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Hypomethylation of CNG targets induced with dihydroxypropyladenine is rapidly reversed in the course of mitotic cell division in tobacco

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Abstract We followed the mitotic transmission of an experimentally induced hypomethylated state of several tobacco repetitive sequences in callus culture and plants. The initial hypomethylation was induced by a hypomethylation drug, dihydroxypropyladenine (DHPA), the competitive inhibitor of cellular S-adenosylhomocysteine hydrolase, which is known to preferentially inhibit methylation at CNG and non-symmetrical motifs while having a negligible effect on methylation at CG motifs. The deprivation of this drug resulted in an almost immediate remethylation of cytosines at CNG motifs (*Msp*I and *Eco*RII sites) leading us to conclude that, the hypomethylation effect of dihydroxypropyladenine is rather transient and differs from that of 5-azacytidine which often induces heritable changes in methylation patterns. The results suggest that de novo methylation of CNG motifs is a rapid and meiotically independent process on DNA sequences with pre-existing CG methylation.

Keywords Plant DNA methylation · S-adenosylhomocysteine hydrolase inhibition · CNG and CG methylation motifs · Sequence remethylation

Introduction

Methylation patterns, once established, are truly mitotically inheritable, thereby providing a convenient molecular tool for cell memory in long-living multicellular organisms. Methylation can provide a signal for various cellular processes, including the control of gene expression and genetic recombination. Despite considerable achievements in isolating and characterizing plant genes involved in methylation processes (for review see Finnegan and Kovac 2000) our knowledge of the mode of de novo generation of methylation patterns is still limited. In *Arabidopsis*, the global demethylation of the genome induced by a mutation in the DDM locus was maintained even after segregation of a mutant gene allele (Kakutani et al. 1999). Antisense MET1 plants maintained hypomethylation after losing a hypomethylation inductor in most sequences even though a centromeric 180-bp repeat was partially remethylated in some plants (Finnegan et al. 1996). 5-Azacytidine, a commonly used hypomethylation drug, incorporates into DNA by replacing cytidine and irreversibly inactivates maintenance methylase. The inheritable effect of 5-azacytidine on plant development has been demonstrated in rice (Sano et al. 1990), tobacco (Vyskot et al. 1995) and *Melandrium* (Janousek et al. 1996). In our previous experiments hypomethylation of the tobacco HRS60 satellite induced with 5-azacytidine was transmitted through mitosis (Koukalova et al. 1994) and partially through meiosis (Vyskot et al. 1995). In polyploid wheat, the hypomethylated ribosomal gene cluster maintained hypomethylation and increased activity after 5-azacytidine treatment (Castilho et al. 1999). Though the results obtained from most genetic and biochemical studies are consistent with the long-term maintenance of hypo-methylation/slow de novo methylation, there are several good examples of reversible methylation that occur during developmental changes (Amasino 1984; Burn et al. 1992), hormonal treatment (Arnholdt-Schmitt et al. 1995; Lo Schiavo et al. 1989) and in stress response (Kovarik et al. 1997). Also, transgenic loci, especially those with a complex genomic organization, have been shown to be powerful inducers of methylation of homologous sequences in trans (Matzke and Matzke 1991; Muskens et al. 2000). A repeat capable of rapid de novo methylation after insertion was isolated from tobacco and *Petunia*, suggesting that the rate of de novo

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methylation could be directed by specific features of a given sequence (Ten Lohuis et al. 1995).

In the present paper we have exploited the previously published ability of dihydroxypropyladenine (DHPA) to specifically hypomethylate CNG (Kovarik et al. 1994) and non-symmetrical sequences in plant DNA (Fulnecek et al. 1998). The drug inhibits DNA methylation through the inhibition of S-adenosylhomocysteine hydrolase, leading to the accumulation of S-adenosylhomocysteine (SAH) (Fojtova et al. 1998; Holy 1975). The apparent sequence specificity of DNA hypomethylation allowed us to study the maintenance of the hypomethylated state at different CNG sequence motifs.

