

## Differential sensitivity of CG and CCG DNA sequences to ethionine-induced hypomethylation of the *Nicotiana tabacum* genome

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Received 11 February 1992

Plant DNA is distinguished from the DNA of all other organisms by its high content of 5-methylcytosine (5mC). 5mC levels may amount to 30% of total cytosines, distributed between the sequences CG and CXG. The results presented here show that the methylation status of CXG sequences could be influenced by culturing tobacco tissues on subtoxic concentrations of ethionine. The hypomethylating effect of ethionine, evaluated as the capability of *MspI* or *HpaII* to cleave the DNA, proved to be rather specific for CCG and differed from that of 5-azacytidine which did not discriminate between CG and CXG sequences.

Plant DNA; CG and CCG DNA sequence; Ethionine-induced hypomethylation; *Nicotiana tabacum*

### 1. INTRODUCTION

In eukaryotic genomes, cytosine methylation often occurs in CG doublets [1]; in plants also in CXG triplets, where X can be any nucleotide [2]. Transient exposure of cells to 5-azacytidine (5-azaC), recognized as cytidine by DNA synthetic machinery, results in the DNA hypomethylation [3] since 5-azacytosine cannot be methylated in the 5-position. It is supposed that DNA methylase binds irreversibly to DNA containing 5-azacytosine [4]. Ethionine (Ethi), an ethyl analog of the essential amino acid methionine, has also been shown to induce hypomethylation of DNA [4,5] and to induce the expression of thymidine kinase in thymidine kinase-deficient Chinese hamster cells [6].

Recently we have shown that CG dinucleotides, and partially also CCG trinucleotides of the family of tandem DNA repeats of the *Nicotiana tabacum* genome (the HRS60 family), were methylated and that upon the action of 5-azaC they became hypomethylated [7]. We here describe an Ethi-induced hypomethylation of plant DNA and present an original finding that Ethi and 5-azaC apparently differ in their inhibitory effects on methylation of the sequences CG and CXG. These re-

sults reveal the existence of a methylation system discriminating these targets in plant DNAs.

### 2. MATERIALS AND METHODS

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

Sterile leaves of *Nicotiana tabacum* L. cv. Vielblatttriger were cut into pieces and transferred to solid MS medium [8] with 2 mg·l<sup>-1</sup> of  $\alpha$ -naphthaleneacetic acid and 0.2 mg·l<sup>-1</sup> of benzylaminopurine to induce callus proliferation. Calli were grown at 26°C at 2000 lx illumination and 12-h light–darkness cycle. After three 4-week subcultures, 500 mg pieces of calli were transferred to the same medium (control tissue) or media supplemented with either 5-azaC (50 mg·l<sup>-1</sup>) or Ethi (50 mg·l<sup>-1</sup>). After 4 weeks cultivation, total DNAs were prepared from freeze-dried calli using the modified CTAB procedure [9].

#### 2.2. Estimation of DNA methylation status

The methylation status of genomic domains, specified by the probes HRS60.1 or R8.1.34, was analyzed using *HpaII* and *MspI*. DNAs were digested with excess of the enzymes (15 U/ $\mu$ g DNA; completeness of cleavage of restriction sites was checked as recommended by Fajkus and Reich [10] using chloroplast DNA as an internal standard). Digested DNAs were subjected to electrophoresis on 0.8% agarose gels. Following electrophoresis, the ethidium bromide-stained gels were photographed, blotted onto nylon membranes (Hybound N, Amersham) and hybridized to <sup>32</sup>P-labelled DNA probes (10<sup>6</sup> d.p.m./ $\mu$ g DNA, oligolabelling kit Amersham) as described by Maniatis et al. [11]. After washing at high stringency conditions (0.2× SSC, 65°C) membranes were autoradiographed.

#### 2.3. DNA probes

The HRS60.1 sequence, a 182 bp member of the HRS60 family of tandem repeats [12] and the R8.1.34 sequence, a 186-bp member of the R8.1 family of dispersed DNA repeats [13] were used. They were <sup>32</sup>P-labelled using a multiprime DNA labelling system.

