Theor Appl Genet (1997) 95:301–306

A. Kovařik · B. Koukalová · M. Bezděk · Z. Opatrný

Hypermethylation of tobacco heterochromatic loci in response to osmotic stress

Received: 26 November 1996 / Accepted: 20 December 1996

Abstract Plants have to cope with a number of environmental stresses which may potentially induce genetic and epigenetic changes and thus contribute to genome variability. In the present study we inspected the DNA methylation status of two heterochromatic loci (defined with repetitive DNA sequences HRS60 and GRS) in a tobacco cell culture exposed to osmotic stress. Investigations were performed on a TBY-2 cell suspension culture, and the stress was elicited with NaCl or D-mannitol. Using the restriction enzymes *Msp*I/*Hpa*II and *Mbo*I/*Sau*3AI in combination with Southern hydridization we observed a reversible hypermethylation of the external cytosine at the CpCpG trinucleotides in cells grown under mild osmotic stress equal to a NaCl concentration of 10 g/l. There were no changes in the methylation of the internal cytosine as the CpG dinucleotides within the CCGG motifs (*Hpa*II sites) appeared to be fully methylated in tobacco DNA repetitive sequences under normal physiological conditions. The data suggest epigenetic changes in the plant genome based on *de novo* methylation of DNA in response to environmental stress.

Key words DNA methylation · Repetitive sequences · Salt stress · Tobacco

Introduction

Abiotic stress such as drought, salinity and cold are the main limitating factors of plant growth, distribution and crop yield. While number of physiological studies have described the pleiotropic effect of environmental stresses on plant metabolism, considerably less information exists on the genetic and epigenetic impacts of stress. There are, however, a few well-described examples of stress-induced variability of plant genomes. In flax rapid genomic changes accompanied by heritable phenotypic changes were induced by environmental stress (Cullis 1986). The ploidy of genome of a highly stress-resistant plant, *Mesembryanthenum crystallinum*, has also been reported to be influenced by stress (Bohnert et al. 1995). DNA methylation polymorphisms of repetitive sequences have been frequently observed during tissue-culture propagation (Kaeppler and Phillips 1993; Smulders et al. 1995) and may contribute to the phenomenon termed as "somaclonal variation" described by Larkin and Scowcroft (1981). The mechanisms leading to such variations remain, however, largely unknown.

The methylation of cytosine residues is by far the most common modification of DNA in higher eukaryotes, forming a molecular basis for epigenetic cell memory (reviewed by Monk 1995). Plant DNA is usually heavily methylated at CpG dinucleotides, CpNpG trinucleotides (Gruenbaum et al. 1981) and occasionally at assymetrical targets (Meyer et al. 1994; Ingelbrecht et al. 1994). It now seems that CpNpG sequences are methylated by a different enzyme than the CpG dinucleotides (Pradhan et al. 1995). DNA methylation provides one mechanism for altering the expression pattern of the gene by affecting the local structure of the chromatin (Lewis and Bird 1991). While hypermethylation in general is correlated with gene inactivity (Matzke and Matzke 1991), hypomethylation, at specific sites or in specific regions, is correlated with active transcription (Jones and Buckley 1990). In plants, experimentally induced hypomethylation has been correlated with a number of phenotypical changes, e.g., onset of flowering (Burn et al. 1993; Ronemus et al. 1996), dwarfism (Sano et al. 1990;

Communicated by R. Hagemann

A. Kovařík (✉) · B. Koukalová · M. Bezděk
Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic

Z. Opatrný
Research Institute of Crop Production, Ministry of Agriculture, Department of Plant Physiology and Molecular Biology, Drnovská 507, 161 00 Prague 6, Czech Republic

Vyskot et al. 1995) and hermaphroditism (Janoušek et al. 1996), thereby suggesting the involvement of DNA methylation in plant development (King et al. 1994).

