DNA Methylation and Embryogenic Competence in Leaves and Callus of Napiergrass (Pennisetum purpureum Schum.)

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ABSTRACT
Quantitative and qualitative levels of DNA methylation were evaluated in leaves and callus of Pennisetum purpureum Schum. The level of methylation did not change during leaf differentiation or aging and similar levels of methylation were found in embryogenic and nonembryogenic callus.

Tissue and cell recalcitrance has been one of the major problems faced in regeneration from cultured cells in the Gramineae (28). Loss of competence or recalcitrance in cells is linked to their genetic/epigenetic status (15, 16). The elucidation of the biochemical and molecular changes which accompany loss of competence, therefore, will be helpful in identifying the underlying mechanisms involved.

Leaves of the Gramineae show a spatial and temporal gradient in morphogenic competence in vitro (16, 28). Only the basal parts of young leaves respond in culture. Because of the distinct gradient in response, this system has provided a model which has been used to study possible correlations between the loss of cell competence and biochemical, physiological, and molecular changes occurring during leaf development and differentiation. Such studies have involved analysis of DNA integrity and quantity, DNA amplification, the cell cycle, mitotic activity, and hormonal and protein changes (2, 3, 11, 13, 18–20, 27).

It has been suggested that recalcitrance in the Gramineae is linked to changes in gene expression, which may be controlled by qualitative or quantitative changes in DNA. One qualitative change in DNA which is believed to participate in the regulation of transcription and differentiation is DNA methylation. Circumstantial and direct evidence which supports the idea that methylation is involved in gene regulation has come principally from studies on mammalian tissue which evaluate quantitative and qualitative changes in the level and pattern of DNA methylation, and the influence of methylation inhibitors, such as 5-azacytidine. These studies indicate that (a) changes in the methylation pattern of genes during differentiation are correlated with changes in gene expression, (b) methylated genes introduced into cells are not expressed when compared with unmethylated genes, and (c) gene expression is depressed after treatment with the methylation inhibitor 5-azacytidine (1, 10, 12).

In view of the putative role played by DNA methylation in the developmental regulation of gene expression, it is possible that methylation may also play a role in plant cell competence by repressing the expression of genes which control a cell’s ability to undergo morphogenesis. This hypothesis has not yet been tested as there are no published reports which assess for the presence or quantify the level of methylation in competent as compared with recalcitrant tissues. Hence, the present study was undertaken to qualitatively and quantitatively evaluate the presence and amount of dinucleotide CG and trinucleotide C-N-G methylation (where N is any nucleotide) occurring in leaves and nonembryogenic and embryogenic callus of Pennisetum purpureum and to determine whether there is any correlation between DNA methylation and cell competence.

MATERIALS AND METHODS

DNA Isolation
Leaves were collected from field grown plants of Pennisetum purpureum Schum. (accession number PP-9 obtained from Dr. S. C. Schank). DNA was isolated from the base, middle, and tip region of leaf numbers 3, 5, and 7 by the method of Eichholtz et al. (5) in December 1986 and June 1987. The leaf number was based on allocation of leaf 1 as the leaf closest to the meristem. The DNA for each leaf sample was derived from 10 to 20 g of tissue. The mean lengths of leaves were 15, 29, and 30 cm, respectively, for leaves 3, 5, and 7. Regions were determined by dividing each leaf into three equal portions.

Qualitative Analysis by Restriction Digests
One μg of DNA from all leaf samples was restricted, according to manufacturer’s instructions, with 10 units of the isoschizomers MspI and HpaII (Boehringer Mannheim Biochemicals), EcoRII (BRL, Bethesda, MD), and BstNI (New England Biolabs). The isoschizomers MspI and HpaII cleave at CCGG sites and show differential sensitivity to the presence of methyl residues. HpaII will not cut DNA where the cytosine residues of the sequence are modified to 5'-CmCCG-3' or 5'-mCCG-3' by methylation. While MspI will cut if the internal C is methylated, methylation of the external C, 5'-
which is insensitive. While the plant of 400 containing EcoRlI, a sensitivity exists. DNA is digested in the presence of the restriction enzyme BstNI, which is insensitive to methylation. In the presence of methylation-sensitive enzymes EcoRII and HpaII, the DNA remains undigested while it is only partially digested in the presence of MspI.

Figure 1. Illustration of the presence of DNA methylation in DNA from the basal, middle, and tip of leaf three. Numbers 1, 2, 3 represent basal, middle, and tip regions, respectively. Number 4 is undigested DNA. DNA is fully digested in the presence of the restriction enzyme BstNI, which is insensitive to methylation. In the presence of methylation-sensitive enzymes EcoRII and HpaII, the DNA remains undigested while it is only partially digested in the presence of MspI.

mCCGG-3' renders MspI inactive (14). Similar methylation sensitivity exists in the isoschizomers EcoRII and BstNI. EcoRII will not cut if the cytosine in the trinucleotide sequence CTG or CAG is methylated while BstNI will not (21). Restricted DNA was electrophoresed on 0.8% agarose gels containing 400 μg/mL ethidium bromide in 1 x TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) at 20 V for 16 h. A 1 kb ladder (BRL) was run with all digests to determine the size of any fragments produced.

