DNA methylation analysis of a male reproductive organ specific gene (*MROS1*) during pollen development

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Abstract: Pollen grains of angiosperm plants represent a good model system for studies of chromatin structure and remodelling factors, but very little is known about the DNA methylation status of particular genes in pollen. In this study, we present an analysis of the DNA methylation patterns of the *MROS1* gene, which is expressed in the late phases of pollen development in *Silene latifolia* (syn. *Meladrium album*). The genomic sequencing technique revealed similar DNA methylation patterns in leaves, binucleate pollen, and trinucleate pollen. Extremely high DNA methylation levels occurred in the CG dinucleotides of the upstream region (99%), whereas only a low level of CG methylation was observed in the transcribed sequence (7%). Low levels of methylation were also observed in asymmetric sequences (in both regions; 2% methylated). The results obtained in the *MROS1* gene are discussed in consequence with the immunohistochemical data showing a hypermethylation of DNA in the vegetative nucleus.

Key words: DNA methylation, genomic sequencing, immunocytology, pollen, *Silene latifolia*.

Résumé : Les grains de pollen chez les angiospermes constituent un bon système modèle pour étudier la structure de la chromatine et les facteurs de modulation, mais bien peu de choses sont connues au sujet de la méthylation de gènes individuels chez le pollen. Dans ce travail, les auteurs ont analysé la méthylation du gène *MROS1*, lequel s'exprime dans les dernières phases du développement pollinique chez le *Silene latifolia* (syn. *Meladrium album*). La technique de séquençage génomique a révélé des motifs de méthylation semblables chez les feuilles, le pollen binucléé et le pollen trinucléé. Un très fort degré de méthylation (99 %) a été observé au sein des nucléotides CG situés en amont du gène tandis que seul un très faible degré (7 %) de méthylation CG a été observé au sein de la région transcrite. Un faible degré de méthylation a été observé au sein de séquences asymétriques (2 % dans les deux régions). Les résultats obtenus pour le gène *MROS1* sont comparés avec les résultats d'analyses immunohistochimiques montrant une hyperméthylation du noyau végétatif.

Mots clés : méthylation de l'ADN, séquençage génomique, immunocytologie, pollen, *Silene latifolia*.

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Introduction

Methylation of cytosine residues is the most common covalent modification of DNA found in vivo. Most of the data concerning the role of DNA methylation have been obtained on animal model systems. The role of DNA methylation in vertebrates includes genomic imprinting, dosage compensation mechanisms, and tissue-specific control of gene expression (reviewed by Bestor 1998). In spite of the fact that plants have not been studied as intensively, a relatively large amount of data has been obtained on the abundance and sequence context of methylated cytosines. As many as 30% of cytosine residues in plant genomes may be methylated. Most methylcytosine residues are found, similarly to vertebrates, in symmetrical CG dinucleotides, but methylation often occurs also in CAG and CTG trinucleotides (Gruenbaum et al. 1981) and in CCG motifs (Bezdek et al. 1992; Fulnecek et al. 1998). The methylation of asymmetric sequences has also been frequently found in higher plants (Meyer et al. 1994). The non-CG methylation has been suggested to be a unique property of plants, but several cases of methylation in asymmetric sites have been recently reported in vertebrates too (e.g., Woodcock et al. 1997).

In contrast to vertebrates, there is little knowledge concerning the role of tissue-specific DNA methylation of plant genes (Finnegan et al. 2000). A high level of DNA methylation in leaves, as a non-expressing tissue, has been observed in the promoter sequence of the maize *Opaque2* gene coding an endosperm-specific B-Zip protein (Rossi et al. 1997) and similar results have been reported for the bar-

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ley B-hordein gene (Sorensen 1991). The expression of these genes is correlated with hypomethylation of their promoters. The endosperm-specific demethylation correlating with expression has also been described in two other groups of genes in maize: the zein gene family (Lund et al. 1995*a*; Sturaro and Viotti 2001) and some genes coding α-tubulin (Lund et al. 1995*b*). In both cases only maternal copies were demethylated, thus indicating the role of DNA methylation in genomic imprinting. The case of an endogenous gene whose inactivation is connected with DNA methylation was described in *Arabidopsis*. The transcription factor encoded by the *FWA* gene, which is involved in the control of flowering, is ectopically expressed in *fwa* mutants and silenced in mature wild-type plants. This silencing is associated with the extensive methylation of two direct repeats in the 5′ region of the gene (Soppe et al. 2000).

