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Meiotic transmission of a hypomethylated repetitive DNA family in tobacco

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Abstract We have recently shown that hypomethylation of cytosine residues in the HRS60 family of repetitive DNA sequences can be induced with 5-azacytidine (5-azaC) in tobacco tissue cultures. We have also proven that such a DNA methylation status is maintained during the recovery of protoplasts, plant regeneration, and vegetative development. In the present paper we follow meiotic transmission of hypomethylated HRS60 DNA. Plants obtained from seeds treated with 5-azaC were either self pollinated or crossed with a non-treated control in a reciprocal way. Analysis of the methylation status of the HRS60 DNA revealed that these sequences were hypomethylated in the progenies up to the extent found in the parental 5-azaC-treated plant. Since no parent-of-origin effect was observed, we presume that both male and female gametes transmit an artificial methylation imprint to a similar extent. This result is supported by methylcytosine evaluation in the total genomic DNA samples. A temporal analysis of 5-azaC effects on germinating seeds and a phenotypic evaluation of 5-azaC-treated tobacco plants are also presented.

Key words 5-azacytidine · DNA methylation
Meiotic transmission · *Nicotiana tabacum* L.
Repetitive DNA sequences

Introduction

A large body of literature has demontrated that DNA methylation is one of the important modifications which is heritable, reversible, and implicated in the control of gene expression. It represents one mode of transmitting epigenetic information in a majority of eukaryotic organisms. Plants often possess large nuclear genomes rich in methylated repetitive DNA sequences. The most common modified base in plant genomes is the cytosine in CG and CNG sequences. Studies on the meiotic inheritance of DNA methylation patterns in plants have so far mainly involved transgenes (for recent reviews see Jorgensen 1993; Matzke and Matzke 1993; Flavell 1994). Foreign genes introduced into plants are often methylated and inactivated, depending on their copy number and site of integration. Generally, with more copies of a sequence, introduced either by sequential transformation steps or by a cross, the likelihood of gene inactivation by methylation increases. DNA methylation as a result of an interaction of homologous DNA sequences can be inherited in the progeny thus transgressing the Mendelian principle that two alleles following passage through the same nucleus appear unaltered in segregating progeny. Data concerning the control of the methylation status of non-transcribed repetitive sequences in the plant genome are as yet lacking.

The most common drug used to modify DNA methylation and to activate silent genes both in mammals and plants is 5-azacytidine (5-azaC). This can be incorporated into DNA instead of cytosine (C) or 5-methylcytosine (mC) and inhibits DNA methylation by a covalent binding of the DNA methyltransferase (Santi et al. 1984). A number of biological effects of 5-azaC have been described in plants. In addition to the activation of silent endogenous genes (Ngernprasirtsiri and Akazawa 1990) and transgenes (Hepburn et al. 1983), changes in the structure of chromatin (Fajkus et al. 1992) and the timing of chromosome replication (Široký et al. 1994), 5-azaC has also been shown to induce new complex phenotypes, such as dwarfism (Sano et al. 1989, 1990; Fieldes 1993), vernalization (Burn et al. 1993) and sex-reversal (Vyskot et al.1995).

We have recently described hypomethylation of repetitive DNA sequences in the tobacco genome induced

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by 5-azaC, ethionine and dihydroxy propyladenine, and have found sequence-specific differential effects of these drugs (Bezděk et al. 1992; Kovařík et al. 1994). This induced hypomethylation status was maintained in the course of protoplast recovery, callus growth, and plant regeneration (Bezděk et al. 1991; Koukalová et al. 1994). In the present paper we demonstrate that 5-azaC-treated tobacco seeds give rise to hypomethylated plants displaying specific phenotypic changes, and that the hypomethylation of repetitive HRS60 DNA sequences is transmitted through both male and female gametes into the progeny.

Materials and methods

Plant material

The plant material used throughout this work was *Nicotiana tabacum* L. cv Vielblättriger, kindly provided by the Tobacco Research Institute, Báb, the Slovak Republic.

