TISSUE-SPECIFIC DNA METHYLATION IN *HAEMANTHUS KATHARINAE* BAK. (AMARYLLIDACEAE)

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ABSTRACT

The level of DNA methylation was compared in root meristem, adult leaf and endosperm of monocotyledonous species, *Haemanthus katharinae*, with the use of HPLC, restriction analysis, Southern blot hybridization and in situ nick-translation driven by restriction enzyme HhaI. The highest level of 5-methylcytosine was observed in adult leaf whose nuclear chromatin is particularly condensed, and the lowest in endosperm. The level of DNA methylation of repetitive HaeIII 400bp sequence follows that of the total DNA.

KEY WORDS: DNA methylation, root meristem, leaf, endosperm, *Haemanthus katharinae*.

INTRODUCTION

The methylation of cytosine in CpG and, in plants, in CpXpG sequences in DNA is one of the possible mechanisms of regulation of gene expression (Razin and Cedar 1991). Tissue-specific level of cytosine methylation has been studied by the methods enabling the estimation of 5-methylcytosine (^{m5}dC) amount (paper chromatography and HPLC) and with the use of restriction enzymes sensitive to cytosine methylation. It has been shown by paper chromatography that the amount of ^{m5}dC in generative organs (inflorescence, flower buds, flowers) is a little higher than that in vegetative ones (shoot, leaf – Chvoyka et al. 1978). Using HPLC method, no differences have been found in DNA methylation between juvenile and mature scions in *Larix decidua* (Greenwood et al. 1989). In *Daucus carota* HPLC analyses performed on 10 different tissues have demonstrated that the level of DNA methylation either decreases or increases during differentiation. Growth and ageing of leaves is accompanied with extensive methylation ranging from 18.5% of ^{m5}dC in the seedlings to 24.0% in the adult plants. In roots, however, during differentiation, DNA methylation decreases from 23.1% in the seedlings to 17.8% in the storage roots (Palmgren et al. 1991). In *Vicia faba* the level of ^{m5}dC,as determined by HPLC, varies from 22.9% in first leaves of seedlings, through the intermediate value in mature leaves (28.8%) to the high level in old leaves (30.7%); cotyledones from seedlings display the highest methylation level – 31.0% (Kaźmierczak 1998). An increase in DNA methylation connected with dedifferentiation has been shown in *Daucus carota* (Palmgren et al. 1991) and by restriction enzymes digestion in *Vicia faba* roots (Frediani et al. 1992). The pattern of restriction enzymes digestion has suggested, similary as in *Daucus carota,* lower DNA methylation in juvenile than in adult leaves of *Corylus avellana* (Diaz-Sala et al. 1995). Recently, with the use of a monoclonal anti-5-methylcytosine antibody and confocal fluorescence microscopy the difference in DNA methylation level between generative and vegetative nuclei in pollen of *Nicotiana tabacum* has been demonstrated. The intensity of staining of the generative nucleus decreases and reaches 1/5 of that of the vegetative nucleus (Oakeley et al. 1997).

It is known that most tissue-specific genes are activated through demethylation (ref. Razin and Cedar 1991). In storage tissue, endosperm, several DNA sequences coding for storage proteins are specifically transcribed. Digestion with methylation-sensitive restriction enzymes and Southern blots have revealed in *Zea mays* endosperm a specific and intensive undermethylation of sequences coding for zeins and gluteins (Bianchi and Viotti 1988, Lund et al. 1995). Similarly in barley endosperm specific demethylation of B-hordein has been found in promoter and adjacent coding region, whereas leaf DNA has been highly methylated at these regions (Sørensen 1992). Organ-specific demethylation occurs at two phytohemaglutinin genes in cotyledons of *Phaseolus vulgaris* (Riggs and Chrispeels 1990). In tomato in spite of the fact that HPLC analyses have not demonstrated any significant differences in overall DNA methylation between green and ripe fruit, Southern blot analyses have revealed a decrease in methylation of sequences containing genes encoding cellulase and polygalacturonidase, enzymes involved in the ripening (Hadfield et al. 1993). In maturing tomato fruit, DNA methylation studied by restriction patterns produced by pairs of izoschizomeres and Southern hybridization, was not detected in DNA sequences containing genes that are actively expressed in chromoplasts (Ngemparasirtsiri et al. 1988).

