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Detection of DNA methylation changes during seed germination in rapeseed (*Brassica napus*)

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Abstract DNA methylation is known to play a crucial role in regulating plant development and organ or tissue differentiation. In this study, we assessed the extent and pattern of cytosine methylation during rapeseed (*Brassica napus* L.) seed germination, and compared the methylation level of various tissues in seedling, using the techniques of methylation-sensitive amplified polymorphism (MSAP) and HPLC separation and quantification of nucleosides. In all, 484 bands, each representing a recognition site cleaved by either or both of the isoschizomers, were amplified by 12 pairs of selective primers in DNA obtained from dry seeds. A total of 76 sites were found to be differentially digested by the isoschizomers, indicating that approximately 15.7% of 5′-CCGG-3′ sites in the genome were cytosine methylated. Four classes of patterns were observed in a comparative assay of cytosine methylation in the dry and germinating seeds; a small number of hypermethylation events occurred at 5′-CCGG-3′ sites in germinating seeds compared with dry seeds, while many more hypomethylation events were detected after seed germination. Differences in DNA methylation level in various tissues were also detected; radicel was less methylated than hypocotyl and cotyledon. These observations were further confirmed by HPLC analysis. In addition, sequencing of eleven differentially methylated fragments and the subsequent blast search revealed that cytosine methylated 5′-CCGG-3′ sequences were equally distributed between coding and non-coding regions. These results clearly demonstrate the power of MSAP technique for large-scale DNA methylation detection in rapeseed genome, and the complexity of DNA methylation change during seed germination. DNA Hypomethylation going with seed germination appears to be a necessary step toward transcriptional activation in gene expression, and may well contribute to the developmental gene regulation.

Keywords: DNA methylation, MSAP, seed germination, *Brassia napus*.

DNA methylation is a common yet important modification of DNA in eukaryotic organisms. DNA methylation, especially methylation of cytosine (m^5C), have both epigenetic and mutagenic effects on various cellular activities such as differential gene expression, cell differentiation, and chromatin inactivation[1]. It modifies access to genetic information but does not alter the primary nucleotide sequence, resulting in gene silencing and morphological changes[2]. DNA methylation is also important in gene regulation in higher plant. Transcriptionally silent genes are generally found to be more methylated than active genes in promoters or certain coding regions[3]. Hypomethylation in transgenic *Arabidopsis* plants would bring on huge morphological changes[4], and heavy methylation of *SUPERMAN* and *AGAMOUS* caused transcriptional silence of these genes with the consequence of floral homeotic transformation[5]. DNA methylation is also connected with plant vernalization. In wheat, winter near-isogenic lines (NILs) are more highly methylated than spring NILs, and vernalization results in DNA demethylation that induces or accelerates flowering[6]. In *Arabidopsis*, vernalization requires epigenetic silencing of *FLC* by histone methylation[7]. Meanwhile, significant differences in DNA methelation level exist among various tissue types in several plant species such as tomato[8], maize[9], rice[10], implying that DNA methylation may also function in tissue or organ differentiation and formation.

Rapeseed is one of the most important oil crops worldwide. Intense investigations have been focused on the biodiversity, molecular markers, physical mapping and genes isolation. There are also some works on DNA methylation such as the evaluation of DNA methylation changes induced by potassium dichromate in genotoxicity study[11] and the investigation of methylation status in plant mobile elements S1Bn SINE in rapeseed[12]. The study on DNA methylation changes during seed germination, however, is not yet documented.

In the present study, we assessed the dynamic changes of DNA methylation during seed germination

in rapeseed, using the techniques of MSAP and HPLC analysis. The major aim of this work is to provide evidences for a better understanding of the role of DNA methylation in rapeseed biological development and tissue differentiation.

1 Materials and methods

1.1 Plant material

Fresh seeds of winter rapeseed variety 'Westar' used in this study were obtained from the State Medium-Term Germplasm Bank of Oil Crops (Wuhan).