5-azaC treatment and plant cultivation

Sterilized seeds were incubated in distilled water containing 0, 10 or 50 μM of 5-azaC (Sigma) for 10 days under gentle shaking and dim light conditions. The solution of 5-azaC was changed every day. Seedlings were finally transferred to soil and cultured under standard greenhouse conditions. The height of plants was measured at the time of flowering, and pollen viability was evaluated by staining with acetocarmine. To prepare seed progenies, plants were castrated when necessary, pollinated with a respective pollen donor, and bag-protected against open pollination.

In a separate experiment, to determine the time interval of maximum sensitivity of the seeds/seedlings to 5-azaC treatment, the drug (50 μM) was applied only during the 1st–3rd, 4th–6th, or 7th–9th days of liquid culture.

Characterization of the DNA methylation status

To characterize the methylation status of the genomic DNA, DNAs were digested with an excess of the restriction enzyme isoschizomers *Hpa*II or *Msp*I, which do not cut the sequences ^{m}CCGG, C^{m}CGG, ^{m}C^{m}CGG, and ^{m}CCGG, ^{m}C^{m}CGG, respectively. Leaves from adult plants were harvested, ground in liquid nitrogen, and total DNA was extracted according to Dellaporta et al. (1983) and then subjected to phenol and chloroform purification. The completeness of DNA digestion was checked according to Fajkus and Reich (1991). DNA fragments were size-separated on 0.8% agarose gels and blotted onto Hybond N (Amersham) membranes. To monitor cytosine methylation in repetitive DNA sequences, the 182-bp monomeric unit HRS60.1 was used as a probe (Koukalová et al. 1989). HRS60.1 was labelled with $\alpha[^{32}P]$-dCTP and Southern hybridizations were performed at high-stringency conditions (Sambrook et al. 1989).

Quantitative evaluation of DNA methylation

In order to quantify the degree of HRS60 methylation, in some of the experiments Hybond N membranes were cut into strips after Southern hybridization and their relative radioacivity measured by scintillation counting. This approach enabled us to follow the size fractions of HRS60 sequences cleavable with *Hpa*II.

To estimate the overall content of mC residues in genomic DNA samples, the method of Cedar et al. (1979) was used. DNAs were cleaved with *Taq*I, the 5′-fragment ends dephosphorylated and then labelled with $\gamma[^{32}P]$ATP. DNAs were hydrolyzed with DNaseI and nuclease P1, the resulting nucleotides were separated by thin-layer chomatography (TLC) on Polygram cellulose. The radioactivity of dCMP and 5-methyl-dCMP spots was measured by scintillation counting. Each sample was analyzed twice.

Results

DNA hypomethylation in plants grown from 5-azaC-treated seeds

The methylation pattern of the HRS60 family of DNA repeats was analyzed using the restriction enzymes *Hpa*II or *Msp*I, which are sensitive to the methylation of cytosine residues. The DNAs were extracted from plants grown from seeds treated with 0, 10, or 50 μM 5-azaC for 10 days; five plants from each treatment were evaluated. Most of the HRS60 DNA from the control plant, cleaved with *Hpa*II, migrated as high-molecular-weight relic DNA (Fig. 1a). The *Hpa*II isoschizomer *Msp*I, sensitive to the methylation of the outer cytosine within the CCGG target, produced a ladder typical for tandemly arranged repetitive sequences. HRS60 DNAs of plants grown from 5-azaC-treated seeds always displayed enhanced susceptibility to *Hpa*II (Fig. 1 b, c). The HRS60 family represents non-transcribed repetitive DNA sequences comprising about 2% of the total tobacco genome (Koukalová et al. 1989). To estimate the overall content of methlated cytosine residues in the bulk DNA, the relative amounts of mC at TCGA sequences were evaluated (Fig. 2). The average decrease of mC content in the 5-azaC-treated plants was 20% compared with the control.

