The gene for domains rearranged methyltransferase (DRM2) in *Arabidopsis thaliana* plants is methylated at both cytosine and adenine residues

Vasili V. Ashapkin*, Lyudmila I. Kutueva, Boris F. Vanyushin

Belozersky Institute of Physical and Chemical Biology, Lomonosov Moscow State University, Moscow 119992, Russia

Received 12 July 2002; revised 28 October 2002; accepted 6 November 2002

First published online 22 November 2002

Edited by Marc Van Montagu

Abstract The methylation patterns of cytosine and adenine residues in the *Arabidopsis thaliana* gene for domains rearranged methyltransferase (DRM2) were studied in wild-type and several transgene plant lines containing antisense fragments of the cytosine DNA-methyltransferase gene *METI* under the control of copper-inducible promoters. It was shown that the promoter region of the *DRM2* gene is mostly unmethylated at the internal cytosine residue in CCGG sites whereas the 3′-end proximal part of the gene coding region is highly methylated. The *DRM2* gene was found to be also methylated at adenine residues in some GATC sequences. Cytosine methylation in CCGG sites and adenine methylation in GATC sites in the *DRM2* gene are variable between wild-type and different transgenic plants. The induction of antisense *METI* constructs with copper ions in transgene plants in most cases leads to further alterations in the *DRM2* gene methylation patterns.

Key words: DNA-methyltransferase; DNA methylation; 5-Methylcytosine; N^6-Methyladenine; Plant; *Arabidopsis thaliana*

1. Introduction

The first complete nucleotide sequence of a plant (*Arabidopsis thaliana*) genome became available by the end of the year 2000 [1]. Its analysis supports the recent view that plants possess three major families of cytosine DNA-methyltransferase genes (*MET*) encoding DNA-methyltransferase proteins that are different in both structure and putative function [2]. The first gene family, *METI*, encodes four proteins closely related to mouse maintenance cytosine DNA-methyltransferase *Dnmt1* [2]. *METI* encodes, in particular, the predominant DNA-methyltransferase that is most highly expressed in meristematic cells in both vegetative and floral plant tissues [3–5]. *METIIa/b* are also transcribed in all tissues but the level of transcripts is about 10 000-fold lower than that for *METI* [5]. The expression of *METIII* has not yet been studied. The predominant protein encoded by *METI* is evidently the major maintenance DNA-methyltransferase that methylates mostly, if not exclusively, symmetrical CpG sequences [6]. The second family of the *A. thaliana* cytosine DNA-methyltransferase genes encodes three so-called chromomethylases unique for plants [7]. One of these chromomethylases (CMT3) seems to be the major non-CpG maintenance DNA-methyltransferase [8,9]. The third family of the *A. thaliana* DNA-methyltransferases consists of one functional gene and several pseudogenes related to animal de novo DNA-methyltransferases *Dnmt3* [10]. This family is distinguished from all known eukaryotic cytosine DNA-methyltransferases by an unusual arrangement of the conserved cytosine DNA-methyltransferase motifs and it is, therefore, named *DRM* (*domains rearranged methyltransferases*). Their function is still unknown though the high level of similarity to animal de novo methyltransferases suggests them to be the plant de novo DNA methyltransferases.

N^6-Methyladenine (m^6A) along with 5-methylcytosine (m^5C) was isolated from total DNA of some higher plants [11]; m^6A was detected in wheat mtDNA [12], and it seems that some nuclear (zein) genes in corn plants may also be methylated at adenine residues [13]. It was found that an open reading frame for a putative protein containing all conservative motifs typical for prokaryotic DNA-(amino)methyltransferases is present in the genome of *A. thaliana* (GenBank, BAB02202.1) as well as of some other eukaryotes [14], and an N^6-adenine DNA-methyltransferase (wadmtase) was recently isolated from wheat coleoptiles [15]. Since m^6A was found in DNA of higher plants [11] and it seems to affect gene transcription and plant development [16–18], it was assumed that methylation at both adenine and cytosine residues may be essential for regulation of gene activity. In particular, methylation of adenine residues in DNA seems to control mtDNA replication in plants [12]. Unfortunately, the nature and character of methylation of DNA-methyltransferase genes themselves in higher plants are unknown.

Recently we produced a number of transgenic lines of *A. thaliana* containing copper-controllable antisense *METI* constructions (to be described in detail elsewhere). As part of a general investigation of these transgenic plants we have studied the methylation patterns of different DNA-methyltransferase genes. In this paper we describe the methylation character of adenine and cytosine residues in the coding and promoter regions of the *DRM2* gene.

