1.0 x 10^-1 M.s. DNA) was denatured and added to the prehybridization mixture itself and hybridization was continued further for 48 hours at 50°C. The probe DNA (specific activity nearly 10^7 cpm/μg) was used at a concentration of 2 x 10^7 cpm for nearly 10 μg of blotted DNA. After 48 hours of incubation, unhybridized probe DNA was washed off sequentially as follows:

i) 2 x SSC + 0.1% SDS, 4 times, 10 min each at room temperature;

ii) 0.1 x SSC + 0.1% SDS, 3 times, 10 min each at room temperature and

iii) 0.1 x SSC + 0.1% SDS, once, 10 min at 40°C.

The above cycle of washings was repeated once again, following which two final washings were given at room temperature of 10 min duration each in 0.1 x SSC alone. The filters were dried and autoradiographed using two intensifying screens.
RESULTS

Standardisation of DNA elution from Agarose gels

The isolated plant DNA show considerable size heterogeneity (Figure V.1a, lanes 1 and 3), necessitating elution of the high molecular weight regions from these DNAs. The elution was performed as described in Materials and Methods. The mobilities of eluted high molecular weight DNAs in agarose gels are shown in Figure V.1a, lanes 2 and 4. The suitability of the eluted DNA for restriction enzyme action and ligation was ascertained by using plasmid pBR 322 DNA, HindIII digest of λ DNA and HaeIII digest of φX174 RF DNA. In the case of plasmid pBR 322, the supercoiled DNA alone was eluted and digested with BamHI. More than 90% of the supercoiled DNA could be linearized in this way clearly indicating that the eluted DNA could be digested with restriction enzymes (data not shown). In the second experiment, the first six bands of HindIII digest and the first 5 bands of φX174 RF-HaeIII digest were separately eluted and then randomly ligated to each other. These data are shown in figures V.1b and c. In both the cases, the random ligation mixtures showed an increase in the sizes of the bands (Figure V.1b lane 8 and figure V.1c lane 6) thereby confirming that the eluted DNAs are amenable to ligation reactions also.
Figure V.1: STANDARDIZATION OF ELUTION

a) Lane 1 Pigeonpea DNA  Lane 2 Eluted high molecular weight pigeonpea DNA
Lane 3 Cowpea DNA  Lane 4 Eluted high molecular weight cowpea DNA

Electrophoresis was carried out in 1.0% agarose gel.

b) Lane 1 A-HindIII digest (the molecular weights of the bands in decreasing order are 23.1, 9.4, 6.4, 4.2, 2.2 and 2.0 kb)
Lane 2 Eluted 23.1 kb band  Lane 3 Eluted 9.4 kb band
Lane 4 Eluted 5.4 kb band  Lane 5 Eluted 4.2 kb band
Lane 6 Eluted 2.2 kb band  Lane 7 Eluted 2.0 kb band
Lane 8 Random ligation mixture of above eluted DNAs

(Gel 1.0% agarose)

c) Lane 1 Ø X 174 RF-HaeIII digest (the molecular weights of the bands in decreasing order are 1.5, 1.08, 0.87, 0.61 and 0.31 kb).
Lane 2 Eluted 1.5 kb band  Lane 3 Eluted 1.08 kb band
Lane 4 Eluted 0.87 kb band  Lane 5 Eluted 0.61 kb band
Lane 6 Random ligation mixture of eluted bands  Lane 7 Eluted 0.31 kb band