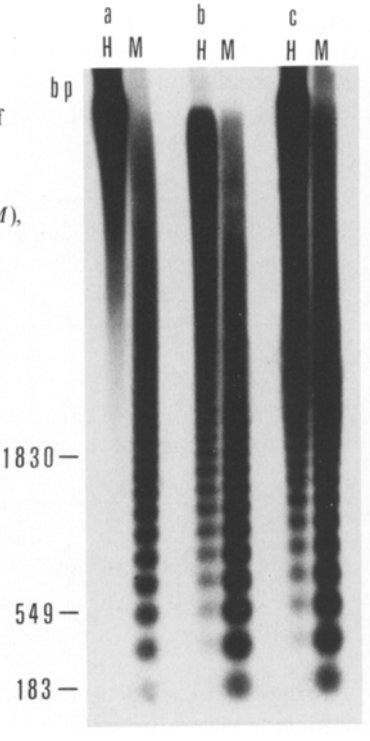

Fig. 1 a–c DNA methylation patterns of plants grown from 5-azaC-treated seeds. DNA samples were prepared from leaves of the control (**a**) and of plants treated with 10 μM, 5-azaC (**b**) or 50 μM 5-azaC (**c**), cut with the restriction enzymes *Hpa*II (*H*) or *Msp*I (*M*), hybridized with the $[^{32}P]$-labelled HRS60. 1 probe, and autoradiographed

plant	WT	5-azaC	5-azaC self	5-azaC x WT	WT x 5-azaC
methyl-dCMP / dCMP					
$\frac{mC}{mC + C}$ [%]	76	58	60	69	72
mC reduction [%]	100	76	78	91	94

Fig. 2 Amounts of mC in typical representatives of the controls (*WT*), the 5-azaC-treated plants (50 μM) and their progenies as revealed by 1D-TLC. The data reflect the overall CG methylation in TCGA targets and are expressed as a percentage of the mC content of the total (C + mC) and the mC percentage normalized to the control plant value

Determination of a period in the seed germination sensitive to 5-azaC

It has been observed that during the very early period of seed germination a rapid decrease in the overall mC content occurs (Follmann et al. 1990) which is apparently connected with the activation of various metabolic pathways. To determine the most sensitive period of seed germination and early plantlet development to the hypomethylating action of 5-azaC, three different 3-day 5-azaC treatments (50 μM) were applied. DNA samples were collected from ten plants from each experimental group. Pooled DNAs were cut with *Hpa*II or *Msp*I and subjected to hybridization with the HRS60.1 probe. As is shown in Fig. 3, the HRS60 repetitive family was most efficiently hypomethylated during the second interval of 5-azaC exposure, i.e. the 4th to 6th days of liquid culture (starting from dry seeds). Results from the Southern-blot analyses of DNA methylation patterns were complemented by TLC quantification of the methylcytosine content at TCGA sequences (Fig. 4). 5-azaC applied during the second interval (4th to 6th days) of seed culture led to the same extent of hypomethylation as the 10-day treatment mentioned above, while there was nearly no 5-azaC effect before or after this interval.

Phenotypic changes induced by 5-azaC

At the stage of flowering a number of prominent phenotypic deviations could be seen in the 5-azaC (50 μM, 10 days)-treated plants. They were significantly smaller (a dwarf phenotype) and suffered from a low pollen viability (Fig. 5). Their most apical flowers were always sterile and displayed drastic whorl malformations (Fig. 6a). The later flowers were nearly normal, but one to five of their stamen(s) were transformed into a petaloid structure with a sterile anther attached (Fig. 6b). Since these changes generally afflicted more than one whorl, they strikingly resembled classical ho-