The importance of DNA methylation patterns for tissue-specific control of expression during plant development is illustrated by studies showing prominent tissue-specific changes in DNA methylation levels (Oakeley et al. 1997; Janousek et al. 2000; Zluvova et al. 2001), aberrant phenotypes induced by a genome-wide hypomethylation (Kakutani et al. 1996; Kakutani 1997), and the role of tissue-specific DNA demethylation in the control of flowering (Sheldon et al. 2000). By analogy to mammals where extensive DNA methylation changes were observed during sperm and early embryonic development (Warnecke and Clark 1999), it might be expected that similar changes could also occur in plants during the period of intense chromatin differentiation when new cell types are formed. A very attractive model system for plant epigenetics is afforded by the pollen grains of angiosperms because they contain only two cell types: either vegetative and generative cells (in binucleate pollen species) or one vegetative cell and two sperm cells (in trinucleate pollen species). At present, significant progress in the study of pollen nuclei has been made, with some genes important to pollen nuclei differentiation having been isolated (Ueda et al. 2000). However, very little is known about the role of DNA methylation at the gene level, because efforts have been primarily concentrated on studies of changes of DNA methylation during pollen development. These studies employed an immunohistochemical approach, which does not afford information concerning the methylation of specific sequences (Oakeley et al. 1997; Janousek et al. 2000). To date, only the auxin-binding protein gene (*Nt-abp1, T85*) has been studied. It was shown that this gene is not methylated in leaves, but that two different patterns of DNA methylation were found in the mature pollen (Oakeley and Jost 1996). Both hypomethylated copies (only one site methylated) and hypermethylated copies were present. This is in accordance with the immunohistochemical data showing the higher DNA methylation of the vegetative nucleus in mature pollen of *Nicotiana* (Oakeley et al. 1997) and *Lilium* (Janousek et al. 2000). From the presence of sequences that escape the prominent DNA hypermethylation taking place during the one X-chromosome inactivation in mammalian females (reviewed by Disteche 1999), it can be predicted that similar exceptions may also occur in plants.

Here we report the DNA methylation patterns of the *MROS1* gene (male reproductive organ specific) isolated from *Silene latifolia* (Matsunaga et al. 1996). *Silene latifolia* represents a model species that attained an interest of researchers from several fields. Because this species is dioecious and possesses well-developed sex chromosomes, it became an important model to study the evolution of sex chromosomes (Charlesworth and Guttman 1999) and various aspects of sex determination (Donnison et al. 1996; Farbos et al. 1999) including the role of DNA methylation (Janousek et al. 1996; Vyskot 1999). The main advantage of this model for pollen biology is also connected with its dioecy. The suppression of female organ development in male flowers facilitates the production of male-specific cDNA libraries from all developmental stages of a gender-specific flower, which is impossible in hermaphrodites (except monoecious species). The *MROS1* gene was isolated from the male specific cDNA libraries independently by two research groups and the pattern of its expression was carefully studied (Matsunaga et al. 1996, 1997; Scutt et al. 1997). Its expression proceeds in the binucleate pollen grains of this trinucleate pollen species and does not occur in other tissues. In situ hybridization patterns indicate that *MROS1* is transcribed only in the vegetative nucleus (Matsunaga et al. 1997) and it has been shown that the haploid genome of *S. latifolia* contains only one copy (Matsunaga et al. 1997), and this copy is autosomally linked (Kejnovsky et al. 2001). The last phase of pollen development resembles the late period of seed development by the reduction of water content, the presence of dehydration-related proteins, and the role of abscisic acid (Osborne and Boubriak 1994; Wolkers et al. 2001; Vicient et al. 2000). Because some genes taking part in seed maturation (i. g. B-hordein gene, Sorensen 1991; zein gene family, Lund et al. 1995*a*) are subject to DNA methylation control of transcription, it is possible that the transcriptional control of some pollen genes is connected with their methylation pattern. These genes could escape the DNA hypermethylation taking place in the vegetative nucleus during the last phase of pollen grain maturation.

We have analysed the DNA methylation status of the *MROS1* gene in binucleate and mature (trinucleate) pollen grains using bisulphite genomic sequencing. The data obtained are interpreted with respect to the immunocytological data comparing DNA methylation levels in the vegetative and sperm nuclei of *S. latifolia*.

