CHAPTER II

ASSESSMENT OF CHANGES IN NATIVE SUGARCANE DNA IN DIFFERENTIATED AND DEDIFFERENTIATED TISSUES
SUMMARY

Physicochemical characterization of native sugarcane DNAs was carried out to assess some gross molecular changes during in vitro shoot differentiation. These studies included determination of certain properties of DNA such as thermal denaturation, buoyant density in CsCl, content of 5-methyl cytosine and pattern of distribution of methylated sequences in total genomic DNA.

The melting temperature of the three sugarcane DNAs from CO-740 callus, CO-7219 callus and CO-740 shoot were in the range of 86.2° to 86.5°C. The first derivative melting profiles were polyphasic and indicated skewing on G + C region. The DNAs from the two callus tissues banded as a single narrow peak with a buoyant density of 1.702 g/cm³ while that from CO-740 shoot banded with a buoyant density of 1.703 g/cm³. CO-740 shoot DNA was more digested with Bam HI, EcoRI, AluI and Hind III as compared with the digestion of two callus DNAs. The three sugarcane DNAs did not show any organised repeat structure with these enzymes. HpaII digested all the three sugarcane DNAs to a lesser extent than the digestion with MspI indicating less methylation of 5'-CCGG-3' sequences. The 5-mC proportion was 4 moles percent for CO-740 callus and CO-740 shoot DNAs; and 9 moles percent in CO-7219 callus DNA. The three sugarcane DNAs thus indicated similarities in melting temperature and buoyant density; and a few changes with respect to digestion with Bam HI, EcoRI, AluI and Hind III and content of 5-mC.
INTRODUCTION

Molecular studies in tissues during development have clearly shown that the differences among specialised cells are due to a differential gene activity. For example, using various nucleic acid hybridization techniques, extensive changes in mRNA populations during differentiation have been reported in many different animal cell types (Ryffel and McCarthy 1975; Hastie and Bishop 1976; Galau et al. 1976; Affara et al. 1977; Blumberg and Lodish 1980; Sheperd and Nemer 1980; Jacquet et al. 1981).

In plants, variation in gene expression during cell development has been studied only recently (Kamalay and Goldberg 1980; Galau and Dure 1981; Goldberg et al. 1981; Vaillant 1983). For example, the work on DNA characterization to understand the molecular basis of cell differentiation has been carried out mainly in leaf and fruit in *Cucumis melo* and *C. sativus* Pearson et al. 1974), suspensor cells in *Tropaeolum* (Nagl 1976; Nagl et al. 1976), epicotyl cells in pea (Van Oostveldt and Van Parijs 1976) and also during phase change from juvenile to adult in ivy (Schaffner and Nagl 1979). These studies have indicated that most of the cells in higher plants do have quantitatively different DNAs in their nuclei (Nagl 1978) in different phases of growth.

In the previous chapter, we have mentioned about the identification of in vitro differentiating system in
sugarcane. In this system, CO-740 callus represents mainly undifferentiated mass of cells while CO-740 shoot is the differentiated tissue. CO-7219 callus is different from CO-740 callus since it loses its ability to differentiate after 7-10 subcultures. We were, therefore, interested in assessing the molecular basis of differentiation in this system. As a first step towards this direction, the DNAs from these three tissues were isolated and characterized with respect to melting temperature, buoyant density in neutral CsCl, methylation status and content of 5 methyl cytosine. This chapter contains the data obtained using the above approaches.
MATERIALS AND METHODS

Chemicals

All the chemicals used throughout the work were of Analytical Reagent (AR) or Guaranteed Reagent (GR) grade. They were obtained from British Drug House (India), E. Merck (India) or SD's (India).

Chloroform and ethanol (Swastik Laboratories Pvt. Ltd., Pune), methanol (Loba Chemie Industrial Co., Bombay) and phenol (SD's Lab. Chem Industry, Bombay) were freshly distilled prior to their use. Other chemicals like sodium dodecyl sulfate (SDS), Trizma base, standard purines and pyrimidines like adenine (A), guanine (G), thymine (T), cytosine (C) and 5-methyl cytosine (5-mC), agarose, calf thymus DNA, and bovine pancreatic ribonuclease A were obtained from Sigma Chemical Co., USA. All restriction endonucleases were obtained either from Bethesda Research Laboratories (BRL), USA or New England Biolabs (NEB), USA. DNA molecular weight marker (DNA digested with Hind III) was obtained from Bio-Rad Laboratories, USA or New England Biolabs., USA.

DNA isolation

The methods of isolation and maintenance of sugarcane tissues have already been described in Chapter I.