1.2 Seed germination

Germination test was performed in plant growing chamber at 20℃. Four replicates for 100 seeds were placed in 15 cm Petri dishes on two layers of filter paper soaked with distilled water. Germination was checked daily and was considered to have occurred when radicel was longer than the diameter of seed. Germination percentage (GP) was calculated 7 d after germination. The mean GP in the present study was as high as 98.6%, indicating that the fresh seeds tested have a strong vigor.

1.3 DNA extraction

Genomic DNA was extracted following the modified SDS method[13]. Seedlings were collected for DNA extraction at 0 (dry seeds), 1, 2, 4 and 8 d after germination, respectively. In addition, tissues of radicel, hypocotyls, and cotyledon were also collected from 8 d old seedlings for DNA extraction.

1.4 HPLC analysis of DNA methylation

HPLC analysis was exactly the same as the procedure described by Chakrabarty *et al.*[14]. The digestion reaction contained the components of 20 μg genomic DNA, 5 U nuclease P1, 10 U alkaline phosphatase, 1× alkaline phosphatase buffer in final concentration. The total reaction volume was bring to 200 μL with digestion buffer (30 mmol/L $NaCH_3$, 0.1 mmol/L $ZnCl_2$, pH 5.3). Hydrolyses were performed at 37℃ for 3 h and stopped by addition of 500 μL absolute alcohol. The samples were centrifuged at 10000 *g* for 15 min, and supernatant was transferred to a new tube, vacuum-dried. The resulting nucleosides were resuspended in 1 mL ddH_2O and filtered through 0.2 μm membranes. HPLC analysis were performed on C18 column (Waters, MA, USA) for 20 min using isocratic elution butter (50 mmol/L KH_2PO_4, 8% methanol，pH 3.5) at a flow rate of 0.8 mL/min. The effluent was monitored at a wavelength of 285 nm. The content of 5mdC was calculated using the formula:

$$5mdC\ (\%) = 100\times[5mdC]/([5mdC] + [dC]),$$

where [dC] and [5mdC] are the respective concentration of two forms of dC, as deduced from the calibration curves for external standards of known concentration.

1.5 MSAP analysis of DNA methylation

MSAP analysis was performed according to the procedure of Portis *et al.*[15] with minor modification. DNA samples were separately restricted with *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I in two tubes. To minimize discrepancy caused by experimental factors, digestion and ligation were performed simultaneously. The digestion-ligation reaction was performed in a volume of 50 μL containing the following components: 500 ng DNA template, 5 U *Eco*R I, 10 U *Hpa* II (or *Msp* I), 2 U T_4 DNA ligase, 5 pmol *Eco*R I adapter, 50 pmol *Hpa* II/*Msp* I adapter and 1× buffer (10 mmol/L Tris-HCl pH 7.5, 10 mmol/L MgAc, 50 mmol/L KAc, 10 mmol/L DTT, 0.2 mmol/L ATP). The mixture was incubated at 37℃ overnight, and then inactivated at 65℃ for 10 min and ten-fold diluted with ddH_2O.

5 μL aliquot of digestion-ligation mixture was taken for preamplification. The resulting products were then 50-fold diluted and used as template for selective amplification. The procedure of selective amplification, denaturing polyacrylamide gel separation and silver stain detection were the same as previously described[13].

1.6 Cloning and validation of MSAP fragments

Several fragments were excited directly from the wet polyacrylamide gels on the plate, using a razor blade. The fragments were rehydrated with 20 μL of ddH_2O, mashed with a tip, heated at 95℃ for 5 min, and store at 4℃. Aliquots of 2 μL supernatant were used as template for re-amplification. PCR reactions were performed using the same primer combination and reaction condition as used in the selective amplification step. After checking 10 μL of PCR product on the agarose gel for the presence of a band, the rest was ligated into the vector pGEM-T (Promega) and transformed into *E. coli* strain JM109. Sequences determinations were carried out at BGI (Beijing, China). Homology search and sequences analysis were performed at the public database NCBI (http://www.ncbi.nlm.nih.gov).

