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 demonstrated 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 (Siroký 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...
by 5-azaC, ethionine and dihydroxy propyladenine, and have found sequence-specific differential effects of these drugs (Bezdéček et al. 1992; Kovařík et al. 1994). This induced hypomethylation status was maintained in the course of protoplast recovery, callus growth, and plant regeneration (Bezděček 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, Bubíň, 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 enzymes isoschizomers *Hpa*II or *Msp*I, which do not cut the sequences *CpCGG*, *CpCGG*, *CpCGG*, and *CpCGG*, *CpCGG*, 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 γ[32P]-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 radioactivity 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 γ[32P]-ATP. DNAs were hydrolyzed with DNaseI and nuclease P1, the resulting nucleotides were separated by thin-layer chromatography (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. 1b, 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 methylated 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.