For DNA isolation, it was always advantageous to use tissues in the exponential growth phase. Callus tissue of 21 day old and multiple shoots of 15 day old were,
therefore, collected, frozen in liquid nitrogen and stored at -70.0°C till further use. Bendich and Bolton's method (1967) was used with modification for DNA extraction. The frozen tissue was powdered in a mortar using liquid nitrogen and the finely powdered tissue was homogenised with a buffer 1% SDS, 0.1 M disodium EDTA, and 3 x SSC (SSC - 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) (1 ml buffer/g of tissue) in a Remi Mixer at a high speed for 1 min. Thick paste of the tissue was transferred to a conical flask and mixed with equal volume of chloroform-isoamyl alcohol mixture (24:1, v/v). The mixture was quickly shaken for 1 min and centrifuged for 1 min at 5000 RPM. The aqueous layer containing DNA was incubated at 62°C for 30 min in a water bath and then cooled to room temperature. Sodium perchlorate was added to this aqueous layer to a final concentration of 1 M and the mixture was shaken with equal volume of chloroform - isoamyl alcohol mixture (24:1, v/v) on a rotary shaker for 30 min. The aqueous layer was collected by centrifugation and was repeatedly subjected to chloroform - isoamyl alcohol treatment till there was no protein interphase. DNA was precipitated from the aqueous layer by addition of two volumes of ethanol and chilling the solution at -20°C for 2 h. The DNA fibres were removed, washed with 70% ethanol, dried and dissolved in 1 x SSC. The DNA solution was incubated with RNase A
(50 μg/ml) at 30°C for 1 h to remove the contaminating RNA. Prior to use, RNase was heated to 80°C for 10 min to make it free from DNase activity. The solution was then deprotenized with chloroform–isoamyl alcohol mixture (24:1, v/v) and DNA was precipitated as mentioned earlier.

This DNA was either stored at -20°C in ethanol as a precipitate or was dissolved in a suitable buffer and stored at 4°C. For thermal denaturation studies and for CsCl centrifugation, the DNA was dissolved in 0.12 M sodium phosphate buffer (pH 6.8). For HPLC analysis, the DNA precipitate itself was used after drying free of all ethanolic traces.

E. coli B (NCIM No.2089) and Micrococcus lysodeikticus (NCIM No.2437) were grown in a nutrient broth (1% peptone, 1% beef extract and 0.5% sodium chloride, pH 6.5 - 7.0) till A600 nm reached 0.8. The cells were harvested and DNA was isolated essentially according to Marmur (1961) except that for M. lysodeikticus, the cells were treated with lysozyme prior to SDS lysis at 62°C.

Criteria of purity of DNAs

(i) UV absorption spectrum: Wave length scanning of sugarcane DNAs was carried out in the range of 220 to 300 nm on a Shimadzu double beam spectrophotometer, Model UV 210A. Only those DNA preparations with an optical density ratio A280/A260 = 0.55 and A230/A260 = 0.45 and an absorbance less
than 0.1 at 300 nm were further used. Figure 2.1 depicts the UV profile of purified sugarcane DNAs of all the three tissues.

(ii) Nativity of DNAs

In order to assess the extent of double strandedness (nativity) of the isolated DNA, hydroxyapatite column chromatography was used. Hydroxyapatite was prepared according to Tiselius et al. (1956). Approximately 400 µg of DNA in 0.12 M sodium phosphate buffer (pH 6.8) was loaded on the hydroxyapatite column (17 x 2.5 cm) which was pre-equilibrated in the same buffer. The denatured DNA was eluted with 0.12 M sodium phosphate buffer (pH 6.8) while native duplex DNA was eluted with 0.4 M sodium phosphate buffer (pH 6.8).

DNA preparations which were more than 95% in duplex form were considered as native and were used for further characterization.

(iii) Hyperchromicity

The purified DNA preparations showed a hyperchromicity of approximately 20% after denaturation. Sharp melting profiles of DNA and absence of a foot or shallow absorbance rise before the actual start of DNA melting (Fig. 2.2) indicated the absence of single stranded DNA and RNA. Only those DNA preparations which exhibited sharp
FIGURE 2.1 UV ABSORPTION SPECTRUM OF SUGARCANE DNAs

The spectra were obtained in the wave length range of 220-320 nm. The DNAs were dissolved in T<sub>10</sub> buffer (10 mM Tris-HCl, pH 7.5)

a. Blank T<sub>10</sub> buffer
b. CO-740 callus DNA
c. CO-740 shoot DNA
d. CO-7219 callus DNA
FIG. 2.1.

WAVELENGTH (nm)

ABSORBANCE

WAVELENGTH (nm)

FIG. 2.1.
FIGURE 2.2 MELTING PROFILES OF NATIVE SUGARCANE DNAs (10 kbp)

The melting of DNA was carried out in 0.12 M sodium phosphate buffer (pH 6.8) at a heating rate of 1.0°C/min.
FIG. 2.2.
melting profiles and hyperchromicities greater than 20% were used for further work.

**Thermal denaturation**

Thermal stability of a DNA gives information about its melting temperature from which the G + C content can be calculated. The most convenient and reliable method for studying the melting of DNA is to monitor the change in its UV absorbance as a function of increasing temperature under precise environmental conditions.

Thermal denaturation of DNA was carried out in a Gilford 250 spectrophotometer equipped with thermo programmer (Model 2527), analog multiplexer (Model 6046) and automatic reference compensator (Ranjekar et al. 1976). Thermal cuvettes were filled with 0.3 ml of DNA solution (25-300 µg/ml) in a 0.12 M sodium phosphate buffer (pH 6.8) and the absorbance was recorded at room temperature. The cuvettes were filled in such a way that the air space was less than 10% of the solution volume. The evaporation of DNA was prevented by using teflon stoppers. The temperature of the cuvettes containing DNA solution was then raised to 98°C at a heating rate of 1°C/min. The absorbance change which occurred during the heating process was monitored with a recorder (Model 6051).

