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Methylation patterns of two repetitive DNA sequences in tobacco tissue cultures and their regenerants

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Abstract

DNA methylation of two repetitive sequences in tobacco nuclear genome was studied in the course of *in vitro* dedifferentiation and differentiation. Using 5-mC sensitive restriction enzymes and DNA/DNA hybridization with 25S-rDNA probe it has been shown that during the early phase of callus induction prominent changes in the methylation pattern occur which are stably maintained during subsequent callus growth. The following protoplast recovery and plant regeneration have again displayed some more modifications of the methylation status. Comparing the patterns of R_0 plants with the original plant material and the calli it can be assumed that both share in the resulting methylation status. The experiments analyzing the HRS60 family of non-transcribed highly repetitive sequences have displayed a quite monotonous methylation status thus indicating no random methylation perturbations in silent DNA sequences.

Introduction

There is an increasing evidence that methylation of cytosine DNA residues (5-mC) plays a controlling role in gene expression in eukaryotic cells (Cedar 1988). The level of DNA methylation in plant nuclei is relatively high reflecting both methylation of cytosine in CpG dinucleotides and CpNpG triplets (Gruenbaum *et al.* 1981). The DNA methylation phenomenon can explain not only a mechanism of regulation of gene expression by preventing a specific transcription factor binding, but it can also elucidate how gene expression patterns are inherited through cell division (Holliday 1987).

Plant cells cultured *in vitro* are subjected to growth stress conditions which often result in a great phenotype heterogeneity in populations of regenerants (Larkin and Scowcroft 1981). This phenomenon called a somaclonal variation can reflect both changes in nucleotide DNA sequences and methylation patterns (Brown 1989). In

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principle, there are two basic processes influencing DNA methylation patterns: maintenance methylases conserve a given methylation status thus enabling its transfer into daughter nuclei, while various putative internal and/or external factors induce *de novo* methylation/demethylation. *In vitro* stress conditions may then cause both errors in the fidelity of maintenance methylases function and induce programmed or unscheduled methylation changes.

In this paper we have tried to detect an evolution of the *in vitro* induced DNA methylation modifications and demonstrate if they are transmitted into regenerated plants. To analyze specific methylation patterns two repetitive DNA sequences were chosen which differ in their frequency, function, and chromosomal localization: the coding sequence of 25S-rDNA represents middle repeated, developmentally regulated transcribed sequences in the nucleolar organizing regions, while the HRS60 is a non-transcribed, highly reiterated family of sequences, predominantly localized in telomers (Koukalová *et al.* 1989).

Materials and methods

Plant material and tissue culture: The plant material used was *Nicotiana tabacum* L. cv. Vielblättriger (kindly supplied by the Tobacco Research Institute, Báb, Slovakia). Leaf pieces were cultured on solid Murashige and Skoog (1962) media with 2 mg l^{-1} α-naphthylacetic acid (NAA) and 0.2 mg l^{-1} benzylaminopurine (BAP) to induce callus proliferation. All the cultures were grown under a light/dark period (12/12 h) at 26 °C. Samples of calli from the first three subcultivations (each of them took 25 d) and leaves from the original tobacco plant were taken off and subjected to DNA analysis. In order to analyze the methylation status in non-chimaeric plant regenerants the resulting callus after the third subcultivation was used as a source of protoplasts. The protoplasts were isolated and regenerated as described by Vyskot *et al.* (1991). After 5 weeks of culture one of the protoclones was randomly chosen and regenerated into plants on MS medium with 0.2 mg l^{-1} NAA and 2.0 mg.l^{-1} BAP. After a rooting phase on MS media without hormones five plantlets were transferred into greenhouse conditions and their leaves were analyzed for the methylation status.

DNA isolation: To isolate total DNA from calli and leaves a modified *CTAB* procedure according to Saghai-Maroof *et al.* (1984) was used. Concentration of DNA was determined according to Labarca and Paigen (1980).

Southern analysis: DNA samples were cleaved with an excess of restriction enzymes, separated in 1 % agarose gels, and transferred to *Hybond N* membrane (*Amersham*) according to Maniatis *et al.* (1989). The following DNA probes were used: an internal 2478 bp (EcoRI) fragment of the 25S-rDNA gene isolated from tomato (Kiss *et al.* 1989), and the 366 bp dimer HRS60.dim1, a member of the HRS60 family of tobacco DNA repeats (Matyášek *et al.* 1989). The probes were labelled using the *Amersham* Multiprime DNA labelling kit with α-^{32}P-dCTP according to the manufacturer's procedure. DNA/DNA hybridizations were made after Maniatis *et al.* (1989).

Results

Changes in DNA methylation patterns of 25S-rDNA during callus growth: To analyze methylation changes during callus induction the following restriction enzymes with a different sensitivity to 5-mC were used: HpaII, MspI, and SmaI possessing respective cleavage sites with sensitivity to methylation indicated according to Kessler and Manta (1990): C^+/C^+GG, C^+/C^0GG, $C^+C^0C^+/GGG$. Since a large proportion of the 25S-rDNA sequences was resistant to these enzymes, the DNA samples were cleaved in the same time with EcoRI to obtain smaller DNA fragments enabling a qualitative analysis. There were no prominent changes in the total digestion patterns on gels between the leaves and the calli if the DNAs were cut with MspI + EcoRI, HpaII + EcoRI, or SmaI + EcoRI (data not shown). However, the hybridization data show that very early, during the initial callus phase (*i.e.* the first subculture), a number of methylation changes has appeared in the 25S-rDNA coding sequences (Fig. 1 A,C).

