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M.-T. Cervera · L. Ruiz-García
J.M. Martínez-Zapater

Analysis of DNA methylation in *Arabidopsis thaliana* based on methylation-sensitive AFLP markers

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Abstract AFLP analysis using restriction enzyme isoschizomers that differ in their sensitivity to methylation of their recognition sites has been used to analyse the methylation state of anonymous CCGG sequences in *Arabidopsis thaliana*. The technique was modified to improve the quality of fingerprints and to visualise larger numbers of scorable fragments. Sequencing of amplified fragments indicated that detection was generally associated with non-methylation of the cytosine to which the isoschizomer is sensitive. Comparison of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I patterns in different ecotypes revealed that 35–43% of CCGG sites were differentially digested by the isoschizomers. Interestingly, the pattern of digestion among different plants belonging to the same ecotype is highly conserved, with the rate of intra-ecotype methylation-sensitive polymorphisms being less than 1%. However, pairwise comparisons of methylation patterns between samples belonging to different ecotypes revealed differences in up to 34% of the methylation-sensitive polymorphisms. The lack of correlation between inter-ecotype similarity matrices based on methylation-insensitive or methylation-sensitive polymorphisms suggests that whatever the mechanisms regulating methylation may be, they are not related to nucleotide sequence variation.

The first two authors contributed equally to this work

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M. T. Cervera · L. Ruiz García · J.M. Martínez Zapater (✉)
Departamento de Genética Molecular de Plantas,
Centro Nacional de Biotecnología, CSIC,
Campus de la Universidad Autónoma de Madrid,
Cantoblanco, 28049 Madrid, Spain
E mail: zapater@cnb.uam.es
Tel.: +34 91 5854687
Fax: +34 91 5854506

M. T. Cervera · L. Ruiz García · J.M. Martínez Zapater
Departamento de Biotecnología, SGIT, INIA,
Ctra. de la Coruña Km 7, 28049 Madrid, Spain

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Introduction

The addition of a methyl group to the C5 position of a cytosine residue is the most common DNA modification in plants and animals. DNA methylation has been associated with numerous biological processes including genomic imprinting, transcriptional regulation of genes and transposable elements, and gene silencing (Jost and Saluz 1993; Finnegan et al. 2000; Hafiz et al. 2001; Martienssen and Colot 2001; Paszkowski and Whitham 2001). It modifies access to genetic information, without altering the primary nucleotide sequence (Holliday 1987). Such modifications are maintained through DNA replication and thereby transmitted to both daughter cells upon mitotic cell division (Holliday 1990).

Patterns of methylation are established by de novo methyltransferases and maintained by maintenance methyltransferase activities. CG and CWG sequences are methylated by CG- and CWG-methyltransferases (Finnegan and Dennis 1993; Houlston et al. 1993; Pradhan and Adams 1995; Cao et al. 2000; Finnegan and Kovac 2000; Lindroth et al. 2001). Changes in methylation patterns occur by de novo methylation, or by passive demethylation due to failure to maintain methylation through DNA replication (Matsuo et al. 1998; Hsieh 1999). Active demethylation has been reported in chicken and mouse (Jost 1993; Weiss et al. 1996; Fremont et al. 1997) but not yet in plants.

Cytosine methylation analysis in plants has been approached by studying either global levels of methylated cytosines (Gruenbaum et al. 1981; Leutwiler et al. 1984; Adams and Burdon 1985; Watson et al. 1987; Vongs et al. 1993) or by examining specific gene sequences (Bender and Fink 1995; Cubas et al. 1999; Luff et al. 1999; Jacobsen et al. 2000; Soppe et al. 2000, Riddle and Richards 2002) using either bisulfite treatment

(Frommer et al. 1992; Sadri and Hornsby 1996) or restriction enzyme isoschizomers that differ in their sensitivity to methylation at their recognition sequences (Vongs et al. 1993). *Hpa*II and *Msp*I are isoschizomers that are frequently used to detect cytosine methylation. Both restriction enzymes recognize the tetranucleotide sequence 5′-CCGG-3′. However, *Hpa*II cannot cleave if one or both cytosines are methylated (in both strands), whereas *Msp*I cleaves $C^{5m}CGG$ but not $^{5m}CCGG$ sequences (Korch and Hagblom 1986; McClelland et al. 1994).

Our understanding of the structure, organization and evolution of plant genomes has advanced tremendously during the last decade thanks to the advent of several techniques for multilocus profiling, such as RAPD, AFLP, SAMPL or ISSR (Karp and Edwards 1998). The AFLP (Amplified Fragment Length Polymorphism) technique has been adapted for the analysis of cytosine methylation in plants (Xiong et al. 1999; Liu et al. 2001) and fungi (Reyna-López et al. 1997), based on the use of isoschizomers that show differential sensitivity to cytosine methylation. This strategy allows the study of anonymous CCGG regions in the genome that are sensitive to methylation. We set out to improve the AFLP protocol in order to reduce fingerprint background and visualise larger numbers of scorable fragments, and to used the modified method for the characterization of variation in methylation in Arabidopsis. Our results indicate that in different Arabidopsis ecotypes approximately one-third of CCGG restriction sites detected showed differential sensitivity to either *Hpa*II or *Msp*I. Interestingly, methylation status of CCGG sites was stable within Arabidopsis ecotypes, while it differed for 24–34% of the amplified fragments between different ecotypes. Cloning and subsequent sequence characterization of amplified fragments revealed the absence of internal *Hpa*II/*Msp*I restriction sites in most of the fragments, implying that their detection indeed indicates the lack of methylation of the cytosine to which the isoschizomer is sensitive. In addition, methylation-sensitive polymorphisms were equally likely to occur in coding and non-coding sequences.

Materials and methods

Plant material and growth conditions

Ten Arabidopsis ecotypes were used in this study. Ecotypes C24, Cvi 0 (Cape Verde Islands), Es 0 (Finland), Ita 0 (Morocco), Kas 1 (India), L*er* (Poland), Mt 0 (Libya) and Shah (Tadjikistan) were provided by C. Alonso Blanco (Madrid, Spain), while Col 0 (USA) and Ko 2 (Denmark) were provided by F. Lehle (Lehle's Seed, USA) and the Nottingham Arabidopsis Stock Centre respectively. Seeds were sterilised for 10 min with 30% commercial bleach containing 0.1% Triton X 100 and rinsed four times with sterile water, before being sown on Petri plates (9 cm diam.) containing solidified GM medium [MS medium (Murashige and Skoog 1962) with 1% sucrose]. Plants were grown in vitro in a growth chamber at 22°C on a 16 h photoperiod.

