Does DNA Methylation Pattern Mark Generative Development in Winter Rape?

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In this paper we report on changes in DNA methylation pattern in rape apices and leaves during transition from vegetative to reproductive stage due to grafting and/or vernalization. Grafted plants of winter rape (*Brassica napus* L., var. "Górczański") (stock from vernalized, scion from non-vernalized plants) were used together with vernalized non-grafted plants. In addition, methylation status was determined also in spring rape (var. "Młochowski") grown under normal and low temperature. The methylation-sensitive amplification polymorphism (MSAP) method with *Eco*RI/*Msp*I and *Eco*RII/*Hpa*II restriction enzymes was employed. The majority (ca. 68%) of analyzed loci (566 in winter and 551 in spring rape) were monomorphic, *i.e.* did not undergo methylation. Both cultivars showed a similar degree of methylation. 188 loci in winter and 176 in spring cultivars expressed changes in the methylation pattern. All differentially amplified fragments resulted from either full methylation of an internal cytosine or from hemi-methylation of an external cytosine. A pair-wise comparison showed that a similar number of loci underwent development-related methylation changes in apices of the winter and spring rape. The majority (80%) of changes were demethylation events in generative (vernalized) apices of the winter cultivar. However, an increased number of demethylated loci was detected in vernalized apices in comparison with generative, non-vernalized ones. In apices of vegetative and generative grafted plants the same number of demethylation events was observed. Overall, 10 MSAP loci were detected that expressed methylation changes in vernalized apices only; among them 7 loci underwent demethylation after vernalization and remained methylated in both vegetative and generative non-vernalized stage. Only 1 locus was demethylated in generative non-vernalized apices. Thus, most of demethylation events can be ascribed to vernalization and not to the generative stage. In leaves of winter rape methylation and demethylation events occurred with similar frequency, while in the spring cultivar more demethylation events were detected. The results show that during vernalization and transition to the generative stage different sets of genes are activated.

Key words: DNA Methylation, Rape, Vernalization

Introduction

The change from vegetative to generative development in response to a prolonged exposure to cold temperature (vernalization) is a useful adaptation for winter-annual and bi-annual plants. While the physiology of vernalization has been extensively studied in many species, the molecular mechanism of this process remains largely unknown (Michaeles and Amasino, 2000). The genetic analysis of genes controlling flowering was started in *Arabidopsis* by Koornneef and co-workers at the early nineties (Koornneef *et al*., 1991). Up to now tens of loci affecting flowering time (FT) have been localized on the genetic map of *Arabidopsis* and some of them even cloned. They include those which are expressed due to perception of environmental stimuli as cold treatment and photoperiod and those which act autonomously. They have been arranged in several interacting pathways of a hierarchical order. Thus, at the moment the complexity of the network of flowering control can be approached in *Arabidopsis* as reviewed *e.g.* by Koornneef *et al*. (1998) or Mouradov *et al*. (2002).

DNA methylation is considered as one of the factors regulating the expression of the genes during the development of eukaryotes (Schwartz and

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Dennis, 1986; Ronemus *et al*., 1996). Some developmental events are closely related with the demethylation pattern. *Arabidopsis* plants that were either cold-treated or treated by the demethylating agent 5-azacytidine (5-azaC) showed a reduced amount of 5-methylcytosine in their DNA which was associated with earlier flowering in both cases. This led to the hypothesis that vernalization is mediated by changes in DNA methylation (Burn *et al*., 1993). Finnegan *et al.* (1998) came to the same conclusion using different molecular approaches. Enzymatic methylation of C residues in the DNA occurs postreplicatively and is confined to cytosine residues, primarily CG dinucleotides and CNG trinucleotides (Lorenz and Groundine, 2001). The extent and pattern of methylation of genomic DNA are species- and tissue-specific, what implies that the pattern of methylation is faithfully inherited in all cells of common lineage within a tissue (Christman *et al*., 1995). Although not all genes are regulated by methylation, the hypomethylation at specific sites or in specific regions in a number of genes is correlated with active transcription (Wada *et al*., 2003).