(Gel 1.4% agarose)
Restriction endonuclease analyses of plant DNAs

The digestion patterns of DNAs of four Vigna species and of Cajanus cajan with BamHI, HindIII, Alul, MboI, Sau3AI and PvuII are depicted in figures V.2a-e; while those with Smal, MspI, HpaII, HaeIII and Hhal (enzymes recognizing a GC rich sequence) are shown in figure V.3a-e. From these two figures, it is clear that a ladder like set of bands are seen only in Cajanus cajan (pigeonpea) DNA digested with MboI and Sau3AI, suggesting an organised repeat structure having sites for these two enzymes. When the sizes of the bands were compared with those of the marker DNAs, it was found that the MboI bands differed from successive members by 0.27 kbp with the smallest visible band being nearly 0.27 kb. In contrast, Sau3AI bands had different molecular weights and were less distinct indicating a relatively lower frequency of Sau3AI sites. The four Vigna plant DNAs did not show any organized repeat structure with any of the enzymes used. They, however, were extensively digested with Sau3AI and Alul which suggests the occurrence of either very small spacing between successive sites for these enzymes or a random distribution of these sites. Surprisingly, though, in direct contrast to pigeonpea, MboI digested all the Vigna DNAs to a lesser extent than Sau3AI suggesting a differential methylation of the same recognition sequence 5'-GATC-3'.
Figure V.2  Restriction endonuclease analysis of cowpea (a), mothbean (b), mungbean (c), urdbean (d) and pigeonpea (e) DNAs with \textit{BamH}I, \textit{MboI}, \textit{Sau3AI}, \textit{AluI}, \textit{PvuI} and \textit{HindIII}.

The control DNAs are for high salt enzymes (HS) and medium salt enzymes (MS). The markers are $\theta$ x 174 RF DNA digested with \textit{HaeIII} (lane 4 in all photographs) and DNA digested with \textit{HindIII} (lane 7) in all the photographs.

Lane 1   HS control
Lane 2   \textit{BamHI} digest
Lane 3   \textit{MboI} digest
Lane 5   \textit{Sau3AI} digest
Lane 6   \textit{AluI} digest
Lane 8   \textit{HindIII} digest
Lane 9   \textit{PvuI} digest
Lane 10  MS control

Electrophoresis in all case was carried out in 1.4\% agarose gels.
Figure V.3  Restriction endonuclease cleavage analysis of cowpea (a), mothbean (b), mungbean (c), urdbean (d) and pigeonpea (e) DNAs with Smal, MspI, HpaII, HaeIII and HhaI.

Lane 1  in all photographs is Smal control
Lane 2  in all photographs is Smal digest
Lane 3  in all photographs is LS control
Lane 4  in all photographs is MspI digest
Lane 5  in all photographs is λ-HindIII digest
Lane 6  in all photographs is HpaII digest
Lane 7  in all photographs is HaeIII digest
Lane 8  in all photographs is φ x 174 RF-HaeIII digest except in (a) and (d) where it is λ-HindIII + BamHI digest
Lane 9  in all photographs is HhaI digest
Lane 10 in all photographs is MS control

Electrophoresis in all cases was carried out in 1.0% agarose gels.
In the previous chapter (Chapter III), I have described the reassociation kinetics of pigeonpea DNA. Since the pigeonpea DNA did not show a kinetically distinct highly repetitive component, Cot. \(1.0 \times 10^{-1}\) M.s. DNA fraction was isolated and was used as a probe for S-blot of genomic digests of pigeonpea, mainly to find out if the discrete banding pattern of pigeonpea DNA obtained with MboI and Sau3AI is due to the presence of repetitive sequences or not and further to identify if the digests of pigeonpea with other enzymes also exhibit homology to Cot 1.0 x \(10^{-1}\) M.s. DNA probes. For this purpose, pigeonpea DNA was first digested with EcoRI, HaeIII, MboI, Sau3AI, HhaI and BamHI and electrophoresed on 1.0% neutral agarose gel (Figure V.4a). Figure V.4b shows the pattern of hybridization of labelled Cot 1.0 x \(10^{-1}\) M.s. pigeonpea DNA to pigeonpea DNA digested with the above restriction enzymes. The pattern of hybridization clearly indicates extensive hybridization with MboI, Sau3AI and BamHI digests. In case of MboI digestion two major size classes of 0.6 to 1.4 kbp and less than 0.3 kbp are seen while in case of Sau3AI, the hybridization regions are 0.9 to 3.1 kbp and upto 0.7 kbp. These hybridization patterns for Sau3AI and MboI digests confirm our earlier observations that the former digests pigeonpea DNA less extensively as compared to the latter. Furthermore, in case of both these enzymes, less intense hybridization ladder is seen for higher sizes with the MboI bands being separated by 2.2 kbp and Sau3AI bands being separated by 4.5 kbp sequences.
Figure V.4 a) Digestion of total pigeonpea DNA with EcoRI (lane 2), HaeIII (lane 3), MboI (lane 5), Sau3AI (lane 7), HhaI (lane 8), BamHI (lane 9) are depicted along with pigeonpea Cot 1.0 x 10^{-1} M.S. DNA (lane 1), total DNA of pigeonpea (lane 10) and markers of pBR 322 DNA-AluI digest (lane 4) and λDNA-HindIII digest (lane 6). Electrophoresis was carried out in 1.0% agarose gel.