Materials and methods

Plant material

Seeds of three strains of *Silene latifolia* Poiret subsp. *alba* (Miller) Greuter et Burdet were obtained from the seed collections of the Department of Frontier Sciences, University of Tokyo, Japan (MAV strain); the Institute of Biophysics, Brno, Czech Republic (CZ1 strain); and the University of North Carolina, Chapel Hill (Gen8c strain). Plants were cultured in greenhouse conditions under a 16 h light : 8 h dark photoperiod to promote flowering. Pollen fertility was tested as described by Janousek et al. (1998). For genomic sequencing studies, pollen of the CZ1 strain was collected from anthers via forming suspension of pollen grains in hexan and centrifugation at 500 *g* for 1 min. To release the binucleate pollen from anthers, the anthers were slightly

crushed using a pipette tip. The purity and developmental stage of pollen was checked using 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei and epifluorescence microscopy.

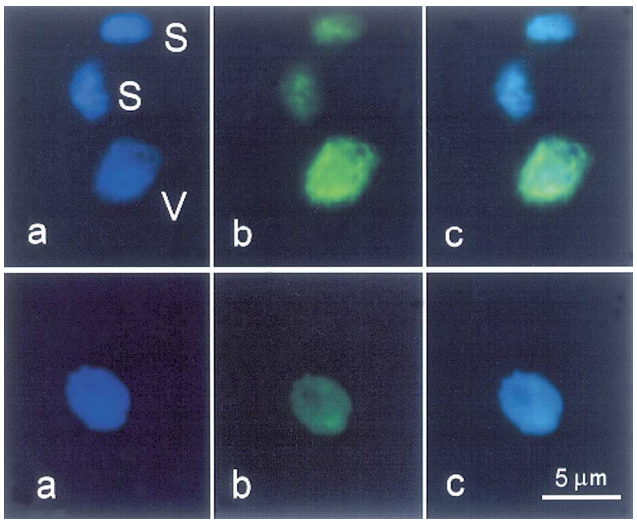
Preparation of sections and squashes for immunostaining

The anthers containing microspores were fixed in ethanol – acetic acid (3:1) overnight at 4°C, embedded in Cryomount (Histolab, Göteborg, Sweden) and sliced into 10-µm thick sections using a cryomicrotome Leica CM 1800 (Leica Instruments GmbH, Wetzlar, Germany). Samples were mounted on slides coated with poly-L-lysine. Pollen grains were squashed in 45% v/v acetic acid and mounted on poly-L-lysine coated slides using the dry ice technique. The postfixation was carried out in the ethanol – acetic acid mixture decribed above and proteins were further extracted in chloroform, 45% v/v acetic acid, and pepsin (100 µg/mL). The slides were denatured in 2 M HCl for 5 min, dehydrated in ascending series of ethanol (50, 70, and 96% v/v) and air dried. The monoclonal antibody against 5-methylcytosine (5-mC) was kindly provided by Dr. Ruffini-Castiglione (University of Pisa) and its preparation and basic properties were described by Podesta et al. (1993). Its specificity was verified using methylated and non-methylated oligonucleotides as described by Oakeley et al. (1997). The immunocytochemical validity of this antibody has also been demonstrated on *S. latifolia* nuclei. DAPI-dense chromocenters in control interphase nuclei displayed strong 5-mC immunosignals. These signals disappeared after a treatment with a demethylating agent, 5-azacytidine (Siroky et al. 1998). After the blocking reaction (1% w/v bovine serum albumin in PBS with 0.5% v/v Tween 20 for 1 h), the anti-5mC antibody (diluted 1 : 1 000) was applied and the slides were incubated at 4°C for 12 h. The FITC-labeled goat anti mouse IgG (Sigma, F-0257) was used as the secondary antibody (dilution 1:100, incubation for 12 h) and the slides were counterstained with DAPI. The accessibility of DNA epitopes for antisera was checked using the mouse anti-DNA monoclonal antibody (No. 1003 399, dilution 1:4, incubation for 12 h, Boehringer Mannheim, Germany). Fluorescence was visualized using an epifluorescence microscope (Olympus AX70, Olympus Optical Co. Ltd., Tokyo, Japan) and signals were evaluated using ISIS software (Metasystems, Sandhausen, Germany). Statistical evaluations were done according to Janousek et al. (2000). The original antibody signal ratios (sum of antibody signals of sperm nuclei/antibody signal of the vegetative nucleus) were corrected for the DNA amount (the antibody signal ratios were divided by DAPI staining ratios). The background fluorescence from the cytoplasm was subtracted.

Isolation of *MROS1* upstream region and comparative analysis of strain differences

The *MROS1* upstream region from the MAV strain was isolated using inverse PCR according to Matsunaga et al. (1999). Based on the sequence of the iPCR product, we have designed the following primers to amplify homologous sequences from the CZ1 and Gen8c strains : F4 primer, 5′-TAC CAG GTC TAC ACT TGA GT -3′; IR1 primer, 5′-TCA GTT GCG AAT GAG AGA GC -3′. The PCR products were cloned into the pZERO II cloning vector (Invitrogen, Carlsbad, Calif.) and sequenced using ALFexpress AutoRead Sequencing Kit (Pharmacia, Piscataway, N.J.).