There are several methods that allow to determine the level of DNA methylation and to observe changes in methylation pattern of the genome. One of the methods, that combines the restriction of DNA with methylation-sensitive enzymes and the PCR reaction, is methylation-sensitive amplification polymorphism (MSAP) (Xiong *et al.,* 1999; Xu *et al*., 2000). This method is a modification of the AFLP technique (amplified fragment length polymorphism, Vos *et al*., 1995) and relies on the restriction of DNA with *EcoR*I enzyme, which is not sensitive to the DNA methylation and recognizes the 5′-GAATTC-3′ sequence, together with one of the isoschisomeres *Msp*I or *Hpa*II, that both recognize the 5′-CCGG-3′ sequence, but differ in their methylation sensitivity. *Msp*I does not cleave the sequence when the external C atom is fully or hemi-methylated, but is active when the internal C atom is fully or hemi-methylated. *Hpa*II is inactive when any of the C atoms is fully methylated, or when the internal C atom is hemi-methylated. *Hpa*II can cut DNA in the case of hemimethylation of the external C atom, however the frequency of restriction is much lower compared to the unmethylated site. Both enzymes do not digest DNA when both C atoms in the CCGG sequence are fully or hemi-methylated. The MSAP technique allows to analyze the methylation pattern and to observe its changes in various plant organs, at different developmental stages or after application of exogenous factors.

In this report, we use the MSAP method to examine changes in the DNA methylation pattern in rape apices during transition from the vegetative to the generative stage. Our earlier experiments proved that flowering in winter rape can be induced either by vernalization or by grafting of vegetative, not vernalized apices on a generative (vernalized) stock (Dubert *et al*., 1988; Filek *et al*., 2003). Rape, with a clearly distinguished stem, is an excellent object for grafting. The aim of the presented studies was to determine whether vegetative and reproductive rape apices differ in their DNA methylation pattern, and to check whether the same methylation changes appear during transition to flowering induced by vernalization and by grafting. As leaves of vegetative and generative rape differ in shape and size, the analyses were performed in both apices and leaves. Additionally, the DNA methylation pattern was determined in vegetative and generative organs of spring rape. In this case, although vernalization is not required for flowering, spring rape plants were subjected to a period of low temperature, similarly to the winter cultivar. All these experiments allowed to speculate whether the DNA methylation may function as a common switch of generative development of rape plant.

Material and Methods

Plant material

The research was carried out with winter rape cv. "Górczański" and spring rape cv. "Młochowski". The seeds, after sterilization and germination on blotting-paper, were placed in pots containing a mixture of peat, soil and sand in the ratio 1:1:1, and transferred into a conditioned chamber at a temperature of 17/15 °C day/night (16 h photoperiod). The plants were grown under these conditions for about 4 weeks, until they reached the stage of 5-leaf rosettes – the vegetative development stage which is optimal for vernalization of winter rape. After this time, plants were vernalized for 8 weeks at the temperature of 5/2 °C day/night (16 h photoperiod). Spring plants do not require a cooling period for the induction of flowering, however they were grown in the same conditions as winter rape with the aim of defining the effect of low temperatures on the DNA methylation chan-

ges. After vernalization, the winter and spring plants were transferred back to the temperature of 17/15 °C day/night (16 h photoperiod) and grown until floral buds appeared on the extended shoots. At this stage, plants become the generative stock for grafting of vegetative apices.

Grafting was done by cutting off the apical part of the stem of a generative stock plant in a place where the stem diameter was similar to the diameter of the graft, on condition that generative leaves have already appeared in that place. The stem segment with the vegetative apex (about 2 cm), without leaves (with the exception of the youngest leaf), was cut on the "cone" and grafted on the generative stem. The connecting surfaces were wrapped with parafilm and the whole upper part of the plant was placed in a plastic bag to ensure moisture. Bags were removed after one week, when both plant parts started to grow. The developing vegetative leaves were systematically removed from the graft. The appearance of generative leaves on the grafted stem (these leaves were not removed) was an indicator of the transition to the generative state.