*Corresponding author. Fax: (7)-095-939 3181.
E-mail address: ashapkin@genebee.msu.su (V.V. Ashapkin).

Abbreviations: DRM2, domains rearranged methyltransferase; METI, cytosine DNA-methyltransferase I; m^6A, N^6-methyladenine; m^5C, 5-methylcytosine; PCR, polymerase chain reaction

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)03711-0

2. Materials and methods

2.1. Plant material

The leaves of *A. thaliana* plants (Columbia ecotype) were used as the source of DNA samples. Transgene plant lines were produced by in planta transformation of Columbia wild-type *A. thaliana* by a recombinant binary vector plasmid pPMB765 [19] containing a 5′-proximal fragment of the *Arabidopsis METI* gene (~0.6 kb polymerase chain reaction (PCR) fragment from +12 to 592 bp of the gene coding sequence [20]) or fragments of *METI* cDNA from recombinant plasmid pYc2 (~2 kb 5′-end proximal part of the full-length *METI* cDNA sequence) and pYc8 (~2.6 kb 3′-end proximal part of the full-length *METI* cDNA sequence) [3] in antisense orientation under the control of copper-inducible promoters from the recombinant plasmids pPMB7066 [21] and pPMB768 [19]. The plants were grown in a Fisons Plant Growth Cabinet (UK) with a 16/8 h day/night cycle at 23°C. For copper induction of transgenes the respective populations of 14 day old plants (denoted +Cu) were grown for 30 days being watered with 10 μM $CuSO_4$ solution.

2.2. Analysis of DNA methylation patterns

The DNA hybridization probes DRM2Pro, complementary to the immediate 5′-flanking sequence of the *DRM2* gene (from +54 up to −755 bp relative to the transcription initiation site), and DRM2Str, complementary to the exon 9 sequence (from +1997 to +2648 bp), were obtained by PCR amplification of the respective segments of *Arabidopsis* genomic DNA. Probes were cloned in a plasmid vector pGEM3Zf+ (Promega), their sequences were verified by complete sequencing of both strands and they were used for Southern blot hybridization experiments.

The DNA samples were first hydrolyzed with *Pst*I and then with a number of methylation-sensitive restriction endonucleases [22]; resulting fragments were fractionated by 0.7% agarose gel electrophoresis and blotted to Hybond-N+ membranes by the alkali-blotting method as recommended by the supplier (Amersham). The blots were first hybridized to the ^{32}P-labeled DRM2Pro probe at 65°C in a hybridization buffer solution containing 5×SSPE, 5×Denhardt's solution, 1% SDS and 100 μg/ml denatured salmon sperm DNA. The membranes were washed at 65°C three times (15 min each) with 1×SSPE, 1% SDS followed by three washes with 0.1×SSPE, 0.1% SDS solution. Wet blots were wrapped in SaranWrap and exposed to X-ray film. After exposure the filters were stripped of labeled DRM2Pro probe by a freshly boiled 0.5% SDS solution and hybridized with a ^{32}P-labeled DRM2Str probe under the same conditions. Of the methylation-sensitive restriction endonucleases used *Hpa*II does not hydrolyze DNA when either of the two C residues in the CCGG recognition site is methylated, whereas its isoschizomeric restriction endonuclease *Msp*I is inhibited by methylation of the external C residue only, and it cleaves DNA irrespective of the methylation of the internal C residue; the cleavage of DNA by restriction endonuclease *Sau*3A is inhibited by methylation of the C but not the A residue in the GATC recognition sequence, whereas its isoschizomeric restriction endonuclease *Mbo*I has opposite sensitivity to methylation, it is insensitive to C methylation and inhibited by A methylation on either or both DNA strands. Their unique isoschizomeric restriction endonuclease *Dpn*I cleaves only GATC sites methylated at adenine residues on both DNA strands.

The restriction fragments of the 5′-flanking and structural regions of the *DRM2* gene produced by each of the restriction endonucleases mentioned were compared with a detailed map of their corresponding recognition sites deduced from the known nucleotide sequence of the *DRM2* gene (GenBank, AL163792). The positions of *DRM2* exons along the complete genomic sequence were deduced from its alignment with the *DRM2* cDNA sequence (GenBank, AF240695 [10]).