Southern blots were conducted using the probes of cloned fragments to verify the methylation polymorphism. A total of 20 μg genomic DNA from different sources was digested separately with two sets of restriction enzymes, *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I, and then resolved on 0.8% agarose gel. Prehybridization, hybridization, and washing were performed as described by Sharpe *et al.*[16]. Probe preparation and detection were carried out using a DIG DNA labeling and detection kit (Roche Diagnostics, Swiss).

2 Results

2.1 DNA methylation profile

Intact genomic DNA is crucial for MSAP analysis. However, the quality of DNA extracted from dry seeds seems to be compromised by the existence of high oil content. Therefore, a preliminary experiment was conducted using DNA template from the source of dry seeds in order to define conditions that would yield distinct banding pattern on the sequencing gel. In this preliminary experiment, a standard MSAP procedure was adopted and optimized. The result showed that the banding quality of dry and germinating seeds was as good as that of a standard AFLP analysis. We also tested the repeatability of this technique and found that almost all the fragments were invariable among several independent reactions, indicating that MSAP can be effectively applied to the study of DNA methylation in rapeseed genome. We screened dozens of primer combinations with three selective nucleotides at each end of *Eco*R I and *Hpa* II-*Msp* I, and twelve primer combinations yielding distinct amplified fragments were selected for further analysis (Table 1). A total of 484 bands resolved by 12 primer combinations were detected in DNAs extracted from dry or germinating seeds, among which 76 were differently amplified from *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I digestions. Thus, approximately 15.7% (76/484) of 5′-CCGG-3′ sites in the rapeseed genome were cytosine-methylated in dry seeds, as detected by the differential recognition of the two isoschizomers.

Hpa II is inactive if either cytosine is fully methylated at 5′-CCGG-3′ sequence, while *Msp* I is sensitive only to methylation at the external cytosine. Therefore, a fragment would be produced from *Eco*R I/*Msp* I digest but not the *Eco*R I/*Hpa* II digest when the internal cytosine is fully methylated. Actually, among the 76 polymorphic fragments detected in dry seeds, the majority were present in the selective amplification products of the *Eco*R I/*Msp* I digest but not in the other. However, a small proportion of bands appeared only in *Eco*R I/*Hpa* II digestion were also detected (Table 1). This could be attributed to the hemimethylation of recognition site, in which the external cytosine is methylated only in one strand[17]. These results showed that full methylation of internal cytosine occur more often than hemimethylation of external cytosine at 5′-CCGG-3′ site in the rapeseed genome.

Table 1 Numbers of bands amplified using various primer combinations in dry seeds and seedlings

Primer pairs[a)]	Number of bands	Number of monomorphic sites	Number of polymorhphic sites	Fully methylated sites[b)]	Hemimethylated sites[c)]
HM+TAA/E+AAC	71	5	3	5	0
HM+TAA/E+ACG	49	3	5	5	3
HM+TAA/E+ACT	62	5	7	5	1
HM+TAA/E+AGT	87	7	8	5	3
HM+TCC/E+AAC	52	7	12	5	2
HM+TCC/E+ACG	28	1	2	1	0
HM+TCC/E+ACT	16	9	3	2	7
HM+TCC/E+AGT	19	5	1	5	0
HM+TTC/E+AAC	40	7	6	5	3
HM+TTC/E+ACG	37	5	1	4	1
HM+TTC/E+ACT	26	7	3	6	1
HM+TCT/E+ACT	45	6	10	6	1
Total	532	67	61	54[d)]	22[d)]

a) The core sequences of primer HM, E are 5′-ATCATGAGTCCTGCTCGG-3′ and 5′-GACTGCGTACCAATTC-3′, respectively. Three selective nucleotides were appended. b) Internal cytosine fully (both strands) methylated at 5′-CCGG-3′ site. c) Methylation of external cytosine only in one strand. d) The data in these two columns are based on analysis of the dry seed only.