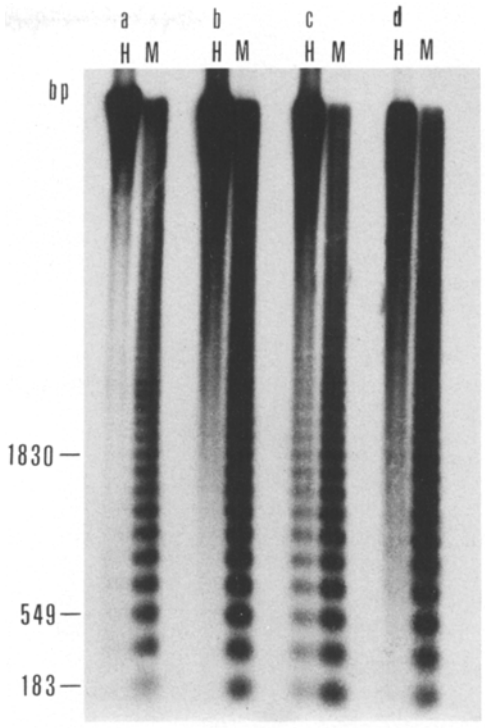

Fig. 3a–d Hybridization patterns of plant DNA samples treated with 5-azaC at different periods of seed culture: **a** control, non-treated plants, **b** 5-azaC applied during the first 3 days, **c** during the second 3 days, and **d** during the third 3 days. DNAs were cut with *Hpa*II (*H*) or *Msp*I (*M*) and hybridized with the HRS60.1 labelled probe. In each variant, DNA was isolated from the leaves of ten young plants (at the stage of 3–4 true leaves) pooled together

plant	WT	intervals		
		1st	2nd	3rd
methyl-dCMP / dCMP				
$\frac{mC}{mC + C}$ [%]	76	72	60	74
mC reduction [%]	100	95	79	97

Fig. 4 Relative amounts of mC in DNA samples from plants treated with 5-azaC at different time intervals of seed germination as described in Fig. 3. The data represent the CG methylation in TCGA targets and are expressed as indicated in Fig. 2

meotic flower mutations. Not all of the stamens within a flower, and not all of the flowers on the same plant, displayed the same extent of transformations and malformations. Similar phenomena occasionally occur among plants regenerated from tissue cultures (e.g. Vyskot et al. 1991) and could be explained by methylation perturbations caused by stress under *in vitro* conditions (Philips et al. 1994).

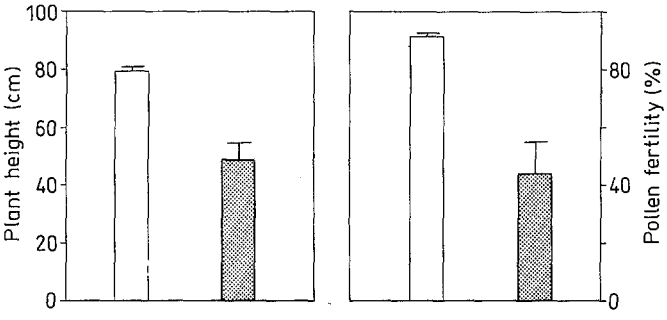

Fig. 5 Comparison of plant heights and pollen fertilities of the controls (empty columns) and the 5-azaC-treated (50 μM, filled columns) plants. Ten plants from each group were evaluated. The differences both in height and fertility are highly significant

Methylation of the HRS60 DNA in the progenies

One of the 5-azaC-treated (50 μM for 10 days) plants with a hypomethylated genome was selfed and used in reciprocal crosses with the control tobacco plant. Five randomly chosen plants from each progeny were analyzed for methylation patterns of the HRS60 DNA. Progeny of the selfed and cross-fertilized 5-azaC-treated plants contained a significantly higher proportion of hypomethylated HRS60 sequences when compared with the control (Fig. 7). Both types of reciprocal crosses yielded plants with HRS60 DNA partially sensitive to *Hpa*II. We did not observe quantitative differences between the reciprocal crosses, either in the bulk HRS60 CCGG methylation or in the total genomic TCGA methylation (Figs. 2 and 7a). Plants grown from the self-pollinated 5-azaC-treated plant contained genomes with different degrees of hypomethylation up to the extent found in the parental 5-azaC plant; two extreme examples are presented as a semi-quantitative analysis of Southern blots after hybridization with the HRS60.1 probe (Fig. 7b). The unexpected variation observed in this case could be explained by a random combination of gametes possessing more/less hypomethylated genomes and by the relatively small plant populations tested (five individuals).