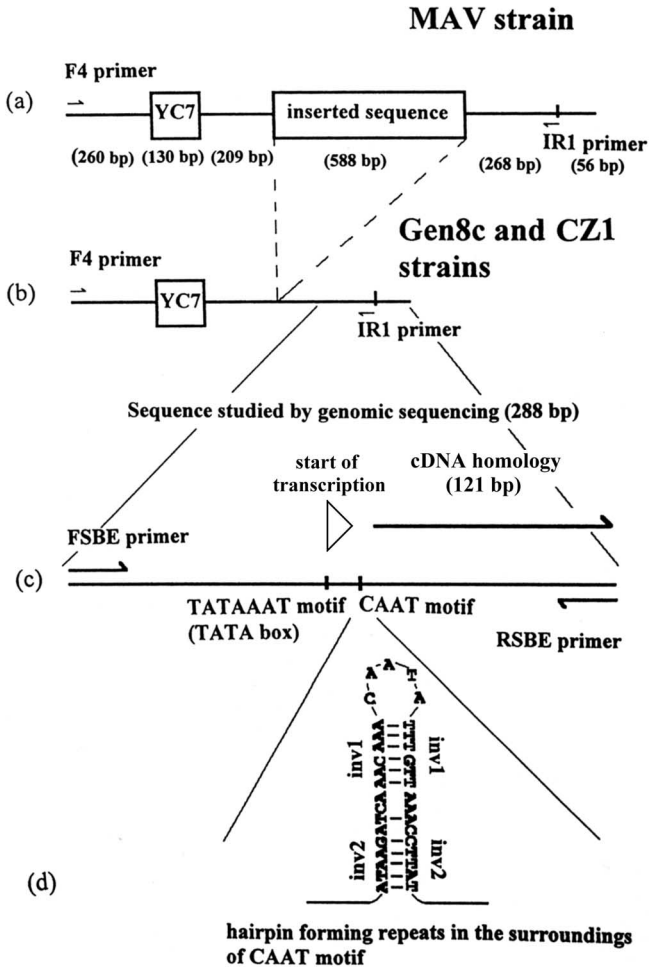
Fig. 1. DNA methylation analysis of mature pollen grain nuclei (top) and microspore nuclei (bottom) as revealed by immunolabeling with the anti-5-mC monoclonal antibody. The secondary antibody was labeled with FITC and visualized by epifluorescence microscopy. (*a*, top) DAPI staining of DNA shows the equal intensity of staining of all nuclei and a decondensed character of the vegetative nucleus. (*b*, top) FITC signal of the secondary antibody shows a prominent signal on the vegetative nucleus, V, in comparison with a low signal on the sperm nuclei, S. (*c*, top) The higher intensity of DNA methylation is visualised as green colour of vegetative nucleus in comparison with blue colour of sperm nuclei on the merged image. (*a–c*, bottom) Labeling of the microspore nucleus with the antibody raised against 5-mC displays a low signal intensity.



DNA methylation analysis using genomic sequencing

Plant DNA was isolated using DNeasy Plant Mini kit (Qiagen, Valencia, Calif.). The sodium bisulphite reaction was carried out according to Olek et al. (1996) with minor modifications. DNA was digested with *Eco*RI, then boiled for 5 min, quickly chilled on ice, and subsequently incubated in 0.3 M NaOH for 15 min at 50°C. The denatured DNA was mixed with two volumes of 2% w/v low melting point agarose dissolved in deionised water and this mixture was pipetted into chilled mineral oil so that 10 µL agarose beads containing 200 ng DNA were formed. Aliquots of 200 µL of 5 M bisulphite solution (2.5 M sodium metabisulphite, 1.25 mM hydroquinone; Sigma, St. Louis, Mo.) were added to each reaction tube containing a single bead. Reaction mixtures were incubated overnight at 50°C in the dark. The treatments were stopped by equilibration against 1 mL of 1× TE (10 mM Tris–HCl, 1 mM EDTA (pH = 7.0), 6 × 15 min), followed by desulphonation in 500 µL of 0.2 M NaOH (2 × 15min). The reactions were neutralized with 1 mL 1× TE, followed by equilibration against 1 mL of deionised water (3 × 15 min). The described process converts all the non-methylated cytosines in the sequence to uracil. The beads were used directly for PCR. The sequence studied was amplified using degenerate primers (FSBE and