DNA methylation profiles were generated by analysis of 10 lanes corresponding to five sources of genomic DNAs (i.e. dry seeds and seedlings of 1, 2, 4, 8 d after germination), each restricted with *Eco*R I/*Hpa* II (H) or *Eco*R I/*Msp* I (M). A total of 532 fragments were produced by 12 primer combinations, each representing a recognition site cleaved by one or both of the isoschizomers. For each primer pair, the number of fragments amplified variated from 16 (HM+TCC/E+ACT) to 87 (HM+TAA/E+AGT), with an average of 44.3 (Table 1). Typically, the resulting MSAP bands could be classified into two major types: (1) Monomorphism, i.e. bands always appearing after digestion with H but not with M or vice versa (Fig. 1(a) and (b)), reflecting the unchanged methylation status at 5′-CCGG-3′ site; there were 67 bands belonging to this category (Table 1). (2) Polymorphism, i.e. MSAP pattern altered during seed germination; some fragments were present in dry seeds after digestion with H and M but no longer detected at a certain stage of germination

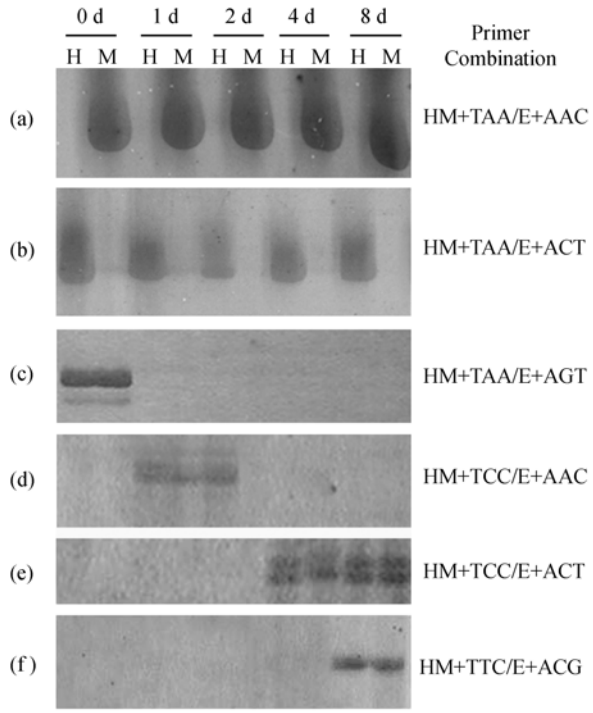

Fig. 1. Examples of some MSAP profile in dry and germinating seeds. H, pattern after digestion with *Eco*R I/*Hpa* II; M, pattern after digestion with *Eco*R I/ *Msp* I. (a) and (b), Examples of fragments whose methylation pattern does not change during germination; (c) examples of fragments detected after both digestion with H and M in dry seed and disappearing at certain stages of germination (pattern C1 in Table 2); (d) fragments show two changes in methylation status during germination (appearance and disappearance of a fragment); (e) and (f), fragments detected after digestion with H and M in germinating seed but not present in dry seed (pattern A1 in Table 2).

(Fig. 1(c)), while others were only detectable after digestion with H and M during germination but not in dry seed (Fig. 1(d)—(f)). There are 61 bands belonging to this kind (Table 1), reflecting the alteration of methylation status at 5′-CCGG-3′ site during germination.

The detected 61 polymorphic bands among dry and germinating seeds fell into a number of patterns that could be grouped into four major classes (classes A, B, C and D in Table 2). The most frequently observed classes were A and B. Class A consisted of 25 sites, the fragments in this class were present in amplification products of both H and M digestions in germinating seeds but absent in either or both digestions in dry seeds, indicating that complete demethylation of cytosines had occurred at 5′-CCGG-3′ sequences. There were 28 fragments belonging to class B, these fragments could not be detected in dry seeds due to the full methylation of cytosines at 5′-CCGG-3′ sequences, but could be detected in germinating seeds in either H or M digestions due to a reduced level of cytosine methylation. Contrarily, three fragments in class C and five fragments in class D reflected the increasing level of cytosine methylation at 5′-CCGG-3′ sequence during seed germination (Pattern 1 in Table 3; see also in Fig. 2).

