

Analysis of Cytosine Methylation Pattern in Response to Water Deficit in Pea Root Tips

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Abstract: The correlation between environmental stress and DNA methylation has been studied by following the methylation status of cytosine residues in the DNA of pea root tips exposed to water deficit. DNA methylation was evaluated by two complementary approaches: (i) immunolabelling by means of a monoclonal antibody against 5-methylcytosine; (ii) MSAP (Methylation-Sensitive Amplified Polymorphism) to verify if methylation and de-methylation in response to water deficit may be related to specific DNA sequences. Immunolabelling showed that water stress induces cytosine hypermethylation in the pea genome. Regarding the CCGG target sequence, an increase in methylation specifically in the second cytosine (about 40% of total site investigated) was revealed by MSAP analyses. In addition, MSAP band profile detected in three independent repetitions was highly reproducible suggesting that, at least for the CCGG target sequence, methylation was addressed to specific DNA sequences.

Key words: *Pisum sativum* L., DNA methylation, water stress, MSAP.

Introduction

The expression of the information contained in the primary DNA sequence can be affected by the addition (or loss) of a methyl group to the ring structure of a cytosine residue. The epigenetic modification, which is mediated by methyl transferases enzymes, alters DNA-protein interaction and prevents cleavage by methylation-sensitive restriction endonucleases (Jost and Saluz, 1993^[15]). In animals, as in plants, the main consequence of methylation is interference with DNA transcription and replication. Effects on the extent of DNA mutation and on chromatin structure change have also been verified (Ehrlich and Ehrlich, 1993^[9]). In human DNA, changes in methylation patterns are recognized as an agent for some diseases (Reik et al., 2001^[26]; Bird, 2002^[2]).

Plant genomes are methylated to a far greater extent than animal genomes: up to 40% of cytosine residues are methylated in plants, as compared with an average of 8% in animals (Adams

and Burdon, 1985^[11]). Research to investigate the extent and role of methylation in plants has shown that: (i) methylation occurs predominantly on the cytosine residues in symmetrical sequences, such as CpG and CpNpG (Guenbaum et al., 1981^[12]; McClell, 1983^[22]), (ii) DNA hyper- or hypomethylation is correlated with gene expression during development (Ulian et al., 1996^[37]; Rossi et al., 1997^[31]; Hoekenga et al., 2000^[13]), and (iii) methylation plays an important role in the regulation of the activity of transposable elements (Martinessen and Richards, 1995^[21]), in paramutation (Hollick et al., 1997^[14]; Walker, 1998^[39]) and in somaclonal variation induced by stress conditions (Cecchini et al., 1992^[5]; Lo Schiavo et al., 1989^[20]; Kaeppler et al., 2000^[17]).

Hypermethylation of tobacco heterochromatic loci in response to osmotic stress (Kovarik et al., 1997^[18]) and in silenced genes in transgenic plants (Meyer et al., 1992^[24]) has been reported; similarly, hypomethylation has been documented in chicory root tips (Demeulemeester et al., 1999^[7]) and in *Arabidopsis thaliana* L. (Finnegan et al., 1996^[9]) when exposed to low temperature. These findings support the hypothesis that variations in genome methylation are due to plant mechanisms for adaptation to abiotic stresses (Kovarik et al., 1997^[18]) and make the investigation of the methylation status a relevant topic in the search for crop improvement.

Water availability is the major limiting factor to plant growth in many environments. In the present work, global changes in DNA methylation in water-stressed pea root tips were determined by immunolabelling with a monoclonal antibody against 5-methylcytosine and the Methylation-Sensitive Amplified Polymorphism (MSAP) technique (Reyna-López et al., 1997^[27]) was used to correlate stress events to epigenetic changes. The results indicate that: i) DNA hypermethylation in pea root tips is induced by water deficiency; and ii) this hypermethylation at the CCGG sequences occurs in specific DNA sequences.