AFLP analysis of methylation sensitive sites

Total genomic DNA was extracted from 15 day old seedlings using the DNeasy kit (Qiagen). The AFLP protocol was performed following the general steps described by Xiong et al. (1999) but with major modifications. For each sample, AFLP analyses were performed using both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I digests. Digestion of 500 ng aliquots of genomic DNA with *Hpa*II was carried out in 25 μl of 10 mM Bis TRIS HCl pH 7.0, 10 mM $MgCl_2$, 1 mM DTT (Buffer 1, New England Biolabs) and 6 U of *Hpa*II (New England Biolabs) for 2 h at 37°C. After digestion, DNA was precipitated and digested with *Eco*RI in 35 μl of 10 mM TRIS acetate pH 7.5, 10 mM magnesium acetate, 50 mM DTT, and 10 U *Eco*RI (Pharmacia) for 2 h at 37°C. *Eco*RI/*Msp*I DNA digestions were carried out in a final volume of 35 μl in 10 mM TRIS acetate pH 7.5, 10 mM magnesium acetate, 50 mM DTT, 10 U of *Eco*RI, 10 U of *Msp*I and 500 ng of genomic DNA for 3 h at 37°C.

Two different adapters, designed to avoid reconstruction of restriction sites, one for the *Eco*RI sticky ends and one for the *Hpa*II/*Msp*I sticky ends, were ligated to the DNA after digestion, by adding to each final digestion 5 μl of a mix containing 5 pmol of *Eco*RI adapter, 50 pmol of *Hpa*II/*Msp*I adapter, 8 mM ATP, 10 mM TRIS acetate pH 7.5, 10 mM magnesium acetate, 50 mM DTT, and 1.4 U of T4 DNA ligase (Boehringer). The ligation was incubated for 3 h at 37°C and overnight at 4°C. The *Eco*RI adapter consisted of the combination of two primers: 5′ CTCGTAGACTGCGTACC 3′ and 3′ CTGACGCATGGT TAA 5′. The *Hpa*II/*Msp*I adapter consisted of the combination of the primers 5′ GACGATGAGTCTCGAT 3′ and 3′ TACTCA GAGCTAGC 5′.

Digested and ligated DNA fragments were diluted fivefold for use as templates for the first amplification reaction. This step consisted of a PCR using primers which are complementary to the *Eco*RI and *Hpa*II/*Msp*I adapters with an additional, selective 3′ nucleotide. The PCRs were performed in a 20 μl volume of 10 mM TRIS HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 30 ng of each primer *Eco*RI + A and *Hpa*II/*Msp*I + A, 0.4 U of Taq DNA polymerase and 3 μl of diluted fragments. PCR amplifications were carried out in a Perkin Elmer 9600 thermocycler for 20 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C.

Preamplified fragments were diluted 15 fold and used as starting material for selective radioactive amplification. For this amplification, only the *Eco*RI primers were labelled; one *Eco*RI and one *Hpa*II/*Msp*I primer, with the same sequences as those used in the pre amplification but with two and three selective nucleotides respectively at the 3′ end, were used in each analysis, selecting 1/64 of the pre amplified fragments. The PCR was performed in a 20 μl volume of 10 mM TRIS HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.08 mM of each dNTP, 4 ng of ^{33}P labelled *Eco*RI primer, 24 ng of *Hpa*II/*Msp*I primer, 0.4 U of Taq DNA polymerase (Boehringer), and 5 μl of diluted pre amplified DNA. The selective amplification was carried out using classical AFLP cycling parameters (Vos et al. 1995). The combination of one EcoRI and one *Hpa*II/*Msp*I primer with 2 and 3 selective nucleotides, respectively, gave an optimum number of scorable polymorphic fragments per primer combination. Eight primer combinations were used to analyse Landsberg *erecta* DNA isolated from individual or pooled plants: *Eco*RI + AC/*Hpa*II/*Msp*I + AAT, *Eco*RI + AC/*Hpa*II/*Msp*I + ATC, *Eco*RI + AC/*Hpa*II/*Msp*I + ACT, *Eco*RI + AA/*Hpa*II/*Msp*I + AAT, *Eco*RI + AA/*Hpa*II/*Msp*I + ATC, *Eco*RI + AA/*Hpa*II/*Msp*I + ACT, *Eco*RI + AG/*Hpa*II/*Msp*I + ATC and *Eco*RI + AT/*Hpa*II/*Msp*I + ACT. At the end of the selective radioactive PCR, samples were denatured by adding an equal volume of formamide buffer (98% formamide, 10 mM EDTA, pH 8.0, 0.05% bromophenol blue and 0.05% xylene cyanol) and heating for 3 min at 94°C. Aliquots (2 μl) of each sample were loaded on 4.5% polyacrylamide gels (acrylamide/bisacrylamide: 19:1) containing 7.5 M urea and 1×TBE gels.

Isolation and characterization of amplified fragments

Amplified fragments were isolated from gels that had been dried on Whatman paper. The excised piece of paper was incubated in 500 µl of high salt buffer (20% ethanol, 1 M LiCl and 10 mM TRIS HCl pH 7.5) for 1 h at 65°C. After centrifugation in a mi crofuge at 13,000 rpm for 5 min, the supernatant was transferred to a new tube and precipitated with 1 ml of ethanol. Eluted DNAs were resuspended in 20 µl of distilled water. A PCR was performed using 3 µl of the supernatant with the same primer combinations as those used in the selective amplification, and a temperature profile similar to either that used for the preamplification (fragments B3 B12; see below) or the selective amplification (fragment B1; see below). Amplified products were purified, using a PCR purification kit (Qiagen), and cloned into the pGEM T Easy Vector (Promega). DNA fragments were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer). Corre sponding sequences in the Arabidopsis genome were identified us ing BLAST. Primers were designed for DNA fragments that did not show sequence homology in searches in Arabidopsis sequence databases, to verify that they indeed originated from Arabidopsis nature. Primers B1F (5′ GAATTCATGGTATTCTTCAGGAA CC) and B1R (5′ CGGACTCGGAAGATGAACCTG), as well as the primers B6′ 2F (5′ AATACGGGCGGACGGCATTTC) and B6′ 2R (5′ AATTCAAGATCAAATGAACGGCA) were used to amplified B1 and B6′ 2, respectively, using total L*er* and Col DNAs as templates.

