DNA METHYLATION AND SOMATIC EMBRYOGENESIS OF ORCHARDGRASS (*DACTYLIS GLOMERATA* L.)

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Summary. Coupled restriction digestion with *Hpa*II/*Msp*I and *Eco*RII/*Bst*NI was performed to detect the methylation level of CCGG and CC(A/T)GG sites, respectively, in DNA of leaf explants, embryogenic and non-embryogenic calli as well as of various morphological structures from different developmental stages of somatic embryogenesis in orchardgrass (*Dactylis glomerata* L.) suspension cultures. DNA methylation was lower as a whole in the most basal leaf portion, from which callus and embryos develop (indirect somatic embryogenesis) when compared to the more distal leaf parts, from which somatic embryos are formed (direct somatic embryogenesis). Significant differences in the DNA methylation level were observed in embryogenic and non-embryogenic calli from the same genotype, cultured under identical conditions. During somatic embryogenesis variations were observed at the level of methylation according to a characteristic pattern. The three morphological structures (microclusters, proembryogenic masses and embryos) showed different levels of methylation, the lowest level being found in the fraction containing the microcluster cells.

Key words: DNA methylation, orchardgrass suspension culture, somatic embryogenesis.

Abbreviations: CTAB - hexadecyltrimethyl-ammonium bromide, E_2 - embryogenic suspension culture, Dicamba - 3, 6-dichloro-o-anisic acid, NE_2 - non-embryogenic suspension culture, PEMs - proembryogenic masses, SH0 - Schenk and Hildebrandt (1972) medium, SH30 - SH medium, supplemented with 30 μM dicamba.

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INTRODUCTION

An important post-replication modification of DNA is the methylation of certain residues, frequently cytosine within a dinucleotide sequence CpG or CpNpG, the latter typically found in plant genomes (Gruenbaum et al., 1981). In plants, as in mammals, DNA methylation has a dual role in the defence against invading DNA and transposable elements, as well as in gene regulation. The implication of cytosine methylation in modulation of gene expression includes inhibition and activation of gene transcription via methylation and demethylation in promoter regions and, normally methylation of a gene inactivates its transcription (Finnegan et al., 1998, 2000). Cytosine methylation has been shown to be negatively correlated with transcriptional activity of several endogenous genes like those encoding photosynthetic proteins (Ngernprasirtsiri et al., 1989), rRNA (Watson et al., 1987; Flavell et al., 1988; Ananiev et al., 2003) or several transgenes (Lambe et al., 1997). Methylation and demethylation are considered to be important mechanisms of cell- and organ-specific expression (Boyes and Bird, 1991). In plants, significant changes in the genomic level of DNA methylation have been observed during zygotic embryogenesis, somatic embryogenesis and dedifferentiation (LoSchiavo et al., 1989; Frediani et al, 1992; Richards, 1997). These studies suggest that genomic cytosine methylation plays a fundamental role in plant development, differentiation and physiology.

Tissue and culture recalcitrance has been one of the major problems of regeneration from cultured cells in *Gramineae*. Leaves of Gramineae show a spatial and temporal gradient in morphogenic competence *in vitro* (Morrish and Vasil, 1989). Orchardgrass (*Dactylis glomerata* L.) leaf segments have a high capacity for direct embryogenesis from mesophyll cells and indirect embryogenesis through callus (Conger et al., 1983). Besides, *D. glomerata* L. is the first cereal grass in which somatic embryos fully developed to a germinable stage in a single liquid medium (Conger et al., 1989). This makes it a useful model system for studying fundamental processes of embryogenesis in *Poaceae*. It has been suggested that recalcitrance in Gramineae is linked to changes in gene expression. In view of the putative role of DNA methylation in the developmental regulation of gene expression, it is likely 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.

The present study was undertaken to evaluate the presence and level of DNA methylation in leaves, embryogenic and non-embryogenic callus and suspension cultures of *D. glomerata* L. and to determine whether there is any correlation between DNA methylation and embryogenic potential.

