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Sequence-specific hypomethylation of the tobacco genome induced with dihydroxypropyladenine, ethionine and 5-azacytidine

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Abstract Higher plant DNA is methylated at CG and CNG targets. In this study we have investigated the tobacco methylation system in tissue culture using the methylation inhibitors 5-azacytidine (5-azaC), dihydroxypropyladenine (DHPA) and ethionine (Ethi), and methylation-sensitive restriction endonucleases *Hpa*II, *Msp*I, *Hha*I, *Eco*RII, *Scr*FI, and *Fnu*4HI. Surprisingly, CAG/CTG sequences, contrary to CG doublets and CCG/CGG triplets, appeared to be refractory to the inhibitory effect of 5-azaC. Thus 5-azaC cannot be considered a general inhibitor of DNA methylation in tobacco cells. On the other hand, DHPA, the inhibitor of *S*-adenosylhomocysteine (SAH) hydrolase, and Ethi caused hypomethylation of both CAG/CTG and CCG/CGG triplets but not of the CG doublets. The sensitivity of triplet-specific methylation to the inhibition of SAH hydrolase suggests the possibility that plant-specific DNA methylation at CNG targets might be modulated by alterations of the SAH/*S*-Adenosylmethionine ratio in plant cells.

Key words: DNA methylation inhibitor; CG and CNG DNA sequence; *Nicotiana tabacum*

1. Introduction

Methylation in higher eukaryotes occurres in CG doublets. Additional methylation of CNG triplets seems to be a characteristic of higher plant genomes [1]. Besides symmetrical methylation at CG doublets and CNG triplets a non-symmetrical cytosine methylation has recently been detected in a plant transgene [2]. Methyl groups are transported to the cytosine residues from *S*-adenosylmethionine (SAM) by specific m^5C-Mtases. Recently cDNA sequences of the mouse [3] and *Arabidopsis thaliana* m^5C-Mtases [4,5] have been cloned, and shown to contain regions of homology with DNAs coding for prokaryotic m^5C-Mtases. As for methylation of CG doublets and CNG triplets in higher plant genomes, it is still not certain whether it is accomplished by a single multispecific m^5C-Mtase or by multiple enzymes. Some of the recently published data suggest the existence of a complex methylation system in plants: (i) DNA-hybridization studies with cloned m^5C-Mtase sequences revealed multiple homologue sites in the genome of *Arabidopsis thaliana* which could reflect the existence of a family of m^5C genes [4,5]; (ii) recent investigation of DNA substrate specificity of a pea DNA methylase indicates that more than one enzyme or its several isoforms may exist [6]; (iii) our previous work showed efficient inhibition of methylation of both CG and CCG sequences by 5-azaC and a selective effect of Ethi on mCCG triplets [7].

In our experiments we have induced DNA hypomethylation of the tobacco genome with 5-azaC, Ethi and DHPA, the latter being an inhibitor of SAH hydrolases and cellular transmethylases [8,9]. The methylation status of the genomic DNA was analyzed using methylation sensitive restriction endonucleases *Hha*I, *Hpa*II, *Msp*I, *Eco*RII, *Scr*FI, and *Fnu*4HI at the sequences HRS60 [10] and R8.1 [11] covering approximately 2.5% of the whole genome. The sequence-specificity of the utilized methylation inhibitors suggests the presence of different m^5C-Mtase activities in tobacco cells.

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2. Materials and methods

2.1. Plant material, treatment with drugs and isolation of DNA

Nicotiana tabacum L. cv. Vielblattriger calli cultures were prepared and cultured on the Murashige–Skoog medium as described by Bezděk et al. [7]. After three 4-week subcultures, 500 mg pieces of calli were transferred to the media supplemented with either 5-azaC (200 μM), Ethi (300 μM) or DHPA (25 μM) and grown for another 4 weeks. 5-azaC and Ethi were from Sigma, DHPA was synthetised in the Institute of Organic Chemistry and Biochemistry Prague. Genomic DNA from control and treated cells was isolated by a modified cethylammoniumbromide method [12].