Materials and Methods

Plant material and seedling treatment

Pea (*Pisum sativum* L. cv. Alaska) seeds were surface sterilised in 10% NaClO and germinated in moistened agriperlite at 25 °C in the dark. After 72 h, the seedlings were transferred to glass dissectors on top of 25% w/v glycerol (approx. 90% relative

humidity) for 3 days in the dark. When pea seedlings are dried at 90% relative humidity for 72 h, roots are able to resume growth quickly after being transferred to water (Bracale et al., 1997^[41]).

Control seedlings were grown in agriperlite in the dark for the same period of time. One hundred seedlings and 100 root tips were weighed after 24, 48 and 72 h of treatment to assess water loss. The relative water content was calculated as the per cent of weight of dehydrated apices versus control apices.

DNA extraction

Root tips (2 mm in length) were excised from seedling dehydrated for 24, 48 and 72 h and from the corresponding control seedlings. At each time, 30 root tips were collected from both control and treated samples and immediately used for DNA extraction with the Plant tissue Kit (Quiagen). This procedure was repeated three times, using three independent control and treated samples.

Slot-blot analysis

DNA extracted from treated and control samples was used for slot-blot analysis. For each sample, 5 ng of DNA were put on a nitocellulose filter, using the PR 600 Slot-Blot apparatus (Hoefer Scientific Instruments). The filter was incubated with a 1 : 1000 diluted mouse monoclonal antibody against 5-methylcytosine (anti-5-mC). The preparation and specificity of this monoclonal antibody have already been described (Podestà et al., 1993^[25]) and were recently used to study DNA methylation dynamics in plants (Zluvovala et al., 2001^[41]). After extensive washing, the filters were treated with a 1 : 9000 diluted anti-mouse alkaline phosphatase conjugated IgG. The secondary antibody was detected using 4-nitroblue tetrazolium chloride (Sigma). Image analysis was performed using the Gel Doc 2000 Image analysis system (Biorad).

DNA extraction and slot-blot analysis experiments were performed in triplicate.

MSAP analysis

DNA (1 µg) was digested for 6 h at 37 °C with 5 U of EcoRI and 5 U of Msp I (New England BioLabs) or 5 U of Hpa II (New England BioLabs) in a final volume of 40 µl containing RL buffer (Gibco-BRL). DNA fragments were ligated (with 1 unit of T4 DNA ligase for 3 h at 37 °C) to the MspI-HpaII adapter (5 pmol) and EcoRI adapter (0.5 pmol) in a final volume of 50 µl, as described (Xiong et al., 1999^[39]).

Pre-amplification was performed in a mixture containing 5 µl of the above mixture, 75 ng of each E + 01/HM + 0 primer (Table 1), 200 µM of each dNTP, 0.5 U of Dynazyme II (Finnzymes) and 5 µl of Dynazyme buffer (Finnzymes) in a final volume of 50 µl. After 2 min at 94 °C, pre-amplification was carried out for 20 cycles of denaturation (45 s at 94 °C), annealing (30 s at 50 °C) and extension (1 min at 72 °C). After a final elongation step (7 min at 72 °C) the pre-amplification product was diluted 1 : 50 with water and used for the selective amplification. This was carried out using one of the selective DNA primers (E32, E33, E34 and E37 of Table 1) complementary to the EcoRI adapter and one of the DNA primers (HM32, HM36,

Table 1 DNA primers for MAPS analysis

Name	DNA sequence
HM + 0	5' - ATCATGAGTCCCTGCTCGG - 3'
E + 01	5' - GACTGCGTACCAATTC - 3'
HM32	5' - ATCATGAGTCCCTGCTCGGACA - 3'
HM36	5' - ATCATGAGTCCCTGCTCGGATT - 3'
HM38	5' - ATCATGAGTCCCTGCTCGGTCC - 3'
HM39	5' - ATCATGAGTCCCTGCTCGGAAT - 3'
E32	5' - GACTGCGTACCAATTCTG
E33	5' - GACTGCGTACCAATTCAG
E34	5' - GACTGCGTACCAATTCAA
E37	5' - GACTGCGTACCAATTC