The aim of this study was to find the differences in DNA methylation level among three different types of organs and tissues with various functions: root meristem, where cells di-

visions occur, adult leaf involved in photosynthesis and developing storage tissue, endosperm.

MATERIAL AND METHODS

Plant material

The object of our study, *Haemanthus katharinae* Bak. is a diploid species (2n=18, Vosa and Marchi 1980) with very large genome size (2C=117±2.24 pg DNA, Olszewska and Osiecka 1982). Plants were grown under natural light conditions. Root meristems (first 3 mm long apical parts) from seedlings, fully developed leaves and endosperm isolated from young seeds when the increase in proteins is the most intense (Marciniak 1993b) were compared. Among tissues used in the experiments DNA endoreplication occurs only in endosperm (Marciniak 1993a; root – Olszewska and Osiecka 1982, leaves – this study).

DNA extraction

Total high molecular mass DNA was prepared from frozen tissues according to Dellaporta et al. (1983). Purification procedure and plasmid DNA preparation were performed as described earlier (Sakowicz et al. 1994).

HPLC analysis

20 µg of purified DNA in TE buffer was enzymatically hydrolised to nucleosides by P1 nuclease (Boehringer Mannheim) and bacterial alkaline phosphatase (Amersham). DNA digestion and deoxynucleosides separation were performed according to Gehrke and Kuo (1984). Modified parameters consisted in flow-rate of 1.0 ml / min at 23°C. dA, dC, ^{m5}dC, dG, dT were monitored at 258 nm, 280 nm, 280 nm, 255 nm , 267 nm respectively. The mol percent of nucleoside was calculated from the areas under peaks using external standards. Peaks were identified by retention times using deoxynucleosides standards (Sigma). Measurements were repeated three times on two independent samples of DNA. Similar procedure was applied to identify ^{m6}adenine.

Restriction endonucleases digestion

DNA was digested as specified by the manufactures (Sigma). The following restriction enzymes have been applied: HpaII (5...CCGG...3, does not cleave when the 5residue is ^{m5}dC), MspI (5...CCGG...3, inactivated by methylation of 5C, but can cleave when the internal residue is methylated), MboI (5...GATC...3, sensitive to A methylation), Sau3A (5...GATC...3, does not cleave when C is methylated) and HhaI (5...GCGC...3, inactivated when 5C is ^{m5}dC). DNA fragments were electrophoretically separated on 1 % agarose gel with 22.0, 9.0, 2.8 and 0.9 kb fragments as standards.

Southern hybridization

From cloned HaeIII repetitive sequence family from *Haemanthus katharinae*, characterized earlier (Sakowicz et al. 1994), the 400 bp fragment present in 8.24×10^5 copies was chosen as a probe; this sequence constitutes 1.62% of the haploid genome. The probe was labelled by the nick-translation procedure with ^{32}P-ATP. After gel electrophoresis DNA was denatured and transferred to a nylon-66 membrane NY 45 (Serva). The stringency of hybridization and washing conditions were as described by Sambrook et al. (1988).

Cytophotometry of nuclear DNA

Apical parts of roots, young and adult leaf, and endosperm were fixed in MAF (methanol, formalin, glacial acetic acid 80:15:15, v/v) for 4h at 4°C, then washed and stored in 70% methanol. MAF fixation instead of ethanol-acetic acid fixation has been applied to avoid an error due to the presence of tannins which interfere with the Feulgen reaction (Greilhuber 1988). All kinds of tissue samples were submitted to the Feulgen procedure together. The material was hydrolized with 4N HCl at room temperature for 1h. The tissue samples were incubated in Schiff's reagent made of pararosaniline (Sigma) for 1h in the dark, at room temperature. After washing in three changes of SO$_2$ water, squashed preparations were made. 2C and 4C values were calculated from telophase and prophase nuclei, respectively. Cytophotometric measurements of 100 nuclei from each series were performed at λ=550 nm with CCD camera based computer Image Processing System IMAL 512. The results are expressed in C values.