Fig. 2. Characterization of the upstream region of *MROS1* and its surroundings. The comparison of the upstream sequences of three different strains — MAV, Gen8c, and CZ1 — reveals the presence of a 588-bp insertion in the MAV strain (*a*) that is absent in Gen8c and CZ1 (*b*). *YC7* (*a* and *b*) represents a region that shows a high homology with the *YC7* repetitive element found previously in *S. latifolia* (Scutt et al. 1997). The description of the region analysed is shown below (*c*). Location of TATA box and CAAT motif in the middle is also outlined. The detailed scheme of the surroundings of the CAAT motif shows the potential of this sequence to form a hairpin (*d*).



RSBE) designed for the study of the bottom strand. Conversion of cytosines to uracil at the binding site prevents annealing of RSBE to template during the first cycle. These primers contained an *Eco*RI recognition site. The sequences of the primers were as follows: FSBE, 5′- CAG CCT GAA TTC CCA AAA TRA TCT RTT CCA A-3′; RSBE, 5′- ATG GCT GAA TTC ATG TAT GGG TYA YTA GGY TG-3′.

The amplification was performed using the following reaction conditions: 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM dNTP, 0.6 μM primers, and 1.5 U *Taq* polymerase (Finnzymes). The temperature profile of the amplification was 1 cycle of 94°C for 3 min and 35 cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 90 s, followed by a 5-min extension step at 72°C. The PCR products were cut with *Eco*RI and cloned into the pZERO II vector. The cloned PCR products were sequenced using ALFexpress AutoRead Sequencing Kit. Assuming the 2:1 ratio of clones coming from the two sperm nuclei or replicated generative nucleus to one from the vegetative nucleus (based on the relative DNA amounts), we have analyzed 10 clones both in binucleate pollen and mature trinucleate pollen. The probability of random absence of the clone coming from a vegetative nucleus (P_{abs}) can be counted according to the formula $P_{abs} = (2/3)^n \times 100\%$, where n is the number of clones analyzed. This probability equals 1.7% for $n = 10$. The control reaction to test the completeness of the modification was performed on 10 pg of plasmid DNA containing the studied region. The sequencing of three modified plasmid clones confirmed the completeness of the modification.

Results and discussion

Immunocytochemical analysis of pollen DNA methylation

The vegetative nucleus differs both from the generative nucleus and sperm nuclei not only by function, but also in chromatin structure. The generative and sperm nuclei show a highly condensed chromatin in contrast with the chromatin of the vegetative nucleus, which is relatively relaxed. It has been shown that at least two types of mechanisms are involved in these differences of chromatin structure. The decrease of histone H1 was demonstrated to be involved in the decondensation of chromatin of the vegetative nucleus (Tanaka et al. 1998). On the other hand, the highly compacted status of the generative nucleus is connected with the presence of specific histone variants (Ueda et al. 2000). The roles of DNA methylation and histone acetylation in pollen differentiation have also been studied. Recent data obtained in *Lilium* suggest that DNA methylation is not involved in the condensation status formation in generative nuclei, but it contributes to the stabilization of the vegetative nuclei in the stage of quiescence, which enables pollen to survive for a long time without any source of energy or nutrients (Janousek et al. 2000).

A statistical evaluation of immunocytochemical data obtained in the mature trinucleate pollen grains of *S. latifolia* (15 pollen grains evaluated) showed that DNA of the vegetative nucleus of *S. latifolia* is highly methylated in comparison with DNA of both sperm nuclei (Fig. 1). The significance of this difference has been proven by the paired Student's t test ($P < 0.05$). The corrected signal ratio of DNA methylation signals (sum of signals of the sperm nuclei corrected according to DNA amount / antibody signal of the vegetative nucleus) is 0.55 (standard error (SE) = 0.05). However, this value needs careful interpretation, because the linearity of the relationship between the signal intensity and DNA methylation was not tested. The comparison with the immunolabeled nuclei of microspores (Fig. 1) indicates that the difference is caused by a DNA hypermethylation of the vegetative nucleus rather than by a hypomethylation of the sperm nuclei. These data suggest that the vegetative nucleus in *S. latifolia* pollen undergoes a similar hypermethylation as we previously described in the binucleate pollen species *Lilium longiflorum* (Janousek et al. 2000), which can be concluded from the data obtained in *Nicotiana tabacum* (Oakeley and Jost 1996; Oakeley et al. 1997). It is unclear if