HM38, HM39 of Table 1) complementary to the MspI-HpaII adapter. The EcoRI primer was end-labelled with $\gamma^{32}\text{P-ATP}$ (Amersham, Italy). The amplification mixture (10 µl, final volume) contained 2.5 µl of the pre-amplification mixture, 3 ng of labelled EcoRI primer, 15 ng of MspI-HpaII primer, 200 µM of each dNTP, 0.5 U of Dynazyme II and 2 µl of Dynazyme buffer. After 2 min at 94 °C (denaturation), amplification was for 36 cycles under the following conditions: denaturation for 30 s at 94 °C; annealing for 30 s at 65 °C in the first cycle, followed by a 0.7 °C decrease in temperature in each of the next 12 cycles, then at 56 °C for the remaining 23 cycles; extension for 60 s at 72 °C. The PCR-amplified mixture (1.5 µl) was added to an equal volume of loading buffer (80% formamide, 1 mg · ml⁻¹ xylene cyanol FF, 1 mg · ml⁻¹ bromophenol blue, 10 M EDTA, pH 8.0), denatured for 5 min at 92 °C, loaded onto a 5% denaturing polyacrylamide gel and electrophoresed in TBE electrophoresis buffer for 3 h at 80 Watt. The gel was finally fixed in 10% acetic acid and exposed to an X-ray film for 24 h. Polymorphic bands were scored by visual inspection of the resulting autoradiograms. Each band represents a recognition site cleaved by one or both of the isoschizomers. The methylation levels were deduced on the basis of DNA polymorphisms detected between the band patterns produced within each DNA preparation after digestion with two different enzymes (MspI and HpaII).

Results

Seedling dehydration and slot-blot analysis

Water content was determined in the whole seedling and in the root tip at 24, 48 and 72 h after dehydration. Results were expressed as per cent of weight in the dehydrated samples versus the control. Weight, and thus water content, decreased considerably in seedlings grown in drought conditions, and was even further decreased in root tips, where, at time 72 h it was 55% of that of the controls (Fig. 1).

With the aim of quantifying DNA methylation variations, genomic DNA was extracted from dehydrated and control root tips, immobilized onto nitro-cellulose membranes and immunolabelled with the anti-5-mC. A representative example of the results obtained by this slot-blot analysis is shown in Fig. 2. After 24 h of dehydration, small differences between control and stressed samples was observed. After 48 h this difference became marked and after 72 h reached 63%. This indicates an

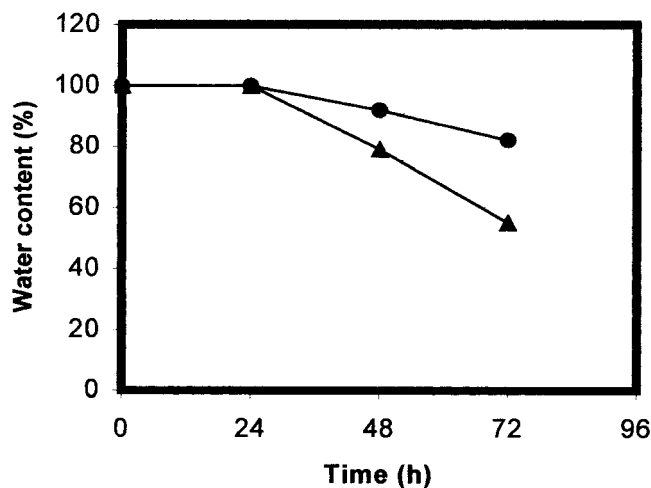


Fig. 1 Water content in pea seedling and root tips at different times after dehydration. Dehydration condition and analysis of water content were as described in Materials and Methods. ●: seedling; ▲: root tips.

extensive hypermethylation of cytosine residues in water stressed pea root tips. In the control lanes the amount of 5 mC remains constant at the different times. This suggests that the DNA methylation level was not influenced by growth condition or plant development state.

MSAP analysis

MSAP analysis was performed to verify the information produced by slot-blot analysis and to establish whether DNA hypermethylation at the CCGG sequence in response to water deficit occurs at random within the genome or is addressed to specific DNA sequences.