Since absorbance by nucleotide bases in the wavelength region around 260 nm is due to π→π* electronic
transmission in purine and pyrimidine bases, the intensity of absorbance increases by 20-40% when the hydrogen bonds between the bases are disrupted. A linear relationship exists between this hyperchromicity and the extent of disruption of base pairs. The hyperchromicity of the DNA sample was calculated using the formula:

\[
H = \frac{A_{260} \text{ at } 98^\circ C - A_{260} \text{ at } 62^\circ C}{A_{260} \text{ at } 98^\circ C}
\]

(Zimmerman and Goldberg, 1977)
(Wimpee and Rawson, 1979)

where \( H \) = Hyperchromicity
\( A_{260} \) = Absorbance at 260 nm.

The total hyperchromicity of DNA was normalised to 100% and the graph of % hyperchromicity vs temperature was plotted. The melting temperature (Tm) of the DNA was the temperature at which half of the total increase in absorbance was obtained. The G + C content of the DNAs was determined by the formula

\[
G + C = (Tm - 69.3) \times 2.44 \quad \text{(Marmur and Doty, 1962)}
\]

The transition interval (\( \Delta T \)) was calculated as

\[
T_a^b = T_b^C \quad (83\% \text{ hyperchromicity}) - T_a^C \quad (17\% \text{ hyperchromicity})
\]

(Mähler and Dutton, 1964)
(Huguët and Jouanin, 1972)

DNA from *E. coli* was used as standard in these experiments.
High resolution thermal denaturation

Thermal denaturation at a heating rate of 1°C/min displays only the overall denaturation pattern of DNA. To enhance the resolution, fine melting or high resolution thermal denaturation (HRTD) analysis was carried out using the heating rate of 0.25°C/min. Such analysis not only tells us about the overall distribution of bases in DNA but also helps to identify discretely melting components which are considered to represent repetitive sequence families at least in case of higher plants and animal DNAs. For high resolution thermal denaturation analysis, the sugarcane DNAs were denatured at a heating rate of 0.25°C/min and absorbance values were recorded at 0.1°C increment from 62°C to 98°C for native DNA. Thus the melting curve consisted of 300-400 experimental data points depending on the melting range of DNA. A large slit width (1 mm) and a high initial DNA concentration (1-1.2 O.D. at 260 nm) helped in reducing the noise levels. Data smoothening and differentiation were performed by computer fitting of ten continuous data points at a time to a simple polynomial by the least squares method. A linear function was used as a fitting polynomial (Wada et al., 1980). The differential curves were obtained by plotting the temperature derivative of the absorbance of DNA against temperature (dA/dT vs T). The reproducibility
of the melting data was checked by using at least five DNA samples each in duplicate for melting and the resulting curves were analysed separately. The limit of resolution of this analysis was 0.001 absorbance and peaks separated by an interval as narrow as 0.4°C (approximately 1% G + C) could be detected reproducibly.

Isopycnic centrifugation in CsCl

CsCl density gradient centrifugation of DNA serves as an extremely powerful tool in differentiating the base composition of DNA. Thus DNA sequences that are enriched in G + C or A + T regions can be readily separated and purified from the bulk DNA.

CsCl density gradient centrifugation of DNA was performed using the procedure of Robkin et al., (1958). CsCl solution of initial density of about 1.700 g/ml was prepared by dissolving 951.8 mg of CsCl, 8-10 µg of sample DNA and about 5 µg of Micrococcus lysodeikticus DNA in 1 ml of SSC (X). The initial density of CsCl was calculated by measuring the refractive index of the solution using Abbe refractometer. This solution (0.4 ml) was loaded in an analytical cell and was spun in a Beckman Spinco (Model E) analytical ultracentrifuge at 36,000 RPM using ANF Ti rotor. Scans were taken at 270 nm at regular intervals of time. Attainment of density equilibrium was
confirmed by the fact that in two consecutive scans with an interval of 1 h, the DNA band had the same width and height. The density equilibrium was normally reached after centrifugation of DNA for 24-26 h. The buoyant density of DNA was calculated using the following equation.

\[ p = p_0 + 4.2 \omega^2 (r^2 - r_0^2) \times 10^{-10} \]

Where \( p \) is the buoyant density of sample DNA, \( p_0 \) is the buoyant density of the marker DNA, \( \omega \) is the angular velocity, \( r \) and \( r_0 \) are the radial distances of the sample and the marker DNA respectively from the axis of rotation. The buoyant density of the DNA was used to calculate \( G + C \) content of DNA using the relationship developed by Schildkraut et al. (1962).

\[ G + C \% = \frac{p - 1.660 \times 100}{0.098} \]

where \( p \) is the buoyant density of the sample DNA

**Sizing of DNA by agarose gel electrophoresis**

The size of the native DNA was measured by agarose gel electrophoresis in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid and 2 mM disodium EDTA, pH 7.8). The DNA solution was loaded in submerged agarose slab gels (1%) and the electrophoresis was carried out at a constant current (20 mA) for 6 h. Following electrophoresis, the gels were stained in
ethidium bromide (1 µg/ml) in 1 x TAE and visualized on 302 nm long wavelength UV transilluminator (Ultra Violet Products, San Gabriel, USA). Hind III digest of αDNA was used as the DNA size marker and the photographs were taken using the Minolta X 700 (35 mm) camera with red filter. The isolated sugarcane DNAs were mostly heterogeneous in size. They showed a main band of approximately 10 kbp along with a trail in the size range of 0.5 to 10 kbp.