Data analysis

Degrees of genetic similarity were estimated in two different ways. Polymorphic amplified fragments among ecotypes that showed common *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I patterns were scored as present (1) or absent (0) in a binary matrix of "methylation insensitive polymorphisms". Amplified fragments that differed in presence/absence or in their intensity between *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I patterns were considered as "methylation sensitive polymorphisms". Methylation sensitive polymorphisms were scored as 1 when polymorphism (presence vs absence or different intensities of the amplified fragment) was detected for the same sample between *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I fingerprints, and 0 when no polymorphism was detected (same amplified fragment intensity or no detection of amplified fragment). Genetic similarity (GS) between pairs was estimated according to Dice (Sneath and Sokal 1973):

$$GS\ (ij) \quad 2a/(2a+b+c),$$

where $GS\ (ij)$ is the measure of genetic similarity between individ uals i and j, a is the number of polymorphic fragments that are shared by i and j, b is the number of fragments present in i and absent in j, and c is the number of fragments present in j and absent in i. Matrix comparisons were made to determine the correlation between methylation insensitive and methylation sensitive similar ity matrices using Mantel's test (Mantel 1967). Relationships among ecotypes based on genetic polymorphism and methylation sensitive polymorphism similarity matrices were established based on Principal Coordinate Analysis (PCOORDA). Analyses were performed using the NTSYS PC software package, version 2.0 (Rohlf 1998).

Results

AFLP analysis based on the application of isoschizomers showing different sensitivity to cytosine methylation (*Hpa*II and *Msp*I) was used to detect methylation-sensitive anonymous restriction sites. AFLPs were performed as described by Xiong et al. (1999) and Liu et al. (2001), but with major modifications designed to increase the number of amplified fragments and improve fingerprint readability. A new *Msp*I/*Hpa*II adapter was designed based on a core sequence described by Vos et al. (1995). Fragment digestion and ligation conditions were improved by reducing the DNA and restriction enzyme concentrations while optimising separate digestion steps (see Materials and methods). In order to avoid the amplification of unspecific fragments, we perform more stringent preamplification and amplification steps. For this purpose, the number of selective nucleotides was adjusted to take account of the size of the Arabidopsis genome and differences of more than two selective nucleotides between two consecutive PCR amplifications were avoided (Vos et al. 1995). In addition, the number of PCR cycles was reduced to 20 during the preamplification step to avoid PCR product saturation (Cervera et al. 2000). Finally, since *Eco*RI-*Hpa*II/*Msp*I AFLP fragments were generally larger than *Eco*RI-*Mse*I AFLP fragments (data not shown), 4.5% polyacrylamide gels were used to increase the number of fragments visualised. As a result, the number of scorable amplified fragments per reaction increased by an average of 20% with respect to previous protocols (Xiong et al. 1999; Liu et al. 2001).

Stability of methylation-sensitive polymorphic sites within Arabidopsis ecotypes

Given the improved efficiency of detection of anonymous methylation-sensitive restriction sites, the first question to be addressed was whether genotypically identical plants would share similar patterns of amplified methylation-sensitive fragments. To answer this question, five plants belonging to the Landsberg *erecta* (L*er*) ecotype and not directly derived from the same plant were analysed independently using three different primer combinations (*Eco*RI + AC/*Hpa*II/*Msp*I + AAT, *Eco*RI + AA/*Hpa*II/*Msp*I + AAT and *Eco*RI + AT/*Hpa*II/*Msp*I + ACT; Fig. 1) to compare their methylation patterns. Only one or two out of a total of 286 fragments were found to be polymorphic on pairwise comparison of randomly selected individuals; i.e., less than 1% of the sites analysed. These results indicate that there is a high degree of homogeneity in the methylation state of specific methylation-sensitive sites among identical genotypes. Based on this result, all additional analyses were performed with DNA samples isolated from pools of 30–50 individuals. Analysis of pooled L*er* plants using eight primer combinations revealed differences between the *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I profiles at, on average, 28% of the analysed CCGG restriction sites (Table 1), with this percentage ranging from 14 to 49 depending on the primer combination analysed. We also detected fragments that differed in intensity between *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I analyses, possibly indicating different methylation states of target restriction sites in different cell types or tissues of the samples analysed. However, the relationship between the

