Cytosine methylation occurs in a CDC48 homologue and a MADS-box gene during adventitious shoot induction in Petunia leaf explants

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Abstract
The DNA methylase inhibitors, 5-azacytidine and 5-aza-2'-deoxycytidine inhibited adventitious shoot induction in Petunia leaf cultures. Cytosine methylation status at CCGG sites in shoot- and callus-inducing culture treatments was analysed by coupled restriction enzyme digestion (HpaII or MspI) and random amplification. Two differentially methylated genomic DNA bands from the PCR products were cloned (OPU9-1 and OPU9-2) and sequenced. The open reading frames contained in OPU9-1 and OPU9-2 showed similarity to CDC48 and MADS-box genes, respectively. Cytosine methylation was restored at CCGG sites when the leaf explants were transferred from medium containing the drugs to medium without the drugs, simultaneously recovering the ability to develop adventitious shoot buds. Furthermore, combined bisulphite treatment and restriction analysis revealed differential methylation of CGCG sites in the drug-treated and control cultures. These results demonstrate that cytosine methylation at CCGG and CGCG sites within a MADS-box gene and a CDC48 homologue, among others, shows strong positive correlation with adventitious shoot bud induction in Petunia leaf explants.
differentiation are members of gene families with many functionally redundant homologues, making single mutant genetic screens a less attractive strategy for further characterization of these genes. An in vitro system capable of inducing de novo organogenesis would thus be of considerable value, not only to identify genes involved in meristem formation and organ differentiation but also to understand the mechanisms of gene regulation related to these developmental pathways.

Recently, it has been shown that a vegetatively expressed MADS-box gene regulates adventitious shoot regeneration in *Paulownia* (Prakash and Kumar, 2002). MADS-box transcription factors have a highly conserved DNA-binding domain and are important regulators of plant growth and development (Ng and Yanofsky, 2001). The majority of the known MADS-box genes control different steps of flower development. However, some of them are also expressed in vegetative tissues (Carmona et al., 1998; Prakash and Kumar, 2002) suggesting that they may regulate several vegetative developmental events as well.

Considerable evidence from both plant and animal systems suggests that methylation of specific DNA-sequences is involved in the regulation of gene expression (Bucherna et al., 2001). Plant nuclear DNA is heavily methylated, which in some cases may include up to 30% of the cytosine residues. Methylation occurs at CG and CNG sequences where N represents any base (Gruenbaum et al., 1981). The pattern of methylation is species- and tissue-specific and may vary during different stages of plant development. In maize, differential methylation has been detected in different plant tissues (Walker, 1998). Methylated cytosine residues have been implicated in the control of eukaryotic gene expression (Bezek et al., 1991; Finnegan et al., 1998). Consequently, there has been considerable interest in cytose sequences analogous as 5-azacytidine (AzaC) and 5-aza-2'-deoxyctydine (AzadC), which inhibit methylation when incorporated into DNA. These drugs lead to a rapid time- and dose-dependent decrease in DNA methylase activity (Juttermann et al., 1994).

Despite the occurrence of a high degree of methylation in plants, there have been very few studies using the demethylating agents. Some examples of methylation-induced silencing of T-DNA in transgenic plants are known (Mandal et al., 1994; Zhou et al., 1996). Additionally, changes in DNA methylation levels induced by mutation or by antisense suppression of the DNA methylase gene resulted in significant morphological and physiological alterations in plants (Jacobsen and Meyerowitz, 1997). These reports indicate a correlation between the level of expression of selected genes and their methylation state.

Adventitious shoot initiation from *Petunia* leaf discs was inhibited when cultured on shoot induction (SI) medium containing \( \geq 20 \mu M \text{ AzaC} \) or \( 5 \mu M \text{ AzadC} \) (Prakash and Kumar, 1997). This was associated with a decrease in cytosine methylation at CCGG sites in the drug-treated explants, which were analysed by Coupled Restriction Enzyme Digestion and Random Amplification (CRED-RA). Further molecular analysis of the effect of the DNA methylase inhibitors (AzaC and AzadC) on adventitious shoot induction in *Petunia* leaf explants is reported here. DNA methylation status was compared between the shoot- and callus-forming explants. Two differentially amplified genomic DNA bands from the control cultures were sequenced and analysed. One of the bands contains an open reading frame (ORF) that encodes for a gene product with similarity to *CDC48*, while another encodes a MADS domain protein, indicating that differential methylation of cytosine residues occurs in selected genes during shoot organogenesis in *Petunia*.