MATERIALS AND METHODS

Plant material

The highly embryogenic genotype Pleven 201 was maintained under greenhouse conditions. The basal 3 cm portions of the innermost leaves were excised and split along the midvein; these leaf pieces were cut transversely into six segments and surface-disinfected as described (Conger et al., 1989). The segments from one half of each leaf were serially explanted onto Petri plates with SH30 medium, and the segments from the other half were stored at - 70°C until DNA isolation and were used as a control. Callus-derived suspension cultures from one embryogenic (E_2) and one non-embryogenic (NE_2) cell line of orchardgrass (*D. glomerata* L.) were initiated according to Conger et al. (1989) and maintained in a liquid SH30 medium essentially as previously described (Tchorbadjieva and Odjakova, 2001). The non-embryogenic suspension culture NE_2 was initiated from segregated non-embryogenic sectors of the callus used for induction of E_2 line, respectively.

Fractionation of suspension cultures

Fractions of globular embryos, PEMs and microclusters from E_2 suspension culture were collected by passing the culture consecutively through a series of 230-, 104-and 60-μm molecular sieves, respectively. After 7 d in culture the cells were separated from the culture media and used as a source of DNA. The microclusters from NE_2 culture were maintained in the same manner.

DNA isolation

Total DNA from leaves, callus and suspension cultures was extracted by modification of the CTAB method of Rogers and Bendich (1988) as described by Sul and Korban (1996). Briefly, a 100 mg of tissue sample was ground in liquid N_2 to a fine powder using a mortar and pestle, and then transferred to a microfuge tube. A 350 μl of 2xCTAB extraction buffer (2% CTAB, 100mM Tris-HCl (pH 8.0), 1.4M NaCl and 1% polyvinylpyrrolidone, Mr 40,000 (PVP) (preheated to 65°C) and 350 μl of 8M LiCl (lithium chloride) were added and the solution was pipeted several times very gently to disperse powder into solution. The tube was then incubated at 65°C for 5 min. A 700 μl of chloroform/isoamyl alcohol (24:1) was added, vortexed 2-5 sec to form an emulsion and centrifuged in a bench-top centrifuge (Microspin 24, Sorvall Instruments, DuPont, Wilmington, USA) for no more than 5 sec. The supernatant was transferred to a new test tube and the steps of deproteinization with chloroform/isoamyl alcohol were repeated twice yielding about 500 μl of solution. A 1 ml of 100 % ethanol was added, mixed gently and centrifuged for 2-3 sec in order to precipitate DNA, and ethanol was decanted. The pellet was washed with 1 ml 70 %

ethanol, the tube was centrifuged for 10 to 15 sec and the ethanol was decanted. This step was repeated twice and finally the pellet was dried at room temperature. A 100 µl TE buffer (10 mM Tris-HCl, pH 8.0 and 1mM EDTA) was added to the dried pellet and the tube was incubated in 65°C for 10 min to dissolve genomic DNA.

DNA restriction analysis

One µg of DNA was restricted, according to supplier's instructions, with 3-5 units of the isoschizomers *Msp*I/*Hpa*II and *EcoR*II/*BstN*I (Stratagene). The isoschizomers *Msp*I and *Hpa*II cleave at CCGG sites and show differential sensitivity to the presence of methyl residues, in the case of *Hpa*II (mC/mCGG, methylation of either cytosine blocks cleavage) or *Msp*I (mC/CGG, methylation of the outer cytosine inhibits cleavage). Similar methylation sensitivity exists for the isochizomers *EcoR*II and *BstN*I. *EcoR*II is not able to cleave the recognition site 5'-CC (AT) GG if the internal cytosine is methylated, whereas *BstN*I cleaves the sequence independently of methylation. Restricted DNA was electrophoresed on 0.8 % agarose gels in 1x TBE (0.089M Tris-borate, 0.089M boric acid, 0.002M EDTA, pH 8.0). Lambda-*Hind* III DNA fragments were used as molecular size markers. To assure that differences between the restriction analysis of leaf explants, callus and suspension culture cells had not been due to incomplete digestion, DNA was tested by addition of lambda phage-DNA as an internal standard in the restriction assay mixture (data not shown).