In the study presented here our aim was to determine whether osmotic stress induces changes in the methylation patterns of repetitive sequences in tobacco. Evidence was obtained for a reversible hypermethylation of repetitive sequences at CpCpG targets in cells treated with salt or mannitol. We propose that stress-induced hypermethylation of a genome might be a part of plant adaptation mechanisms to abiotic stresses.

Materials and methods

Plant material and stress treatments

In most of the experiments tabacco suspension cell culture TBY-2 cells were used (detailed description of the cell line is given in Nagata et al. 1992). Cells were cultivated in standard liquid MS media (Murashige and Skoog 1962) supplemented with 3% sucrose, KH_2PO_4 (200 mg/l), thiamine (1 mg/l), Myo-inositol (100 mg/l) and 2,4-D (0.2 mg/l) in 100-ml Erlenmeyer flasks under constant shaking (130 rpm) at 27°C. Suspension cultures were subcultured every 1–2 weeks at 1:20 dilutions. For non-ionic osmotic and ionic stress treatments D-mannitol (171 m*M*) or NaCl (5 or 10 g/l, i.e. 171 m*M* and 342 m*M*, respectively) was added to media containing freshly subcultured cells, and the treatments lasted 7 days.

DNA was isolated by a modified cetylammonium bromide method (Saghai-Maroof et al. 1984). TBY-2 cells were sedimented and freeze-dried prior to homogenization in liquid nitrogen. DNA after the isopropanol precipitation step was treated with RNase-A (40 μg/ml) for 10 min at 37°C and then with Protease-K (0.1 μg/ml) for 2 h at 52°C. Enzymatic treatments were terminated with phenol-chloroform extraction and ethanol precipitation. DNA concentration was estimated by measuring the optical density at 260 nm.

Restriction enzyme analysis and Southern blot hybridization

The methylation status of genomic domains specified by the probes HRS60.1 (Koukalová et al. 1989) or GRS1.3 (Gazdová et al. 1995) was analysed using the restriction enzymes *Msp*I, *Hpa*II, *Mbo*I and *Sau*3AI. DNA was digested with an excess of these enzymes (10 U/μg DNA) added at two 3-h intervals to the reaction mixture. Digested DNAs (3–4 μg/lane) were subjected to electrophoresis on 0.8% agarose gels. Following electrophoresis, the ethidium bromide-stained gels were photographed, blotted onto membranes (Pall, USA) and hybridized to [^{32}P] labelled DNA probes ($>10^8$ dpm/μg DNA, Decaprime kit, Ambion). After washing under high-stringency (HRS) or medium-stringency (GRS) conditions membranes were autoradiographed for 4–16 h (Sambrook et al. 1989). For the detection of chloroplast genome the pTB29 probe carrying the *rbc*L gene and part of the *atp*B gene (Sugiura et al. 1986) was used in Southern hybridizations.