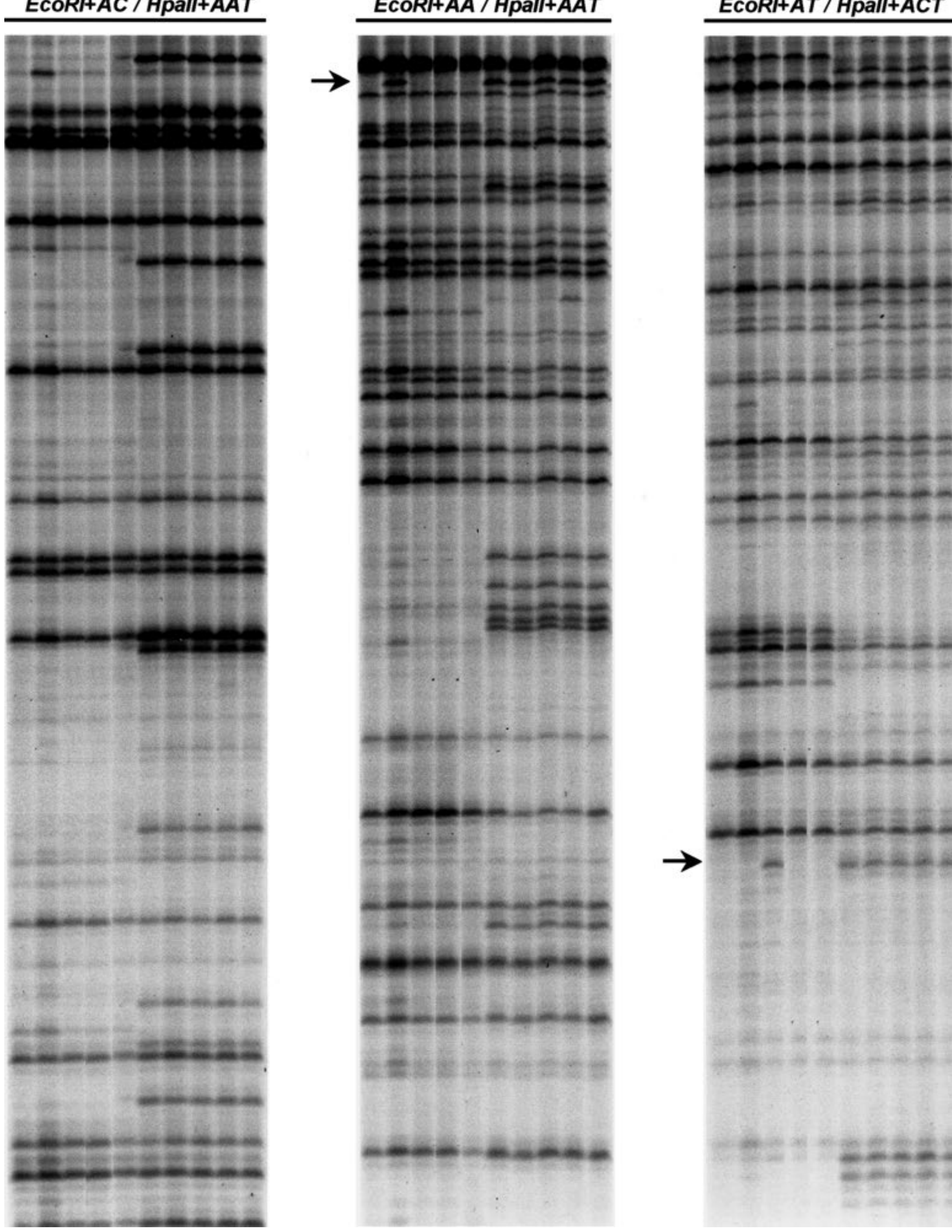


Fig. 1 Methylation sensitive AFLP analysis of five individual plants of the ecotype Landsberg *erecta*. DNA fingerprints were generated with the primer combinations *Eco*RI + AC/*Hpa*II *Msp*I + AAT, *Eco*RI + AA/*Hpa*II *Msp*I + AAT and *Eco*RI + AT/*Hpa*II *Msp*I + ACT. The *arrows* indicate polymorphic bands

Table 1 Total numbers of amplified fragments and methylation sensitive polymorphisms detected with the eight primer combinations used to analyse the Landsberg *erecta* ecotype

Primer combination	Methylation sensitive polymorphisms (%)	Total number of fragments amplified
*Eco*RI + AC/*Hpa*II/*Msp*I + AAT	49.1	118
*Eco*RI + AC/*Hpa*II/*Msp*I + ATC	23.3	60
*Eco*RI + AC/*Hpa*II/*Msp*I + ACT	15.2	59
*Eco*RI + AA/*Hpa*II/*Msp*I + AAT	40.7	113
*Eco*RI + AA/*Hpa*II/*Msp*I + ATC	16.2	74
*Eco*RI + AA/*Hpa*II/*Msp*I + ACT	16.7	108
*Eco*RI + AG/*Hpa*II/*Msp*I + ATC	13.6	66
*Eco*RI + AT/*Hpa*II/*Msp*I + ACT	28.0	107
Total	27.8	705

presence or absence of an amplified fragment and its methylated or non-methylated state cannot be established by this analysis.

To obtain more information on the sequences that are targeted by these AFLP analyses, we isolated and sequenced seven fragments (B3, B4, B5, B6, B6′-1, B7,

B9) that were differentially amplified in *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I assays, and five fragments that did not show polymorphism between the two amplifications (B1, B6′-2, B10, B11, B12). As shown in Table 2, sequences of these fragments were equally distributed between coding and non-coding regions. Furthermore, eleven fragments did not show any internal (CCGG) *Hpa*II/*Msp*I recognition sites, indicating that fragment detection resulted, in most cases, from the lack of methylation of the cytosine to which the isoschizomer is sensitive. Two of the analysed fragments did not show any sequence similarity to Arabidopsis sequences in the databases. However, the corresponding sequences were amplified from genomic L*er* and Col DNA using specific primers, thus confirming that they derived from Arabidopsis. These sequences could be located in centromeres or other highly repetitive chromosomal regions, which have not yet been sequenced completely by the Arabidopsis sequencing consortium (The Arabidopsis Genome Initiative 2000).

Methylation-sensitive polymorphisms among different Arabidopsis ecotypes

The existence of variation in methylation profiles among different ecotypes can be of significance for the understanding of the genetic control of DNA methylation. In order to characterize this variation, we analysed DNA from plants belonging to 10 different ecotypes using three primer combinations (*Eco*RI + AC/*Hpa*II/*Msp*I + AAT, *Eco*RI + AA/*Hpa*II/*Msp*I + AAT and *Eco*RI + AT/*Hpa*II/*Msp*I + ACT). For each ecotype, the percentage of methylation-sensitive polymorphisms revealed by differential sensitivity to *Hpa*II or *Msp*I was calculated; the percentage variation ranged from 35% of the total fragments in Es-0 to 43% in Cvi-0 (Table 3). Pairwise comparisons of amplified fragment profiles between two randomly selected ecotypes showed differences in approximately one-third (range 24–34%) of the differentially restricted fragments.