3. Results and discussion

The schematic structure of the *DRM2* gene in *A. thaliana* is shown in Figs. 1C and 2C. The analysis of the cytosine and adenine methylation patterns was limited to a 5.3 kb fragment of *A. thaliana* chromosome 5 DNA located between two recognition sites for restriction endonuclease *Pst*I and containing the entire coding sequence of the *DRM2* gene together with some flanking sequences. There are three recognition sites for *Hpa*II/*Msp*I in this region: one of them is located in the promoter region of the gene (~0.5 kb upstream of the transcription initiation site), another is found just a few bp downstream of the transcription initiation site, and the last one is found in the 3′-end proximal half of the gene inside the ninth exon. Just one fragment of the expected length (5.3 kb) was cut out from genomic DNA upon its hydrolysis with restriction endonuclease *Pst*I (lines P in Figs. 1A,B and 2A,B). This means that none of the *DRM2* homologous genomic sequences are detectable under the stringent hybridization/washing conditions used. Further digestion of *Pst*I-digested genomic DNA with either *Hpa*II or *Msp*I restriction endonucleases completely reduces the 5.3 kb *Pst*I fragment to smaller fragments (Fig. 1A,B). Therefore, all DNA molecules of the genomic DNA samples contain some unmethylated CCGG sequences inside the 5.3 kb *Pst*I fragment. The methylation degree of individual *Hpa*II/*Msp*I recognition sites in different DNA samples may be deduced from the length of fragments detected with the use of the DRM2Pro and DRM2Str probes. The predominant DRM2Pro hybridizing fragment is 1.2 kb long (Fig. 1A). This band evidently corresponds to a segment of the *DRM2* 5′-flanking region confined between the upstream *Pst*I site (−1.7) and the promoter *Hpa*II site (−0.48) (Fig. 1C). Since this band seems to be predominant in the hybridization patterns for all DNA samples investigated, the promoter CCGG site is significantly (pYc2-7066, pYc2-768, pYc2-768+Cu and pYc8-7066+Cu DNA samples) or completely (Col-WT, PCR-7066, PCR-7066+Cu and pYc8-7066 DNA samples) unmethylated at both external and internal cytosine residues. The cleavage of DNA at the *Hpa*II (−0.48) and *Hpa*II (+0.02) sites would produce a short (0.5 kb) DRM2Pro hybridizing fragment that is not visible since it has evidently migrated out of the gel. On the other hand, the state of *Hpa*II (+0.02) site methylation may be deduced from the presence or absence of the 2.6 kb *Hpa*II (−0.48)–*Hpa*II (+2.1) or still longer *Hpa*II (−0.48)–*Pst*I (+3.6) fragments. Since no such fragments were detected, the CCGG site at the 5′-end of the *DRM2* coding sequence is also completely unmethylated at both external and internal cytosine residues. With some DNA samples (pYc2-7066, pYc2-768, pYc2-768+Cu and pYc8-7066+Cu) we did observe a number of longer (1.7, 2.1 and 2.6 kb) bands. As can be easily seen in Fig. 1C, the 2.1 kb band is produced by the cleavage of *DRM2* at the *Hpa*II (+0.02) and *Hpa*II (+2.1) sites. The chance detection of this band with the DRM2Pro probe seems to be the trivial consequence of their very short overlapping sequence (37 bp). Indeed, the 2.1 kb band was readily observed upon hybridization of the same blots with the DRM2Str probe that have a more substantial overlapping sequence with the *Hpa*II (+0.02)–*Hpa*II (+2.1) fragment (105 bp) (Fig. 1b). The presence of 1.7 and 2.6 kb bands indicates that there is some level of methylation of the *Hpa*II (−0.48) and *Hpa*II (+0.02) sites in the respective DNA samples. The methylation state of the *Hpa*II (+2.1) site may be easily deduced from the DRM2Str probe hybridization patterns. The cleavage of the *DRM2* gene at this site (Fig. 1C) produces a 1.5 kb *Hpa*II (+2.1)–*Pst*I (+3.6) fragment readily detectable by hybridization with the DRM2Str probe, and longer *Hpa*II (+0.02)–*Hpa*II (+2.1), *Hpa*II (−0.48)–*Hpa*II (+2.1) or *Pst*I (−1.7)–*Hpa*II (+2.1) fragments (2.1, 2.6 and 3.8 kb) that have a shorter overlapping sequence with the DRM2Str probe