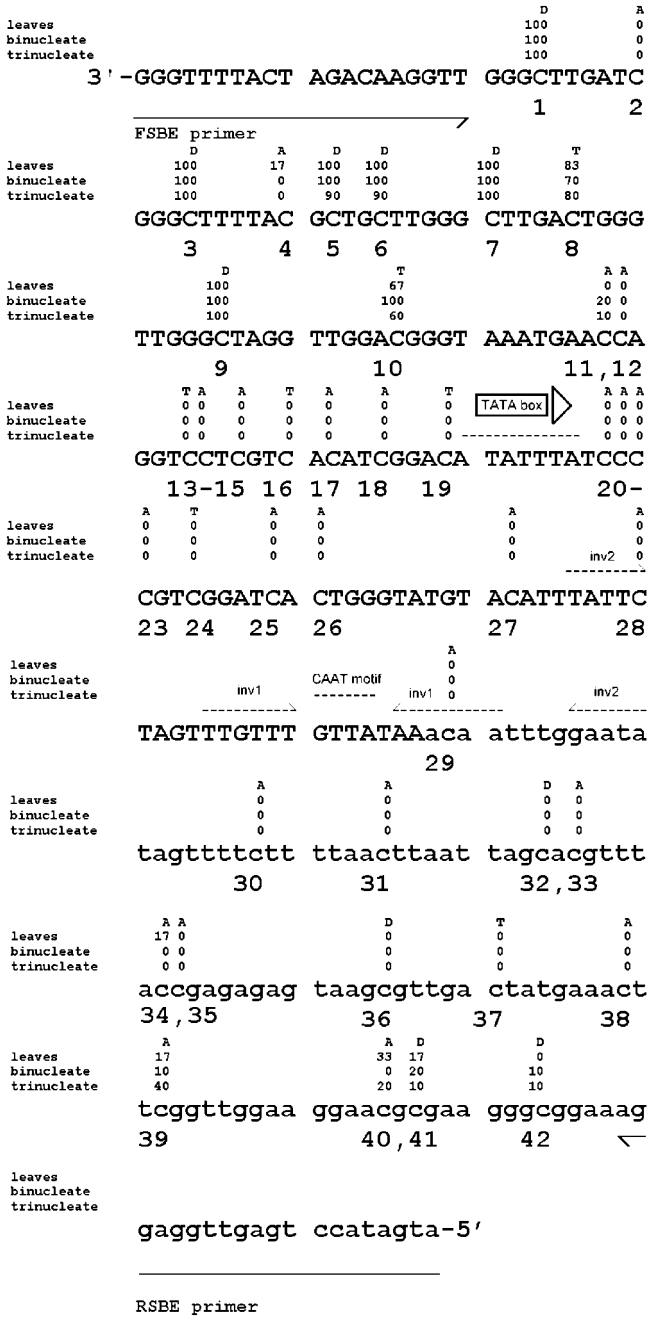


Fig. 3. DNA methylation analysis of the part of the *MROS1* sequence in leaves, binucleate pollen, and mature trinucleate pollen performed by bisulphite genomic sequencing of the bottom strand. First row always marks the sequence context of a given site: A, asymmetric; D, CG dinucleotide; and T, C(A/T)G trinucleotide. The percentage of cytosine methylation at particular methylatable sites in leaves (six clones analyzed) is shown in the second row. The third and fourth rows show the values obtained in binucleate and mature trinucleate pollen (10 clones analyzed each). The sequence of the region showing homology to the cDNA is written in lower case. The positions of the TATA box, CAAT motif, and inverted repeats (inv1 and inv2) are indicated. The presence of a highly methylated CG-rich region was revealed in all 26 clones studied. The frequencies of DNA methylation of particular sites in leaves and binucleate and mature trinucleate pollen show very similar patterns. High methylation of cytosine site Nos. 1, 3, and 5–10 occurs in all the three kinds of samples. The rest of the upstream region, as well as the transcribed region, is characterized by a low level of DNA methylation. The presence of the long non-methylated region (site Nos. 12–38) revealed by all the clones derived from DNA of mature trinucleate pollen suggests that this sequence is not influenced by the hypermethylation of the vegetative nucleus.

these DNA methylation changes occur in the whole nuclear genome or if some sequences can escape hypermethylation.