In situ nick-translation

For in situ nick-translation HhaI was chosen as this restriction enzyme produced the most spectacular differences in DNA digestion among the studied tissues (Fig. 2). The procedure was based on the protocoles developed by Sperling et al. (1985) and Pramora and Ferraro (1990). After fixation and rehydration material was macerated with 20% pectinase and 2% cellulase (Sigma, citric buffer, pH 7.0). Next the samples were squashed under the cover slip with the use of dry ice method. Air-dried preparations were submitted to digestion with HhaI (Sigma), 0.5U/ml in the appropriate buffer, for 6h at 37°C; control slides were treated with the buffer alone. After washing, the preparations were air-dried and placed in the nick-translation mixture, containing dATP, dCTP, dGTP (Promega) and ^{3}H-TTP (Amersham). After 1h incubation slides were washed and dry preparations were coated with LM1 emulsion (Amersham) and exposed for 6 days. After development preparations were stained with Mayer's hematein. Grain counting was performed with the use of ocular square lattice and calculated per 100 µm^2 as the nuclei under study differ in DNA content: 2-9C in root meristem, 2C in leaves (Table 2) and 3-24C in endosperm (Marciniak 1993 a). The number of grains in the background was detracted from that found over nuclei.

RESULTS

HPLC

Comparison of the level of DNA methylation as measured by HPLC in root meristem, adult leaf and developing endosperm (Fig. 1, Table 1) indicates that the highest amount of ^{m5}dC is present in an adult leaf and the lowest in endosperm. In meristem ^{m5}dC content approaches that in endosperm but is a little higher (Table 1).

TABLE 1. Base composition of various DNA samples from *Haemanthus katharinae* in mol% and methylation level – m.l. in %

Tissue/organ	dC	^{m5}dC	dG	dT	dA	m.l.
Root meristem	11.9	7.8	19.7	30.3	30.3	39.5
Adult leaf	10.7	9.3	19.7	30.2	30.1	46.8
Endosperm	12.4	7.3	19.7	30.3	30.3	37.1