The apices (0.3 cm of apical part of stem) and leaves of winter rape in the vegetative (not vernalized) and generative (vernalized) stage, as well as generative apices and leaves obtained after grafting were used as plant material for DNA analysis. For both cultivars, the apices and leaves at the vegetative stage were collected when plants reached a 5-leaf rosette, while materials at the generative phase of development were collected at the stage preceding the appearance of flower buds. Additionally, the organs from spring rape plants subjected to the 8-week period of low temperatures (cooled) were taken for DNA analysis. The following abbreviations were applied for the examined samples: WAV and WLV for winter rape (W) apices (A) and leaves (L) in the vegetative (V) phase; WAGv and WLGv for winter (W) apices (A) and leaves (L) in the generative (G) phase resulting from vernalization (v); WAGg and WLGg for winter (W) apices (A) and leaves (L) in the generative phase after grafting (g); SAV and SLV for spring rape (S) apices (A) and leaves (L) in the vegetative (V) phase; SAG and SLG for spring (S) apices (A) and leaves (L) in the generative (G) phase; SAVc and SLVc for spring (S) apices (A) and leaves (L) in the vegetative (V) phase after a period of cooling (c). For each sample, apices or leaves from 5 plants at proper stage of development were bulked together for the DNA extraction.

DNA extraction

The apices and leaves at each developmental stage were collected using liquid nitrogen. The DNA was extracted using the micro C-TAB method, according to Doyle and Doyle (1987) slightly modified at the Department of Genetics, Silesian University.

MSAP procedure

The differences in the level of DNA methylation were analyzed by the use of the MSAP technique.

Table I. Sequences for *Msp*I/*Hpa*II and *Eco*RI adaptors and primers used in the MSAP analysis.

Name		Sequence (5′–3′)
*Msp*I/*Hpa*II adaptor, upper strand		GACGATGAGTCTAGAA
*Msp*I/*Hpa*II adaptor, lower strand		CGTTCTAGACTCATC′
Primer	*Msp*I/*Hpa*II – T	GATGAGTCTAGAACGGT
	*Msp*I/*Hpa*II – TAC	GATGAGTCTAGAACGGTAC
	*Msp*I/*Hpa*II – TAG	GATGAGTCTAGAACGGTAG
	*Msp*I/*Hpa*II – TCA	GATGAGTCTAGAACGGTCA
	MspI/*Hpa*II – TGA	GATGAGTCTAGAACGGTGA
	*Msp*I/*Hpa*II – TGC	GATGAGTCTAGAACGGTGC
*Eco*RI adaptor, upper strand		CTCGTAGACTGCGTACC′
*Eco*RI adaptor, lower strand		AATTGGTACGCAGTCTAC
Primer	*Eco*RI – A	GACTGCGTACCAATTCA
	*Eco*RI – ACG	GACTGCGTACCAATTCACG
	*Eco*RI – ACT	GACTGCGTACCAATTCACT
	*Eco*RI – ACC	GACTGCGTACCAATTCACC
	*Eco*RI – AAG	GACTGCGTACCAATTCAAG
	*Eco*RI – AAC	GACTGCGTACCAATTCAAC