Many MSAP primer combinations (Table 1), with two or three selective nucleotides at the *EcoRI* end and one to three selective nucleotides at the *HpaII*-*MspI* end were tested for their

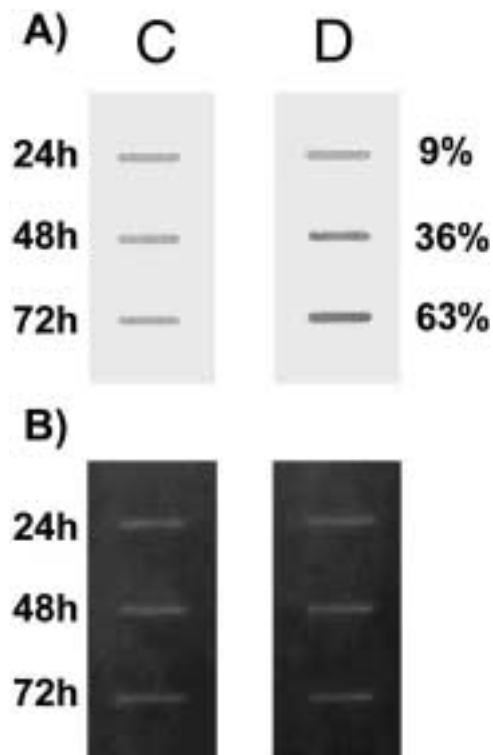


Fig. 2 (A) Slot-blot analysis of DNA from control (C) and dehydrated (D) root tips at different times after dehydration. Per cent refers to the increase in band intensity in the DNA from dehydrated root tips as compared with DNA from the control sample. Value was calculated by Image Analysis System Gel DOC 2000 Biorad. (B) DAPI-stained filters loaded with the same quantity of DNA as in A to check the amount of DNA loaded in each well.

ability to produce distinct scorable bands on the analysis gel. Of these, 10 DNA primer combinations (Table 2) were finally selected and used. For each combination, the number of bands visible on the gels varied from 45 to 68. Fig. 3 shows a repre-

Table 2 Number of amplified bands and methylation or hemi-methylation in CCGG site detected by each DNA primer pair. Total band detected in *EcoRI*-*MspI* digestion lane. Number of full methylation sites or methylation in first cytosine were deduced by comparison pattern of *EcoRI*-*MspI* digestion lane detected in dehydrated pea root tips versus control. Methylation in second cytosine was assessed by calculating number of fragments in the amplification product of *EcoRI*-*MspI* lane absent in *EcoRI*-*HpaII* lane. Finally, hemi-methylated site was indicated as the band present in *EcoRI*-*HpaII* lane but not in *EcoRI*-*MspI*, because *HpaII* digests hemimethylated sites while *MspI* does not

Primer pair	Control root tips			Dehydrated root tips		
	Total band	Methylation in second cytosine	Hemimethylated sites	Total band	Methylation in second cytosine	Hemimethylated sites
HM32/E32	59	17	2	58	25	3
HM32/E33	64	11	2	60	21	2
HM36/E32	39	11	0	38	19	0
HM36/E33	55	18	4	55	27	4
HM36/E34	66	13	3	60	19	4
HM38/E32	57	11	0	57	22	0
HM38/E34	45	10	1	45	15	0
HM38/E37	57	13	0	53	25	0
HM39/E34	68	12	2	66	29	2
HM39/E37	61	13	0	61	24	0
TOT	571	139	14	553	226	15

sentative MAPS analysis with one of the selected primer combinations.

The stress experiments were repeated three times. The banding patterns produced were always clear and reproducible; i.e., 95% of bands with both enzymes were identical. These MSAP bands were rigorous reproducibility in control and dehydrated samples, indicating direct methylation activity addressed to specific DNA sequences.

The MSAP approach was based on different methylation sensitivity of two enzymes *HpaII* and *MspI*. *HpaII* digests only unmethylated CCGG sites or hemi-methylated sites (only one DNA strand methylated) but not sites that have either of the two cytosines methylated, while *MspI* digests CCGG and CmCCG sites but not mCCGG or mCmCCG sites (McClelland et al., 1994^[23]).