Materials and methods

Plant material

Plants and leaf-derived calluses of *Nicotiana tabacum* L. cv Vielblattriger were used. Calluses were cultured in 50-ml Erlenmeyer flasks (cultivation area) containing MS (Murashige and Skoog 1962) agar supplemented with sucrose and hormones. They were subcultured by cutting callus into small pieces and placing them onto fresh medium. The pieces represented in total about one-tenth of the original callus (or 1–1.5 g). Each passage lasted for 4 weeks. In order to obtain information about division time, we weighed the calluses (wet weight) at the time of transfer and at 4-week intervals of growth. On the basis of data averaged from three independent experiments, the doubling time was estimated to be 9–10 days.

Drug treatments

(S)-9-(2,3-Dihydroxypropyl) adenine prepared according to the described procedure (Holy 1975) was used as a hypomethylating drug. For calluses, the cell culture medium contained 25 μ*M* or 100 μ*M* DHPA. After a 4-week cultivation period, a portion of the calluses was used for methylation analysis, while the remainder was further subcultivated in medium without the drug. Calluses from the first, second and third passages, each lasting for 4 weeks, were analyzed. To induce hypomethylation in the plant, we incubated sterilized tobacco seeds in distilled water containing 20 μ*M* DHPA for 10 days under gentle shaking and dim light conditions. Seedlings were finally transferred to soil and cultivated under standard conditions. For analysis, the seedlings and leaves of adult plants were used.

DNA probes

The cloned tobacco DNA repeats, HRS60 (Koukalova et al. 1989), NTRS (Matyasek et al. 1997), 5S rDNA (Fulnecek et al. 1998) and GRD5 (Bejarano et al. 1996) were used as probes. The subtelomeric HRS60 and intercalary NTRS satellites comprise about 2% and 0.2% of the tobacco genome, respectively; there are about 2,000 copies of 5S rDNA and about 100 copies of endogenous geminiviral GRD5 sequences in the genome.

Southern blot hybridization

Genomic DNA was digested with a fivefold excess of restriction enzymes (2 × 2 h) and subjected to electrophoresis on agarose gels. Approximately 1–2 μg and 5–10 μg of digested genomic DNA per lane was loaded for the detection of high- and medium-copy repeats, respectively. Following electrophoresis, the ethidium bromide-stained gels were photographed, blotted onto membranes (Hybond N+) and hybridized to the [^{32}P]-labelled DNA probes (>10^8 dpmμg^{-1} DNA, Fermentas, Dekaprime kit). Southern hybridization was carried out in 0.25 *M* Na-phosphate buffer, pH 7.0, supplemented with 7% sodium dodecyl sulfate (SDS) at 65 °C for 16 h followed by washing with 2× SSC (1 × SSC = 150 m*M* NaCl, 15 m*M* Na_3-citrate, pH 7.0), 0.1% SDS (twice for 5 min each), then 0.2× SSC, 0.1% SDS (twice for 15 min each). The membranes were exposed to X-ray film for 4–48 h. For quantification of the hybridization bands, the amount of radioactivity in each lane was determined using a PhosphorImager (Storm, Molecular Dynamics) and analyzed using ImageQuant. Completion of enzyme digestion was monitored by Southern hybridization with a chloroplast probe.

Determination of intracellular S-adenosylhomocysteine, S-adenosylmethionine and dihydroxypropyladenine levels

Tobacco callus cells were extensively lyophilized and disintegrated in liquid nitrogen. The biomass was extracted using 0.25 *M* perchloric acid at 4 °C and clarified by centrifugation. The acid-soluble extract was analyzed in a Waters HPLC system (996 PDA Detector, PDA Software Millenium, version 3.05, 616 Pump with 600S Controller) equipped with 15 cm × 3.9 mm Nova-Pak C8, 60-Å, 4-μm reverse-phase column. A three-step gradient at a flow rate 0.75 ml·min^{-1} was used. With (1) solvent A, 50 m*M* sodium phosphate pH 3.2, 10 m*M* heptanesulfonic acid, 50% acetonitrile; (2) solvent B, 50 m*M* sodium phosphate pH 3.2, 10 m*M* heptanesulfonic acid. The program consisted of (1) 9–11% A, 5 min (convex curve no. 3); (2) 11–17% A (concave curve no. 8), 10 min; (3) 100% A, 5 min (curve no. 11, isocratic). Peaks of DHPA, SAH and S-adenosylmethionine (SAM) were identified (UV-spectra library) and quantified with the aid of external standards. The concentration of DHPA and both cofactors was determined relative to the protein content.