**Materials and methods**

**Plant material and culture conditions**

*Petunia hybrida* cv. Dazzler shoots that served as the explant source were maintained as nodal cuttings on Murashige and Skoog (MS; Murashige and Skoog, 1962) medium in Magenta GA7 containers. Segments (10×5 mm) from fully expanded leaves were excised (leaf discs) and cultured on shoot induction (SI) medium [MS+4.4 μM BA+0.53 μM NAA containing 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) Gelrite (Sigma, St Louis, USA)] (Horsch et al., 1985). Different concentrations of the demethylating agents AzaC and AzadC were filter-sterilized and incorporated into the autoclaved medium (Prakash and Kumar, 1997). Explants on SI medium without the demethylating agents were maintained as controls. For callus induction (CI), MS medium was supplemented with 10 μM 2,4-D. The pH of the culture media was adjusted to 5.7±0.1 before adding 2 g l\(^{-1}\) Gelrite and autoclaving at 1.1 kg cm\(^{-2}\) for 20 min. Cultures were incubated at 26±2 °C and 12 h photoperiod (25–30 μmol m\(^{-2}\) s\(^{-1}\))

**Scanning electron microscopy**

*Petunia* leaf discs on SI medium were examined by scanning electron microscopy. About 2 mm wide strips of the shoot-forming edges were excised from the explants cultured in the presence or absence of the drugs (AzaC or AzadC) from different culture periods (4, 7 and 12 d after culture initiation). The excised tissues were fixed in 2% glutaraldehyde in 0.025 M sodium phosphate buffer (pH 7.2) overnight at 4 °C, and transferred to 1% osmium tetroxide in the same buffer for 4–6 h at room temperature.

The fixed tissues were dehydrated through a graded ethanol series (50%, 70%, 80%, 90%, and twice at 100%) for 15 min in each step and critical point dried in liquid carbon dioxide in a Samdri™-780A (Tousimis Rockville, USA). The shoot regeneration region was mounted on aluminium stubs and coated with gold in a JEOL fine coat ion sputter JFC-1100 (JEOL, Japan). Scanning electron microscopy was performed with a JEOL scanning electron microscope (JEOL JSM-T220A) and images were photographed.

**Analysis of DNA methylation status**

Genomic DNA was isolated by the modified CTAB method (Prakash and Kumar, 1997) from 1-week-old control and drug-treated (20 mM AzaC or 5 mM AzadC) explants. About 2 mm wide strips of the shoot-forming edges were used for DNA isolation. Differences in cytosine methylation of genomic DNA were assessed between 7-d-old SI and CI cultures. Also, the shoot-forming region (2 mm...
wide strip) was collected from 2-week-old explants that were cultured on SI medium with 20 μM AzaC for the first week and subsequently without the drug. One set (20 explants cultured in five Petri dishes) of explants was maintained in the presence of the drug for 2 weeks. Cytosine methylation status was analysed by CRED-RA (Cai et al., 1996; Prakash and Kumar, 1997). Genomic DNA (2 μg) from SI, CI and the drug-treated samples were restricted with 10 U of methylation-sensitive enzymes (HpaII or MspI; New England Biolabs) and subjected to PCR using arbitrary 10-mer primers as described earlier (Prakash and Kumar, 1997).

**Cloning and sequence analysis of differentially amplified DNA bands**

The PCR products from the CRED-RA that were differentially amplified in the control (SI) reaction were cloned into pBluescript SKII (Stratagene, CA, USA). Two of these clones, designated as pOPU9-1 (1.1 kb) and pOPU9-2 (1.2 kb), were sequenced using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Corporation, CA, USA). DNA sequences obtained were analysed using DNASIS v2.5 software (Hitachi Software Engineering Co. Ltd, Japan).

**DNA and RNA gel blot analyses**

The PCR products from the CRED-RA reaction were fractionated by agarose gel electrophoresis (0.8% w/v) and transferred to nylon membranes (Ausubel agarose gel electrophoresis (0.8%) and transferred to nylon membranes (Ausubel et al., 1993). A MADS-box gene probe was generated by restricting pOPU9-2 and was labelled with the Random Prime labelling kit (Amer sham Pharmacia Biotech, Sweden). Radiolabelled probes were purified with QIAquick nucleotide removal kit (Qiagen). Membranes were prehybridized in hybridization buffer (Ausubel et al., 1993) for 2 h prior to adding the [α-32P]dCTP labelled probe. High stringency hybridization conditions were achieved by hybridizing the membranes at 60 °C for 16 h and washing twice with 0.1 SSC and 0.1% SDS at 60 °C for 30 min followed by one wash with 0.1× SSC and 0.1% SDS at 60 °C for 15 min before exposure to X-ray films.