Results

Stress-induced hypermethylation of non-transcribed heterochromatic loci

DNA was isolated from tobacco TBY-2 cells grown for 7 days in media containing different concentrations of NaCl or mannitol, and the methylation status of two families of highly repetitive non-transcribed DNA sequences, HRS60 and GRS (Koukalová et al. 1989; Gazdová et al. 1995), was investigated. To study methylation of DNA in these sequences, we digested the DNA with isoschisomeric pairs of restriction enzymes differentially sensitive to cytosine methylation: *Msp*I/*Hpa*II cutting at CCGG and *Mbo*I/*Sau*3AI cutting at GATC sequences. *Msp*I will not digest DNA that is methylated at the external C, whereas *Hpa*II will not digest DNA methylated at either C (Nelson et al. 1993). *Mbo*I, in contrast to *Sau*3AI, is entirely insensitive to cytosine methylation. The digestion of control DNAs with *Msp*I (Figs. 1 and 2) produced ladders typical of repetitive sequences, indicating the absence of methylation at the external C at some cutting sites. In contrast, DNA isolated from cells treated with NaCl (Fig. 1) or mannitol (Fig. 2A) migrated mostly as high-molecular-weight relic DNA due to the inhibition of cleavage with this enzyme. This observation suggests that many sites originally methylation-free underwent "*de novo*" methylation under stress. *Sau*3AI digested control DNA to fragments of less than 3 kb with essentially no high-molecular-weight fraction (Figs. 1 and 2A). On the other hand, a lengthy smear of high-molecular-weight fragments more than 3 kb in length was detected in Southern hybridizations with DNA from stressed cells, suggesting the increased methylation of C within the GATC recognition site. Quantitative evaluation of the samples in Fig. 2A, lanes 7 and 8, showed that the monomeric band corresponding to the methylation-free units of the HRS60 sequences had been reduced by 24% in DNA from mannitol-treated cells (Fig. 2B). In contrast, the same DNA clearly yielded a higher proportion of longer fragments (starting from peak 9) due to the higher frequency of *Sau*3AI refractory sites. Methylation-insensitive *Mbo*I izoschisomere cleaved DNAs from control and osmotically stressed cells equally well, indicating the completed digestions. The methylation status of C within the GATC sequence will depend on the flanking sequence. The sequence at the restriction site in the HRS60 sequences is GATCCG (Koukalová et al. 1989), and therefore the cytosine might be methylated as a part of a CpCpG trinucleotide – cytosine methylation in plants occurs most frequently at the CpG or CpNpG sequences (Gruenbaum et al. 1981). Digestion with *Hpa*II did not reveal any differences between control and osmotically stressed cells, probably due to almost complete methylation of the internal cytosine within the CCGG sequence (Fig. 1A). A similar lack of cleavage with *Hpa*II (digests were indistinguishable from nondigested controls) has also been observed for GRS and other tobacco highly repetitive sequences (Kovařík et al. 1994; Gazdová et al. 1995). There were no differences in the *Msp*I/*Hpa*II and *Mbo*I/*Sau*3AI cleavage patterns in hybridizations using the pTB29 probe, indicating the absence of methylcytosine in the