Results

Rapid remethylation of CNG motifs in tobacco repeated sequences

The hypomethylation of the tobacco genome was induced in fully dedifferentiated callus culture with dihydroxypropyladenine (DHPA) as previously described (Kovarik et al. 1994). Cells were then transferred to drug-free medium and grown without the hypomethylation drug. Each passage lasted 4 weeks and was equivalent to about three cell divisions. The growth of the cells was not affected by the drug, which easily penetrated cells and was not significantly metabolized (Fojtova et al. 1998). After the transfer of cells into drug-free medium, the intracellular concentration of both DHPA and SAH decreased and normal levels of metabolites were re-established (Table 1, Fig. 3).

To determine the level of CNG methylation we digested DNA with the methylation-sensitive *Msp*I and *Eco*RII restriction enzymes. *Msp*I cannot digest CCGG sites when the outer C is methylated; *Eco*RII is inhibited by methylation of the second C within the CCWGG site (Nelson et al. 1993). To study methylation at different genomic loci, we hybridized the Southern blots against the HRS60, 5S rDNA, GRD5 and NTRS probes (Figs. 1, 2). Digestion of the tandem repeats to the monomeric units indicated the absence of cytosine methylation at the

Table 1 Intracellular changes of SAH and SAM concentrations in dihydroxypropyladenine-treated *Nicotiana tabacum* calluses. The cells were grown on MS agar in the presence/absence of 100 µ*M* dihydroxypropyladenine for 4 weeks. About 1 g of callus tissue was transferred onto drug-free agar and grown for another 4 (Passage l), 8 (Passage II), 12 (Passage III) or 16 (Passage IV) weeks. Wet weight increased about eight-to-ten fold within 4 weeks, which corresponds to approximately three to four cell divisions. Total cell extracts were prepared from total callus mass and subjected to HPLC analysis on C8 reverse phase column

Sample	DHPA (nmol/mg)[a]	SAH (nmol/mg)	SAM (nmol/mg)	SAH/SAM
Control	–	0.792	1.24	0.638
100 µ*M* DHPA	55.556	219.46	2.56	85.714
Passage I	7.102	16.52	1.10	14.99
Passage II	0	2.21	1.51	1.517
Passage III	0	1.49	1.054	1.413
Passage IV	0	0.814	0.724	1.125

[a] Concentrations of individual metabolites are given relative to the protein content

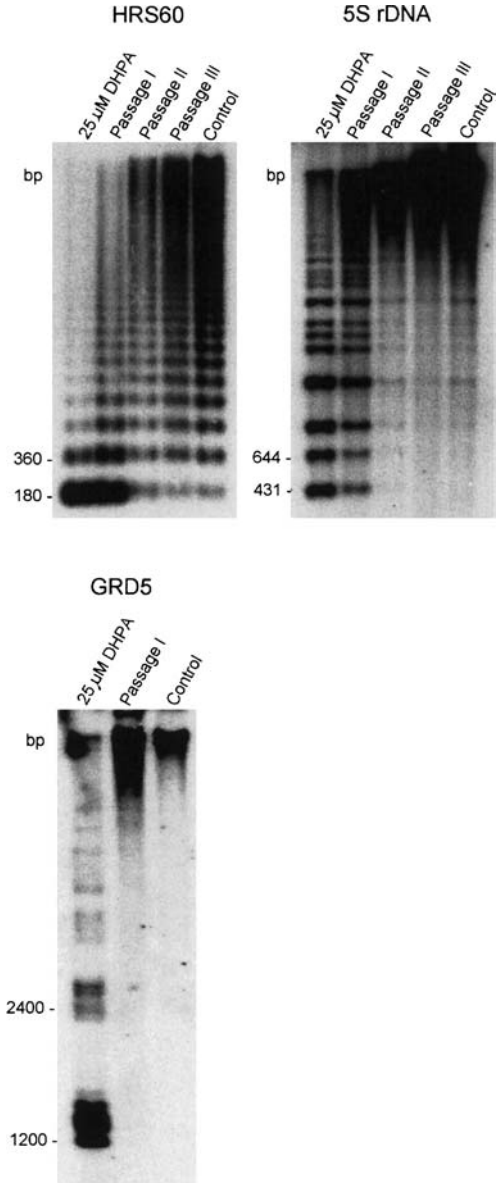

Fig. 1 Induction of hypomethylation and remethylation of CCG DNA motifs in tobacco calluses. Genomic DNA was digested with *Msp*I and hybridized against the HRS60, 5S rDNA and GRD5 probes. Passages I, II, III correspond to 4, 8 and 12 weeks of growth after the removal of DHPA, the methylation inhibitor