Two restriction systems were utilized: *EcoR*I/*Msp*I and *EcoR*I/*Hpa*II. In this analysis, the *Msp*I/*Hpa*II adaptors and primers were designed as proposed by Xu *et al.* (2000) (Table I), while adaptors and primers for *EcoR*I recognition site were the same as in the standard AFLP procedure (Vos *et al.*, 1995). Genomic DNA was digested for 3 h at 37 °C in a total volume of 20 μl containing: 250 ng of DNA, 2.5 U of each of restriction enzyme (New England BioLabs, Ipswich, USA), 1 × bovine serum albumin (BSA), 50 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 10 mM $MgCl_2$, 1 mM 1,1-bis(*p*-chlorophenyl)-2,2,2,-trichloroethane (DDT). Then 30 pmol of *Msp*I/*Hpa*II adaptor and 3 pmol of *EcoR*I adaptor were ligated with the use of 1 U of T4 DNA ligase (Fermentas, Burllington, Canada) at 37 °C overnight. The ligation reaction was used as a template for the preamplification reaction with *Eco*+A and *Msp*I/*Hpa*II+T primers, 7.5 ng of each, in a solution of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each NTPs and 0.2 U of polymerase (Finnzymes, Espoo, Finland). PCR reaction was performed using the T-Gradient Thermocycler (Biometra, Goettingen, Germany) for 20 cycles with 30 s denaturation at 94 °C, 30 s annealling at 56 °C and 1 min extension at 72 °C. Preamplification reaction was diluted six times and used for selective amplification. In this step, a total volume of 8 μl containing: 1.25 μl of preamplified DNA, 0.6 pmol of fluorescence labelled *EcoR*I+3 IRD 800 primer, 15 ng of *Mse*I/*Hpa*II+3 primer, 0.2 mM dNTPs, 0.25 U of polymerase (Finnzymes), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100 and 2.125 mM $MgCl_2$ was applied. PCR reaction was performed in the following conditions: 12 cycles with annealling from 68 °C to 59.6 °C (decreasing 0.7 °C in each cycle), followed by 23 cycles with annealling at 59 °C. In each cycle denaturation and elongation steps were the same – 94 °C, 30 s and 72 °C, 1 min, respectively. This PCR reaction was optimized for detection of products using a Li-Cor automated sequencer (Lincoln, USA).

Table II. Primer combinations used in the MSAP analysis.

Selective nucleotide *EcoR*I	*Msp*I/*Hpa*II
ACG	TGC
ACG	TCA
ACG	TGA
ACG	TAG
ACT	TAC
ACT	TCA
AAG	TAC
AAG	TCA
ACC	TCA
AAC	TAG

Ten primer combinations giving clear, distinct band profiles were used in the analysis (Table II). The MSAP procedure, starting from restriction of genomic DNA, was performed in two independent replications to exclude some possible errors originated from non-specific digestion of the DNA.

Electrophoresis

After PCR amplification, the electrophoresis was performed in denaturing polyacrylamide gel containing 6% acrylamide/bis-acrylamide 19:1 solution (Sigma), 7 M urea (Amersham Pharmacia), 1 × TBE (134 mM Tris-HCl, 45 mM boric acid, 2.5 mM EDTA). Electrophoresis was performed for 4 h using a Li-Cor automated sequencer (Lincoln) under following conditions: 1300 V, 30 mA, 30 W and medium speed of laser scanning.

Data analysis

When a sample of genomic DNA is digested with two combinations of restriction enzymes: *EcoR*I/*Msp*I and *EcoR*I/*Hpa*II, there are four theoretical combinations of MSAP bands in each locus:

- two bands present after digestion with both enzyme combinations.This pattern indicates that no C atom is methylated in the CCGG sequence;
- the type 1/0 polymorphic pattern, when a band is present after *Msp*I digestion but not in the *Hpa*II lane. This pattern indicates that the external C atom is not methylated (what allows digestion by *Msp*I) and the internal C atom is fully methylated (what prevents restriction by *Hpa*II);
- the type 0/1 polymorphic pattern, when a band is present after *Hpa*II digestion but is lacking in the *Msp*I lane. This pattern indicates that the external C atom is hemi-methylated and the *Msp*I enzyme can not cut the CCGG sequence;
- the lack of bands after digestion with both enzyme combinations indicates that both C atoms are fully methylated, one of them is fully and another hemi-methylated, or both are hemi-methylated. These loci can be discovered only in comparison with the second DNA sample where one of C atoms is not methylated.

Table III. The possible band patterns and methylation events that can be observed in the MSAP analysis after digestion of two samples with *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II systems.