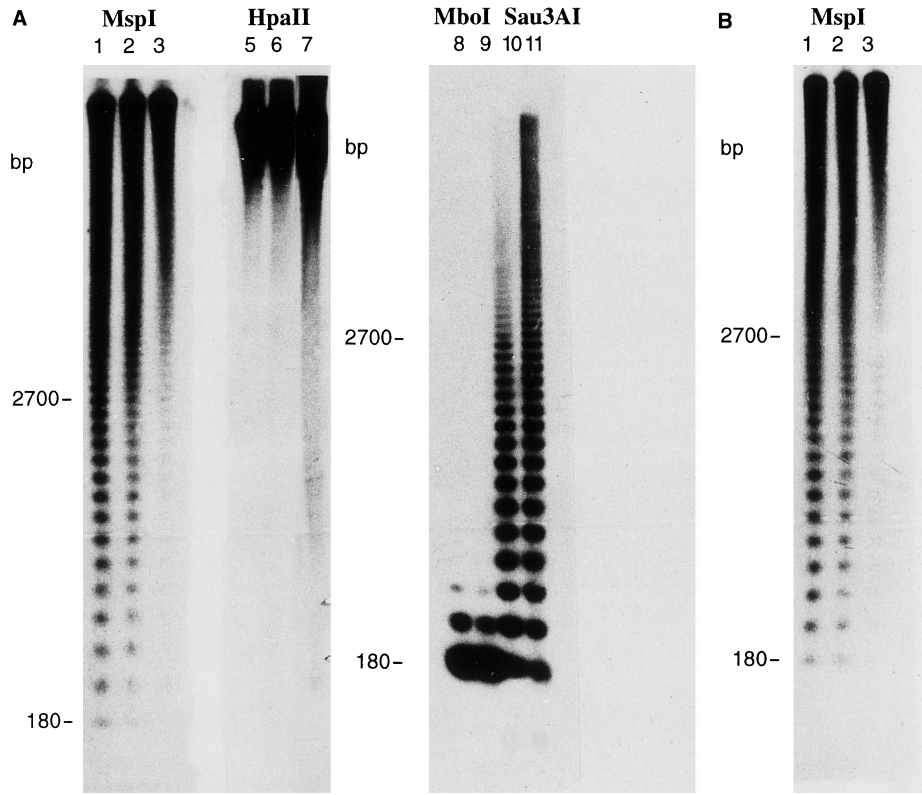


Fig. 1A, B Southern hybridization of DNA from TBY-2 cells exposed to NaCl stress. *Lanes 1, 5, 8, 10* Control DNA, *lanes 2, 6* NaCl (5 g/l), *lanes 3, 7, 9, 11* NaCl (10 g/l) **Panel A** Hybridization with the tobacco subtelomeric HRS60 probe; **panel B** hybridization with the pericentric GRS probe

chloroplast genome in both control and stressed cells (Fig. 3).

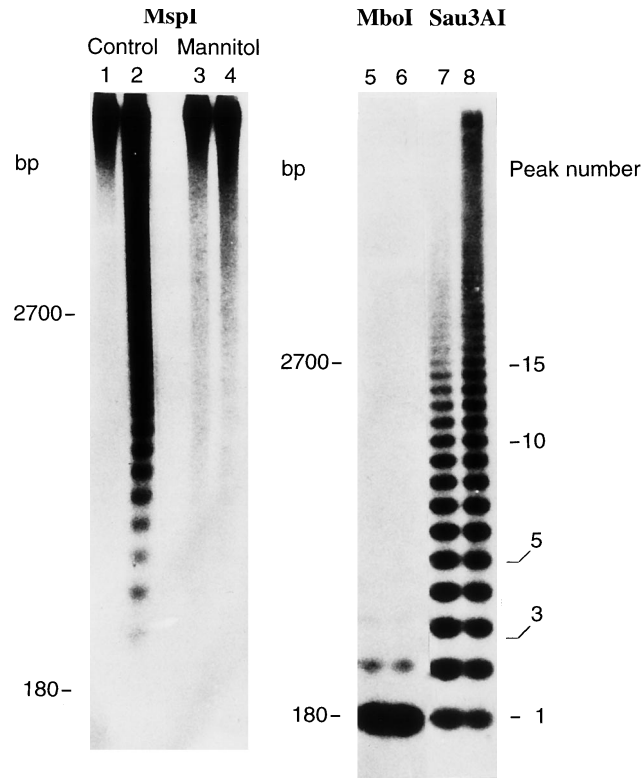
Stressed-induced hypermethylation is reversible

It was of interest to find out whether the hypermethylation of DNA was reversible after the transfer of cells to normal growth conditions. Cells were grown under salt stress for 30 days, and then approximately 5×10^6 cells were transferred to 50 ml of MS growth medium. Cells were grown for 1 week and then subcultured. Figure 4 shows that DNA from cells transferred to standard medium was digested with *Msp*I to a similar extent as DNA of control cells, suggesting the progressive loss of methylated cytosine when normal growth conditions are reestablished.

Discussion

We describe here the increased methylation of tobacco repetitive sequences in response to osmotic stress. Since the investigated loci represent about 3% of the nuclear genome (Koukalová et al. 1989; Gazdová et al. 1995) we conclude that a significant part of nuclear genome could undergo "*de novo*" methylation. On the other hand, two chloroplast genes remained unmethylated, irrespective of growth conditions. This may suggest that the observed hypermethylation of tobacco DNA is rather specific to nuclear sequences. The removal of the stress by transferring the cells into the standard medium resulted in demethylation and the establishment of methylation patterns comparable with those of control DNA, demonstrating reversibility of the described phenomenon.