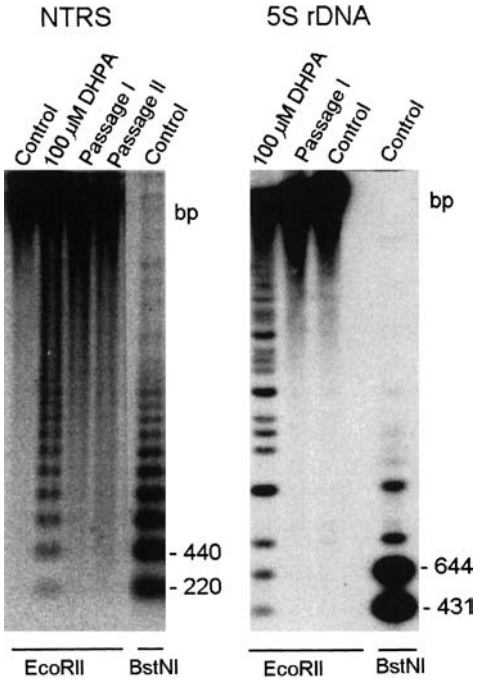

Fig. 2 Induction of hypomethylation and remethylation of CWG DNA motifs in tobacco calluses. Genomic DNA was digested with *Eco*RII and *Bst*NI (methylation insensitive isoschizomere) and hybridized against the NTRS and 5S rDNA probes

target site. In the control non-treated cells most DNA migrated as a high-molecular-weight fraction consistent with the high level of CNG methylation. The HRS60 repeats were more highly digested with *Msp*I than the other sequences, reflecting some variability in methylation levels between loci (Kovarik et al. 2000). Treatment of the cells with DHPA resulted in a considerable increase in the digestibility of DNA with both enzymes. Most HRS60 and GRD5 repeats were digested into the monomeric units with *Msp*I, indicating an almost complete demethylation of the outer C within the CCGG motifs (Fig. 1). The extent of CWG hypomethylation was less pronounced, as indicated by the *Eco*RII "ladders" of fragments (Fig. 2). The transfer of cells into the drug-free medium resulted in a dramatic shift of the ladders of bands upward indicating a decrease of DNA digestibility

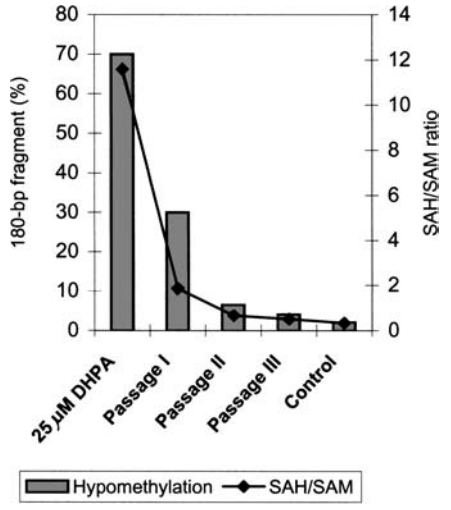

Fig. 3 Correlation of CCG hypomethylation and SAH/SAM ratio in tobacco calluses. The *left y-axis* indicates relative hypomethylation of the HRS60 repeats expressed as the amount of radioactivity in the monomeric band versus total radioactivity in each lane (Fig. 1). The *right y-axis* indicates methylation index (SAH/SAM ratio) in cell extracts. The SAH/SAM values were obtained from an experiment in which 25 μ*M* DHPA was used