Due to the differential DNA cleavage properties of these enzymes, different DNA fragment profiles were observed in control and dehydrated samples. In particular, using the 10 primer pairs selected, a total of 571 scorable bands were obtained from the control sample *EcoRI* – *MspI* digest and 553 bands from dehydrated *EcoRI* – *MspI* digest. On the other hand, in the *EcoRI* + *HpaII* digests of DNA from control and dehydrated root tips, a total of 139 and 226 bands, respectively, were absent (Table 2).

The results demonstrate that, during growth in normal conditions, a portion of the DNA is methylated in the second cytosine of the CCGG sequence (about 25% of the total site investigated) and that this portion shows an increase to 40% in the site investigated in dehydrated root tips. This increase is due to methylation in specific DNA sequences, as assessed by the change in band pattern of the MSAP analysis observed in the different samples. On the other hand, methylation in the first cytosine and hemi-methylation do not change and are not substantially influenced by water stress, as shown in Table 2.

Discussion

The results of the slot-blot and MSAP analysis performed in this work show extensive hypermethylation of cytosine residues in water-stressed pea root tips. This is in agreement with data on DNA hypermethylation of tobacco and potato cell cultures in response to osmotic stress (Sabbah et al., 1995^[32]; Kovarik et al., 1997^[18]). In pea, an increase of methylation of both internal and external cytosine on the CCG sequence was detected in regenerated plants. Finally, in maize DNA methylation/demethylation was described in response to environmental stress (Steward et al., 2002^[35]). These data suggested that epigenetic change at the DNA methylation level might play an important role in plant adaptation to several forms of stress. In this work, we show that the same is true for plant response to stress induced by water deficit.

Bracale et al. (1997^[4]) described an arrest of the cell cycle in pea root tips under similar water stress conditions to those which induced hypermethylation in our experiments. This fact allowed us to postulate the existence of a connection between drought stress, cell division and DNA methylation. A relationship between water stress and reduction of plant development has been demonstrated in *Arabidopsis thaliana* and wheat (Ric-

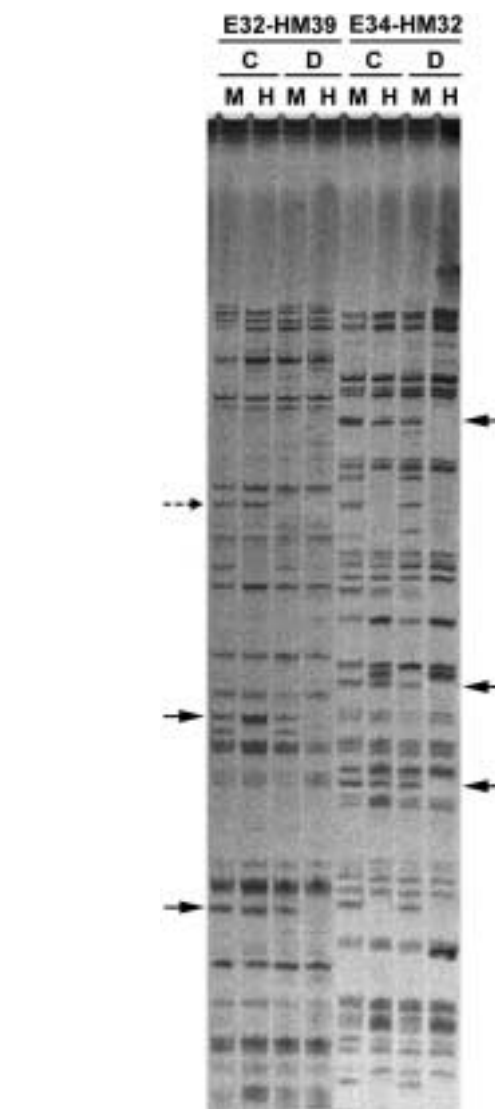


Fig. 3 Examples of methylation pattern detected from comparing DNA from control (C) and dehydrated (D) root tips, using two primer combinations: HM32-E32 and HM39-E34. H and M refer to digestion DNA with *EcoRI*-*HpaII* and *EcoRI*-*MspI*, respectively. \rightarrow : Hypermethylation in the second cytosine induced by water stress; $--\rightarrow$: Hypermethylation in both cytosines induced by water stress.