### 3. RESULTS AND DISCUSSION

The inhibitory effects of Ethi and 5-azaC were stud

*Abbreviations:* 5mC, 5-methylcytosine; Ethi, ethionine; 5-azaC, 5-azacytidine; MTase, DNA methyltransferase; SAM, S-adenosyl-L-methionine; MS medium, Murashige-Skoog's medium; CTAB, cetyltrimethylammoniumbromide; Dam, DNA adenine methyltransferase.

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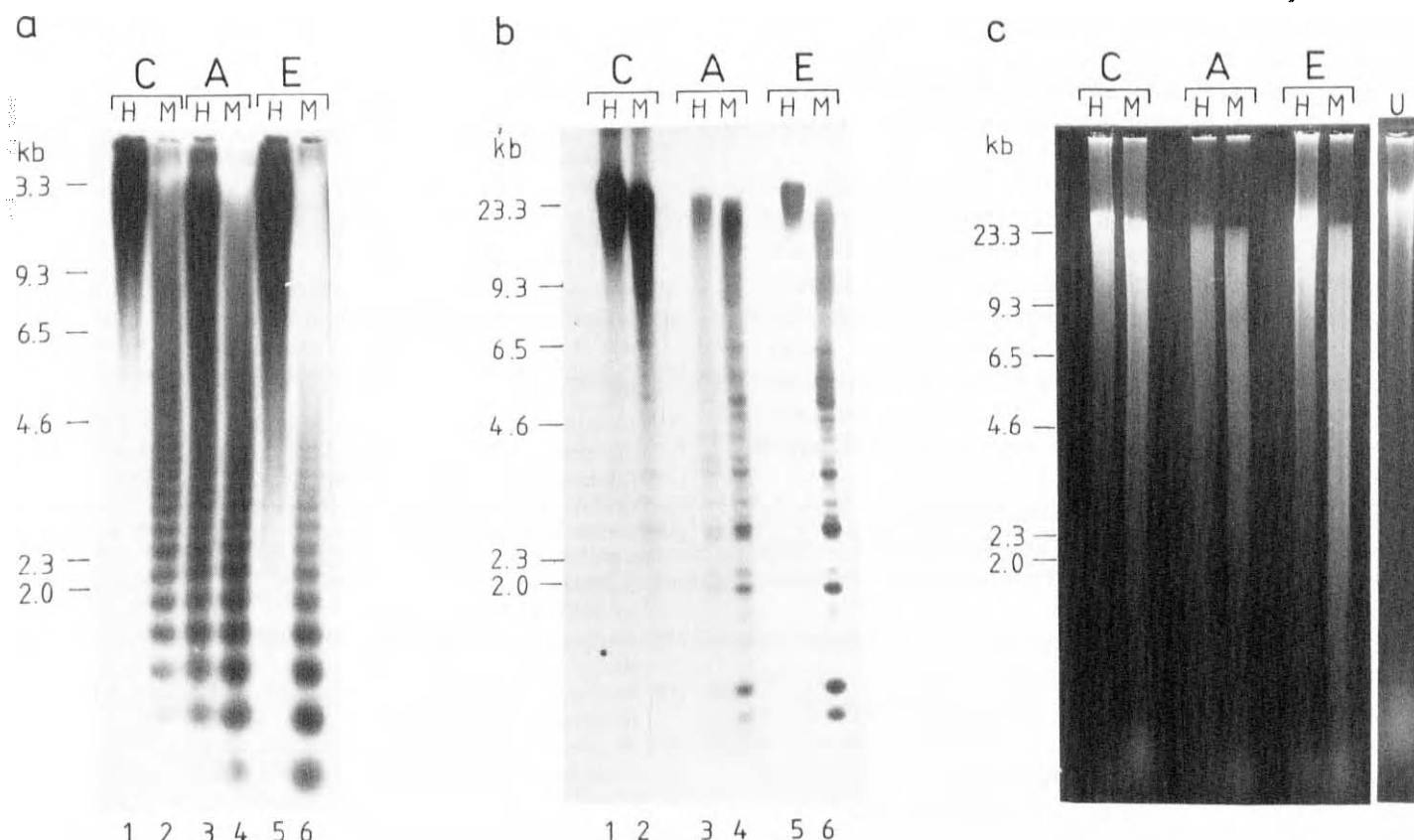