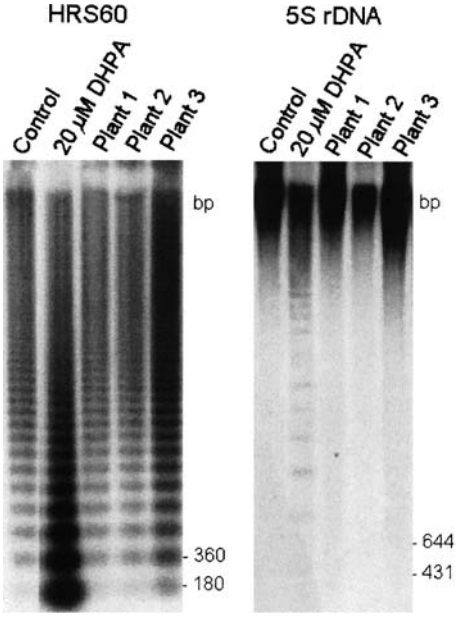

Fig. 4 Drug-induced DNA hypomethylation in tobacco seeds and remethylation during plant growth. DNA was extracted from about 20 seedlings (treated and non-treated controls) and from adult plants (60 days old) germinated in the presence of the drug for 10 days. DNA was digested with *Msp*I and *Eco*RII and hybridized against the HRS60 and 5S rDNA probes, respectively

with both enzymes. A single passage onto the drug-free medium resulted in *Eco*RII patterns indistinguishable from that of the controls. For the HRS60 locus, the remethylation was slower and a small proportion of units remained hypomethylated at *Msp*I (Fig. 1) and *Hpa*II sites (not shown) even after three passages. The extent of hypomethylation was quantified by measuring the radioactivity of bands corresponding to monomeric units relative to the total radioactivity in individual lanes (Fig. 3).

The above data document a rapid remethylation of CNG triplets in callus culture. In order to determine the fate of the hypomethylation state in differentiated plants, we induced hypomethylation in seeds by germinating them in the presence of 20 μ*M* DHPA for 10 days. The viability of the seedlings was not significantly affected by concentrations of the drug lower than 20 μ*M*. Thereafter, plants were grown for 2 months in the absence of inhibitor. In some cases the drug-treated plants displayed reduced growth and aberrant leaf morphology (not shown). The DNA was isolated from both seedlings and adult plants and subjected to methylation analysis. In DHPA-treated seedlings the *Msp*I digested HRS60 sequences mostly into monomers and dimers, while in adult plants high-molecular-weight oligomers appeared (Fig. 4). This indicated that similar to what occurred calluses, the hypomethylation of the outer C within CCGG sites was rather transient and followed by remethylation during plant growth.

Discussion

In the investigation reported here we studied de novo methylation of several tobacco sequences following experimentally induced hypomethylation. The overall hypomethylation was induced with dihydroxypropyladenine, a drug that has previously been shown to elevate S-adenosylhomocysteine levels, thus causing a rather specific hypomethylation of CNG (Kovarik et al. 1994) and non-symmetrical motifs (Fulnecek et al. 1998) in tobacco but leaving the methylation of CG sites relatively intact. Almost 100% of CCG and 30–50% of CWG motifs could be demethylated in 5S rDNA repeats (Fulnecek et al. 1998). The pattern of DNA hypomethylation induced with DHPA was very similar to that observed in chromomethylase mutants in *Arabidopsis* (Lindroth et al. 2001) and maize (Papa et al. 2001) in which the CNG sites underwent hypomethylation while methylation at the CG sites remained largely unaffected. Furthermore, outcrossing of a mutated *Zmet*2 locus resulted in partial restoration of DNA methylation patterns in maize (Papa et al. 2001). However, since the outcrossing procedure requires at least one meiotic cycle it was not possible to determine at which stage of plant development remethylation occurred. In the present investigation we were able to show that remethylation of CNG sites does not require meiosis and that almost complete re-establishment of methylation state is achieved within few mitotic cycles in tobacco callus culture. We demonstrated this for restriction sites for high-copy satellite repeats (HRS60, NTRS), a cluster of endogenous geminiviral sequences (GRD5) and for transcribed 5S rDNA repeats. In plants also CCG hypomethylation was detectable in seedlings that had been germinated in the presence of the drug but not in later developmental stages when the drug was al-

ready diluted, suggesting substantial remethylation in somatic cells. The reversibility of DHPA-induced hypomethylation differs from our previous studies on maintenance of hypomethylation induced with 5-azacytidine. In these studies the *Hpa*II digestibility of HRS60 repeats appeared to be maintained in calluses and regenerated plants (Koukalova et al. 1994), and even in the progeny of the hypomethylated plants (Vyskot et al. 1995). Also genetic studies in *Arabidopsis* methylation mutants are consistent with long term maintenance of hypomethylated state (Kakutani et al. 1999). There is a question of how to interpret differential inheritance of hypomethylation at CG and CNG motifs. There might be several not necessarily mutually excluding explanations.