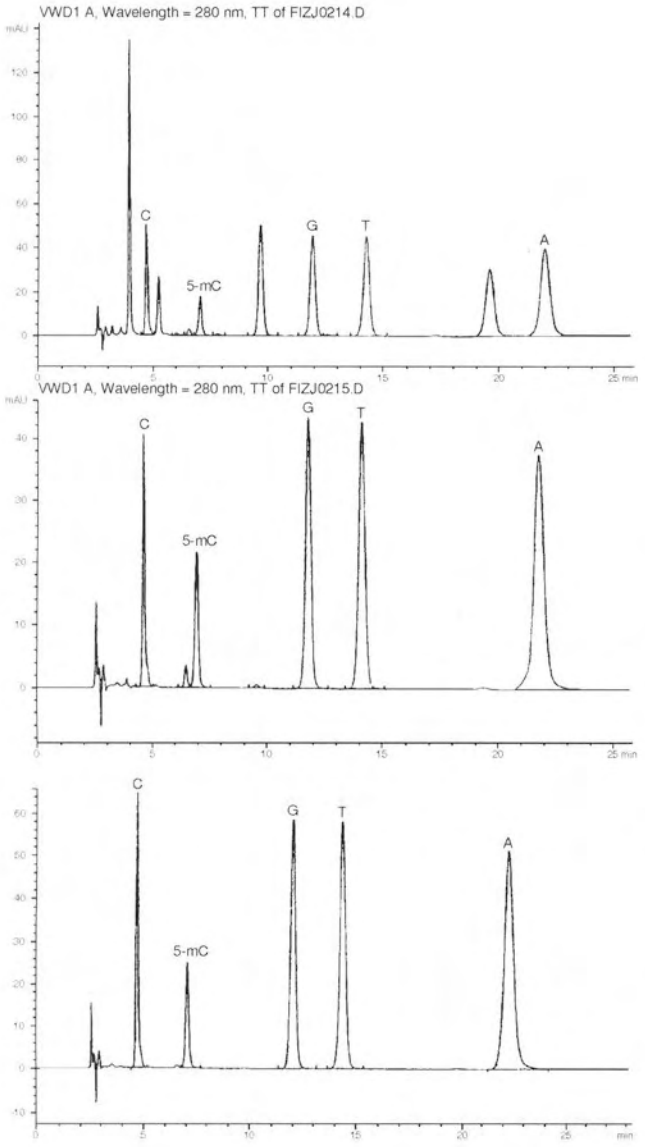

Fig. 1. Separation of DNA deoxynucleosides from a) root meristem, b) adult leaf, c) endosperm.

Restriction analysis and Southern hybridization

A characteristic smear of high-molecular weight hybridization to the probe can be observed in lanes 1,2 – 6,7 – 11, 12 (HpaII and MspI digest – Fig. 2). Intense hybridization signals are obtained with DNA isolated from endosperm, without distinct differences between HpaII and MspI (lanes 1 and 2). Similar results are visible on DNA digests from meristem (lanes 6 and 7) however with MspI the smear is longer (lane 7). In contrast to the above, hybridization signals in lanes 11 and 12 (leaf) are distinctly different. Much shorter DNA fragments are present in lane 12 (MspI). The occurrence of a smear at the high molecular weight region after HpaII digestion (lane 11) suggests extensive 5C methylation in CCGG sequence in leaf DNA. The extensive digestion of DNA with Sau3A (lanes 3, 8, 13) and MboI (lanes 4, 9, 14) results in DNA fragments of approximately 3.7-0.6 kb (meristem, leaf), while in lane 3 hybridization smear moves down to 2.8-06. Kb (endosperm). This pattern demonstrates low level of

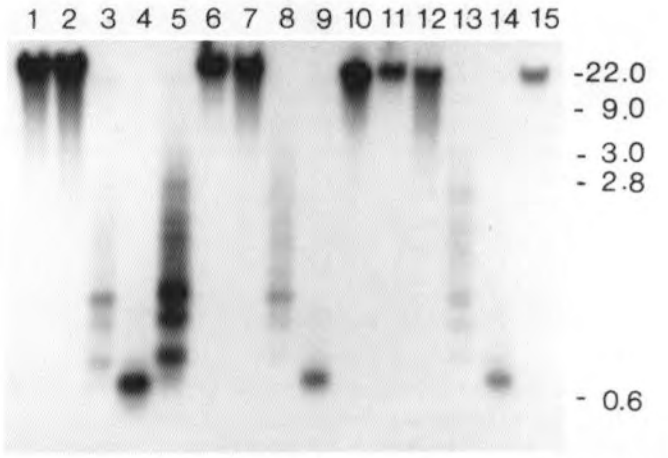

Fig. 2. Southern hybridization of repetitive sequence HaeIII 400 bp from *Haemanthus katharinae* as a probe to digestion products from endosperm (lanes 1-5), root meristem (lanes 6-10) and adult leaf (lanes 11-15); lanes 1, 6, 11 – HpaII, lanes 2, 7, 12 – MspI, lanes 3, 8, 13 – Sau3A, lanes 4, 9, 14 – MboI, lanes 5, 10, 15 – HhaI

methylation in GATC sequences, particularly in endosperm. Moreover, the signal in this band is more intense after MboI (lane 4) as compared to the MboI digestion products in lanes 9 and 14 which could indicate low adenine methylation in GATC sequence in endosperm. However, HPLC analysis does not demonstrate the presence of methyl-6-adenine. The most spectacular results are obtained with HhaI (lanes 5, 10, 15). The restriction patterns of the tested DNAs show in endosperm the presence of fragments ranging in size from 9.0 to 0.6 kb (lane 5) from 22.0 to 3.0 kb in meristem (lane 10) and from 22.0 to 9.0 kb in leaf (lane 15). These results show that DNA from leaf is extremely resistant to HhaI indicating extensive methylation of the internal C in GCGC sequence. On the contrary, DNAs from meristem, and particularly from endosperm, are deeply digested by this restriction enzyme.