For northern analysis, RNA was isolated from leaf explants after different culture periods on SI medium (Ausubel et al., 1993). Total RNA was also obtained from CI, shoot and root apices, and AzacC-treated cultures. Equal amounts of total RNA samples (10 μg per lane) were denatured with formamide/formaldehyde, fractionated and transferred to nylon membranes (Ausubel et al., 1993). Genomic Southern blots were performed using the standard protocols (Ausubel et al., 1993). Probe labelling, hybridization and washing steps were carried out as mentioned above.

**Cladistic analysis**

Derived amino acid sequences for the CDC48 homologue and the MADS-box gene were compared with sequences from the databases. The conserved sequences for the CDC48 homologue (315 amino acids) and the MADS domain proteins [MADS domain, linker and K-box (MLK) regions—about 120 amino acids] were considered for the analysis. Clustal W was used to create an input file of aligned sequences (Thompson et al., 1994). Bootstrap analysis was performed with 100 independent re-samplings of the original data matrix generated with SEQBOOT, and the bootstrap values above 50% are shown at the nodes of the tree. PROTDIST generated a distance matrix and the Neighbor-Joining algorithm was used to generate the tree (Saitou and Nei, 1987). The resulting phenogram was drawn with ‘Treeview’ program (Rod, 1996).

**Combined Bisulphite Restriction Analysis (COBRA)**

Approximately, 1 μg of genomic DNA was used for the sodium bisulphite reaction that converts non-methylated cytosine to uracil, while methylcytosine remains unchanged. The reaction was carried out as described by Xiong and Laird (1997). PCR reactions were performed using arbitrary 10-mer primer OPU9 (Operon Technologies Inc., California, USA) and 2 U of DyNAzyme™ (Finzyme OY) in a TouchDown™ thermal cycling system (Hybaid Ltd, UK) as described earlier (Prakash and Kumar, 1997). The PCR parameters were: 94 °C for 2 min, initial denaturation followed by 40 cycles of 94 °C for 1 min, 37 °C for 3 min, 72 °C for 2 min and a final extension at 72 °C for 10 min.

An aliquot of the PCR products was restricted with BstUI, which recognizes the CGCG sequence. Cleavage will occur only if the CGCG sequence has been retained during the bisulphite conversion by the methylated status of the cytosine residue.

**Results**

**Effect of demethylating agents on adventitious shoot induction**

The origin of shoot primordia, which resembles a cluster of cells arising from the epidermal and sub-epidermal tissue possessing meristematic activity, was observed in the (7–12-d-old) control explants by scanning electron microscopy (Fig. 1). No meristematic structures were observed in the 4-d-old control and AzacC- or AzadC-treated cultures (data not shown). Also, the pattern of inhibition of shoot bud induction appears to be similar in both AzacC and AzadC-treated explants, as no shoot bud initiation was observed even on day 10, suggesting the involvement of cytosine methylation in adventitious shoot induction. The most advanced structures observed from the 7-d-old control explants were nodule-like meristematic domes with no apparent differentiation of leaf primordia (Fig. 1A). There was no meristematic activity or presence of nodule-like structures in the 7-d-old 20 μM AzacC-treated explants (Fig. 1B). A similar inhibition of meristematic activity was also observed with 5 μM AzadC. After 2 weeks in culture, the meristematic domes in the control explants differentiated into well-defined shoot buds (Fig. 1C). However, formation of shoot primordia was significantly lower and delayed at this time in the drug-treated explants of the same age (Fig. 1D). Shoot regeneration was almost completely blocked when the leaf discs were maintained on SI medium supplemented with either AzacC or AzadC (Fig. 1E). These explants could regenerate shoots if they were transferred to SI medium without the drugs.