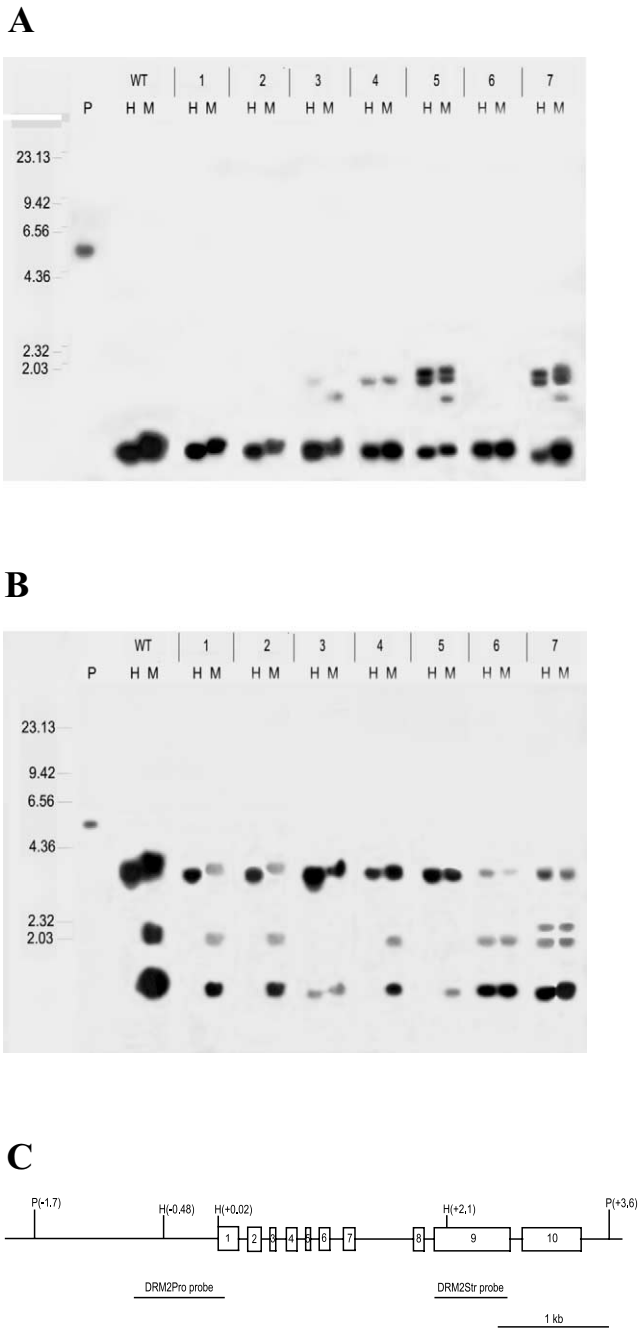

Fig. 1. Methylation patterns of *Hpa*II/*Msp*I recognition sites in the *DRM2* gene of *A. thaliana*. A: Methylation patterns of the promoter region. Samples of genomic DNA isolated from leaves of Columbia wild-type and seven transgenic plant lines were digested with restriction endonuclease *Pst*I (line P) and further digested with restriction endonucleases *Hpa*II (H) or *Msp*I (M) and analyzed by Southern blot hybridization with probe DRM2Pro. The DNA samples are from: WT: Columbia wild-type plants; 1–7: transgenic lines containing antisense constructions: 1, 5′-proximal PCR fragment of the *Arabidopsis METI* gene (~0.6 kb from +12 to 592 bp of the gene coding sequence), under the control of copper-inducible promoter from pPMB7066 plasmid; 2, 5′-proximal PCR fragment, plants grown in the presence of copper ions; 3, fragments of *METI* cDNA from recombinant plasmid pYc2 (~2 kb 5′-end proximal part of the full-length *METI* cDNA sequence), under the control of the pPMB7066 promoter; 4, pYc2 fragment, under the control of the pPMB768 promoter; 5, pYc2 fragment, under the control of the pPMB768 promoter, plants grown in the presence of copper ions; 6, pYc8 (~2.6 kb 3′-end proximal part of the full-length *METI* cDNA sequence) fragment, under the control of the pPMB7066 promoter; 7, pYc8 fragment, under the control of the pPMB7066 promoter, plants grown in the presence of copper ions. The positions of marker fragments (λ phage DNA cleaved with restriction endonuclease *Hin*dIII) are shown on the left. B: The blotting membrane shown in A was stripped of labeled DRM2Pro probe and rehybridized with labeled DRM2Str probe. C: Schematic representation of the *DRM2* gene structure. Exons are shown as numbered open boxes. The positions of the restriction sites for *Pst*I (P) and *Hpa*II/*Msp*I (H) are shown in kb with respect to the transcription initiation site. The relative positions of the DRM2Pro and DRM2Str probes are shown underneath. 1 kb bar is shown for scale.