2.2. Estimation of DNA methylation status

The methylation status of genomic domains, specified by the probes HRS60.1 or R8.1.34, was analysed using *Bst*NI, *Eco*RII, *Fnu*4HI, *Scr*FI, *Hha*I, *Msp*I and *Hpa*II restriction enzymes. DNAs were digested with an excess of the enzymes (10 U/μg DNA; completeness of cleavage of restriction sites was verified according to Fajkus and Reich [13]. 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 nylon membranes (Hybond N, Amersham, UK) and hybridized to ^{32}P-labelled DNA probes (> 10^8 dpm/μg DNA, Megaprime kit, Amersham). After washing at high stringency conditions (0.2 × SSC, 65°C) membranes were autoradiographed for 1–3 days.

2.3. DNA probes

The HRS60.1 sequence, a 182-bp member of the HRS60 family of tandem DNA repeats [10] and the R8.1.34 sequence, a 186-bp member of the R8.1 family of dispersed DNA repeats [11] were used.

3. Results

3.1. The effect of 5-azacytidine on ^{m}CG and ^{m}CNG motifs

Data presented in Fig. 1A show the methylation status of tandemly arranged HRS60 sequences. DNA from control plant calli (lanes 1) could be digested with methylation insensitive *Bst*NI and partially sensitive *Msp*I restriction enzymes. In contrast, the corresponding methylation sensitive izoschizomers *Eco*RII and *Hpa*II did not cut the DNA and most material containing HRS60 sequences migrated as high-molecular-weight 'relic' DNA. DNA, isolated from the 5-azaC treated

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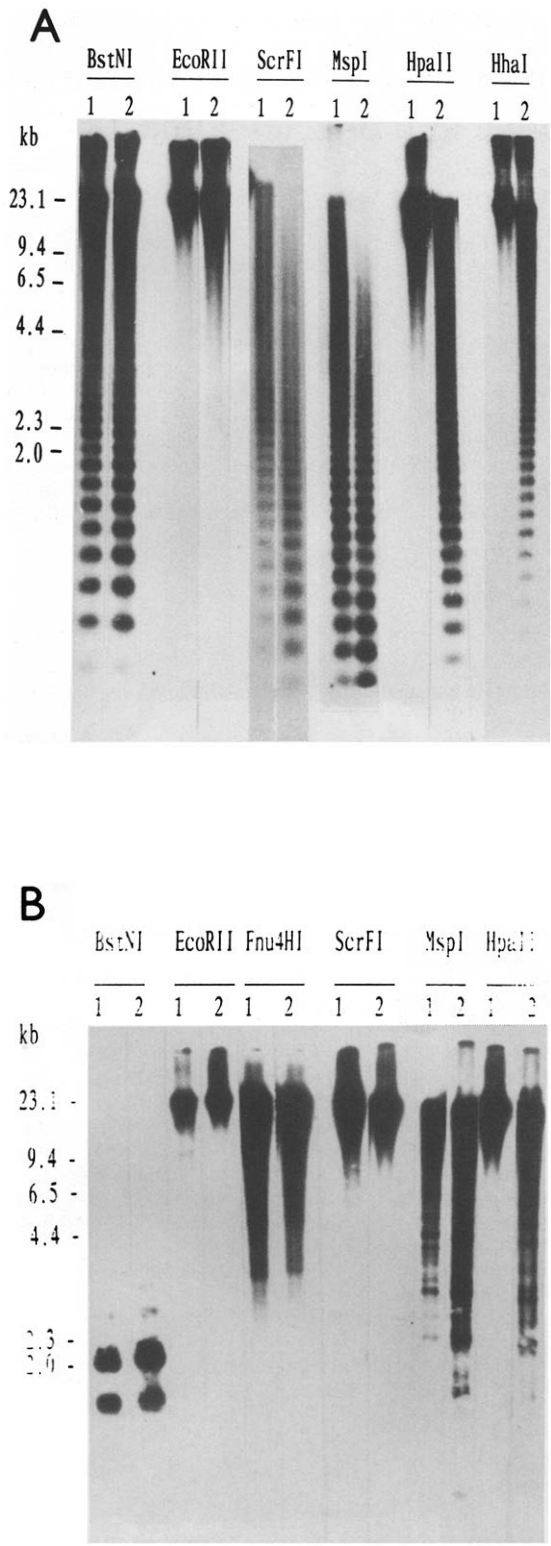