Isolation and characterization of *MROS1* promoter

To study DNA methylation changes at the gene level, we have chosen the upstream region of the *MROS1* gene, which begins to be transcribed in the binucleate pollen grains (Matsunaga et al. 1997). For comparison, we also included a part of the transcribed region. Because the upstream region of the *MROS1* gene was not known, we cloned it from the Japanese material (MAV strain) using inverse PCR. To find the most conserved region putatively involved in the control of transcription, we studied the sequence polymorphism in the region amplified using the F4 and IR1 primers. The analysis of the cloned PCR products of two other laboratory strains (CZ1 and Gen8c) showed that, in contrast to the MAV material, which displayed a 1455-bp-long PCR product, these strains formed an almost identical 867-bp-long PCR product. The comparison of the MAV sequence with those obtained from the Gen8c and CZ1 strains shows that the difference is caused by the insertion of a 588-bp sequence into the *MROS1* upstream region (Fig. 2). Because these strains all showed a normal phenotype and a similar level of pollen fertility (about 90%), we deduced that the inserted region is not crucial for the control of expression of *MROS1*. Because the distal part of the PCR product included a region with a high homology to a repetitive DNA element (*YC7*) (Scutt et al. 1997), the region located more proximal to the coding region was chosen for further study. Sequence analysis revealed the presence of several conserved motifs (TATA and CAAT) likely involved in transcription control. Moreover, the CAAT motif is surrounded by inverted repeats with potential to form a hairpin (Fig. 2*d*). Such types of secondary structures often serve as binding sites for regulatory factors (for a review see Wadkins 2000). The possible role of DNA methylation in this process was suggested by the presence of the CG-rich part.

Methylation patterns in different tissues

Analysis of DNA methylation in leaf samples enabled us to divide the studied region into two parts: a highly methylated C- and G-rich region, characterized by the presence of four CCCG motifs (3′-GGGC-5′ on the bottom strand), and an undermethylated part including the TATA box and the beginning of the transcribed region. Several methylated sites were also found in the transcribed region, but their frequency was much lower in comparison with the CG-rich region (Fig. 3; for details see MethDB database at http://www.methdb.de). The hypermethylated state of the CG-rich region of *MROS1* in non-expressing tissues suggested that the transcription of this gene could be controlled by methylation. Because

Table 1. Comparison of the DNA methylation frequencies of the studied region of the *MROS1* gene in leaves, binucleate pollen, and mature pollen with respect to sequence context.

		Frequency of cytosine methylation (%)			
Site context	No. of sites	Leaves	Binucleate pollen	Mature pollen	Cumulative
CGA	2	0	0	0	0
CGC	2	58	60	50	56
CGG	5	80	82	82	82
CGT	1	100	100	90	96
CG cumulative	10	62	63	60	62[a]
CAG	4	38	43	35	38
CTG	3	0	3	0	1
C(A/T)G cumulative	7	21	26	20	23[b]
CAA	5	7	4	6	5[c]
CAT	3	11	0	0	3
CAC	3	0	0	0	0
CCA	2	0	0	0	0
CTA	4	0	0	0	0
CTC	1	0	0	0	0
CTT	3	6	3	13	8[d]
CCT	2	0	0	0	0
CCC	2	0	0	0	0
Asymmetric cumulative	25	3	1	3	2
Total	42	20	20	19	20

Note: DNA methylation data obtained at particular methylatable sites in leaves (6 clones analysed), binucleate pollen (10 clones analysed), and mature trinucleate pollen (10 clones analysed) were grouped according to sequence context.

[a]Methylation in CG dinucleotides was significantly more abundant in comparison both with the methylation in C(A or T)G trinucleotides ($P < 0.0001$) and asymmetric sites ($P < 0.0001$) as tested using contingency tables.

[b]The methylation level in C(A or T)G trinucleotides was significantly higher if compared with asymmetric sites ($P < 0.0001$).

[c]The DNA methylation frequency in CAA trinucleotides was significantly higher in comparison wiht two other similarly abundant sites: CAC ($P < 0.05$) and CTA ($P < 0.05$).

[d]CTT trinucleotides showed significantly higher frequency of cytosine methylation in comparison with even more types of context: CAC ($P < 0.05$), CCA ($P < 0.05$), CCT ($P < 0.05$), CCC ($P < 0.05$), and CTA ($P < 0.01$).