Elution of high molecular weight DNA from agarose gels

For restriction enzyme analysis, it was essential to have a high molecular weight sugarcane DNA. A modified method of Dharmalingam (1985) involving direct elution of high molecular weight DNA from agarose gel was, therefore used. Briefly, 11 cm x 10 cm x 1 cm slabs of 0.7% agarose in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 7.8 to 8.1) were prepared. The edges of the glass plates were lined with PVC tapes 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, 600-700 µg of DNA was loaded while 2-4 µg of DNA was used in the analytical slot. The electrophoresis was carried out in 1 x TAE buffer at a constant current of 20 mA for 15-16 h. After electrophoresis, the analytical track was cut off from the slab,
stained with ethidium bromide at 1 µg/ml concentration for 30 min and visualised 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 h. Subsequent to this, frozen gel pieces and phenol were thawed out gradually to 4-10°C and centrifuged at 10,000 RPM for 20 min at 4°C using Sorvall SE12 rotor. The aqueous layer was collected and freed of traces of phenol by treatment with chloroforoform - isoamyl alcohol mixture (24:1, v/v). Prior to DNA precipitation with 2 volumes of chilled ethanol, the sodium ion concentration of the aqueous solution was adjusted in 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 then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).
Restriction endonuclease digestions and agarose gel electrophoresis

Because of site specific cleavage of DNA, the restriction endonucleases have proved to be extremely useful in studying the overall distribution of base composition of total DNA, in identification of repeated sequence families and in detecting specific methylated sequences (Fuchs and Blakesley 1983; Watson and Thompson, 1986).

For all digestions, 3-5 μg of DNA was incubated with 6-10 units of enzyme at 37°C for 15-18 h in appropriate buffers (Maniatis et al., 1982). The enzyme - DNA ratio and enzyme volume to reaction volume ratio were carefully adjusted to avoid non-specific activity of the enzymes. Following incubation, the enzyme digestion was stopped by addition of 1/10th volume of a 10 x reaction stop solution (50% glycerol, 10 mM EDTA and 0.25% bromophenol blue) and digestes were analysed by agarose gel electrophoresis on 1% or 1.4% neutral agarose gels in (IX) TAE buffer, pH 7.8 at a constant current of 20 mA for 5-6 h. The gels were then stained, visualized and photographed as described earlier.

High performance liquid chromatography

This is an extremely powerful and sensitive technique for analysis of various nucleic acid constituents. Among
the various HPLC types, reversed phase (RP) and ion pairing chromatography (IPC) have recently been used with considerable success in quantitation of modified bases in DNA hydrolysates as well as normal bases in body fluids as markers for disorders.

In the HPLC experiments, DNAs were hydrolysed with 60-70% perchloric acid for 1 h at 100°C. Following hydrolysis, perchloric acid was neutralised by 10 N potassium hydroxide and the potassium perchlorate precipitate was centrifuged out. Aliquots of the neutralised hydrolysate were applied to the reverse phase RP-18 column (10 µm, HP No.79916B, 250 x 4.6 mm with a 2 µm frit pore). The column was pre-equilibrated in 0.4 M ammonium phosphate buffer (pH 4.3) containing 5% methanol. The chromatography was carried out on Hewlett-Packard HPLC system (Model 1082B) interfaced with a Hewlett-Packard LC terminal (No.79850B) and equipped with an automatic sample injection system. The UV detector was fixed at 254 nm wave length. The chromatographic profiles were obtained at a chart speed of 1 cm/min and a flow rate of 0.5 ml/min. The elution was isocratic in the above pre-equilibrium buffer. The instrument was pre-calibrated with authentic bases namely adenine, guanine, thymine, cytosine and 5-methyl cytosine prior to injection of DNA hydrolysates. The identification of bases was
determined in terms of retention times and in case of 5-methyl cytosine, by peak enhancement due to co-injected authentic sample. The amount of 5-methyl cytosine was determined from the area of the peak in the above profiles.
RESULTS

Method of DNA extraction and yield

To assess the most suitable method for DNA isolation, three different methods namely, Bendich and Bolton's method (1967) with modifications as described in 'MATERIALS AND METHODS', Batchman and Price's method (1977) and Marmur's method (1961) were employed. The criteria of the best method was yield and hyperchromicity of the DNA. In this respect, Bendich and Bolton's method with modification was found to be the most effective and gave good DNA yield (Table 2.1). As the tissue was grown under aseptic conditions, the possibility of microbial contamination could be eliminated. Moreover, tissues with microbial contamination, if any, were discarded.

Thermal denaturation of native DNA (1°C/min.)