An interesting feature of stress-induced hypermethylation was the preferential methylation of the external cytosine at the CpCpG trinucleotides. Internal cytosine residues are extensively methylated, even under normal growth conditions, in the HRS60 and GRS sequences (Kovařik et al. 1994). It is therefore possible that the CpCpG trinucleotides represent the only methylatable targets capable of "*de novo*" methylation in tobacco highly repetitive sequences. Methylation of both cytosines in the CpCpG targets has also been detected in repetitive sequences of other plant species, e.g. Petunia (Prols and Meyer 1992), alfalfa (Xia et al. 1995) and tomato (Smulders et al. 1995). Interestingly, genomic sequencing of the tissue-specific hordein promoter failed to reveal methylation at CpNpG targets even in the transcriptionally inactive state (Sorensen et al. 1996). These data show apparent compartmentalization of CpG and CpNpG methylation sites in plant genomes and preferential localization of the CpNpG methylation in highly repetitive sequences. Based on our results we propose that the methylation



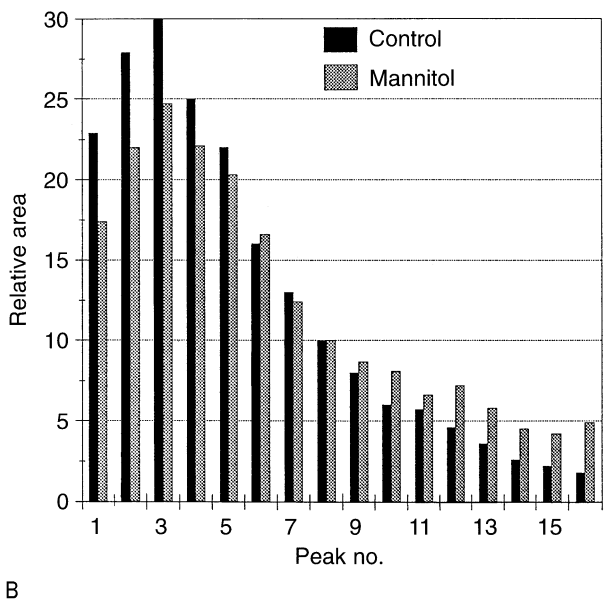


Fig. 2A, B Southern hybridization of DNA from TBY-2 cells treated with 171 m*M* mannitol. **A** *Lane 1, 3* Undigested samples showing integrity of DNA, *lanes 2, 5, 7* digestions of control DNA, *lanes 4, 6, 8* digestions of DNA from mannitol-treated cells. Hybridizations were performed with the HRS60 probe. **B** Densitometric evaluation of hybridizations shown in *lanes 7, 8*. The autoradiogram was scanned on the Molecular Dynamics densitometer, and the results were analysed using the ImageQuant software (line integration method). The relative area has been plotted versus peak numbers. The peaks possessed a 180-bp periodicity corresponding to the sequence periodicity of the HRS60 tandem repeat. Peak 1 represents the monomeric unit as is indicated on the *right margin* of Fig. 2A. The graph represents average values from two independent experiments

Fig. 3 DNA methylation analysis of chloroplast genome in TBY2 cell. *Lanes 1, 3, 5, 7* Controls, *lanes 2, 4, 6, 8* 10 g/l NaCl (7 days). The digested DNAs were hybridized to the pTB29 probe (Sugiura et al. 1986).

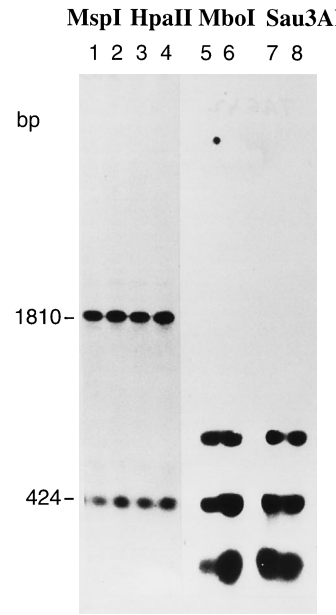
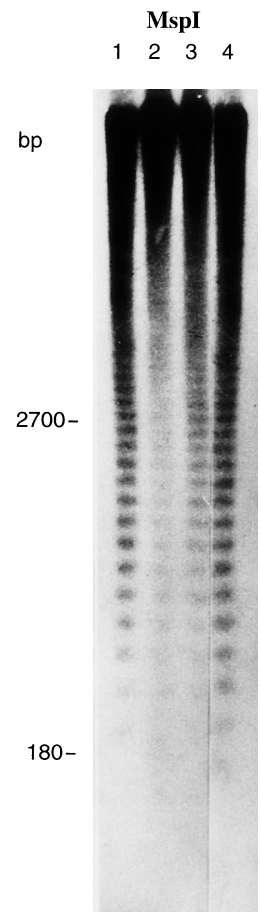