Plasmid DNA Isolation

An Escherichia coli plasmid pMON806 (22) was used in experiments to test the activity of enzymes sensitive to DNA methylation and for an analysis of potential inhibitory influences of plant DNA extracts on enzyme activity. In the absence of plant DNA this plasmid was restricted in the presence of the enzymes MspI and HpaII but was not digested by EcoRII due to methylation of the EcoRII restriction sites. To produce plasmids which lacked methylation in the EcoRII restriction sites, pMON806 was isolated from a Dcm- strain of E. coli (GM271, F-, leucB6, dcm-6, his G4, thi-1, hsdR2, ara-14, lacY1, galK2, galT22, xyl-5, mtl-1, rpsL136, tonA31, Txs-78, λ-, supE44 provided by Dr. Harry Nick). The Dcm- strain lacks the enzyme dcm methylase which methylates the sequence CCGG. Plasmid DNA was isolated using a Triton/SDS procedure (4) and purified on a CsCl gradient. The DNA was quantified spectrophotometrically.

Quantitative Analysis of m5Cyt

The HPLC methods of Gehrke et al. (6, 7) were used in the analysis of 1 to 3 μg of DNA from all samples.

Callus Culture

The production of embryogenic callus was used to establish the embryogenic competence of cells in young, basal regions of leaves. In December 1986 and June 1987, leaf segments from the basal portions of leaves were sterilized and cultured on a callus induction medium according to procedures outlined by Haydu and Vasil (9). After two monthly subcultures embryogenic and nonembryogenic calli were separated. A total of 5 g of each callus type was used for DNA isolation. DNA isolation and qualitative and quantitative analysis of
DNA methylation in these calli was conducted as outlined above.

RESULTS AND DISCUSSION

Restriction digests of DNA isolated from leaves collected from plants in December 1986 and July 1987 both indicate that DNA from all of the *Pennisetum purpureum* leaves and leaf regions tested were methylated. As indicated in Figure 1, the leaf DNA samples were not digested by the methylation sensitive enzymes *Hpa*I and *Eco*RII and only partial digestion occurred in the presence of *Msp*I. The activity of the enzymes *Eco*RII, *Hpa*I, and *Msp*I, and the absence of inhibitory influences of plant DNA extracts on digestion, was confirmed by the digestion of DNA from plasmid pMON806 both in the presence and absence of plant DNA (Fig. 2). These results indicate that the cytosine residues of *P. purpureum* DNA are methylated in both CCGG and CCGG sequences. This corroborates previous studies on restriction digests of plant DNA (8) which indicate that unlike mammalian DNA, DNA methylation in plants occurs at both dinucleotide CG and trinucleotide C-N-G (where N is any nucleotide) sequences.

The quantitative analysis of mSCyt content using HPLC also demonstrated the presence of methylation in all samples (Table 1). The percentage of methylated residues ranged between 38 and 40% in leaf samples and no appreciable differences were detected between different leaf regions or leaves of different ages. This high level of methylation contrasts sharply with that found in mammalian tissue which ranges between 2 and 7% (6, 7) but is similar to that found in other plants (25). The presence of methylation in trinucleotide sequences and the higher frequency of CG residues found in plant (3–4%) as compared with animal DNA (0.5–1%) is believed to account for the high level of methylation found in plant DNA (24).

When compared with callus, the level of methylation found in the leaves was 4% higher. However, the E and NE callus did not show any major variation in DNA methylation. A parallel analysis for the presence of m6Ade indicated that this base modification is not present in leaf or callus samples. Results from HPLC analysis also allowed an evaluation of the mole % concentration of deoxynucleosides present in leaf samples. As indicated in Table 1 there were no major differences between samples.

The present study shows that the DNA of the grass *Pennisetum purpureum* is highly methylated and that loss of embryogenic competence is not linked to a gross alteration in DNA methylation. It is possible, however, that cell competence may be controlled by changes in DNA methylation patterns which were not detected by the techniques employed here. In other plants, putative correlations between methylation and gene expression have been evaluated by using specific gene probes (17, 21, 23, 26). Hence, the elucidation of the putative role played by DNA methylation in cell competence awaits the identification and cloning of specific embryogenic genes which could be used as specific probes, a task which is the objective of many studying plant embryogenesis.

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LITERATURE CITED

3. Beauleau GC, Rogers SO, Bendich HJ (1985) DNA extraction from wheat leaves is highly degraded: a possible basis for the difficulty in establishing leaf culture cells in the Gramineae (abstract 03-11). First International Congress on Plant Molecular Biology, p 11.