Discussion

In addition to the epigenetic control of metabolic pathways, DNA methylation seems also to be involved in the control of the condensed state of chromatin. HRS60 monomeric units are organized in long tandem arrays as the DNA component of subtelomeric heterochromatin in all S chromosomes of the allotetraploid tobacco genome, and most of the HRS60 DNA is constitutively methylated both in CG and CNG sequences (Koukalová et al. 1989; Kenton et al. 1993). We have been able to induce hypomethylation of the tobacco genome with inhibitors of methylation (Bezděk et al. 1992) and have shown that the hypomethylated state of HRS60 sequences is maintained in the course of in vitro growth and plant regeneration (Koukalová et al. 1994).

Provided that the role of cytosine methylation is important for ordered cell functioning, the question arises as to how growing and differentiating plant cells overcome the hypomethylation of a substantial part of their genome. In our experiments we observed a 20% decrease of mC in the DNA of hypomethylated plants, compared with the control. Thus plants, in contrast to mammals, seem more capable of tolerating hypomethylation of the genome. However, the majority of

Fig. 6 a, b Typical floral homeotic-like changes in 5-azaC (50 μM)-treated tobacco plants. **a** The upper flower displaying serious malformations; **b** the most frequent flower transformation, petaloid stamens

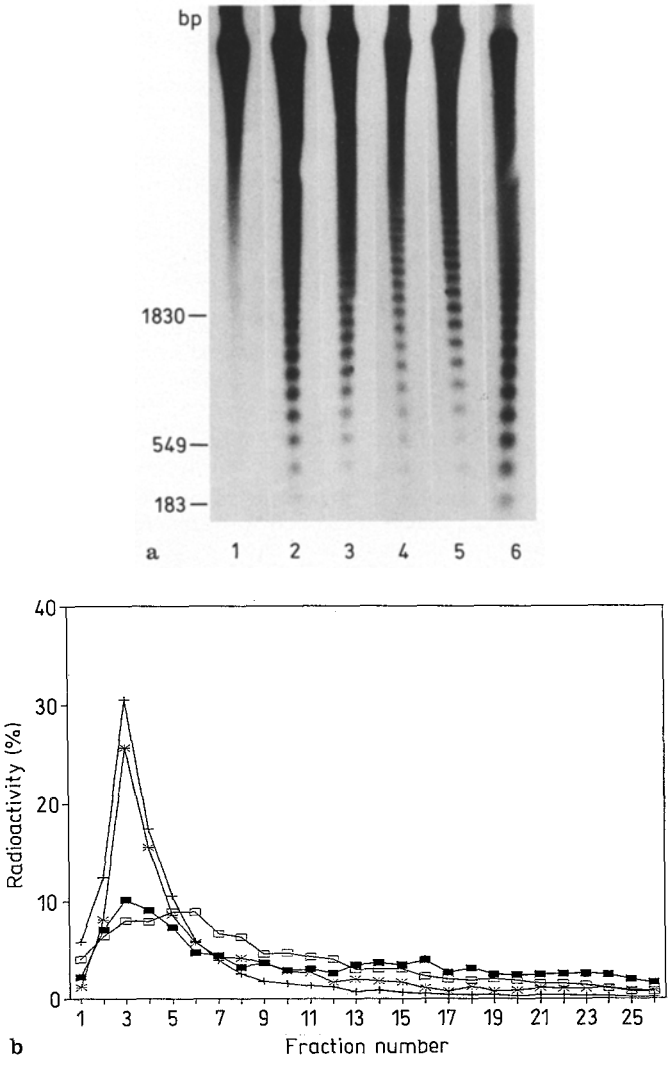

Fig. 7 a, b Inheritance of CG hypomethylation of the HRS60 DNA repeat in representative progeny of a reciprocal cross-fertilization (the 5-azaC-treated plant crossed with a non-treated plant) and selfing. Plant DNAs were cut with *Hpa*II and hybridized with the HRS60.1 probe. The radioactivity signals on membranes were detected either by autoradiography (**a**) or by scintillation counting (**b**). **a** Hybridization patterns as revealed by autoradiography: *1* control parent plant; *2 and 3* two plants from the progeny of the control pollinated with the 5-azaC plant; *4 and 5* two plants from the progeny of the reciprocal cross; *6* the 5-azaC-treated parental plant. **b** Graphic presentation of relative radioactivity counts in strips cut from the hybridization membranes. Fractions are numbered from the start (#1, the heavily methylated, largest fragments) to the end (#26, the hypomethylated, smallest fragments) of gels. The data relate to two representatives of the self progeny of the 5-azaC plant (−x−, −□−), the 5-azaC parent (−■−), and the control parental plant (−+−)