Locus type[a]	Sample 1[b]				Sample 2[b]				Changes in sample 2 in relation to sample 1[b]
	Band pattern		Methylation pattern		Band pattern		Methylation pattern		
	*Msp*I	*Hpa*II	External C	Internal C	*Msp*I	*Hpa*II	External C	Internal C	
M	1	1	no	no	1	1	no	no	–
P1	1	0	no	full meth	1	0	no	full meth	–
P2	0	1	hemi-meth	no	0	1	hemi-meth	no	–
P3a	1	1	no	no	0	0	meth[c]	meth[c]	meth both C
P3b	1	1	no	no	1	0	no	full meth	meth int C
P3c	1	1	no	no	0	1	hemi meth	no	meth ext C
P3d	1	0	no	full meth	0	0	meth[c]	full meth	meth ext C
P3e	0	1	hemi-meth	no	0	0	meth[c]	meth[c]	meth both or int C
P4a	0	0	meth[c]	meth[c]	1	1	no	no	demeth both C
P4b	0	0	meth[c]	full meth	1	0	no	full meth	demeth ext C
P4c	0	0	meth[c]	meth[c]	0	1	hemi-meth	no	demeth both or int C
P4d	0	1	hemi-meth	no	1	1	no	no	demeth ext C
P4e	1	0	no	full meth	1	1	no	no	demeth int C

[a] M, monomorphic loci, not methylated; P1–P4, polymorphic loci reflecting various numbers of methylated C atoms in the CCGG sequence; P1, P2, the same methylation pattern in both analyzed samples; P3, P4, different methylation patterns in two analyzed samples.
[b] 1, presence of band; 0, absence of band; meth, hemi-meth and full meth, methylated, hemi-methylated or fully methylated C atoms at the CCGG sequence.
[c] The following methylation patterns are possible for lack of both bands in one sample: both C atoms are fully methylated; both C atoms are hemi-methylated; one C atom is fully methylated and the other hemi-methylated.

When two or more DNA samples representing different genotypes, organs and/or developmental stages are digested with methylation sensitive restriction enzymes, several band patterns can be observed in their amplification profiles. For two DNA samples, there are 13 possible band combinations in a single locus (Table III). These patterns can be divided into three groups:

- monomorphic for both samples and enzymes (designated M in Table III);
- polymorphic for enzymes but the same in both samples (designated P1 and P2);
- polymorhic for samples or both enzymes and samples (P3 and P4).

The first type of pattern (M) refers to monomorphic loci, where amplification occurred in each sample and in both restriction systems, what indicates that no methylation is present at such locus. Type P1 and P2 polymorphic patterns allow identification of the MSAP loci that are methylated in the same way in both samples, whereas P3 and P4 polymorphic patterns describe loci methylated differentially (Table III). Types P3a–e polymorphic band patterns indicate loci where the higher number of methylated CCGG sequences occurs in sample 2 than in sample 1. They can result either from *de novo* methylation of one or both C atoms of the CCGG sequence in sample 2 or demethylation of the respective positions in sample 1. Types P4a–e band patterns represent loci characterized by a higher number of methylated sequences in sample 1 than in sample 2. These patterns reflect demethylation events in sample 2 or *de novo* methylation of the respective positions in sample 1.

In order to describe the pattern of the DNA methylation in analyzed samples, all clear bands, in a range from 100 to 500 bp for each primer combination were counted. Band patterns were analyzed regarding to the presence or lack of the amplification product in both restriction systems used.

Results and Discussion

In the presented studies, the MSAP profiles of six samples representing apices and leaves from vegetative, generative vernalized and generative grafted stems of the winter variety "Górczański" were compared together with six samples representing apices and leaves of the spring cultivar "Młochowski". Ten primer combinations used in the analysis allowed the amplification of total 566 and 551 bands for the winter and spring variety, respectively (Table IV). The majority of analyzed loci (about 68%) was monomorphic, *i.e.* did not undergo methylation in any of the analyzed samples. Both cultivars showed approximately the same overall degree of methylation. In total, 188 loci in winter rape, and slightly less, 176, loci in the spring cultivar expressed changes in the methylation pattern. Most of the observed polymorphism referred to the lack of a band in one restriction system and its presence in the second one in all analyzed samples. All differentially amplified fragments resulted from either full methylaton of internal cytosine, preventing restriction of the DNA by *Hpa*II, but not by *Msp*I, or from hemi-methylation of external cytosine, that allows digestion only by the *Hpa*II enzyme, though at a lower rate compared to the restriction of unmethylated sequences. This type of polymorphism indicated that a methylation event, common for all samples, was not related to the vernalization or flowering processes. While about 25% of loci showed a band after *Msp*I and a lack of band after *Hpa*II digestion (type P1 polymorphism), and only about 2% of loci had the opposite band pattern (type P2), it can be concluded that full methylation of the internal C atom was much more frequent than hemimethylation of the external C atom in all samples analyzed (Table IV).