(105 bp) and would, therefore, be detectable as weaker bands. The methylation of the *Hpa*II (+2.1) site may be detected by the presence of the 3.6 kb fragment *Hpa*II (+0.02)–*Pst*I (+3.6) or still longer fragments. As shown in Fig. 1B, the *Hpa*II (+2.1) site is completely resistant to *Hpa*II cleavage in DNA samples from Col-WT, PCR-7066, PCR-7066+Cu, pYc2-768 and pYc2-768+Cu plants, it is mostly resistant in DNA from pYc2-7066 plants and less than 50% resistant in DNA from pYc8-7066 and pYc8-7066+Cu plants. *Msp*I cleaves this site much more readily as compared to *Hpa*II but not completely in all DNA samples. Thus, the CCGG site located in the ninth exon of the *DRM2* gene is completely methylated at the internal cytosine residue in DNA of Col-WT, PCR-7066, PCR-7066+Cu, pYc2-768 and pYc2-768+Cu plants; it is highly methylated in DNA of pYc2-7066 plants and partially ($<50\%$) methylated in DNA of pYc8-7066 and pYc8-7066+Cu plants. In all DNA samples this site is partially methylated at the external cytosine residue but always to a lesser extent compared with the methylation at the internal cytosine residue.

The location of GATC sites recognized by isoschizomeric restriction endonucleases *Sau*3A, *Mbo*I and *Dpn*I is shown in Fig. 2C. The close spacing between these sites makes it rather difficult to correlate the bands observed with the methylation state of the individual GATC sites, but some deductions are still possible. The most prominent DRM2Pro hybridizing band in *Sau*3A and *Mbo*I digests (Fig. 2A) is probably the product of DNA cleavage at GATC sites (−0.93) and (+0.21) (Fig. 2C), which are, therefore, mainly unmethylated at both cytosine and adenine residues. The presence of longer fragments in digests of DNA from pYc8-7066 and pYc8-7066+Cu plants shows that one or both of the bases mentioned are partially methylated in these plants. The partial resistance of DNA cleavage to *Sau*3A is not at all unusual since methylation of cytosine residues in plant DNA may easily affect a share of the GATC site population because the C residue may be a part of canonical CG or CNG sites. In contrast, the resistance of DNA to *Mbo*I is totally unexpected since the cleavage by this restriction endonuclease is reported not to be affected by C5 methylation and it is inhibited by the adenine methylation only. Nevertheless, it is not improbable that some unmethylated GATC sites might be refractory to *Mbo*I cleavage due to some peculiar sequence context. The incubation of *Pst*I-digested DNA with restriction endonuclease *Dpn*I in most cases does not lead to further cleavage of DNA (Fig. 2A,B). Since *Dpn*I cleaves DNA only at Gm^6ATC sites this insensitivity of DNA to hydrolysis with *Dpn*I could indicate that DNA of *A. thaliana* plants is mostly devoid of adenine-methylated GATC sites. Unexpectedly, we did observe *Dpn*I cleavage of DNA from Col-WT and to a much lesser degree of DNA from pYc8-7066 and pYc8-7066+Cu plants. The length of DRM2Pro hybridizing with *Dpn*I-specific restriction fragments (~1.1–1.3 kb) indicates that the adenine methylation may occur at some of the GATC sites in the nearest 5′-flanking region (−0.93, −1.06, −1.2) and in the first three exons of the *DRM2* gene.

The most prominent band produced by *Sau*3A and *Mbo*I digestion in DRM2Str hybridization patterns is ~0.8 kb long (Fig. 2B). This is probably due to cleavage of the *DRM2* gene at GATC sites (+2.22) in the ninth exon and (+3.0) in the tenth exon (Fig. 2C). The presence of larger fragments (~0.9–1.2 kb) seems to indicate that there is some level of methylation of GATC sites in exons 9 and 10 at both cytosine and adenine residues. The adenine methylation in this region is further confirmed by its partial cleavage by *Dpn*I. Quite consistent with this notion the cleavage by *Dpn*I is most ob-