Fig. 4 Reversibility of stress-induced hypermethylation. DNAs were digested with *Msp*I and subjected to Southern hybridization using HRS60 probe. *Lane 1* Control, *lane 2* 10 g/l NaCl (30 days), *lane 3* subculture without stress (7 days), *lane 4* subculture without stress (14 days)



system catalysing cytosine modification at CpNpG trinucleotides may be rather flexible, thereby enabling plants to "buffer" environmental changes at the DNA level. This hypothesis is supported by several studies showing changes in DNA methylation levels in response to alternations in levels of phytohormones (Lo Schiavo et al. 1989; Arnholdt-Schmitt et al. 1991). Interestingly, "*de novo*" methylation of CpNpG motifs has been observed in carrot during initiation of primary cultures (Arnholdt-Schmitt et al. 1995). Cellular differentiation/dedifferentiation and environmental changes may thus be interrelated, both inducing gross changes in the genome, e.g. differential methylation.

In conclusion, we found that tobacco heterochromatic repetitive sequences undergo hypermethylation after exposure of the cells to osmotic and salt stress. We suggest that epigenetic changes at the DNA methylation level might play an important role in plant adaptation mechanisms to environmental stress.

Acknowledgements The authors thank Dr. B. Vyskot for helpful discussions and Mrs. E. Koudelková and Mrs. L. Jedličková for technical assistance. Financial support for this work was provided by the Grant Agency of the Czech Republic, Grant No. 506/95/0353.

References

Arnholdt-Schmitt B, Holzapfel B, Schillinger A, Neumann K-H (1991) Variable methylation and differential replication of genomic DNA in cultured carrot root explants during growth induction as influenced by hormonal treatments. Theor Appl Genet 82:283–288

Arnholdt-Schmitt B, Herterich S, Neumann KH (1995) Physiological aspects of genome variability in tissue culture. I. Growth phase-dependent differential DNA methylation of the carrot genome (*Daucus carota* L.) during primary culture. Theor Appl Genet 91:809–815

Bezděk M, Koukalová B, Kuhrová V, Vyskot B (1992) Differential sensitivity of CG and CCG DNA sequences to ethionine-induced hypomethylation of the *Nicotiana tabacum* genome. FEBS Lett 300:268–270

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptation to environmental stresses. Plant Cell 7:1099–1111

Burn JE, Bagnall DJ, Metzger JD, Dennis ES, Peacock WJ (1993) DNA methylation, vernalization, and the initiation of flowering. Proc Natl Acad Sci USA 90:87–291

Cullis CA (1986) Phenotypic consequences of environmentally induced changes in plant DNA. Trends Genet 2:307–309

Gazdová B, Široký J, Fajkus J, Brzobohatý B, Kenton A, Parokonny A, Heslop-Harrison JS, Palme K, Bezděk M (1995) Chromosome Res 3:245–254

Gruenbaum Y, Naveh-Many T, Cedar H, Razin A (1981) Sequence specificity of methylation in higher plant DNA. Nature 292:860–862

Ingelbrecht I, Van Houdt H, Van Montagu M, Depicker A (1994) Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc Natl Acad Sci USA 91:10502–10506