Table IV. Number and types of MSAP loci detected in the analysis of winter and spring rape cultivars.

Cultivar	Total no. of loci analyzed	Monomorphic		Polymorphic							
		M		P1		P2		P3 + P4		Total polymorphic	
		No.	%	No.	%	No.	%	No.	%	No.	%
"Górczański" (winter)	566	378	66.8	140	24.7	13	2.3	35	6.2	188	33.2
"Młochowski" (spring)	551	375	68.1	138	25.0	10	1.8	28	5.1	176	31.9

See Table III for description.

Table V. Changes of DNA methylation pattern observed between pairs of samples representing different physiological stages of apices in winter and spring rape plants.

Compared samples[a]		Number of loci showing changes in methylation pattern											
Sample 1	Sample 2	Total	Increased methylation in sample 2[b]						Decreased methylation in sample 2[b]					
			P3a	P3b	P3c	P3d	P3e	Total	P4a	P4b	P4c	P4d	P4e	Total
WAV	WAGv	16	1	–	2	–	–	3	1	4	1	2	5	13
WAV	WAGg	15	2	1	1	3	–	7	–	5	–	1	2	8
WAGg	WAGv	16	1	1	1	1	–	4	5	1	2	1	3	12
SAV	SAG	14	2	6	–	–	–	8	2	–	–	–	4	6
SAV	SAVc	14	2	3	–	1	–	6	2	1	–	1	4	8
SAVc	SAG	21	3	7	1	1	–	12	3	2	–	–	4	9

[a] WAV, winter (W) apices (A) in vegetative (V) phase; WAGv, winter (W) apices (A) in generative (G) phase after vernalization (v); WAGg, winter (W) apices (A) in generative phase (G) after grafting (g); SAV, spring (S) apices (A) in vegetative (V) phase; SAG, spring (S) apices (A) in generative (G) phase; SAVc, spring (S) apices (A) in vegetative (V) phase after cooling (c).

[b] P3a–e and P4a–e, types of polymorphic band patterns; see Table III for details.

Two other classes of polymorphic loci represented band patterns that varied between samples. Their presence indicated that different methylation events occurred in at least one sample out of six analyzed for each cultivar. Such bands represented 6.2% of total loci examined in the winter cultivar and slightly less, 5.1% of loci analyzed in the spring cultivar (Table IV).

A pair-wise comparison of two samples representing two developmental stages (*e.g.* vegetative and generative apex) makes it possible to use the MSAP profiles for analysis of methylation events occurring during plant development and estimate the number of loci that underwent *de novo* methylation or demethylation in one or both C atoms in the CCGG sequence.

Data presented in Table V show that a similar number of loci underwent development-related methylation changes in apices of winter and spring rape, but the direction of changes was evident only in the winter cultivar. The pair-wise comparisons clearly indicated that the majority (more than 80%) of changes observed between vegetative, not vernalized and generative, vernalized apices of winter rape (WAV – WAGv) referred to demethylation events in the generative apex (Table V). The increased number of demethylated sites in vernalized apices (75%) was also detected in comparison to the generative but not vernalized (grafted) organs (WAGg – WAGv). Contrary to this observation, the analysis of vegetative and generative grafted apices showed approximately the same number of methylation and demethylation events (WAV – WAGg).

Transition to the generative stage in the spring variety "Młochowski" was also accompanied by changes in the DNA methylation pattern, but the direction of changes was not apparent. In apices of the spring cultivar, approximately the same number of demethylation and *de novo* methylation events was identified after passing to the generative stage (SAV – SAG) or after exposure of plants to a 6-week period of low temperature (SAV – SAVc) (Table V).