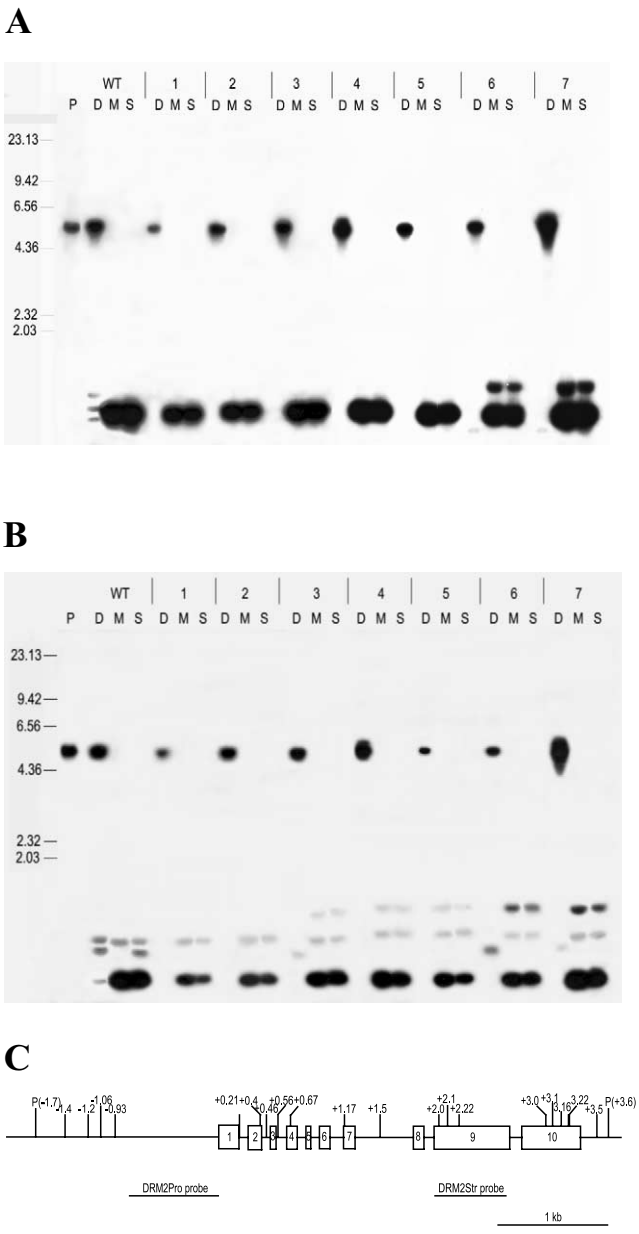

Fig. 2. Methylation patterns of *Sau*3A/*Mbo*I/*Dpn*I recognition sites in the *DRM2* gene of *A. thaliana*. A: Methylation patterns of promoter region. The samples of genomic DNA isolated from leaves of Columbia wild-type and seven transgenic plant lines were digested with restriction endonuclease *Pst*I (P) and further digested with restriction endonucleases *Dpn*I (D), *Mbo*I (M) or *Sau*3A (S), and analyzed by Southern blot hybridization to probe DRM2Pro. The designations of DNA samples are the same as in Fig. 1. The positions of marker fragments (λ phage DNA cleaved with restriction endonuclease *Hin*dIII) are shown on the left. B: The blotting membrane shown in Fig. 1A was stripped of labeled DRM2Pro probe and rehybridized with labeled DRM2Str probe. C: Schematic representation of the *DRM2* gene structure. The positions of the restriction sites for *Pst*I (P) and *Sau*3A/*Mbo*I/*Dpn*I are shown in kb with respect to the transcription initiation site. The relative positions of the DRM2Pro and DRM2Str probes are shown underneath. 1 kb bar is shown for scale.

vious in DNA samples that are most resistant to *Mbo*I cleavage (DNA from plants Col-WT, pYc8-7066, pYc8-7066+Cu).

The major finding of our work concerning the methylation patterns of the *DRM2* gene at CCGG sites is quite straightforward. The 5′-end proximal part of the gene represented by the *Hpa*II sites (−0.48) and (+0.02) is mostly unmethylated, whereas the 3′-end proximal gene part (*Hpa*II (+2.1) site) is mostly methylated at the internal and partially methylated at the external cytosine residues. Interestingly, the methylation degrees of the *Hpa*II sites in the *DRM2* gene are different in DNA of the wild-type Columbia plants and that of the transgenic lines containing the antisense constructs of the *METI* gene under the control of copper-inducible promoters. Moreover, the induction of these transgenic constructs with copper ions in most cases leads to further alterations in *DRM2* gene methylation patterns. We suggest that the activity of the *METI* gene may somehow affect the expression of the *DRM2* gene (and probably genes for other methyltransferases). It should be noted that this influence may be rather complex, involving both direct effects of *DRM2* gene promoter methylation by METI methyltransferase and secondary effects through changed activity of other methyltransferases (including DRM2 itself), chromatin remodelling proteins and other factors. As a matter of fact the existence of a complicated network of mutual interactions of different DNA-methyltransferases active in the plant cells seems to be quite plausible. The possibility of such interactions should be taken into account when analyzing the effects of inactivation of the individual DNA-methyltransferase genes.