Taking the data for all the ecotypes, the three primer combinations identified a total of 663 fragments, ranging in length from 100 to 700 bp (Fig. 2A). To further characterize inter-ecotype variation, 228 of these, representing easy scorable amplified fragments, were considered. A total of 111 out of the 228 showed similar digestibility in *Hpa*II and *Msp*I assays, but differed in their presence or absence among different ecotypes. The presence of the fragment in this case suggests the existence of a non-methylated CCGG restriction site, while its absence could be due either to variation of the CCGG nucleotide sequence or to its full methylation. In any case, we considered these to be methylation-insensitive polymorphisms (MIP in Fig. 2B). In addition, 117 differentially restricted fragments were identified upon comparing *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II fingerprints among ecotypes. Out of this group, 63 polymorphic methylation-sensitive fragments were found with both isoschizomers (MSP1 in Fig. 2B), while 54 were detected with only one of the isoschizomers (MSP2 in Fig. 2B). Of these 54 fragments, 16 represented *Eco*RI/*Msp*I and 38 *Eco*RI/*Hpa*II methylation-specific polymorphisms, respectively.

If variation in methylation profiles among different ecotypes was related to nucleotide sequence variation, one would expect that a similarity matrix constructed on the basis of methylation-sensitive polymorphisms would be correlated with the similarity matrix obtained from methylation-insensitive polymorphisms. Alternatively, the lack of such a correlation would suggest the existence of differential regulation of CCGG-site methylation in different ecotypes. To address this question we compared methylation-insensitive polymorphisms with methylation-sensitive polymorphisms revealed by *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II patterns. Results were scored in two independent binary matrices for each Arabidopsis ecotype (see Materials and methods). Similarity among the different ecotypes was calculated independently for each of the matrices using the Dice coefficient (Fig. 3A). For methylation-insensitive polymorphisms, the Dice coefficient takes into account the presence of shared bands but not their absence. However, given our scoring of methylation-sensitive polymorphisms, the Dice coefficient would take into account the polymorphic bands between *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I fingerprints and not the non-polymorphic markers. No correlation ($r = 0.17$, $P > 0.15$) was found between methylation-insensitive and methylation-sensitive similarity matrices, suggesting the existence of differential regulation of methylation in different ecotypes. Relationships among ecotypes, using either methylation-insensitive or methylation-sensitive polymorphism similarity matrices were also analysed by Principal Coordinate Analysis (PCOORDA), which identifies the principal components defining relationships among ecotypes and represents the three most relevant ones in a three- dimensional space (Fig. 3B). The results show a lack of relationship between the components defining genetic relatedness and components defining methylation patterns. In fact, ecotypes that are genetically close, such as Shah-1 and Kas-1 (GS = 0.88), were not grouped together on the basis of the analysis of methylation-sensitive polymorphism. However, other less genetically related ecotypes, such as L*er*-0 and Col-0 (GS = 0.57), Mt-0 and Ko-2 (GS = 0.57), or L*er*-0 and Mt-0 (0.57), displayed more similar patterns of methylation-sensitive polymorphisms.

Discussion

Identification of sequence-specific methylation using AFLP

We have used AFLP analysis to study methylation of CCGG motifs within the Arabidopsis genome. The technique had previously been applied to plants in order

Table 2 Sequence analysis of *Eco*RI/*Msp*I *Eco*RI/*Hpa*II AFLP fragments

Fragment	Restriction pattern	Sequence homology	Restriction map
B1	weak *EcoRI/MspI* - weak *EcoRI/HpaII* fragment	NF[a]	*EcoRI* ↓ 70 bp ↓ *HpaII / MspI*
B3	*EcoRI/MspI* - weak *EcoRI/HpaII* fragment	AF076275[b] 92125-92164[c] BAC T15F16 Chromosome 4 n.c.r.[d]	*EcoRI* ↓ 140 bp ↓ *MspI*
B4	*EcoRI/MspI* - weak *EcoRI/HpaII* fragment	AL161502[b] 61434-61746[c] contig fragmentN°14 Chromosome 4 n.c.r.[d]	*EcoRI* ↓ 312 bp ↓ *MspI*
B5	*EcoRI/HpaII* fragment	ATCHRIV56[b] 54160-54353[c] contig fragment N°56 Chromosome 4 Putative gene	*EcoRI* ↓ 193 bp ↓ *HpaII* 22 bp ↓ *HpaII*
B6	*EcoRI/MspI* fragment	AC006219[b] / AE002093[b] 66421-66559[c] Clones T14C8, F7B19 Chromosome 2 Putative gene	*EcoRI* ↓ 138 bp ↓ *MspI*
B6'-1	*EcoRI/MspI* - weak *EcoRI/HpaII* fragment	AC005313[b] 58637-58780[c] Clones T18E12 Chromosome 2 n.c.r.[d]	*EcoRI* ↓ 146 bp ↓ *MspI*
B6'-2	weak *EcoRI/MspI* - weak *EcoRI/HpaII* fragment	NF[a]	*EcoRI* ↓ 147 bp ↓ *HpaII / MspI*
B7	*EcoRI/MspI* fragment	AC003671[b] 87641-87888[c] BAC F1707 Chromosome1 Serine/ threonine protein kinase	*EcoRI* ↓ 247 bp ↓ *MspI*
B9	*EcoRI/MspI* fragment	AC022314[b] 18455-18725[c] BAC F9C16 Chromosome1 n.c.r.[d]	*EcoRI* ↓ 270 bp ↓ *MspI*
B10	*EcoRI/MspI* - *EcoRI/HpaII* fragment	AB009053[b], BA000015[b] 10029-10242[c], Clone MQB2 Chromosome 5 n.c.r.[d]	*EcoRI* ↓ 213 bp ↓ *HpaII / MspI*
B11	*EcoRI/MspI* - *EcoRI/HpaII* fragment	AC010927[b] 61966-62046[c] BAC T22K18 Chromosome 3 Putative gene	*EcoRI* ↓ 80 bp ↓ *HpaII / MspI*
B12	*EcoRI/MspI* - *EcoRI/HpaII* fragment	AC023673[b] 58898-58928[c] BAC F21D18 Chromosome 1 n.c.r.[d]	*EcoRI* ↓ 80 bp ↓ *HpaII / MspI*

[a]: No homology detected to Arabidopsis genome sequence in databases
[b]: Accession number
[c]: Homologous region
[d]: Non-coding region

[a]The sequence Accession No. is given, together with the position of the homology within the accession (in *parentheses*), the source clone(s), and the chromosomal assignment. n.c.r., non coding region; NF, no homology detected to Arabidopsis genome sequences in databases

[b]The sequenced AFLP fragment contains an internal *Hpa*II site

to study genome methylation in rice and cotton by Xiong et al. (1999) and Liu et al. (2001) respectively, using a protocol adapted from that of Reyna-López et al. (1997). We have modified several steps in the protocol to reduce fingerprint background and increase the number of scorable amplified bands.