A DNA melting curve indicates one of the simplest forms of transition from a double helical configuration to a distorted coil (Marmur and Doty, 1959). It is obtained by plotting increase in absorbance of DNA at 260 nm (hyperchromicity) against temperature and is characterized by three main features: (i) Tm - which is the temperature corresponding to mid point of absorbance rise (Marmur
TABLE 2.1 METHOD OF EXTRACTION OF DNA, IT'S YIELD AND HYPERCHROMICITY

<table>
<thead>
<tr>
<th>Method</th>
<th>DNA (mg)/100 g of fresh tissue</th>
<th>Hyperchromicity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batchman and Price (3)</td>
<td>847 ± 78</td>
<td>23.24 ± 1.49</td>
</tr>
<tr>
<td>Marmur (3)</td>
<td>330 ± 85</td>
<td>15.56 ± 1.19</td>
</tr>
<tr>
<td>Bendich and Bolton (3)</td>
<td>1566 ± 58</td>
<td>23.45 ± 2.40</td>
</tr>
</tbody>
</table>

Figure in parenthesis indicates the number of experiments performed.

The hyperchromicity of the DNA samples was calculated using the following formula

\[
H = \frac{A_{260}(100\degree C) - A_{260}(62\degree C)}{A_{260}(100\degree C)}
\]

(Zimmerman and Goldberg, 1977)

(Wimpeé and Rawson, 1979)

where \( H \) is the hyperchromicity and \( A_{260} \) is the absorbance of the DNA sample at 260 nm. The total hyperchromicity was normalised to 100%.
and Doty, 1962). It is linearly dependent on G + C content of DNA and higher G + C content confers higher thermal stability of DNA; (ii) $\Delta T$ - which indicates the sharpness or the breadth of DNA melting (Mahler and Dutton, 1964; Huguet and Jouanin, 1972) and (iii) hyperchromicity - which represents the total increase in absorbance of DNA at 260 nm after complete denaturation (Zimmerman and Goldberg, 1977; Wimpee and Rawson, 1979).

Figure 2.2 depicts the melting curves of *E. coli* DNA, calf thymus DNA and high molecular weight DNA (10 kbp) of sugarcane CO-740 callus, CO-740 shoot and CO-7219 callus. The DNAs in these experiments were heated at the rate of 1°C/min. As it is clear from Fig. 2.2, the melting curves of *E. coli* DNA and sugarcane DNAs are smooth and monophasic. The melting data obtained by analyzing such curves are summarized in Table 2.2. The melting parameters such as Tm, G + C content and transition width of *E. coli* DNA compare well with the corresponding reported values (Blake and Lefoley, 1978). In case of three sugarcane DNAs, these three parameters vary in a narrow range of 86.2° - 86.5°C, 41.2% - 41.9% and 7.9° - 8.3°C respectively.
TABLE 2.2 THERMAL DENATURATION PROPERTIES AND BUOYANT DENSITY OF NATIVE SUGARCANE DNAs

<table>
<thead>
<tr>
<th>Species</th>
<th>Tm (°C)⁴</th>
<th>G + C content²</th>
<th>Hyperchromicity²</th>
<th>Δ2/3 (°C)⁴</th>
<th>Buoyant density g/cm³</th>
<th>G + C content²</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>90.8 ± 0.96</td>
<td>52.54</td>
<td>21.30 ± 0.81</td>
<td>4.6 ± 2.31</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CO-740 callus (8)</td>
<td>86.5 ± 0.24</td>
<td>41.96</td>
<td>23.49 ± 0.57</td>
<td>7.90 ± 0.12</td>
<td>1.702</td>
<td>42.85</td>
</tr>
<tr>
<td>CO-shoot (8)</td>
<td>86.2 ± 0.30</td>
<td>41.23</td>
<td>24.01 ± 1.72</td>
<td>8.32 ± 0.18</td>
<td>1.703</td>
<td>43.87</td>
</tr>
<tr>
<td>CO-7219 callus (8)</td>
<td>86.5 ± 0.08</td>
<td>41.96</td>
<td>23.89 ± 0.73</td>
<td>7.95 ± 0.17</td>
<td>1.702</td>
<td>42.85</td>
</tr>
</tbody>
</table>

Figure in parenthesis indicates the number of thermal denaturation experiments performed.

⁴Temperature that corresponds to 50% hyperchromicity

²G + C content has been calculated using the formula: G + C content = (Tm - 69.3) x 2.44 (Marmur and Doty, 1961)

³Calculated using the formula: H = \frac{A_{260}(100°C) - A_{260}(62°C)}{A_{260}(100°C)} (Zimmerman and Goldberg, 1977; Wimpee and Rawson, 1979)

⁴Calculated using the equation: Δ2/3 = Temperature that corresponds to 83.33% hyperchromicity - temperature that corresponds to 16.67% hyperchromicity.

⁵Calculated using the equation: % G + C content = \frac{p - 1.660}{0.098} x 100 where p is the buoyant density of the sample DNA.
Fine melting of native sugarcane DNA (0.25°C/min)

Eukaryotic DNAs are generally very heterogeneous. However, this heterogeneity is not immediately apparent from a simple curve relating the percent hyperchromicity and temperature. It can, however, be readily detected by plotting "derived" melting curves where the ratio of hyperchromicity over a small rise in temperature (e.g. 0.1°C) to the total hyperchromicity is plotted against temperature. Such studies have revealed an ordered and non-random distribution of base sequences in most organisms.