Fig. 1. 5-Azacytidine induced hypomethylation of the HRS60 (A) and R8.1 (B) DNA repeats. Genomic DNAs (2–4 μg) from control (lanes 1) and 5-azacytidine-treated (lanes 2) tobacco cells were digested with different restriction enzymes and analysed by Southern blot hybridization using HRS60 or R8.1 probes. The enzymes used do not cut following restriction sites containing m^5C: *Eco*RII: $C^mC(A/T)GG$; *Fnu*4HI: G^mCNGC, $GCNG^mC$; *Scr*FI: C^mCNGG; *Msp*I: ^mCCGG; *Hpa*II: ^mCCGG, C^mCGG, *Hha*I: G^mCGC, GCG^mC [18].

calli (lanes 2) showed increased sensitivity to cleavage with *Hpa*II, *Hha*I, *Msp*I and *Scr*FI. However, in the *Eco*RII tract no apparent enhancement of the DNA digestion was observed. Sequences of the HRS60 family comprise a large part of tobacco subtelomeric heterochromatin [14]. We were interested whether other parts of the genome responded in a similar manner. To address this question the methylation status of a middle repetitive dispersed sequence R8.1 was investigated (Fig. 1B). It is evident, that similar to the HRS60 sequences, 5-azaC treatment increased sensitivity of the DNA to digestion with *Hpa*II and *Msp*I.

3.2. *Hypomethylating effects of ethionine and dihydroxypropyladenine*

We have previously reported that Ethi prevented methylation of an external cytosine in the sequences mCCG. In this study we were concerned with the question whether DHPA, an inhibitor of SAH hydrolase could also exert a selective effect via feedback inhibition of m^5C-Mtase by increased levels of cellular SAH. Data presented in Fig. 2 demonstrate that treatment of cells with either DHPA or Ethi resulted in an increased sensitivity of the genomic DNA to digestion with *Eco*RII, *Fnu*4HI, *Scr*FI and *Msp*I restriction enzymes, cutting at CNG motifs. On the other hand, neither of these drugs affected CG specific methylation as is demonstrated by the resistance of the DNA to *Hpa*II or *Hha*I cleavage. In general the effect of DHPA was more pronounced than the effect of ethionine in all restriction enzyme digests.

4. Discussion

Our previous experiments revealed that the methylation inhibitors 5-azaC and Ethi exerted differential hypomethylating effects on tobacco DNA [7]. The results presented in this paper can be summarized as follows. (i) Hypomethylation induced with 5-azaC caused increased cleavage of the DNA with *Hpa*II, *Hha*I and *Msp*I enzymes, but not with *Eco*RII. Since *Eco*RII does not cut the sequence CmC(A/T)GG, the mC(A/T)G triplet apparently remains methylated even in the presence of 5-azaC. (ii) Contrary to 5-azaC, DHPA and Ethi did not enhance the *Hpa*II or *Hha*I cleavage but stimulated digestion of the DNA with *Eco*RII, *Msp*I, *Scr*FI, and *Fnu*4HI. Since the latter enzymes are sensitive to methylation of CNG triplets within their recognition sequences, we interpret this result as a triplet-specific inhibition of DNA methylation. The fact, that the DNA became sensitive to *Msp*I but remained resistant to *Hpa*II further suggests that mCmCG and CmCG motifs were more frequent than mCCG motifs, within both HRS60 and R8.1 DNA repeats at least. Based on this observation we speculate that methylation of the external cytosine might depend on the methylation of the internal CG doublet within the CCG sequence.