1) The density of the methylation template might affect the remethylation efficiency in cis. The overall density of CG and CCG methylation along the repeats can be approximately estimated from the sequence. Within the 182-bp to 184-bp HRS60 monomeric unit 10–11 CG dinucleotides and two to three CCG trinucleotides occur (Koukalova et al. 1989). DHPA treatment reduces CCG methylation to almost zero (Fig. 1) while leaving CG methylation almost unchanged (Kovarik et al. 1994), being reduced by 5% at maximum as estimated by genomic sequencing (Fulnecek et al. 1998). It follows that the total CG and CCG methylation of the HRS60 tandems is reduced by about 15%. This might represent a critical threshold for hypomethylation reversibility. In vitro experiments wherein the activity of mammalian methyltransferase was greatly stimulated by methylation of specific sites in oligonucleotide templates (Christman et al. 1995) may lend support to this hypothesis.
2) Remethylation at CNG sites may require interaction with homologous sequences in trans within the framework of the nucleus (Matzke and Matzke 1991). In plants this interaction often seems to rely on RNA molecules (Depicker and Montagu 1997; Wassenegger et al. 1994). However, we have no evidence so far of transcription activity of any of the described repeats except of 5S rDNA. Remethylation induced with de novo methylation ascribed to DNA–DNA pairing has also been described during meiosis in *Ascobolus* (Maloisel and Rossignol 1998). However, extensive DNA–DNA pairing is unlikely to occur within the few mitotic cycles that are sufficient to rebuild methylation of CNG motifs in tobacco callus culture.
3) Finally, it has to be considered that the de novo methylating capacities of CG and CNG methylation enzymes may differ. It has recently been established that CNG methylation in *Arabidopsis* (Lindroth et al. 2001) and maize (Papa et al. 2001) is accomplished by a group of specific enzymes called chromomethylases. Though a tobacco analog of these enzymes has not yet been isolated, it is likely that a similar gene is functional in tobacco. Hypermethylation of a part of the *SUPERMAN* gene in the *Arabidopsis* methylation-deficient mutant (*ddm1*) (Jacobsen and Meyerowitz 1997) could be consistent with an increased capacity of chromomethylase to methylate originally methylation-free DNA. However, in contrast to the *SUPERMAN* locus, de novo methylation of either CG or CNG motifs apparently did not occur in numerous endogenous repeated sequences even after outcrossing of the hypomethylation-inducing *ddm* locus (Kakutani et al. 1999). In 5-azaC-treated tobacco also, some HRS60 units retained increased digestibility with *Hpa*II and *Msp*I for a number of generations in both calluses (Koukalova et al. 1994) and plants (Vyskot et al. 1995). As *Hpa*II is sensitive to methylation of all four cytosines within the CCGG/CCGG quadruplet, this digestion indicates complete demethylation of the target site. We speculate that such completely demethylated sites (and perhaps larger sequences flanking the site) are inefficiently remethylated by both CG and CNG enzyme activities. Dieguez et al. (1997) proposed that methylation at symmetrical sites could provide a signal for overall methylation in the region. Our experiments could support this original model, assuming a pivotal role of mCG in directing methylation of CNG and perhaps nonsymmetrical sites. Once the basic methylation at CG is established, further methylation to non-CG sites may spread rapidly in a highly cooperative manner. The chromatin state dictated by preexisting CG methylation could possibly recruit CNG-type DNA chromomethyltransferase activity to the particular genomic region.

In conclusion, we show that the maintenance of DNA hypomethylation may vary with respect to a dependence on sequence motifs. The specific hypomethylation of CNG motifs without affecting methylation at CG sites leads to rapid reestablishment of the original methylation state.

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