Since fine melting analysis of DNA is a very sensitive method, several precautions were taken to avoid artefacts. Firstly, high molecular weight DNAs (10 kbp size), free from detectable levels of polysaccharides, single stranded DNA and RNA, were used. Secondly, the experimental conditions such as salt concentration (0.18 M Na+) and DNA concentration (50-60 µg/ml) were kept constant. About ten experiments (five preparations, each in duplicate) were carried out with the DNAs of each tissue. The number and position of all components in each preparation were reproducible in all the cases. The first derivative analysis of DNAs of E. coli and calf thymus (Fig. 2.3) which served as controls compared well with the reported values (Blake and Lefoley, 1978; Mayfield 1977, Huguet and Jouanin, 1972).
FIGURE 2.3 THE FIRST DERIVATIVE MELTING PROFILES OF NATIVE SUGARCANE DNAs (10 kbp)

Thermal denaturation of native DNAs in 0.12 M sodium phosphate buffer (pH 6.8) obtained with a heating rate of 0.25°C/min. The absorbance was recorded continuously at 0.1°C temperature intervals. The temperature of the absorbance was determined as described in MATERIALS AND METHODS.

E. coli (-----) and calf thymus (——) DNAs HRTD profiles (-) and 1°C/min heating rate (0-0-0).
FIG. 2.3.
The high resolution derivative melting profiles of the three sugarcane DNAs are depicted in Fig. 2.3. From this figure, it can be observed that the actual melting of native DNAs starts at about 77°C and that the profiles are very heterogeneous consisting of 20-27 melting components. These melting components are divided into two groups. The first group consists of thermal components up to 86.4°C which are mostly A + T rich while the second group consists of components from 86.4°C to 98°C which are mostly G + C rich. Three to four maxima peaks are present in the Tm region (± 1°C difference) in both the callus cultures. In CO-740 shoot DNA, the maxima peak does not coincide with Tm value. The high resolution derivative melting profiles of the three sugarcane DNAs consist of eight to twelve components in A + T rich region and twelve to fifteen components in G + C rich region indicating a skewing on G + C side (Table 2.3).

A qualitative comparison of the derivative melting profiles of CO-740 callus, CO-740 shoot and CO-7219 callus native sugarcane DNAs was made in order to find out the number of components shared by them. For this purpose, a graph was plotted indicating the positions of all the melting peaks (Fig. 2.4). A temperature span of 0.2°C was considered to estimate the number of shared components and these data are summarized in Table 2.3). From Table 2.3, it can be seen
### TABLE 2.3: DISTRIBUTION OF THE COMPONENTS IN THE FINE MELTING PROFILES OF NATIVE DNA ON EITHER SIDE OF 86.4°C

<table>
<thead>
<tr>
<th>DNA</th>
<th>Number of components</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A+T rich side</td>
<td>G+C rich side</td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO-740 callus</td>
<td>9</td>
<td>15</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO-740 shoots</td>
<td>12</td>
<td>15</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO-7219 callus</td>
<td>8</td>
<td>12</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Components shared by 3-sugarcane tissues</td>
<td>3</td>
<td>7</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Components shared by CO-740 callus and shoot</td>
<td>6</td>
<td>8</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Components shared by CO-740 callus and CO-7219 callus</td>
<td>4</td>
<td>8</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 2.4 THE POSITIONS OF MELTING COMPONENTS OBSERVED
IN THE DERIVATIVE MELTING PROFILES OF THREE
SUGARCANE DNAs
FIG. 2-4.
that in a temperature range of 0.2°C, ten components are common in all the three DNAs, three in A + T rich region and seven in G + C rich region. Among the callus cultures of CO-740 and CO-7219, four components are common in A + T rich region while eight components are common in G + C region. When CO-740 callus and shoot DNAs are compared, six components in A + T rich region and eight components in G + C rich region are common among both the cultures.

**Buoyant density of DNA in CsCl**

Figure 2.5 shows the CsCl profiles of the DNAs of two callus tissues namely CO-740 and CO-7219, and CO-740 shoot along with the reference DNA of *Micrococcus lysodeikticus* which has a buoyant density of 1.731 g/cm³ and G + C content of 72%. The DNAs from the two calli band with a buoyant density of 1.702 g/cm³ while that from CO-740 shoot bands with a buoyant density of 1.703 g/cm³. The presence of satellite DNA component is not seen in any of the three DNAs (Fig. 2.5). The G + C content is estimated from the buoyant density of each DNA and is of the order of 42.85 to 43.87% (Table 2.2). These G + C content values compare well with those obtained from the Tm data of sugarcane DNAs.
FIGURE 2.5 ANALYTICAL ULTRACENTRIFUGATION PROFILES OF SUGARCANE DNA's IN NEUTRAL CsCl

Micrococcus lysodeikticus DNA of buoyant density 1.731 g/cm$^3$ used as a marker. For all the profiles, the density increase is from left to right.
CO-7219 CALLUS

CO-740 CALLUS

CO-740 SHOOT

ABSORBANCE

DENSITY (g/cm³)

1.731
1.702

1.731
1.702

1.731
1.703

FIG. 2.5.
Behaviour of DNA towards restriction endonucleases

After determining the thermal denaturation behaviour and buoyant density values, restriction enzyme analysis of sugarcane DNAs was carried out using a few enzymes namely, AluI, Bam HI, EcoRI, Hind III, Hpa II and MspI. The objective of this work was to assess sequence specific difference in the DNAs of the three sugarcane tissues.