To explain the differential effect of 5-azaC on CG and CAG/CTG sequences we propose that these targets might be methylated by two distinct enzyme activities. The enzyme activity acting on CG targets appears to be more prone to the irreversible inactivation by 5-azaC. Moreover, the rate of de novo methylation might be different at different DNA targets.

The triplet-specific inhibitory effect of DHPA and Ethi could be explained by the allosteric properties of the enzyme. We propose that the affinity of the enzyme for the triplet targets could be increased by the binding of a cofactor or its analogue,

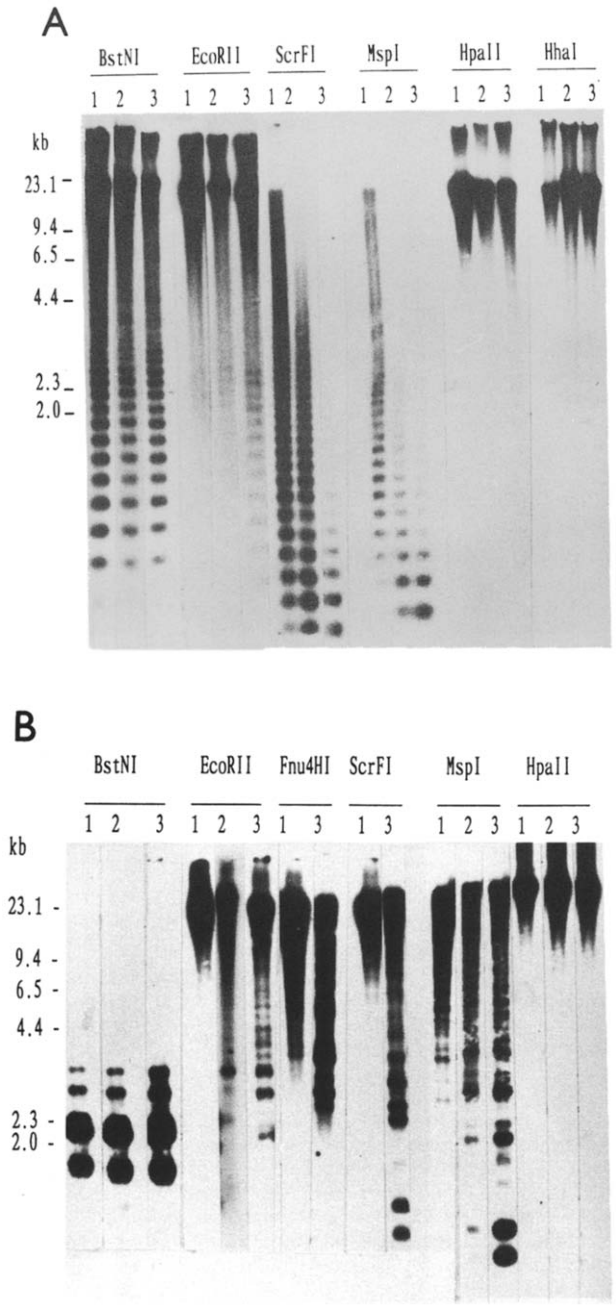

Fig. 2. Hypomethylation of the HRS60 (A) and R8.1 (B) DNA repeats induced with ethionine and dihydroxypropyladenine. DNAs from control (lanes 1), ethionine (lanes 2) and DHPA (lanes 3) treated plant cells were digested with restriction enzymes and analyzed by Southern blot hybridization. Experimental conditions and probes were the same as in Fig. 1.

as previously shown for some procaryotic Mtases [15,16]. The enzyme charged with SAH or S-AdoEthi could then reversibly block triplet methylation. It has been recently reported that tobacco cytokinin-binding protein possesses significant homology with SAH hydrolases from different species [17]. This fact together with our finding that the inhibition of SAH hydrolase leads preferentially to triplet specific hypomethylation of tobacco DNA point to the possibility that some of the effects of cytokinins are mediated through DNA methylation/demethylation.

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