DNA content

DNA content in meristem ranges from 2C to 4C, thus corresponding to G_1, S and G_2 phases of the cell cycle (Table 1). In young leaf nuclei with DNA content below 2C value appear and such nuclei constitute dominant population in an adult leaf (Table 2).

TABLE 2. DNA content (C) in different tissues/organs of *Haemanthus katharinae*.

	Number of nuclei			
Tissue/organ	1-2C	2C	2-4C	4C
Root meristem	0	28	46	26
Young leaf	23	77	0	0
Adult leaf	67	33	0	0

Nick-translation

Results of nick-translation in situ with the use of HhaI indicate relative number of target sequences in which 5C is not methylated (positive signals, i.e. silver grains in autoradiograms) or methylated (lack of silver grains, Fig. 3). The higher number of grains the lower is ^{m5}C methylation. Number of grains over 100 μm^2 of nuclear area is the highest in

TABLE 3. Number of grains per 100 mm^2 after HhaI-driven in situ nick-translation in different tissues/organs of *Haemanthus katharinae*.

Tissue/organ	Number of grains
Root meristem	23.3 ± 0.8
Adult leaf	9.8 ± 0.5
Endosperm	40.6 ± 1.5

All the differences statistically significant at P

endosperm and the lowest in adult leaf (Table 3). In control preparations silver grains were scarce and present mainly in the background. Therefore, it can be concluded that in GCGC sequence 5C is extensively methylated in adult leaf, number of grains being about four times lower than that in endosperm and about more than two times lower than that in meristem. Difference between meristem and endosperm amounts to less than twice.

DISCUSSION

We have assessed by three methods the differences in cytosine methylation in root meristem, adult leaf and developing endosperm. The results are compatible when HhaI digestion patterns and in situ nick-translation are compared (Fig. 2 and 3, Table 3). HPLC method, however, shows less spectacular differences between meristem and endosperm (Table 1) which can be caused by cytosine methylation/demethylation in other sequences than the target one (GCGC). Such differences are discussed in Results. Paralelly with methylation status of the total genomic DNA, methylation/demethylation of the repetitive sequence 400bp takes place, and the differences are the most evident after HhaI digestion (Fig. 2 lanes 5, 10 and 15). It is noteworthy that this sequence, similarly to other members of HaeIII repetitive family, is present in all three tissues studied (Sakowicz et al. 1994).

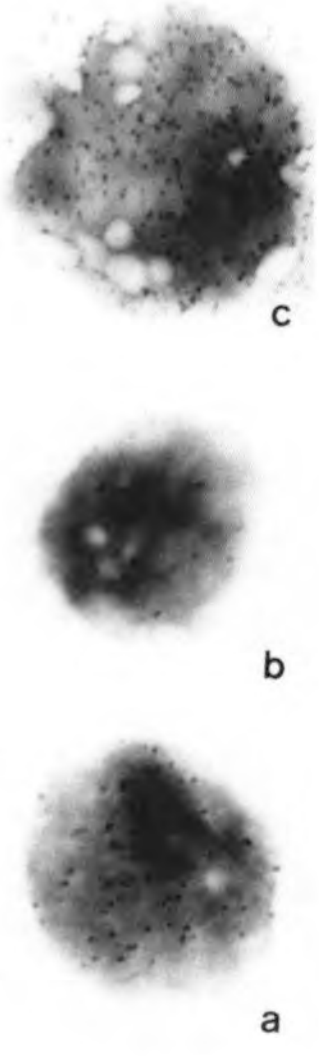

Fig. 3. HhaI-driven nick-translation in situ with ^3H TTP in nuclei from a) root meristem, b) adult leaf, c) endosperm; x 1000