MROS1 begins to be expressed at the late binucleate stage of pollen development (DNA of generative nucleus is already replicated), it can be predicted that one third of the clones represent original vegetative DNA, and thus if methylation controls activity, all of these should be hypomethylated. However, the data obtained in all the clones analyzed showed that the CG-rich region remained highly methylated with a very low variation among the clones studied (Fig. 3; for details see the MethDB database). There was nearly no difference if compared with the leaf DNA methylation pattern. This fact suggests that DNA methylation of the CG-rich region of the putative *MROS1* promoter is not involved in its transcriptional control. The presence of such a highly and stably methylated region in close proximity to the transcription start site in plant genes is rare, because promoters in plants are often characterized by a very low level or even the absence of methylated cytosines (Antequera and Bird 1988; Jacobsen and Meyerowitz 1997; Cubas et al. 1999; Jacobsen et al. 2000). However, the biological role of these hypermethylated sites cannot be quite excluded because it has also been shown that some transcription factors can specifically bind to methylated DNA sites (Rossi et al. 1997).

The presence of the large undermethylated part enabled us to test if this sequence escapes the hypermethylation that takes place in the vegetative nucleus during trinucleate pollen maturation. Because we have not found any significantly hypermethylated clone (see MethDB database) in comparison with both the leaf and binucleate pollen DNA clones, it can be deduced that the undermethylated region in the putative *MROS1* promoter is not influenced by the extensive DNA hypermethylation observed by immunocytochemistry. It is possible that the hypermethylated region serves as an insulator to prevent the spreading of DNA methylation to the rest of the gene. Transcription of this gene probably continues during the period when most genes are transcriptionally inactive and DNA of the vegetative nucleus becomes hypermethylated. The expression could be terminated by changes in the cytoplasm and nucleus in consequence with the formation of so-called molecular glasses that block metabolism on the cytoplasm and nucleoplasm level (Buitink et al. 1999). A similar timing of transcription was described in some genes involved in the late period of seed development (Comai and Harada 1990). Our data indicate that a group of genes involved in the last phase of pollen development is not influenced by the DNA hypermethylation of the vegetative nucleus.

Because there were no prominent differences in DNA methylation distribution in the leaf, binucleate pollen, and trinucleate pollen samples, we combined these data to analyse the DNA methylation context (Table 1). DNA methylation in all the samples occurred mainly in the symmetric sites CG or CNG, but also in the asymmetric sites CTT, CAT, and CAA.

To compare the DNA methylation patterns of the upstream region and the transcribed region, we used the TATA box as a landmark (Table 2). The comparison of the TATA

Table 2. Comparison of the DNA methylation patterns in the part located upstream from the TATA box and the part located downstream from the TATA box.

	TATA box upstream region		TATA box downstream region	
Site Context	No. of sites	Cytosine methylation (%)	No. of sites	Cytosine methylation (%)
CGA	0	—	2	0
CGC	1	96[a]	1	15
CGG	4	100[a]	1	8
CGT	1	96	0	—
CG cumulative	6	99[a]	4	7
CAG	3	51[a]	1	0
CTG	2	2	1	0
C(A or T)G cumulative	5	31[a]	2	0
CAA	1	12	4	4
CAT	1	4	2	2
CAC	1	0	2	0
CCA	1	0	1	0
CTA	2	0	2	0
CTC	1	0	0	—
CTT	0	—	3	8
CCT	1	0	1	0
CCC	0	—	2	0
Asymmetric cumulative	8	2	17	2
Total	19	40[a]	23	3

Note: The data obtained in leaves, binucleate pollen, and mature trinucleate pollen (26 clones in total) were pooled to compare the DNA methylation status of the 89-bp-long region located upstream from the TATA box and the 152-bp-long region located downstream from the TATA box of the *MROS1* gene.

[a]Significantly higher level of methylation at this sequence context in the part located upstream from the TATA box if compared with the part located downstream from the TATA box as revealed by the analysis of the contingency tables (for CGC, CGG, CG cumulative, CAG, C(A or T)G cumulative and total $P < 0.001$).

box downstream region (transcribed) and the upstream region revealed a difference in the relative frequency of methylation in various trinucleotide sequences. A significantly higher level of methylation was present both in CG dinucleotides and CNG trinucleotides, whereas the level of methylation in asymmetric sites showed no difference in both parts. In contrast to the DNA methylation patterns in the upstream region, the transcribed region showed a relatively high variation. The results, however, suggest that even the methylation of asymmetric sites is not quite random because the CAA and CTT sites are preferred in the *MROS1* gene. This preference can be connected with position of the preferred site and (or) it can reflect effects of local DNA structure. Preferential methylation of the CAA context has already been reported (Goubely et al. 1999).

The stable DNA methylation status of *MROS1* described here suggests that there are sequences that are not influenced by the DNA hypermethylation of the vegetative nucleus in angiosperm plant pollen. The mechanisms leading to the extensive hypermethylation of the large part of the genome of the vegetative nucleus and how parts of the genome escape this modification are presently unknown.

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