Another and a rather more intriguing finding of our study is the methylation of the *DRM2* gene at adenine residues. The presence of this methylation in the cells of *A. thaliana* is evidenced by both partial resistance of DNA to cleavage with restriction endonuclease *Mbo*I (known to be sensitive to adenine methylation in GATC recognition sites) and detectable though minor cleavage of the same DNA with restriction endonuclease *Dpn*I that is known to cleave only Gm^{6}ATC sites. However, the partial resistance of single GATC sites to *Mbo*I cleavage irrespective of their methylation state due to some kind of unusual sequence context cannot be ruled out. But this explanation for the observed partial resistance of the *DRM2* gene to *Mbo*I cleavage seems unlikely, since it would similarly affect all DNA samples investigated, which is evidently not the case. The appearance of additional DNA hybridizing fragments in the *Pst*I+*Dpn*I cleavage patterns as compared to those of *Pst*I cleavage is evidently not a result of a spurious hybridization of the DRM2Pro and DRM2Str probes to some homologous *Pst*I fragment of the *A. thaliana* genomic DNA. First, the length of these additional fragments is exactly the same as that of some *Mbo*I and *Sau*3A restriction fragments. That seems to be too much of a coincidence if these fragments are not produced due to DNA cleavage at the same GATC sites. Second, we never observed such 'spurious' bands in the *Pst*I hybridization patterns. Last, but not least, Blast-N search against the complete sequence of *A. thaliana* genome revealed no sequences of significant homology to the DRM2Pro probe except for the *DRM2* gene itself, whereas two additional sequences with significant homology to the DRM2Str probe were found. The first, located in an intergenic region of chromosome 1 (GenBank, AC012375), consists of two nearby DNA segments (184 and 169 bp) with ~80% homology to the DRM2Str probe sequence; the second, located in chromosome 5 (GenBank, AL353993.1), consists of a short (67 bp) segment of a protein coding sequence with ~85% homology to the DRM2Str probe. These sequences could possibly give some additional DRM2Str hybridizing bands in *Pst*I digests of *A. thaliana* DNA but the lengths of corresponding *Pst*I fragments (calculated from the DNA sequence) are >8 and 4.64 kb, respectively, whereas the length of 'additional' bands in *Pst*I+*Dpn*I digests observed in our study is ~1 kb or less. All these considerations convince us that there are indeed some adenine residues in the genomic DNA of *A. thaliana* that are methylated. These methylated adenine residues are found in Gm^{6}ATC sites that could be a constituent part of a sequence T**GATC**A recognized and methylated by wheat adenine DNA-methyltransferase (wadmtase) [12]. Unfortunately, we do not know whether adenine DNA-methyltransferase in the cells of *A. thaliana* has the same site specificity as it has in wheat plants.

Though the functional significance of cytosine DNA methylation as a regulatory mechanism of gene transcription in plants is well established [23], whether the adenine DNA methylation is essential for regulation of plant gene expression or some other genetic processes is still unclear. The presence of a gene for putative adenine DNA-methyltransferase in the genome of *A. thaliana* [14], the detection of m^{6}A residues in the *DRM2* gene (this paper) and the adenine DNA-methyltransferase found in wheat coleoptiles [15] suggest that adenine DNA methylation may play a significant role in plant cells. The effects of artificial adenine methylation on plant gene expression [16–18] further support this notion. The adenine methylation introduced into transgenic tobacco plants via expression of the bacterial *dam* methylase was found to be directly proportional to expression levels of the *dam* construct, and methylation of all GATC sites was observed in a highly expressing line [16]. Increasing expression levels of the enzyme in different plants correlated with increasingly abnormal phenotypes affecting leaf pigmentation, apical dominance, and leaf and floral structure. The methyladenine produced by *dam* methylation in vitro was shown to increase gene expression from constructs based on the *Arabidopsis* alcohol dehydrogenase (Adh1), maize ubiquitin (Ubi1) and rice actin (Act1) genes in transient assays in protoplasts and microprojectile-bombarded whole tissues [17]. The increase in gene expression ranged from three-fold for Ubi1 and Adh1 in protoplasts to 50-fold for Act1 in bombarded wheat tissues. Similarly modification with *dam* methylase was found to be necessary and sufficient for the high levels of GUS expression driven by the promoters of genes which belong to the Pathogenesis Related family (PR-1a and PR-2d) in tobacco protoplasts [18]. Since bacterial *dam* methylase recognizes the same sites that we have found to contain methylated adenine in *Arabidopsis* (GATC) the methylation of plant DNA by endogenous adenine DNA-methyltransferase may well be a mechanism of transcription regulation. However, this line of reasoning seems to be far from conclusive since all the above cited examples of plant gene expression modulation by adenine methylation were obtained in experimental situations involving artificial plant DNA methylation at adenine residues to levels that are much higher than those detected in wild-type plants.