cardi et al., 1998^[28]). In the same stress conditions, a decrease in mitotic activity in meristematic cells has been reported in maize and sunflower (Robertson et al., 1990^[29]; Silk 1992^[34]; Sacks et al., 1997^[33]). This, and the results of our experiment point to the conclusion that DNA methylation represents a powerful tool for the cell within a morphogenetic programme, depending on external or internal factors. It could be considered an example of cell potentiality and an important mechanism correlated to plant growth and development in response to biotic and abiotic environmental stresses.

The molecular mechanism by which methylation and demethylation take place in specific DNA target sequences involved in plant growth and development is not yet clear. DNA methylation changes could be directed to specific sequences or

could be a random response to stress (Kaeppeler and Phillips, 1993^[16]); they could be the primary cause of gene inactivation or a secondary consequence of some other process that has resulted in transcriptional inactivation (Finnegan et al., 1998^[10]; Cocciolone et al., 1993^[6]). In animal systems, methylated cytosine can serve as a substrate for methyl-CpG-binding proteins. Some of these proteins are found as complexes with histone deacetylases and function as key components of the remodeling machinery responsible for chromatin condensation (Bird and Wolffe, 1999^[3]). In this case, it is the variation in chromatin structure and not the direct methylation change of specific sequences that leads to changes: (i) in gene expression, (ii) in recombination rates and (iii) in the timing of DNA replication, perhaps causing chromosome breakage (Kaeppeler and Phillips, 1993^[16]). On the other hand, many papers have reported that, in both animals and plants, some genes are specifically silenced or activated by methylation (Furner et al., 1998^[11]; Steward et al., 2000^[36]).

A powerful tool for ascertaining whether methylation in response to water stress is directed to specific DNA sequences is the recently developed MSAP technique (Reyna-Lopez et al., 1997^[27]). It is a modification of the Amplified Fragment Length Polymorphism (AFLP; Vos et al., 1995^[38]) approach set up for the detection of 5'-methylcytosine in fungal DNA. This technique is a powerful tool to discover specific CCGG target sequences of methyltransferase enzymes.

In our study, MSAP served to indicate the methylation mechanism involved in normal and water stressed plant development. MSAP rigorous reproducibility in control and in stressed samples demonstrated direct methylation/demethylation activity addressed to specific DNA sequences. On the basis of this greater reproducibility of MSAP results, it is reasonable to propose a primary and direct role of hypermethylation in reducing the metabolic cell activity assessed by Bracale et al. (1997^[4]) in pea root tip after 72 h of water stress. The epigenetic control of expression of specific genes by methylation mechanisms is supported by the recent identification of several class of methyltransferase (Finnegan et al., 1996^[9]; Ronemus et al., 1996^[30]) directed to specific functional and structural genes characterized by CpXpG or CpG sequences and involved in the maintenance of this methylation (Lindroth et al., 2001^[19]). Recently, a study suggested a model where different loci may depend preferentially or either CpGpC or CpG methylation as the main mechanism of gene silencing and gene expression (Lindroth et al., 2001^[19]). Our understanding of the relationship between DNA methylation and transcriptional control is growing fast, but is still far from complete. Ongoing molecular and biochemical analysis of the growing number of components of the DNA methylation system will strengthen the link between DNA methylation and mainstream transcriptional mechanisms. Regulation of gene expression is complex and the emerging evidence hints that the roles of DNA methylation will be too. We consider that MSAP analysis provides the possibility of identifying the DNA sequences methylated during normal plant development and in response to environmental stresses. The characterization of these sequences and the study of their gene expression will clarify which type of genes were regulated by methyltransferase enzymes and the relationship between DNA methylation and gene expression or silencing.

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