b) The above DNAs and DNA digest were transferred to nitrocellulose membrane. The figure shows pattern of hybridization of labelled Cot 1.0 x 10^{-1} M.S. probe DNA of pigeonpea mixed with labelled pBR322 and λDNAs, to the above blotted DNAs. The order of samples is as above. The photograph is a positive print of the X-ray autoradiogram.
FIG. V.4.
Since the extent of hybridization of these two groups of bands to Cot 1.0 x 10^{-1} M.s. probe is different, it can be inferred that the organisation of Cot 1.0 x 10^{-1} M.s. sequences in the above two groups is different with a greater proportion of Cot.1.0x10^{-1} M.s. sequences being confined mainly to short and intermediate size classes. This is also inferred from the pattern of hybridization of Cot 1.0 x 10^{-1} M.s. DNA to itself, wherein, eventhough three size classes of greater than 3kbp, 0.6 to 1.8 kbp and 0.18 to 0.4 kbp are distinguishable, the higher size class (greater than 3.0 kbp) hybridizes to a much lesser extent to Cot 1.0 x 10^{-1} M.s. probe than the other two. The DNA digests with EcoRI and HaeIII on the other hand are relatively less homologous to Cot 1.0 x 10^{-1} M.s. probe while bulk of HhaI digest and undigested pigeonpea DNA hybridize strongly to the above probe.

From the above studies, it appears at the preliminary level at least, that a family(ies) of repeated sequences recognised at either ends by MboI and/or Sau3AI exists in pigeonpea and is apparently organized in clusters of different complexity and reiteration frequencies. Since sequences reassociating by Cot 1.0 x 10^{-1} M.s. are selected as probe, the family of sequences recognised by this probe is of highly repetitive type. An actual estimate of the copy number of these repeats awaits further studies.
Methylation status of the five plant DNAs

From figures V.2 and V.3, based on the extent of digestion with the restriction enzymes, some information can be collated regarding the presence of methylated sequences. Of the enzymes that we have used, the most important is an isoschizomeric pair, MspI and HpaII, both recognizing the sequence 5'-CCGG-3' but showing differential sensitivity to whether outer C or inner C is methylated or not. From Table 1 and Figure V.3, it is clear that in case of all the five plant DNAs, MspI digests the DNAs to a much lesser extent than HpaII while with SmaI the digestion is less than that by MspI and HpaII. These data clearly suggest that most of the CCGG sequences are of the type mCCGG rather than CmCGG.