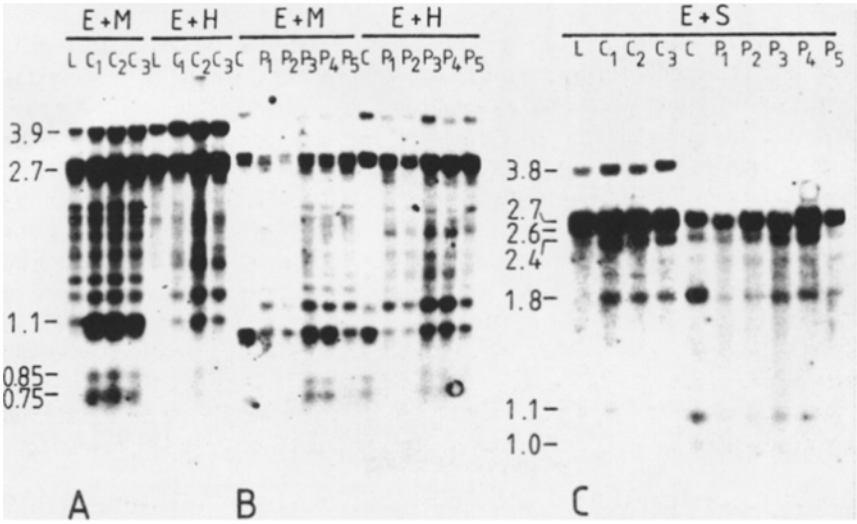

Fig.1. Methylation changes of the 25S-rDNA in the course of dedifferentiation and differentiation. Comparison of restriction patterns of the DNA samples probed with the 25S-rDNA after digestion with the enzymes indicated: (E+M) - EcoRI + MspI; (E+H) - EcoRI + HpaII; (E+S) - EcoRI + SmaI. Molecular masses in kb are indicated.
A. The DNA samples: L - the original leaf, C1 - the 1st callus subculture, C2 - the 2nd subculture, C3 - the 3rd subculture.
B. The DNA samples: C - the protoplast-derived callus, P1 to P5 - the R_0 plants (numbers 1 - 5).
C. The DNA samples: L - the original leaf, C1, C2, C3 - the 1st, the 2nd, and the 3rd callus subculture, C - the protoplast-derived callus, P1 to P5 - the R_0 plants (numbers 1 - 5).

The modifications in the 25S-rDNA methylation patterns were revealed by all the 5-mC sensitive restriction enzymes tested and they did not change in the course of the subsequent cultivation (*i.e.* the 2^{nd} and 3^{rd} subculture). The differences between

the leaves and the calli after digestion with MspI + EcoRI and those between the patterns after cleavage with MspI + EcoRI and HpaII + EcoRI show that both CpG and CpNpG methylation changes can be induced by environmental factors. The digestion with only EcoRI in the control experiment did not show any difference (data not present). A higher number and amount of smaller diffuse fragments in the DNAs isolated from calli in comparison with the leaves show a partial demethylation of the 25S-rDNA sequences probably connected with an increased ribosomal activity.

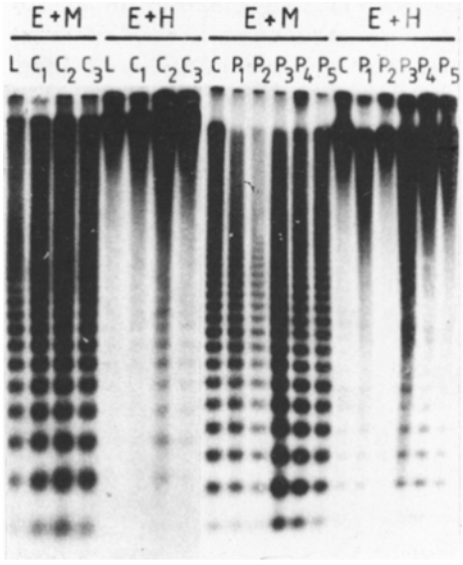

Fig.2. Analysis of methylation patterns of the HRS60 family during callus growth, protoplast recovery and plant regeneration after hybridization with the HRS60.dim1 DNA. The total genomic DNAs were cut with EcoRI + MspI (E+M) or HpaII + EcoRI (E+H). The DNA samples: L - the original leaf, C1 to C3 - the 1st to 3rd callus subculture, C - the protoplast derived callus, P1 to P5 - the R_0 plants (numbers 1 to 5).