Comparison between *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I patterns allowed the identification of methylation-sensitive polymorphisms. This approach does not allow us to distinguish non-methylated CCGG sequences from fully methylated mCmCGG sequences or hemi-methylated CmCGG sequences. However, cloning and sequencing of twelve *Eco*RI-*Hpa*II/*Msp*I AFLP fragments showed that many AFLP fragments (11 out of 12) resulted from the presence of non-methylated CCGG sequences. Thus, most of the visualised fragments (all of them of small size, ranging from 100 to 700 nt) appear to be generated by the lack of cytosine methylation. Moreover, these AFLP fragments derived in equal measure from coding and non-coding sequences. This is in contrast to *Eco*RI/*Mse*I AFLP fragments, which are mostly associated with non-coding sequences (P. Rouzé, personal communication). Thus, the use of this technique to analyse sequenced genomes, such as the Arabidopsis genome, allows the identification of differentially methylated genes.

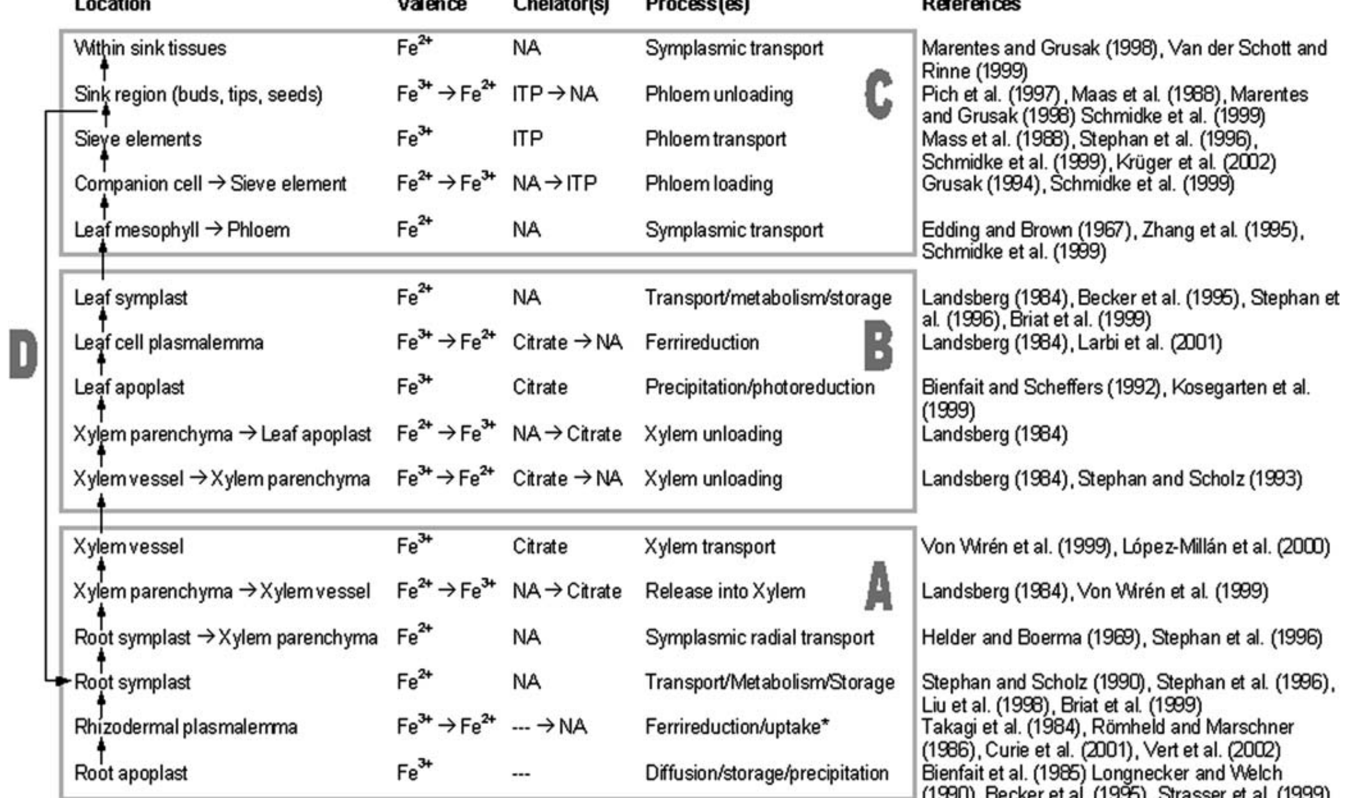
Based on nucleotide composition and genome complexity, the Arabidopsis genome is expected to contain an average of 45,000 *Eco*RI sites. Since two *Msp*I/*Hpa*II restriction sites should flank each of these *Eco*RI restriction sites, a total of 90,000 *Eco*RI/*Hpa*II or *Eco*RI/*Msp*I fragments could theoretically be detected using different selective nucleotides, although only CCGG sequences that lie close to *Eco*RI restriction sites (from 100–700 bp away) can be detected. In this study we used three primer combinations targeting 338 *Msp*I/*Hpa*II sites, which represent 0.37% of those 90,000 CCGG sites. This shows the power of the technique, as a single primer combination enables the analysis of 0.1% of CCGG restriction sites and new sets of *Msp*I/*Hpa*II restriction sites can be analysed by changing the selective nucleotides.