The changes in methylation pattern related to plant development were also detected in the DNA isolated from leaves of both cultivars. Due to their different size and shape, leaves of a rape plant being at the vegetative stage can be easily distinguished from leaves developing after induction of flowering. The transition to the generative phase was accompanied by more changes in the DNA methylation in leaves of the winter than the spring cultivar (Table VI). However, in winter rape, both methylated and demethylated loci appeared with a similar frequency in vegetative and generative leaves (WLV – WLGv; WLV – WLGg), whereas in the spring cultivar, the majority of changes detected in leaves of plants that passed to the generative phase or were exposed to low temperatures referred to *de novo* methylation events (SLV – SLG; SLV – SLVc) (Table VI). These data are in agreement with observations of other authors (Cedar and Razin, 1990; Hsieh, 2000; Zluvova *et al.*,

Table VI. Changes of the DNA methylation pattern observed between pairs of samples representing different physiological stages of leaves in winter and spring rape.

Compared samples[a]		Number of loci showing changes in methylation pattern											
Sample 1	Sample 2	Total	Increased methylation in sample 2[b]						Decreased methylation in sample 2[b]					
			P3a	P3b	P3c	P3d	P3e	Total	P4a	P4b	P4c	P4d	P4e	Total
WLV	WLGv	16	3	3	1	–	2	9	5	–	–	–	2	7
WLV	WLGg	20	5	3	1	–	2	11	6	–	–	–	3	9
WLGg	WLGv	12	2	3	–	–	1	6	3	–	1	1	1	6
SLV	SLG	8	3	2	–	1	–	6	–	1	–	–	1	2
SLV	SLVc	13	1	7	1	1	–	10	–	1	1	–	1	3
SLVc	SLG	12	2	1	–	1	1	5	1	–	–	1	5	7

[a] WLV, winter (W) leaves (L) in vegetative (V) phase; WLGv, winter (W) leaves (L) in generative (G) phase after vernalization (v); WLGg, winter (W) leaves (L) in generative phase after grafting (g); SLV, spring leaves (L) in vegetative (V) phase; SLG, spring (S) leaves (L) in generative (G) phase; SLVc, spring (S) leaves (L) in vegetative (V) phase after cooling (c).

[b] P3a–e and P4a–e, types of polymorphic band patterns; see Table III for details.

2000) and confirm that DNA methylation is a dynamic process. Methylation patterns result from *de novo* methylation, demethylation and maintenance of existing methylated sites. Although not all genes are regulated by methylation, the hypomethylation at specific sites or in specific regions of a number of genes is correlated with active transcription (Christman *et al*., 1995).

When the methylation status of the same loci were compared for winter rape apices representing three different physiological stages (*i.e.* vegetative, generative not vernalized and generative vernalized), the changes in DNA methylation related specifically to vernalization or to grafting could be identified. In the presented studies, 10 MSAP loci were detected that expressed methylation changes in the vernalized apices only; among them there were 7 loci that underwent demethylation after vernalization, but remained methylated both in the vegetative and generative not vernalized stage (Table VII). Contrary to this, only 1 locus was demethylated in generative not vernalized (grafted)apices. The number of loci that underwent specific *de novo* methylation was similar for both, generative vernalized (3 loci) and generative not vernalized (2 loci) apices. While only two same loci demethylated in both types of generative apices were identified, it can be concluded that most of the demethylation events occurring during transition to the generative phase can be attributed to the process of plant vernalization. None of the *de novo* methylated loci, common for vernalized and grafted samples, was identified.

Contrary to these results, in generative apices of the spring cultivar, a higher number of MSAP loci underwent *de novo* methylation (7 loci) than demethylation (3 loci) during transition to flowering (Table VII). These changes were present only in generative apices, so they can be directly related to the passing of apex to a new developmental stage. The number of loci that changed specifically their methylation status in the response to low temperature was equal for the demethylation (4) and *de novo* methylation (4) events. Additionally, 3 loci were demethylated and only one was *de novo* methylated in both, generative and cooled apices.