tobacco plants grown from 5-azaC-treated seeds showed an uniform altered phenotype: dwarf stem, reduced pollen fertility, and floral transformations and malformations. Some of these phenotypic features have also been reported in other plant species exposed to hypomethylating agents – maize (Sano et al. 1989), rice (Sano et al. 1990), and flax (Fieldes 1993). In rice, moreover, both undermethylation and dwarfism were heritable when the plants were self-pollinated (Sano et al. 1990). Interestingly, very similar phenotypes were obtained when a rice MADS box gene, OsMADS1 (An 1994), and a maize MADS box gene, Zmhox1b (W. Werr, personal communication), were introduced and constitutively expressed in transgenic tobacco plants. Therefore, we can speculate that 5-azaC may alter the functioning of some homeotic genes, though this remains to be proven.

It was of interest to follow the transmission of hypomethylated DNA *via* generative propagation. The results reveal that hypomethylated, highly repetitive HRS60 DNA is transmitted through both male and female meiosis. It should be stressed that, due to the high copy number of this sequence, the methylation analysis reflects a bulk of fragments only, and not the fate of individual copies separately. In accordance with the observed transmission of hypomethylated HRS60 sequences, the overall decrease in the mC content of the tobacco genome is also maintained in the progenies of genetic crosses involving hypomethylated and normal plants as parents. As documented in Fig. 1, a fraction of the HRS60 DNA remained resistant to cleavage with *Hpa*II, i.e. it escaped the treatment with 5-azaC; thus, not all HRS60 sequences were hypomethylated in the plants used in crosses. In such a situation, a co-ordinate methylation of most of the HRS60 multicopy DNA sequences could be expected based on their mutual homology. The global results obtained do not support this kind of epigenetic interaction, but some methylation perturbations in individual HRS60 sequences cannot be excluded. It seems that plants do not efficiently recognize and restore an induced alteration of DNA methylation at non-transcribed repetitive DNA sequences.

In some cases the Mendelian rule on the identity of reciprocal crosses is known to be violable. In addition to genes linked to organelle genomes or to sex chromosomes, their parent-of-origin (gametic imprinting) could be a causal factor. Contrary to mammals, which regularly imprint a number of genes and chromosomal domains (for a recent review see Driscoll 1994), gametic impriting in plants has only been reported in endosperm (Kermickle and Alleman 1990). In addition, according to studies on transgenes in mammals, the paternal genome may have evolved to be subject to a greater diversification of functions during development, while the maternal genome seems to play a more suppressive role (Surani et al. 1988). In our experiments no difference in the inheritance of a new methylation pattern between the reciprocal crosses have been detected. This might indicate a similar tolerance of male and female meiosis to a newly acquired epigenetic change in this hermaphroditic plant species.

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