Results and discussion

Leaf cultures of Pleven 201 genotype produced a large number of somatic embryos due to their high embryogenic potential. This system included also a gradient of embryogenic response within the basal 30 mm of the youngest/innermost leaves. After 4-6 weeks on induction medium (SH30) the younger basal tissue (segment 1) gave rise to callus in addition to embryo formation (indirect somatic embryogenesis), while the cells in the distal regions (segment 2) appeared to produce only direct embryos (direct somatic embryogenesis) (Fig. 1, panel a).

Somatic embryos from orchardgrass suspension cultures fully developed on a hormone-containing medium before being transferred to a hormone-free medium for regeneration. Two-three days after inoculation of induction medium competent single cells in E_2 suspension culture started to divide intensively and formed microclusters. Proembryogenic masses (PEMs) containing centers of embryonic growth were formed a week later and after 2 weeks the globular embryos began to differentiate from the cell masses (Fig. 1, panel b). Single cells from NE_2 culture then divided to form microclusters whose further development was blocked (Fig. 1, panel c).

The level of DNA methylation can be evaluated by the extent of site-specific methylation in the recognition sequence of a methylation-sensitive enzyme. It was of interest to compare the DNA methylation pattern in leaf segments which give rise to

callus and embryos to those from which direct embryos develop. For this purpose, DNA isolated from the corresponding segments before plating the explant (t_0) and after 25 days (t_{25}) on induction medium (SH30) was digested with the isoschizomers *Msp*I and *Hpa*II.

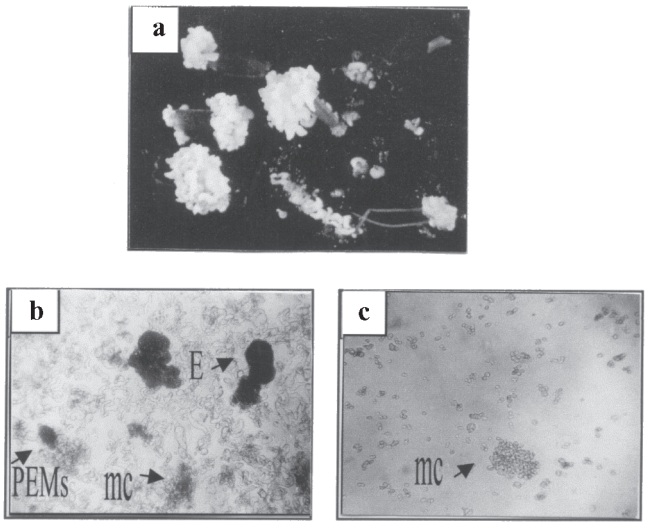

Fig. 1. Somatic embryogenesis of *Dactylis glomerata* L. Direct somatic embryogenesis from leaf explants (segment 2) after 30 days on induction (SH30) medium (a), E_2 embryogenic (b) and NE_2 non-embryogenic (c) suspension culture 7 days after subculture. mc-microclusters; PEMs-proembryogenic masses; E-globular embryos.