**Cloning and sequence analysis of differentially amplified bands**

In order to understand the molecular aspects of cytosine methylation in relation to shoot meristem formation better, the differential methylation status of CCGG sites in the genomic DNA from the control (SI) and drug-treated explants was examined. Five distinct bands were amplified in the control DNA subjected to CRED-RA, but they were absent in the DNA from the drug-treated tissues where shoot induction was inhibited (Prakash and Kumar, 1997). These bands were amplified from the genomic DNA of the
control, but not the drug-treated tissues even after the template was digested with MspI. Two of these bands, namely, OPU9-1 and OPU9-2 were cloned and sequenced to determine the presence of CCGG and CGCG sites and their methylation status. The OPU9-1 band contains 1 CCGG and 2 CGCG sites and the OPU9-2 band has 3 CCGG and 1 CGCG towards their carboxyl termini. The sequence analysis of the ORF in these two DNA bands showed that they have high similarity to CDC48 (Fig. 2) and a MADS-box gene (Fig. 3), respectively.

The OPU9-1 clone consists of a 945 bp ORF with similarity to CDC48 and other members of the AAA (ATPase associated with diverse cellular activities) family of ATPase. Multiple alignment of this sequence to FtsH from Nicotiana (76% identities), E. coli (70% identities) and Saccharomyces (32% identities, 50% positives) is shown (Fig. 2A). To examine the sequence data further, amino acids were aligned for similarity to the distinct subfamilies of the AAA protein family. OPU9-1 clone clustered with the Walker-type ATPase subfamily, which
has two consensus motifs, Walker A and B. Additionally, AAA proteins have a conserved region within the ATPase domain that is known as the second region of homology (SRH). All these conserved motifs are present in OPU9-1 (Fig. 2A). Phylogenetic analysis of a 315 amino acid region confirms the observed high similarity to FtsH, which is a membrane-bound ATP-dependent protease belonging to the AAA family members. The putative α helices and β sheets (in bold letters) are indicated based on the NSF-D2 crystal structure. (B) A phenogram based on a 315 amino acid region was constructed using distance matrix generated with the Neighbor-Joining algorithm. Abbreviations and accession numbers for the clones used are: CDC B.s, Bacillus subtilis (P37476); YME-1, human (NP0550781); FtsH P.p, Porphyra purpurea (P51327); FtsH S.sp., Synechocystis sp. (NP_442160); FtsH A.t, Arabidopsis thaliana (AL353993); FtsH N.t, cell division cycle protein, Nicotiana tabacum (AF117339).

The OPU9-2 band contains an ORF of about 480 bp coding for a MADS domain protein. The MADS-, K-box and the linker region (MLK region) from the deduced amino acid sequence of the OPU9-2 clone was used for comparison with similar proteins from the database (Fig. 3A). The MADS-box of the Petunia OPU9-2 protein has high similarity to short vegetative phase protein (SVP) and to JOINTLESS, which are expressed in the vegetative parts of Arabidopsis and tomato, respectively (Hartmann et al., 2000; Mao et al., 2000) (Fig. 3B). Also, the OPU9-2 clone shows similarity to the vegetatively expressed MADS-box proteins including PkmADS1, StMADS11,
StMADS16, and other members of the StMADS11-like group of MADS-box genes (Prakash and Kumar, 2002). The MADS-box genes expressed in the vegetative tissues form a separate clade from their counterparts expressed in the floral tissues (e.g. AP1, PI, AG, and AP3).

Expression of OPU9-1, the CDC48 homologue
The Petunia genome has several copies of this gene or closely related genes as indicated by the multiple bands in a genomic Southern blot (Fig. 4A). Total RNA gel blot analyses show a transcript size of 2.4 kb (Fig. 4B), which is the reported size of several other CDC48 homologues in the database. There was basal level signal in 0-, 10-, 12-d-old SI, and 10-d-old CI cultures, and shoot and root apices. No signal was detectable in the 7-d-old AzaC-treated tissues cultured on SI medium, whereas increased expression of the CDC48 homologue was observed in the control 7-d-old SI cultures.

Expression of OPU9-2, the MADS-box homologue
Genomic Southern blot indicated that OPU9-2 exists as a single copy in the Petunia genome (Fig. 5A). Expression of the OPU9-2 clone was studied by RT-PCR. Primers for the OPU9-2 clone (MADS1, 5'-CTGTTCAAGTGGAAA-...
were designed to amplify a 500 bp DNA fragment that includes the MADS-box and a part of the non-conserved region. The expected signal was detected from 7-d-old SI explants and shoot apices (Fig. 5B), but not from CI, 0- or 7-d-old AzaC-treated explants, or flower and root apices. The PCR blot was probed with the OPU9-2 clone (data not shown), which hybridized to the amplified bands from 7-d-old SI cultures and shoot apices, confirming that the amplified product corresponds to the MADS-box.