Interestingly the adenine methylation of the *DRM2* gene observed in our study is most prominent in wild-type plants and appears to be diminished by the presence of antisense

METI transgenes. Since METI does not possess adenine DNA-methyltransferase activity its action on adenine methylation is evidently a secondary effect mediated through adenine DNA-methyltransferase or some other factors.

Acknowledgements: Plasmids pYc2 and pYc8 were a generous gift of Drs. E. Jean Finnegan and Elizabeth S. Dennis (CSIRO, Plant Industry, Canberra, Australia); the copper-inducible vector system for plant transformation was a generous gift of Drs. Vadim L. Mett and Paul H.S. Reynolds (Horticulture and Food Research Institute of New Zealand). This work was supported in part by the Russian Foundation of Fundamental Research (Grants 02-04-48005, 00-15-97920).

References

[1] The Arabidopsis Genome Initiative (2000) Nature 408, 796–815.
[2] Finnegan, E.J. and Kovac, K.A. (2000) Plant Mol. Biol. 43, 189–201.
[3] Finnegan, E.J. and Dennis, E.S. (1993) Nucleic Acids Res. 21, 2383–2388.
[4] Ronemus, M.J., Galbiati, M., Ticknor, Ch., Chen, J. and Dellaporta, S.L. (1996) Science 273, 654–657.
[5] Genger, R.K., Kovac, K.A., Dennis, E.S., Peacock, W.J. and Finnegan, E.J. (1999) Plant Mol. Biol. 41, 269–278.
[6] Kishimoto, N., Sakai, H., Jackson, J., Jacobsen, S.E., Meyerowitz, E.M., Dennis, E.S. and Finnegan, E.J. (2001) Plant Mol. Biol. 46, 171–183.
[7] Henikoff, S. and Comai, L. (1998) Genetics 149, 307–318.
[8] Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S. and Jacobsen, S.E. (2001) Science 292, 2077–2080.
[9] Bartee, L., Malagnac, F. and Bender, J. (2001) Genes Dev. 15, 1753–1758.
[10] Cao, X., Springer, N.M., Muszynski, M.G., Phillips, R.L., Kaeppler, S. and Jacobsen, S.E. (2000) Proc. Natl. Acad. Sci. USA 97, 4979–4984.
[11] Vanyushin, B.F., Kadyrova, D.Kh., Karimov, Kh.Kh. and Belozersky, A.N. (1971) Biokhimiya 36, 1251–1258.
[12] Kirnos, M.D., Alexandrushkina, N.I., Zagorskaya, G.Ya., Kireev, I.I. and Vanyushin, B.F. (1992) FEBS Lett. 298, 109–112.
[13] Pintor-Toro, J.A. (1987) Biochem. Biophys. Res. Commun. 147, 1082–1087.
[14] Shorning, B.Yu. and Vanyushin, B.F. (2001) Biochemistry (Moscow) 66, 753–762.
[15] Fedoreyeva, L.I. and Vanyushin, B.F. (2002) FEBS Lett. 514, 305–308.
[16] Van Blokland, R., Ross, S., Corrado, G., Scollan, C. and Meyer, P. (1998) Plant J. 15, 543–551.
[17] Graham, M.W. and Larkin, P.J. (1995) Transgenic Res. 4, 324–331.
[18] Brodzik, R. and Hennig, J. (1998) Plant Physiol. Biochem. 36, 401–406.
[19] Mett, V.L., Podivinsky, E., Tennant, A.M., Lochhead, L.P., Jones, W.T. and Reynolds, P.H.S. (1996) Transgenic Res. 5, 105–113.
[20] Kutueva, L.I., Ashapkin, V.V. and Vanyushin, B.F. (1996) Biochem. Mol. Biol. Int. 40, 347–353.
[21] Mett, V.L., Lochhead, L.P. and Reynolds, P.H.S. (1993) Proc. Natl. Acad. Sci. USA 90, 4567–4571.
[22] Roberts, R.J. and Macelis, D. (2001) Nucleic Acids Res. 29, 268–269.
[23] Finnegan, E.J., Genger, R.K., Peacock, W.J. and Dennis, E.S. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 223–247.