The digestion patterns of DNAs of CO-740 callus, CO-740 shoot and CO-7219 callus with Bam HI, Hind III, Alu I and EcoRI are depicted in Fig. 2.6. The three sugarcane DNAs are extensively digested with these four restriction endonucleases. However, some differences in the extent of their digestion are noticed. CO-740 shoot DNA, for example, is more digested with Bam HI, EcoRI, Alu I and Hind III as compared to the digestion of CO-740 callus DNA and CO-7219 callus DNA. The three sugarcane DNAs do not show any organised, repeat structure with any of the enzymes used. This suggests the occurrence of either very small spacing between successive sites for these enzymes or a random distribution of these sites.

Of the enzyme pairs that can be used to study the methylation status, the most important is an isoschizomeric pair, MspI and Hpa II, both recognising the sequence 5'-CCGG-3' but showing differential sensitivity to whether outer C or inner C is methylated or not. Msp I,
FIGURE 2.6 RESTRICTION ENZYME ANALYSIS OF SUGARCANE DNAs

CO-740 callus (Lane 2, 5, 8 and 11), CO-740 shoot (Lanes 3, 6, 9 and 12) and CO-7219 callus (Lanes 4, 7, 10 and 13) DNAs.

Lanes 2, 3 and 4. Bam HI digest
Lanes 5, 6 and 7. Eco RI digest
Lanes 11, 12 and 13. AluI digest
Lanes 1 and 14. Hind III digest of λ DNA as a molecular weight marker (23.3 kbp, 9.5 kbp, 6.4 kbp, 4.2 kbp, 2.2 kbp and 1.8 kbp)

Electrophoresis was carried out in 1.4% agarose gel.
for example, cleaves CmCGG but not mCCGG while Hpa II cleaves mCCGG but not CmCGG. From Fig. 2.7, it is clear that in case of all the three sugarcane DNAs, Hpa II digests the DNAs to a lesser extent than MspI. These data clearly suggest that most of the CCGG sequences are of the type CmCGG. Among the three sugarcane DNAs, CO-740 shoot DNA is more extensively digested with Msp I in contrast to CO-740 callus DNA and CO-7219 callus DNA.

Content of 5-methyl cytosine in native DNA

The restriction enzyme analysis with methylation specific enzymes gives an overall information about the methylation status of bases. The HPLC helps to detect the presence of normal as well as modified bases. The HPLC was performed using a silica banded non-polar stationary phase (hydrocarbons of various carbon chain length) and a polar mobile phase. As the stationary phase is less polar than the eluent, it is generally referred to as reversed phase chromatography. In such systems, solvophobic forces are responsible for the binding of a solute (substances to be resolved by chromatography) to the stationary phase so that polar substances elute first. Solute retention is mainly determined by solvent effects, in particular the polarity of the solvent. In order to ensure the reproducibility of the retention time of the solute, solvent
FIGURE 2.7 DIGESTION OF TOTAL SUGARCANE DNAs WITH Msp I AND Hpa II

CO-740 callus (Lanes 2 and 5), CO-740 shoot (Lanes 3 and 6) and CO-7219 callus (Lanes 4 and 7)

Lanes 1 and 8. Hind III digest of λ DNA as a molecular weight marker (23.3 kbp, 9.5 kbp, 6.4 kbp, 4.2 kbp, 2.2 kbp and 1.8 kbp)

Lanes 2, 3 and 4. Msp I digest

Lanes 5, 6 and 7. Hpa II digest

Electrophoresis was carried out in 1.4% agarose gel.
FIG. 2.7.
polarity is rigidly maintained by a critical pH adjustment and addition of specific proportions of organic solvents (Harvath, 1981).

By the use of appropriately designed instrument and micro particulate packed columns, free bases of DNA hydrolysate are identified by their retention time. The method of DNA hydrolysis employed in these studies ensures formation of all free bases so that it should be possible to detect all modified bases in addition to normal A, T, G and C.

The HPLC elution profiles of calf thymus DNA, and sugarcane DNA hydrolysates and of a standard mixture of authentic samples of adenine (A), guanine (G), thymine (T), cytosine (C) and 5-methyl cytosine (5-mC) are depicted in Fig. 2.8. The proportions of 5-mC as calculated from the area of the peak in the above profiles are given in Table 2.4. Our results show that 5-methyl cytosine content is 4% (molar basis) in CO-740 shoot and callus DNA while it is 9.6% (molar basis) in CO-7219 callus DNA.
FIGURE 2.8  HPLC ANALYSIS OF SUGARCANE DNAs

Standard mixture contains authentic bases namely cytosine (C), 5-methyl cytosine (5 mC), guanine (G), thymine (T) and adenine (A).

\[ \downarrow \] indicates 5 mC peak.
FIG. 2.8.
<table>
<thead>
<tr>
<th>DNA</th>
<th>Moles percent of 5-methyl cytosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO-740 callus</td>
<td>4.2</td>
</tr>
<tr>
<td>CO-740 shoot</td>
<td>4.1</td>
</tr>
<tr>
<td>CO-7219 callus</td>
<td>9.6</td>
</tr>
</tbody>
</table>

*Proportions of mole percentage are calculated from peak area.