Table 3 Methylation sensitive polymorphisms found in different Arabidopsis ecotypes

Ecotype[a]	Methylation sensitive polymorphisms	Total fragments
Espoo (Es 0)	34.7	314
Martuba (Mt 0)	34.9	320
Shahdora (Shah)	39.9	346
Landsberg *erecta* (L*er*)	39.3	338
C 24	35.9	314
Columbia (Col)	36.2	337
Cape Verde Islands (Cvi 0)	43.3	353
Kashmir (Kas 1)	34.9	365
Ithaca (Ita 0)	35.2	323
Copenhagen (Ko 2)	35.1	353

[a] The analysis is based on a comparison of *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II AFLP fingerprints for each ecotype

Fig. 2A, B Methylation sensitive AFLP analysis of 10 Arabidopsis ecotypes. **A** DNA fingerprints were generated with the primer combinations *Eco*RI + AC/*Hpa*II *Msp*I + AAT and *Eco*RI + AT/*Hpa*II *Msp*I + ACT, using as templates preamplifications obtained from *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I digests. The *arrows* indicate positions of size markers. **B** Detail of the *Eco*RI + AT/*Hpa*II *Msp*I + ACT fingerprint. The *arrows* labelled MIP correspond to methylation insensitive polymorphisms, while MSP1 and MSP2 correspond to methylation sensitive fragments found with both isoschizomers or with only one of them, respectively

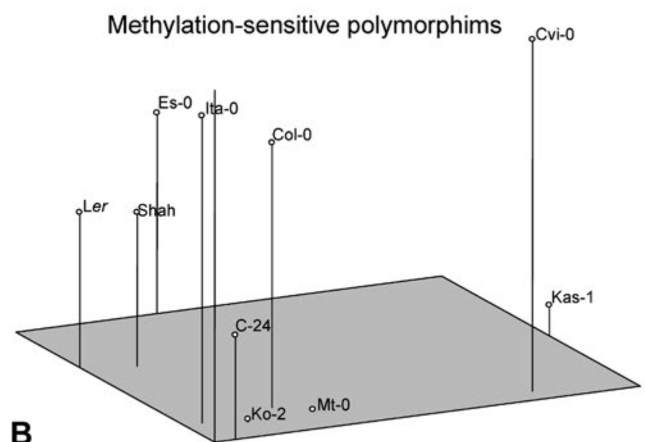


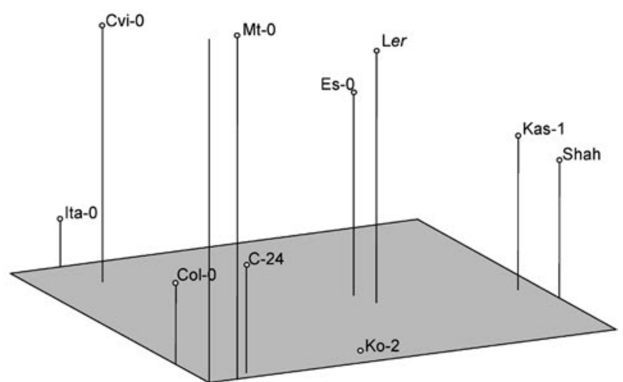
Location	Valence	Chelator(s)	Process(es)	References
Within sink tissues	Fe^{2+}	NA	Symplasmic transport	Marentes and Grusak (1998), Van der Schott and Rinne (1999)
Sink region (buds, tips, seeds)	$Fe^{3+} \rightarrow Fe^{2+}$	ITP → NA	Phloem unloading	Pich et al. (1997), Maas et al. (1988), Marentes and Grusak (1998) Schmidke et al. (1999)
Sieve elements	Fe^{3+}	ITP	Phloem transport	Mass et al. (1988), Stephan et al. (1996), Schmidke et al. (1999), Krüger et al. (2002)
Companion cell → Sieve element	$Fe^{2+} \rightarrow Fe^{3+}$	NA → ITP	Phloem loading	Grusak (1994), Schmidke et al. (1999)
Leaf mesophyll → Phloem	Fe^{2+}	NA	Symplasmic transport	Edding and Brown (1967), Zhang et al. (1995), Schmidke et al. (1999)
Leaf symplast	Fe^{2+}	NA	Transport/metabolism/storage	Landsberg (1984), Becker et al. (1995), Stephan et al. (1996), Briat et al. (1999)
Leaf cell plasmalemma	$Fe^{3+} \rightarrow Fe^{2+}$	Citrate → NA	Ferrireduction	Landsberg (1984), Larbi et al. (2001)
Leaf apoplast	Fe^{3+}	Citrate	Precipitation/photoreduction	Bienfait and Scheffers (1992), Kosegarten et al. (1999)
Xylem parenchyma → Leaf apoplast	$Fe^{2+} \rightarrow Fe^{3+}$	NA → Citrate	Xylem unloading	Landsberg (1984)
Xylem vessel → Xylem parenchyma	$Fe^{3+} \rightarrow Fe^{2+}$	Citrate → NA	Xylem unloading	Landsberg (1984), Stephan and Scholz (1993)
Xylem vessel	Fe^{3+}	Citrate	Xylem transport	Von Wirén et al. (1999), López-Millán et al. (2000)
Xylem parenchyma → Xylem vessel	$Fe^{2+} \rightarrow Fe^{3+}$	NA → Citrate	Release into Xylem	Landsberg (1984), Von Wirén et al. (1999)
Root symplast → Xylem parenchyma	Fe^{2+}	NA	Symplasmic radial transport	Helder and Boerma (1969), Stephan et al. (1996)
Root symplast	Fe^{2+}	NA	Transport/Metabolism/Storage	Stephan and Scholz (1990), Stephan et al. (1996), Liu et al. (1998), Briat et al. (1999)
Rhizodermal plasmalemma	$Fe^{3+} \rightarrow Fe^{2+}$	--- → NA	Ferrireduction/uptake*	Takagi et al. (1984), Römheld and Marschner (1986), Curie et al. (2001), Vert et al. (2002)
Root apoplast	Fe^{3+}	---	Diffusion/storage/precipitation	Bienfait et al. (1985) Longnecker and Welch (1990), Becker et al. (1995), Strasser et al. (1999)