The low level of DNA methylation can be related to high transcriptional potential. In different organs of *Vicia faba* sensitivity to DNase I digestion which shows the potential transcriptional activity is reversely related to the DNA methylation level (Kaźmierczak 1998 and ref.). In *Haemanthus katharinae* both in root meristem and in endosperm at the studied stage of its development cell divisions take place. Similarly, in suspension culture of *Daucus carota* the fraction enriched in meristematic cells has lower level of DNA methylation (22.1%) than the fraction enriched in vacuolised cells (25.3%, Palmgren et al. 1991). In endosperm along with cells divisions, DNA endoreplication and extensive protein synthesis occurs (Marciniak 1993 a and b), thus the transcriptional activity should be higher than in root meristem. The lowest level of DNA methylation in endosperm may be partly involved in regulation through demethylation of specifically transcribed sequences coding for storage proteins, as it has been demonstrated in maize (Bianchi and Viotti 1988, Lund et al. 1995) and barley (S_rensen 1992) endosperm, but also could be related in general to the functional status of this storage tissue.

The above rules, however, were not always observed in in vitro conditions. In *Nicotiana tabacum* the highly repetitive sequence HRS60 once demethylated by 5-azacytidine treatment is maintained in hypomethylated state at various stages of differentiation, e.g. protoplast regeneration, non-differentiated callus growth, and plant regeneration (Koukalova et al. 1994). In tobacco suspension cultures under osmotic stress reversible hypermethylation of the external cytosine at CpCpG trinucleotides, but not at CpG dinucleotides, takes place in non transcribed heterochromatic sequences (Kovarik et al. 1997). In *Daucus carota* tissue cultures (root explants) in proliferative tissue conditioned by kinetin an extensive reduction in DNA methylation has been demonstrated by restriction pattern (Arnholdt-Schmitt et al. 1991).

In adult leaf of *Haemanthus katharinae* the number of nuclei with DNA content below 2C is greater than in young ones (Table 2). Similar observations have been made in other perennial and annual monocotyledonous species (Kuran 1993 and ref.) and in dicotyledonous species (Olszewska et al. 1986 and ref.). The common feature of nuclei from adult and old leaves is chromatin compactness, characteristic for ageing tissues. It is known that the more condensed chromatin is the longer hydrolysis time is necessary to reveal the Feulgen reaction product (Olszewska et al. 1986 and ref.). Chromatin condensation is due to DNA methylation related to increased binding of histone H1 (Adams 1995). In *Haemanthus katharinae* the highest level of DNA methylation occurs in a mature leaf (Table 1 and 3) where nuclei are highly condensed (Fig. 3). Taking into account that the number of nuclei with compact chromatin and with DNA content below 2C increases with ageing, one might suppose that DNA methylation increases as well, in agreement with the results presented by

Palmgren et al. (1991), Diaz-Sala et al. (1995) and Kaźmierczak (1998).

In conclusion, our results show that in *Haemanthus katharinae* various tissues display different levels of DNA methylation and these differences can be related to the cell function. It is possible that methylation of cytosine might be involved in the epigenetic control of plant development.

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TKANKOWO SPECYFICZNA METYLACJA DNA U *HAEMANTHUS KATHARINAE* BAK. (AMARYLLIDACEAE)

STRESZCZENIE

Porównano poziom metylacji DNA w merystemie korzeniowym, dojrzałych liściach i bielmie jednoliściennej rośliny (*Haemanthus katharinae*). Do oceny stopnia metylacji wykorzystano HPLC, analizę restrykcyjną, hybrydyzację typu Southern i nick-translację in situ po trawieniu restryktazą HhaI. Najwyższą zawartość ^{m5}C stwierdzono w dojrzałych liściach, gdzie chromatyna jądrowa wykazuje szczególnie wysoki stopień kondensacji, natomiast najniższą w bielmie. Potencjalny poziom metylacji sekwencji repetytywnej HaeIII 400 pz odpowiada poziomowi metylacji w całkowitym DNA.

SŁOWA KLUCZOWE: metylacja DNA, merystem korzeniowy, liść, bielmo, *Haemanthus katharinae*.