The analysis of MSAP profiles in leaves of winter rape revealed only two methylation changes that can be attributed specifically to the process of vernalization (Table VIII). Both these changes referred to the *de novo* methylation events. None locus, specifically demethylated in leaves of vernalized plants was identified. The majority of changes observed in generative leaves was common for samples collected from vernalized and grafted (not vernalized) stems, and included the equal number of methylation (6) and demethylation (6) events. Additionally, 7 loci that underwent specific methylation changes, related to the transition to the generative phase induced by grafting, but not connected with the vernalization process, were identified in this analysis. These results are

Table VII. Number of MSAP loci expressing changes of DNA methylation in apices of winter and spring rape cultivars subjected to vernalization and/or grafting.

Cv. "Górczański"					Cv. "Młochowski"				
Methylation changes specific for	Methylation pattern			No. of loci	Methylation changes specific for	Methylation pattern			No. of loci
	WAV	WAGg	WAGv			SAV	SAVc	SAG	
Vernalization	M	M	D	7	Induction of flowering	M	M	D	3
	D	D	M	3		D	D	M	
Grafting	M	D	M	1	Cooling	M	D	M	4
	D	M	D	2		D	M	D	4
Both	M	D	D	2	Both	M	D	D	3
	D	M	M	–		D	M	M	1
Total				15	Total				22

M, at least one methylated C atom at the CCGG recognition site; D, no methylation at the CCGG recognition site. See Table V for other abbreviations.

Table VIII. Comparison of methylation status at particular MSAP loci in leaves of winter and spring rape cultivars at different developmental stages.

Cv. "Górczański"					Cv. "Młochowski"				
Methylation changes specific for	Methylation pattern			No. of loci	Methylation changes specific for	Methylation pattern			No. of loci
	WLV	WLGg	WLGv			SLV	SLVc	SLG	
Vernalization	M	M	D	–	Induction of flowering	M	M	D	–
	D	D	M	2		D	D	M	3
Grafting	M	D	M	3	Cooling	M	D	M	–
	D	M	D	4		D	M	D	6
Both	M	D	D	6	Both	M	D	D	1
	D	M	M	6		D	M	M	2
Total				21	Total				12

M, at least one methylated C atom at the CCGG recognition site; D, no methylation at the CCGG recognition site. See Table VI for other abbreviations.

in contrast to the DNA methylation changes detected in apices.

A much lower level of differentially methylated loci was detected in leaves of the spring cultivar. In total, only 12 loci expressed various methylation states. *De novo* methylation events in plants exposed to a 6-week cooling accounted for 50% of the observed changes. Only 3 loci were specifically methylated in leaves of plants induced to flowering and none locus showed demethylation specific to this stage of plant development. This observation, although gained by different approaches, is in agreement with the conclusion by Burn *et al.* (1993) and Finnegan *et al.* (1998) that vernalization may rest at molecular level in demethylation of DNA. The lack of the same demethylation pattern in apices of plants reaching the generative state by grafting indicates the possibility of activation of an alternative pathway and/or acting at some point down the regulatory chain leading to flowering. It is assumed that by grafting (*e.g.* Chailakhyan, 1988) a florigenic stimulus, *i.e.* the final outcome of controlling pathways, is transferred to activate the cells of shoot apical meristem. We can speculate that flowering stocks of rape producing such a signal already passed *e.g.* the step of control by the central FLC gene, repressor of flowering, which is alleviated by vernalization. Similar interpretation may be applied when comparing methylation pattern and demethylation pattern between reproductive apices of vernalized rape plants and that of reproductive spring ones. The data presented by Lízal and Relichová (2001) on different responses of *Arabidopsis* genotypes to vernalization and demethylation treatment by 5-azaC suggest the possibility of at least two different pathways promoting flowering. The methylation status of leaves developed at reproductive apices of vernalized rape plants and those developed at vegetative ones, although morphologically differing, did not reveal the same features as apices of the same developmental stage. This may be due to heterogeneity of the shoot apical meristem and positional effect, which determines tissue and/or organ specific developmental fate of cell lineages.

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