	Es-0	Mt-0	Shah	*Ler*	C-24	Col-0	Cvi-0	Kas-1	Ita-0	Ko-2
Es-0	1.00									
Mt-0	0.72	1.00								
Shah	0.80	0.78	1.00							
Ler	0.92	0.80	0.82	1.00						
C-24	0.69	0.78	0.77	0.81	1.00					
Col-0	0.76	0.75	0.73	0.83	0.78	1.00				
Cvi-0	0.72	0.71	0.69	0.69	0.71	0.75	1.00			
Kas-1	0.73	0.72	0.68	0.69	0.65	0.68	0.71	1.00		
Ita-0	0.70	0.70	0.75	0.79	0.72	0.75	0.70	0.53	1.00	
Ko-2	0.72	0.80	0.72	0.81	0.78	0.75	0.67	0.70	0.75	1.00

	Es-0	Mt-0	Shah	*Ler*	C-24	Col-0	Cvi-0	Kas-1	Ita-0	Ko-2
Es-0	1.00									
Mt-0	0.56	1.00								
Shah	0.64	0.51	1.00							
Ler	0.69	0.57	0.65	1.00						
C-24	0.57	0.72	0.57	0.59	1.00					
Col-0	0.63	0.67	0.53	0.57	0.75	1.00				
Cvi-0	0.68	0.57	0.50	0.62	0.58	0.64	1.00			
Kas-1	0.69	0.53	0.88	0.69	0.60	0.53	0.57	1.00		
Ita-0	0.56	0.43	0.45	0.52	0.55	0.56	0.68	0.52	1.00	
Ko-2	0.67	0.57	0.63	0.65	0.71	0.73	0.53	0.65	0.50	1.00

A





B

Fig. 3A, B Relationships among 10 Arabidopsis ecotypes based on Dice similarity matrices derived from data for methylation sensitive and insensitive polymorphisms (**A**) and represented using principal coordinate analysis (**B**)

The probability that a methylated CCGG site will be digested by *Hpa*II is lower than for *Msp*I based on the different sensitivities of the two isoschizomers to cytosine methylation. Furthermore, total Arabidopsis DNA has been observed to be more sensitive to *Msp*I than to *Hpa*II in Southern blots hybridized with different probes, such as the 180-bp centromere repeat or the 5S rDNA sequence (Martínez-Zapater et al. 1986; Vongs et al. 1993; Finnegan et al. 1996; Ronemus et al. 1996). Based on these observations, we would expect a larger number of *Eco*RI-*Msp*I fragments than *Eco*RI-*Hpa*II fragments in the AFLP profiles. However, contrary to those expectations, we generally observe larger numbers of amplified *Eco*RI-*Hpa*II fragments than *Eco*RI-*Msp*I fragments among the methylation-sensitive fragments found. This apparent discrepancy could result from the fact that the lower methylation sensitivity of *Msp*I results a higher number of amplified fragments that are shorter than 100 bp, which are not resolved under our electrophoretic conditions.

Natural variation in methylation patterns

Comparison of genotypically identical plants indicated the existence of a specific methylation pattern characterising each ecotype, with intra-ecotype variation being minimal (less than 1%). However, methylation patterns varied between different ecotypes, showing differences in the methylation state at one-third of the common detectable CCGG restriction sites. Thus, while methylation patterns are maintained in genotypically identical plants, differences in the methylation patterns accumulate between ecotypes. In agreement with these results, the methylation level of NOR regions has been found to be conserved in individuals belonging to the same ecotype but significantly variable among different Arabidopsis ecotypes (Riddle and Richards 2002). Whether these differences are spurious and accumulate as a consequence of random methylation or passive demethylation through failure of maintenance methylation over time, or reflect the existence of different regulatory mechanisms as specific adaptations to the life style of, and the environmental conditions encountered by, each ecotype is unknown. The lack of correlation between methylation-sensitive and methylation-insensitive polymorphism similarity matrices among ecotypes would suggest that whatever the regulatory mechanisms controlling methylation may be, they are not related to sequence variation around the CCGG motifs.

Natural variation in methylation profiles can be genetically controlled. In fact, a genetic analysis designed to identify the genetic determinants responsible for variation in the methylation level of the NOR region using Recombinant Inbred Lines has shown that this variation results from a combination of genetic and epigenetic mechanisms (Riddle and Richards 2002). Apart from the possible inheritance of parental methylation patterns contributing to the maintenance of natural epigenetic variation, this study identifies three

different QTLs which act in *trans* to generate the different methylation patterns (Riddle and Richards 2002).

Significant differences in the level of cytosine methylation have been observed among different organs in several plant species. In tomato, the level of DNA methylation was higher in seeds than in mature leaves, and was lower in young seedlings than in mature leaves (Messeguer et al. 1991). Similarly, a higher level of DNA methylation was detected in seedlings than in flag leaves of rice (Xiong et al. 1999). Different Arabidopsis ecotypes have different environmental requirements for flowering, and therefore they are expected to be at different developmental stages when grown for the same time under similar environmental conditions. Thus, some of the methylation differences found among ecotypes could reflect differences in the developmental stage attained. In fact, the percentage of methylation-sensitive polymorphisms differentially digested by *Hpa*II or *Msp*I seems to be lower in early flowering ecotypes like Cvi-0 (43.3%), Shah (39.9%) or L*er* (39.3%) than in late flowering ones such as Ita (35.2%), Ko-2 (35.1%), Kas-1 and Mt-0 (34.9%) and Es-0 (34.7%). A further analysis of methylation profiles during the course of Arabidopsis development will be required to confirm this possibility.

Finally, environmental conditions have also been reported to affect methylation levels, and could result in different methylation patterns (Burn et al. 1993; Schmitt et al. 1997; Finnegan et al. 1998). Although probably important, these environmental effects can be disregarded as a possible source of the variation in methylation patterns in our experiments, since all the ecotypes tested had been grown for several generations under the same controlled environmental conditions.

In conclusion, we have shown that it is possible to analyse variation in the methylation status of anonymous CCGG restriction sites using a modified AFLP technique based on differential digestibility with *Msp*I or *Hpa*II. The application of this approach to Arabidopsis ecotypes reveals an important level of natural variation in methylation profiles of anonymous DNA sequences, the biological significance of which remains to be determined. Further experiments will be required to characterize whether this variation is the result of random or genetically controlled methylation changes that could be inherited as epigenetic variation, and to determine whether this variation is selectively neutral or represents a component of adaptation to different environments.

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