**Differential cytosine methylation during shoot and callus induction**

When cytosine methylation status at the CCGG sites in *Petunia* leaf discs undergoing shoot and callus induction was investigated, distinct profiles of DNA amplification...
products (CRED-RA) were observed for SI and CI cultures (Fig. 6). Among more than 15 bands present in the SI treatment, at least four bands, ranging from approximately 0.4 to 1.0 kb, were absent in the MspI digested DNA from CI tissue, but two of these bands (0.7 and 0.9 kb) were present in the HpaII digested DNA from CI. These four bands were present in the products amplified from SI tissue, suggesting the occurrence of differential methylation in the CCGG sites during shoot induction. In addition, four bands (below 300 bp) were present in MspI digested DNA of CI tissue, but these were absent in the HpaII digested DNA (Fig. 6). From the data, it appears that the outer or both C residues in the CCGG sites in specific regions of DNA remain methylated during shoot regeneration.

Restoration of cytosine methylation at CCGG sites after removal of the drug treatment

Reversal of cytosine methylation of CCGG sites was examined in the genomic DNA isolated from the explants that were transferred after 1 week of culture from AzaC- or AzadC-containing to the drug-free SI medium for an additional week (Fig. 7). Restoration of cytosine methylation in the genomic DNA isolated from such explants (transferred from AzaC-containing medium to medium without the drug) was revealed by the appearance of at least three amplified DNA bands. These bands were absent in the PCR products from explants maintained on AzaC-containing medium for the 2 week culture period (Fig. 7). Similar PCR profiles were obtained in the undigested genomic DNA obtained from both treatments. In an earlier report, restoration of normal shoot regeneration was demonstrated in the explants that were transferred after 1 week of culture from the medium containing the drugs to medium without the drugs (Prakash and Kumar, 1997; also Fig. 1E). The simultaneous restoration of shoot regeneration and cytosine methylation in specific DNA segments identified in this study further corroborate the finding that site-specific methylation at CCGG in certain genes is involved in adventitious shoot bud formation in Petunia.

Detection of cytosine methylation at CGCG sites by COBRA

The methylation status at CGCG sites in the genomic DNA isolated from the control and drug-treated explants was examined by COBRA, where sodium bisulphite converts cytosine to uracil, but methylcytosine remains unchanged. Similar amplification profiles were obtained in the DNA of control and drug-treated explants before digestion of the PCR products with BstUI, which recognizes CGCG sites (Fig. 8A). At least three distinct bands were lost in the control after digestion. The PCR gel blot was hybridized with the MADS-box probe, which showed two smaller bands at approximately 650 and 440 bp instead of a single 1.1 kb band in the control PCR products digested with BstUI (Fig. 8B). The single signal was retained even after the BstUI digestion of the PCR products from the drug-treated tissues.

**Fig. 6.** Differential methylation of cytosine in SI and CI cultures revealed by CRED-RA. Genomic DNA samples, undigested or digested with HpaII or MspI, from the SI and CI cultures were used as the template for random amplification with OPU9 primer. Arrows indicate the absence of two amplified bands in the CI DNA digested with HpaII and MspI. Asterisks indicate the presence of two amplified bands in the CI DNA digested with HpaII, but absent in the MspI digested DNA. Arrowheads indicate the presence of four amplified bands in the MspI digested CI DNA and absent in the HpaII digested DNA.

**Fig. 7.** Reversal of cytosine methylation at CCGG sites when AzaC-treated cultures were transferred to drug-free SI medium. Genomic DNA from 2-week-old Petunia leaf discs on SI medium treated with 20 μM AzaC for 2 weeks continuously (+) or from the explants that were treated with 20 μM AzaC for 1 week followed by an additional week on drug-free SI medium (+/-) were used as templates for random amplification (CRED-RA) with OPU9 primer. Three DNA bands that were amplified in the cultures transferred from AzaC to control medium (but absent in AzaC-treated cultures) are indicated by arrows.
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Different developmental stages in rice (Xiong et al., 1995). Significant differences in the expression (Boyes and Bird, 1991; Renckens et al., 1992). Further, there is evidence that methylated DNA sequences regulate the activity of other sequences in trans (Matzke and Matzke, 1995). Significant differences in the levels of cytosine methylation have been revealed between different developmental stages in rice (Xiong et al., 1999). While these findings demonstrate the significance of cytosine methylation in the regulation of various molecular processes, little is known about the specific role of cytosine methylation on shoot meristem differentiation and development.