DNA from segment 1 appeared to be highly hypomethylated before plating (Fig. 2, A), which can be revealed by an increased accessibility of methylation-sensitive restriction sites to *Hpa*II. After a 25-day induction period on SH30 medium DNA showed higher methylation level, as judged by the prevailing high molecular mass DNA, non-digested by *Hpa*II (Fig. 2, B). Restriction digests of DNA isolated from segment 2 showed that DNA from both non-induced (t_0) and induced (t_{25}) leaves was highly methylated. As indicated in Fig. 2, C, the leaf DNA samples at t_0 were not digested by the methylation-sensitive *Hpa*II and only partial digestion occurred in the presence of *Msp*I. The methylation level was considerably higher for the induced leaf segments 25 d after induction (Fig. 2, D) as was demonstrated by the almost total lack of *Hpa*II digestion. Evidence is accumulating in support of the view that there are fundamental similarities between direct and indirect somatic embryogenesis (De Jong et al., 1993). There is a striking difference, however, in the methylation level of DNA from segment 1 and segment 2 before induction (compare Fig. 2, A and C). Many of the observed differences in the DNA methylation pattern can be attributed to the different level of differentiation of the cells in the segments. Generally auxin causes hypermethylation of DNA (LoSchiavo et al., 1989; Carman 1990). This could

explain the higher level of DNA methylation in both segments after 25 d on induction medium (in the presence of dicamba). It is possible however that selective hypomethylation also occurs which may activate genes for embryogenesis. The higher level of "basic" methylation in segment 2 could be explained by the differentiated state of the cells. Apparently, the gradient of embryogenic response coincided with the gradient of leaf tissue differentiation. The younger basal tissue with more actively dividing cells was prone to callus formation while the cells in more differentiated distal regions, where the rate of cytokinesis was reduced, appeared to produce only direct embryos.

Next, we compared the methylation pattern of DNA from E_2 and NE_2 calli and that of E_2 and NE_2 microclusters - the first morphological structures which appear after initiation of suspension cultures. DNA from E_2 callus used to initiate the E_2 suspension culture showed a relatively high level of methylation as judged by the high molecular mass fragments of DNA in gel (Fig. 3, A). Except for *Hpa*II, *Msp*I also did not cleave its DNA indicating that the external cytosine was methylated, too. In embryogenic microclusters DNA was cleaved by *Msp*I and partly by *Hpa*II suggesting that DNA was hypomethylated compared to the embryogenic callus (Fig. 3, B). *Msp*I and *Hpa*II cleaved DNA from NE_2 callus and NE_2 microclusters more extensively than DNA isolated from their embryogenic counterparts showing that methylation of 5' cytosine in the sequence CCGG was also reduced (Fig. 3, C and D). The extent of demethylation was comparable for both NE_2 callus and NE_2 microclusters. Interestingly, the two types of calli used were from the same genotype and had arisen from the same cultural conditions, so the expression of abnormal (non-embryogenic) callus type should reflect the parameters of a presumably epigenetic nature. In support of this idea protein patterns have been shown to be quite different between embryogenic and non-embryogenic calli (Tchorbadjieva et al., 2004). Methylation changes occur at a sufficiently high frequency to be an important source of tissue culture-induced variation (Jaligot et al., 2000). In contrast, Morrish and Vasil (1989) did not observe differences in DNA methylation between embryogenic and non-embryogenic callus of *Pennisetum purpureum*, though both were hypomethylated relative to whole-plant tissues. The level of DNA methylation was equally low in the NE_2 callus and NE_2 microclusters and did not change during development. Evidence has been currently accumulating for an involvement of DNA methylation disfunctions in the emergence of developmental abnormalities in plants (Finnegan et al., 1996; Phillips et al., 1994; Richards, 1997; Santos et al., 2002). This constant low level of DNA methylation in the non-embryogenic line could possibly explain the loss of embryogenic competence. It is possible that some key regulatory genes of embryogenic competence might be demethylated and inactivated during the selection of non-embryogenic callus. The loss of totipotency during culture may reflect the progressive elimination of cells capable of differentiation. The relative constant low level of DNA methylation in non-embryogenic callus may reflect the acti-