Represents an average value of 3 to 5 independent experiments.
DISCUSSION

In the present study, an attempt has been made to assess the differences at the total DNA level in three different sugarcane tissues namely, CO-740 callus, CO-740 shoot and CO-7219 callus using four independent approaches, namely, thermal denaturation at a heating rate of 1°C/min and 0.25°C/min, buoyant density determination in neutral CsCl, restriction enzyme analysis and HPLC. In the literature, scattered attempts have been made to assess DNA differences during cell differentiation. For example, thermal denaturation analysis has indicated differences in the melting temperatures of DNAs from different tissues and stages in pea epicotyl cells (Van Oostveldt and Van Parijis, 1976), developing protocorms of cymbidium (Nagl and Rucker, 1976), leaves of juvenile and adult phases in ivy (Kessler and Reches, 1977) and differentiating root systems of *Allium cepa* and *Vicia faba* (Durante et al., 1977; Bassi et al., 1984). Changes have also been observed in DNA methylation patterns of *Cucumis melo* (Grisvard, 1985), *Vicia faba* (Negrük et al., 1986) and soybean (Quemada and Lark, 1987); and in buoyant density of satellite DNA in *Vicia faba* (Natali et al. 1986) carrot (Schäfer et al., 1978) and *N. glauca* (Durante et al., 1982) in differentiated and dedifferentiated tissues. Ours is probably the first effort to use as many as four approaches at a time to tackle the problem
of *in vitro* differentiation at the DNA level.

From Figs. 2.2 and 2.5 and Table 2.2, it is clear that thermal denaturation analysis and buoyant density measurement have not revealed any significant differences among the DNAs of CO-740 callus, CO-740 shoot and CO-7219 callus. However, some difference is seen with reference to the DNA methylation status of these three different tissues. Here, although the DNAs of all the three tissues are more digested with MspI than with HpaII, the extent of digestion with each of these two enzymes is different. For example, the digestion of CO-740 shoot DNA is significantly more than that of CO-740 callus DNA with both MspI and HpaII. Such a difference is clearly due to the variations in the number of 5'-CCGG-3' sequences in these tissues; the number being higher in shoot DNA than that in callus DNA. The second important difference among these three tissues is the content of 5-mC as revealed by HPLC measurements. The latter have indicated that CO-7219 callus DNA contains 9.6% of 5-methyl cytosine on molar basis as against 4.2% in CO-740 callus and shoot DNA. It can be seen from Table 2.5 that 5-methyl cytosine content of CO-740 shoot DNA and CO-740 callus DNA is comparable to that of other plants. CO-7219 callus DNA is rather unique in having a high proportion of 5-mC. As said earlier, CO-7219 callus tissue is different from CO-740 callus in the fact that it loses its differentiating ability
**TABLE 2.5: 5-METHYL CYTOSINE CONTENT IN DIFFERENT PLANTS**

<table>
<thead>
<tr>
<th>Plant</th>
<th>5 methyl cytosine (Mol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daucus carota</td>
<td>6.0</td>
</tr>
<tr>
<td>Cucurbita pepo</td>
<td>3.7</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>5.2</td>
</tr>
<tr>
<td>Gossypium hirsutum</td>
<td>4.6</td>
</tr>
<tr>
<td>Triticum sp.</td>
<td>6.0</td>
</tr>
<tr>
<td>Zea mays</td>
<td>6.2</td>
</tr>
<tr>
<td>Allium cepa</td>
<td>5.4</td>
</tr>
<tr>
<td>Cymbidium pumilum</td>
<td>3.5</td>
</tr>
<tr>
<td>Sugarcane CO-740 shoot (Present Study)</td>
<td>4.1</td>
</tr>
<tr>
<td>Sugarcane CO-740 callus (Present Study)</td>
<td>4.2</td>
</tr>
<tr>
<td>Sugarcane CO-7219 callus (Present Study)</td>
<td>9.6</td>
</tr>
</tbody>
</table>

*J.A. Brant (1976)*
after 10 subcultures. CO-740 callus, on the other hand, has retained its capacity for differentiation even after 100 subcultures. In view of its high content of 5-\textit{MC} in CO-7219, it is likely that 5-\textit{MC} may have some role in its inability for differentiation after specific number of subculturing. Since it is known that actively transcribing genes are generally under methylated, methylation may lead to the inactivation of certain genes in CO-7219 callus as a result of which it loses its ability to differentiate.
REFFERENCES


Galau, G.A., Klein, W.H., Davis, M.M., Wold, B.J.,
Goldberg, R.B., Hoscheck, G., Ditta, G.S. and Breidenbach,
   Harbor Laboratory Press, New York.