The results presented here show that maintenance of methylation in specific genes is positively correlated with adventitious shoot regeneration in Petunia leaf explants. Reversal of inhibition of shoot regeneration when the demethylating agents are withdrawn from the culture medium supports the view that the inhibition is not due to general toxicity (Prakash and Kumar, 1997). These results are consistent with an earlier report of an increase in cytosine methylation in auxin-treated carrot cell cultures, where the addition of AzaC blocked somatic embryogenesis (Loschiavo et al., 1989). By contrast, the presence of hypomethylating agents during the first three days of culture led to enhanced androgenesis in barley microspores, but a dramatic reduction in androgenesis was observed when the drug treatment was longer than three days (Li et al., 2001), demonstrating the complex regulation of methylation of specific genes required for the induction and maintenance of developmental events.

It is noteworthy that cytosine methylation levels were generally low in young seedlings, while genomic DNA from apical buds was highly methylated in Pisum sativum (Watson et al., 1987). Genomic DNA from young seedlings was also methylated to a greater extent than that of the flag leaves in rice, which was assessed by the methylation-sensitive amplified polymorphism (MSAP) technique (Xiong et al., 1999). Genes involved in the apical meristem formation might require selective maintenance of cytosine methylation at specific sites for tight regulation of gene expression and for maintaining the highly organized meristem structure and its function of continual organogenesis.

The techniques used to observe changes in the cytosine methylation patterns between the control and drug-treated and SI and CI explants of Petunia in this study can detect methylation status only in the CCGG or CGCG sites. Many of the other possible methylated cytosine residues in the genome, which might also be critical for regulating the observed developmental events, will not be identified. Further, the possibility of global hypomethylation by AzaC or AzadC causing activation or inhibition of other genes that may affect development cannot be ignored. Nevertheless, this study is significant, because it demonstrates the effective use of a relatively simple technique for identifying some of the genes that are likely to be involved in the shoot bud induction process.

DNA hypomethylation induced by the drugs was heritable even in the absence of further drug treatment in some species. However, the methylation levels could be restored after the removal of the drug to an extent that varied with the cell type (Finnegan et al., 1998). This study demonstrated that the cytosine methylation is highly reversible under culture conditions inducing shoot formation in leaf discs.

Shoot meristem formation is mediated by a complex interaction of different genetic elements. Recent studies have identified a number of new molecular regulators involved in the control of cell division, meristem formation and floral organ differentiation (Clark, 2001; Ng and Yanofsky, 2001). Zhang et al. (1998) also demonstrated that the expression of CDC2Zm, a homologue of the
cyclin-dependent kinase gene CDC2, was induced during the formation of adventitious meristems in maize and barley. The results presented here show that the CDC48 homologue encoding an AAA member of the ATPase genes, and OPU9-2, that encodes for a MADS-box protein are likely to be involved in shoot meristem formation in Petunia.

Although most of the MADS-box genes identified in plants are involved in the control of floral patterning and development of reproductive structures, some also have roles in vegetative development. For example, JOINTLESS in tomato is critical for abscission (Mao et al., 2000), while PkMADS1 is required for vegetative shoot development and adventitious shoot regeneration in leaf cultures of Paulownia (Prakash and Kumar, 2002). The ORF encoded within the OPU9-2 genomic DNA shows high similarity to these genes. The results of the preliminary expression studies in this investigation clearly show that OPU9-2 is specifically expressed in the shoot meristem-forming explants and in the vegetative SAM, suggesting the involvement of this MADS-box gene in shoot meristem formation in Petunia in a manner similar to that of PkMADS1 mentioned above. However, it is not known whether OPU9-2 is required for the maintenance of undifferentiated stem cells of the SAM or if it is involved in channelling the progeny cells towards development of organ primordia. This can only be concluded by functional analysis of the gene, either by mutant analysis or by a transgenic plant approach, which is beyond the scope of the present investigation.

The identification of genes and molecular events that are critical to the formation and maintenance of shoot meristem and their activities under in vitro conditions will greatly enhance understanding of the regulation of meristem differentiation and morphogenesis in plants. In this context, the results presented here are significant, because they have clearly established a link between cytosine methylation in specific genes and the shoot regeneration process. Initiation of a highly organized and novel developmental pathway such as de novo meristem formation from a differentiated tissue (e.g. leaves) would involve significant changes in gene expression, which could be partly attributed to the altered chromatin conformation by cytosine methylation. The results of this study imply that selective maintenance of cytosine methylation in CCGG and CGCG sites in specific genes is involved in adventitious shoot production in Petunia leaf cultures.

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