Janoušek B, Široký J, Vyskot B (1996) Epigenetic control of sexual phenotype in a dioecious plant *Melandrium album*. Mol Gen Genet 250:483–490

Jones AP, Buckley JD (1990) The role of DNA methylation in cancer. Adv Cancer Res 54:1–23

Kaeppler SM, Phillips RL (1993) Tissue culture-induced DNA methylation variation in maize. Proc Natl Acad Sci USA 90:8773–8776

King GJ (1994) The role of DNA methylation in modulation of floral initiation. FNL 18:21–25

Koukalová B, Reich J, Matyášek R, Kuhrová V, Bezděk M (1989) A *Bam*HI family of highly repetitive DNA sequences of *Nicotiana tabacum*. Theor Appl Genet 78:77–80

Kovařík A, Koukalová B, Holý A, Bezděk M (1994) Sequence-specific hypomethylation of tobacco genome induced with dihydroxypropyladenine, ethionine and 5-azacytidine. FEBS Lett 353:309–311

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Lewis J, Bird A (1991) DNA methylation and chromatin structure. FEBS Lett 285:155–159

Lo Schiavo P, Pitto L, Guiliano G, Torti G, Nuti-Ronchi V, Marazzati D, Vergera R, Orselli S, Terzi M (1989) DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor Appl Genet 77:325–331

Matzke MA, Matzke AJM (1991) Differential inactivation and methylation of a transgene in plants by two suppressor loci containing homologous sequences. Plant Mol Biol 16:821–830

Meyer P, Niedenhof I, ten Lohuis M (1994) Evidence for cytosine methylation of non-symmetrical sequences in transgenic *Petunia hybrida*. EMBO J 13:2084–2088

Monk M (1995) Epigenetic programming of differential gene expression in development and evolution. Dev Genet 17:188–197

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. Int Rev Cytol 112:1–30

Nelson M, Raschke E, McClelland M (1993) Effect of site specific methylation on restriction endonucleases and DNA modification methyltransferases. Nucleic Acids Res 21:3139–3154

Pradhan S, Adams RLP (1995) Distinct CG and CNG DNA methyltransferases in *Pisum sativum*. Plant J 7:471–481

Prols F, Meyer P (1992) The methylation patterns of chromosome integration regions influence gene activity of transferred DNA in *Petunia hybrida*. Plant J 2:465–475

Ronemus MJ, Galbiati M, Ticknor C, Chen J, Dellaporta SL (1996) Demethylation-induced developmental pleitropy in *Arabidopsis*. Science 273:654–657

Saghai-Maroof MS, Soliman KM, Jorgensen SA, Allard RW (1984) Ribosomal DNA spacer/length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Sambrook J, Fritsch FE, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Sano H, Kamada I, Youssefian S, Katssumi M, Wabiko H (1990) A single treatment of rice seedlings with 5-azacytidine induces heritable dwarfism and undermethylation of genomic DNA. Mol Gen Genet 220:441–447

Smulders MJM, Rus-Kortekaas W, Vosman B (1995) Tissue culture-induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants. Theor Appl Genet 91:1257–1264

Sörensen MB, Müller M, Skerritt J, Simpson D (1996) Hordein promoter methylation and transcription activity in wild type and mutant barley endosperm. Mol Gen Genet 250:750–760

Sugiura M, Shimozaki K, Zaita N, Kusuda M, Kumano M (1986) Clone bank of the tobacco (*Nicotiana tabacum*) chloroplast genome as a set of overlapping restriction endonuclease fragments: mapping of eleven ribosomal protein genes. Plant Sci 44 : 211–216

Vyskot B, Koukalová B, Kovařík A, Sachambula L, Reynolds D, Bezděk M (1995) Meiotic transmission of a hypomethylated repetitive DNA family in tobacco. Theor Appl Genet 91 : 659–664

Xia X, Du S, Erickson L (1995) A moderately repetitive sequence in alfaalfa is transcribed in a floral-specific manner. Genome 39 : 9–16