The second set of enzymes is HaeIII and HhaI recognizing 5'-GGCC-3' and 5'-GCGC-3' sequences respectively. In cowpea, mothbean and mungbean, HhaI digests the DNAs to a greater extent than HaeIII while in case of urdbean converse is the case. The former data are interpreted to indicate that HhaI site containing CpG dinucleotide is not methylated since methylation of either C in GCGC is reported to block the cleavage by HhaI (Table 1). In the case of urdbean, however, it appears that most of the HhaI sites contain methylated C. In the case of pigeonpea, apparently both HaeIII and HhaI give only a limited digestion suggesting a greater extent of methylation of these sites.
The third set of enzymes useful in recognizing site-specific methylation is \textit{MboI} and \textit{Sau3AI}. While \textit{MboI} digestion is adversely affected if A is methylated in 5'-GATC-3' \textit{Sau3AI} does not digest only if C is methylated. The four \textit{Vigna} DNAs are rather unique in that they show limited \textit{MboI} and extensive \textit{Sau3AI} digestion indicating that many GATC sites contain methylated A. In contrast, pigeonpea DNA shows extensive digestion with \textit{MboI} as compared to that by \textit{Sau3AI} suggesting that some of the cytosine is methylated in the sequence 5'-GATC-3'.

\textbf{DISCUSSION}

The southern hybridization studies of \textit{MboI} digested pigeonpea DNA to Cot 1.0x10^{-1} M.s. DNA have revealed the occurrence of a large proportion of small size repeats and a small proportion of long repeats. In this context it must be pointed out that in case of lily (23) and garlic (25) repeat elements of 7 kb and 1 kb respectively have been identified while in rye (26) smaller repeat sizes are observed. The pigeonpea repeats on the other hand, are both long fragment clusters and short fragment clusters.

The restriction endonuclease cleavage analyses reveal information about site-specific methylation in DNA. In these studies, analyses of methylation of cytosine of CpG dinucleotides is of considerable importance. In case of animals, in general,
CpG is the sole site of methylation of cytosine. The other methylated dinucleotides together account for less than 10% of the methylated site (15), with a few exceptions (76-78). In contrast, plants show a more diverse distribution of methylated dinucleotides other than CpG and trinucleotides CpXPpG where X is any base (29). Furthermore, methylation of sequences in plants has been attributed to be a factor which prevents digestion of plant DNAs with restriction endonucleases (72,73). Our studies in the present five species for base composition analyses have revealed a substantial amount of 5-methylcytosine. The presence of the latter has also been quantitated by HPLC as described in Chapter II. However, in case of Vigna DNAs, digestion with MboI and Sau3AI have suggested occurrence of methylation of some adenine as well. It is, therefore, of interest to note that in our HPLC analyses in case of Vigna DNAs, at least two unidentified peaks probably corresponding to odd bases are present while in case of pigeonpea no odd base was apparently detectable. These unidentified peaks may correspond to methylated adenine also. In thus appears that in the Vigna DNAs at least, methylated residues other than 5-methylcytosine also occur.

When the five plant DNAs were digested with PvuI, SmaI, HpaII and HhaI (enzymes which are sensitive to CpG methylation at C) or with MspI (an enzyme which digests when CpG of its
recognition sequence is methylated), it becomes obvious that
C_pG methylation is relatively rare in these plants in general.
In fact our data on digestion pattern for MspI and HpaII clearly
indicate that more of methylation may be present in C_pC
dinucleotides. Furthermore, digestion of the Vigna DNAs with
MboI and Sau3AI have also suggested the presence of adenine
methylation. These data of ours are in good agreement with
those for other leguminous DNA sequences by McClelland (30)
C_pG dinucleotides were observed at only 49% of expected
frequencies while C_pG and C_pA + T_pG were observed at 114%
and 126% of the expected frequencies. Our data are, however,
in disagreement to some extent with the trend suggested by
Gruenbaum et al. (29). The latter have suggested that greater
than 80% methylation occurs in dinucleotide C_pG and trinucleotides
C_pA_pG or C_pT_pG in wheat and tobacco.

Based on our HPLC data and restriction enzyme analyses
data, we infer that methylation is not only extensive in the
present five species, but is also not restricted to cytosine
nucleotides and C_pG dinucleotides alone.
REFERENCES