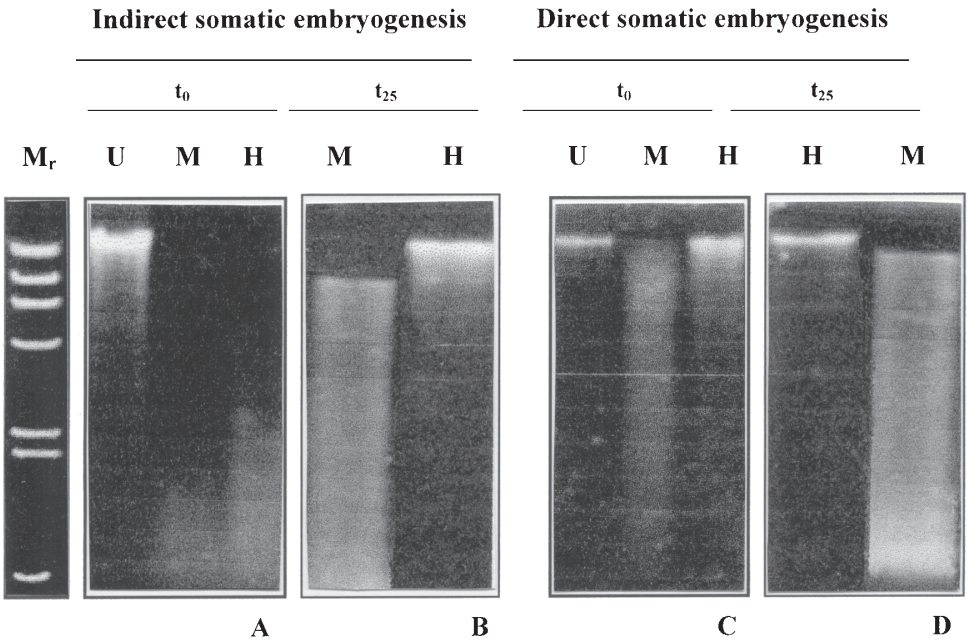

Fig. 2. Gel electrophoresis of DNA extracted from leaf segment 1 and leaf segment 2 before (t_0) (A, C) and 25 days after (t_{25}) (B, D) plating the explant on induction medium, respectively. U, uncut DNA; M-*Msp*I digest; H-*Hpa*II digest. M_r –lambda phage DNA digested with *Hind* III as DNA fragment size marker.

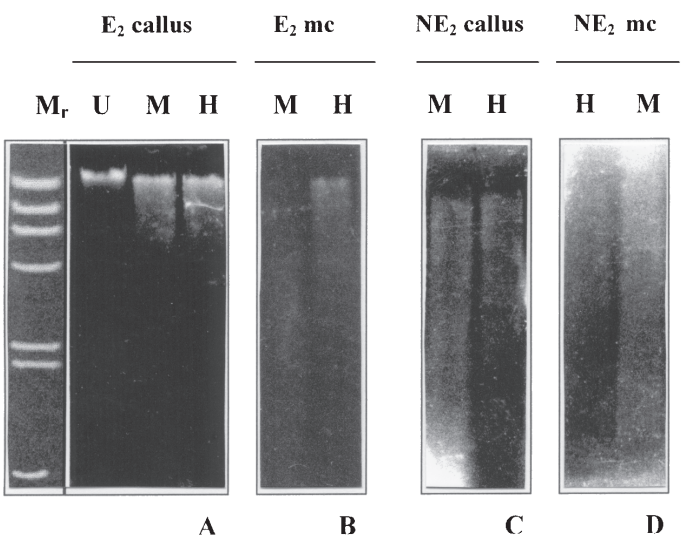

Fig. 3. Restriction pattern of DNA from: E_2 embryogenic callus (A) and microclusters (B), NE_2 non-embryogenic callus (C) and microclusters (D). U, uncut DNA; M-*Msp*I digest; H-*Hpa*II digest; mc-microclusters. M_r –lambda phage DNA digested with *Hind* III as fragment size marker.

vation of otherwise inactive genes, thus changing an established equilibrium necessary for the cells to undergo embryogenesis.