Methylation status after protoplast recovery and plant regeneration: In order to follow the fate of the methylation patterns after protoplast regeneration and plant differentiation the same analyses were also performed with the DNAs isolated from one randomly selected protoplast-derived callus as well as its regenerated plants. The results show prominent differences in the strength or position of some hybridization signals between the protoplast-derived callus and the regenerants, but the restriction patterns of the five regenerants are not strictly the same as in the original leaf sample (Fig. 1 B, C). The most striking evidence of methylation perturbations could be seen in the SmaI + EcoRI digests where during the regeneration of the protoplast clone a demethylation of one restriction site in a large DNA fragment (3.8 kbp) occurred and was stably transmitted into all five regenerants (Fig. 1C). The results indicate that a

regulated recovery of the DNA methylation pattern of the 25S-rDNA coding sequence may be subjected to a stress induced modulation.

Methylation stability of the HRS60 family during *in vitro* growth and differentiation: The HRS60 is a highly repeated BamHI family of slightly diversified short sequences (Koukalová *et al.* 1989) which are regularly heavily methylated (Bezděk *et al.* 1991). After digestion with a number of restriction endonucleases it forms a typical hybridization "ladder" which is more or less blocked in the case of 5-mC sensitive enzymes leaving "relic" DNAs. To look for changes in methylation patterns during callus growth and plant regeneration the DNAs were cut with MspI or HpaII, in each case also with EcoRI. Using these enzymes the restriction patterns after hybridization with the HRS60.dim1 probe are completely the same in all the DNA samples including the relative frequencies of the individual "mers". It can be concluded that the methylation status of this repetitive DNA family is highly stable at any stage of tissue culture as well as in the leaves of both the original plant and the protoplast-derived regenerants (Fig.2). Thus in this case the stress *in vitro* conditions during dedifferentiation and differentiation do not seem to cause any random methylation perturbations. Both the digestions with MspI + EcoRI and HpaII + EcoRI display some non-cleaved DNA, but comparing the amounts of relic DNAs it is obvious that the methylation of the inner cytosine in the CCGG target sequence is much higher than the outer one. The digestion only with EcoRI forms a hardly visible ladder with a huge amount of relic DNA thus demonstrating that only a tiny proportion of members of the HRS60 family has an EcoRI recognition site (data not shown).

Discussion

It has been recently shown that plant tissue cultures undergo transitory DNA methylation changes which can be influenced by hormones and reflect a level of differentiation (Lo Schiavo *et al.* 1989, Vergara *et al.* 1990, Arnholdt-Schmitt *et al.* 1991). There are some ambiguous data showing that during an early period of tissue culture establishment (the dedifferentiating phase) an amplification of some repetitive DNA sequences as well a changed rate of total cytosine methylation occur (Durante *et al.* 1982, Arnholdt-Schmitt *et al.* 1991, Palmgren *et al.* 1991). These changes have been also detected as a restriction fragment length polymorphism in regenerated plants and their progeny and may be responsible for epigenetic and/or genetic variation (Brown 1989).

The nuclear ribosomal genes are usually highly methylated (Steele-Scott *et al.* 1984), but in the active nucleolar organizers specific sites in the non-transcribed intergenic spacer are regularly hypomethylated (for a review see Flavell 1986). There is so far only a little information about changes in the rDNA methylation patterns in relation with growth conditions and gene activity. A correlation has been shown between a stage of plant development and a methylation status of pea nuclear ribosomal RNA genes (Watson *et al.* 1987). In some experiments environmental conditions induced discrete changes in the methylation status of rDNA genes in radish (Delseny *et al.* 1984), while in others even so contrast differences as aerobic

vs. anaerobic conditions did not cause any methylation change (Olmedilla *et al.* 1984). It is also a question whether the methylation of a coding region of abundant ribosomal genes plays any role in their expression.

The differences in methylation patterns in the 25S-rDNA between leaves and leaf-derived calli presented in this paper are not surprising, but it can hardly be distinguished if they reflect a changed rDNA activity or a stress induced variation. It seems to be that regular changes in DNA methylation patterns should be strictly reversible contrary to unscheduled methylation perturbations which may be corrected during meiosis or even transmitted to sexual progeny. From this point of view the methylation of the 25S-rDNA coding region is regulated, but partly influenced by growth conditions which is maintained through mitoses during *in vitro* organogenesis. It is obvious that plants tolerate even an undesirable methylation inactivation of some rDNA copies, which can reflect the fact that they have a considerably greater number of rDNA genes than it is required to sustain ribosome synthesis (Flavell 1986). In this work we have detected some methylation fluctuations in tobacco which has the additive genome of *N. sylvestris* and *N. tomentosiformis* thus containing a double dosage of rDNA genes. Studying the methylation patterns of calli it should be mentioned that they represent a heterogeneous population of cells so the results obtained reflect an average sample. Moreover, these limitations are stressed in the analysis of R_0 plants where regenerants from the only one randomly selected protoplast clone were checked. Thus the partial reversibility of the 25S-rDNA methylation status can be interpreted not only as reflecting specific physiological requirements for callus growth or random perturbations, but it is also possible that during protoplast recovery and plant differentiation in callus cultures a selection for cells with a sustainable methylation pattern occurs.

The presented results confirm that the stress tissue culture conditions can affect the DNA methylation status of cells and that this effect can be transmitted into regenerated plants.

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