Fig. 1. Drug-induced hypomethylation of two families of DNA repetitive sequences of tobacco genome. DNAs were prepared from leaf-derived calli, digested with either *HpaII* (H), lanes 1, 3, 5, or *MspI* (M), lanes 2, 4, 6, size-separated on agarose gels and Southern blot hybridized. The hybridization probes used were a, 184 bp HRS60.1, b, 186 bp R8.1.34. (C) control calli cultured on MS solid medium. (A) calli cultured in the presence of 5-azacytidine, (E) calli cultured in the presence of ethionine. c. Undigested DNA (U) and digested DNAs stained with ethidium bromide following agarose gel electrophoresis.

ied in two different classes of tobacco DNA repeats, HRS60 and R8.1. Non-transcribed highly repetitive DNA sequences of the family HRS60 comprise about 2% of the nuclear tobacco genome [12] and are located mostly in telomeric regions [7]. They occur presumably in the *N. sylvestris* component of the *N. tabacum* amphitetraploid genome [14]. The family R8.1 of non-transcribed dispersed repeats represents about 0.3% of nuclear tobacco DNA [13].

To inhibit the DNA methylation, 5-azaC or Ethi in subtoxic doses were applied to cultured tobacco leaf-derived calli. Total DNAs were then isolated, cleaved with restriction nucleases *MspI* or *HpaII*, and Southern blot analysis was carried out as described in section 2. Both *MspI* and *HpaII* have the common recognition CCGG sequence, but they differ in their sensitivity to methylation: CmCCGG can be digested with *MspI*, but not with *HpaII*, while the sequences mCCGG and mCmCCGG cannot be digested with either of these enzymes.

The CCGG sequence has been found in all members of the HRS60 family of DNA repeats sequenced until now [12,15], but only in some members of the R8.1 family (Kuhrová et al., in preparation). For example, the DNA probe R8.1.34 contains only four methyla-

tion-prone sites, convertible to the sequence CCGG by one nucleotide substitution.

DNAs isolated from untreated control calli were resistant to cleavage with *HpaII* in DNA sequences homologous to both probes, HRS60.1 and R8.1.34. The results shown in Fig. 1, lanes a1, b1 and lanes a2, b2 reflect higher levels of methylation in CG doublets than in CCG triplets. It is also obvious that more *MspI* cleavable sites are methylated in R8.1 dispersed repeats than those in the HRS60 family (Fig. 1, lanes a2, b2).

As expected, the treatment of calli with 5-azaC led to hypo-methylation of CG doublets as revealed by digestion with *HpaII* (Fig. 1, lanes a3, b3) and CCG triplets as revealed by digestion with *MspI* (Fig. 1, lanes a4, b4).

Since the mode of action of Ethi, compared with that of 5-azaC, is entirely different [4], it occurred to us that the comparison of hypomethylation effects induced by these two drugs could supply more information about the plant DNA methylation system. This expectation has proven to be correct. In contradistinction to 5-azaC, Ethi exerted a profound inhibitory effect upon the methylation of the CCG triplet within the CCGG tetranucleotide recognition sequence (Fig. 1, lanes a6, b6) while it had only a slight effect on the methylation status of the CG doublet, as revealed by the resistance to

digestion with *HpaII* (Fig. 1, lanes a5, b5). Fig. 1c shows that essentially the same conclusion can be drawn from the comparison of the degree of digestion of whole DNA samples following electrophoresis and ethidium bromide staining.

The asymmetric inhibitory effect of EthI implies non-randomness in the decision which of the two cytosines in the CCGG sequence is to be modified. The data reported so far show that only one MTase activity, which does not discriminate between CG and CXG sequences, can be detected in plant nuclei [16]. To explain the specific inhibitory effect of EthI we propose the analogous allosteric interaction of SAM with eukaryotic MTases as described by Bergerat et al. for Dam of *E. coli* [17].

Allosteric interaction of the plant enzyme with S-adenosyl-ethionine could shift the affinity of the enzyme complex in favour of CXG and could bias the methylation escape of these sequences.

Anyhow, our finding that ethionine could efficiently inhibit methylation of trinucleotide targets in various regions of the tobacco genome opens the possibility to elucidate the role of methylation of CXG sequences in the control of plant gene expression.

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