The embryogenic suspension culture E_2 with its well defined morphological structures which develop in the course of somatic embryogenesis is a good model system to study the changes of DNA methylation during development. DNA from PEMs and globular embryos was digested with *Hpa*II/*Msp*I and *EcoR*II/*Bst*NI. *EcoR*II profiles were used to detect the differences in the level of site-specific methylation of the motif –CNG- of different morphological structures. The PEMs showed a lower methylation level as compared to globular embryos (Fig. 4). However, it was higher compared with that of the microclusters (Fig. 3, B). The pattern of methylation differed between the two morphological structures. CG sites (tested with *Hpa*II) as well as CAG and CTG sites (monitored with *EcoR*II) were cleaved more extensively in DNA from PEMs than in globular embryos. *Msp*I cleaved DNA from PEMs more extensively showing that methylation of the 5' cytosine in the sequence CCGG was also reduced in PEMs compared to globular embryos (Fig. 4).

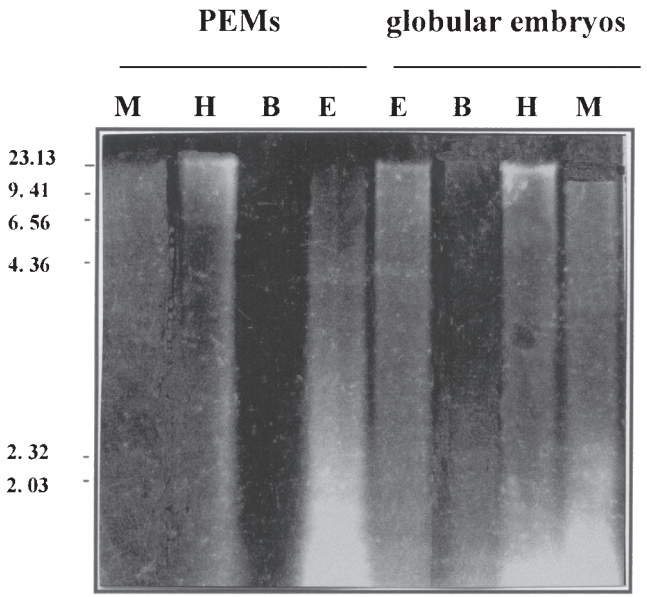

Fig. 4. Comparison of the methylation patterns of DNA isolated from proembryogenic masses (PEMs) and globular embryos. M- *Msp*I digest; H-*Hpa*II digest; B- *BstN*I digest; E-*EcoR*II digest. The sizes (in kb) of *Hind*III-cleaved lambda phage DNA standard markers are shown to the right.

Thus it seems that embryogenesis starts with an initial drop in the level of methylation followed by a rise in the later embryo stages. This supports the idea that a hypomethylated state of DNA is essential for the acquisition of embryogenic poten-

tial of somatic cells. Our data are in agreement with those of Munksgaard et al. (1995) who found that DNA of the first developmental stages of somatic embryos contained lower levels of methylated DNA than the later stages. LoSchiavo et al. (1989) observed an initial decrease in methylation, followed by an increase during late embryogenesis. These authors found also that this "basal level" of methylation with its temporal variation during embryogenesis could not be changed without impairing the process. Palmgren et al. (1991) showed that in *D. carota* suspension cells, the cell population that was the precursor of somatic embryos contained a lower level of DNA methylation than the suspension as a whole. Arnoldt et al. (1995) reported that the changes in DNA methylation pattern were part of the normal cell physiology of dividing cells in tissue culture.

Acquisition of embryogenic competence largely relies on dedifferentiation, a process whereby existing transcriptional and translational patterns are altered in order to allow cells to set a new developmental program. Our results suggest that the disruption of a defined basic level of DNA methylation in *D. glomerata* L. callus leads to a loss of its embryogenic potential. Besides, there is a considerable change in the methylation pattern during development (somatic embryos are more methylated than microcluster cells) and in this way DNA methylation may be involved in the regulation or maintenance of differentiation.

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