2.2 Alterations of cytosine methylation during seed germination

A total of 61 fragments differently methylated in dry seeds and germinating seeds were detected (Table 2). Among these sites, three were unmethylated in dry seeds but *de novo* methylated in germinating seeds (pattern C1, C2 and C3 in Table 2), while 25 were methylated in dry seeds but completely demethylated after seed germination (pattern A1, A2 and A3 in Table 2). The number of demethylation events was 7 times higher than the *de novo* methylation during seed germination. These results showed that although both methylations and demethylations occur during seed germination, demethylations seem to be predominant. Fig. 2 showed the changes of DNA methylation and demethylation during seed germination. It clearly demonstrated that the number of methylation sites were almost invariable while the demethylation sites increased rapidly during seed germination, leading to the accumulation of net global methylation sites.

2.3 Tissue specificity of cytosine methylation

Two methods viz. MSAP and HPLC were used to

Table 2 Changes in patterns of cytosine methylation during seed germination revealed by MSAP analysis

Dry seeds[a]		Germinating seeds		Pattern	Changes of methylation status[b]		Number of bands
H	M	H	M		before germination	after germination	
–	–	+	+	A1	CCGG GGCC	CCGG GGCC	22
–	+	+	+	A2	CCGG GGCC	CCGG GGCC	1
+	–	+	+	A3	CCGG GGCC	CCGG GGCC	2
–	–	–	+	B1	CCGG GGCC	CCGG GGCC	10
–	–	+	–	B2	CCGG GGCC	CCGG GGCC	18
+	+	–	–	C1	CCGG GGCC	CCGG GGCC	1
+	+	+	–	C2	CCGG GGCC	CCGG GGCC	1
+	+	–	+	C3	CCGG GGCC	CCGG GGCC	1
–	+	–	–	D1	CCGG GGCC	CCGG GGCC	2
+	–	–	–	D2	CCGG GGCC	CCGG GGCC	3

a) H, pattern after digestion with *Eco*R I/*Hpa* II; M, pattern after digestion with *Eco*R I/*Msp* I; "+", presence of a band; "–", absence of a band. b) Underlined cytosine is methylated.

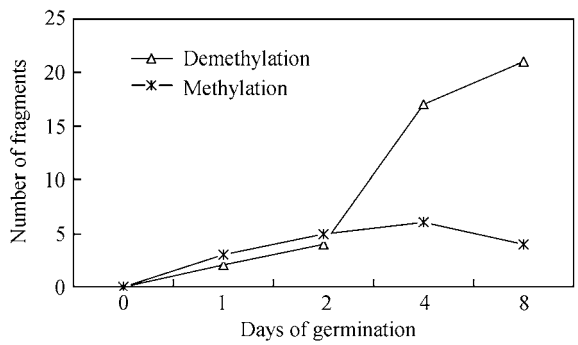

Fig. 2. Trend of methylation and demethylation changes during seed germination.

assess the extent of DNA methylation in various tissue types from seedling. A total number of 513, 503 and 494 fragments resolved by 12 primer combinations were amplified from radicel, hypocotyls and cotyledon, respectively. Analysis of the three tissues using 12 primer pairs revealed differences between H and M profiles at 16.8%—20.2% of the tested 5′-CCGG-3′ sequences. Cotyledon was the most methylated, hypocotyls second, and radicel the least (Table 3). These results were further confirmed by HPLC separation and quantification. In HPLC analysis, 31.7% of cytosines were methylated in cotyledon and radicel was methylated the least (20.6%).

Table 3 Methylation level in different tissues detected by MSAP and HPLC

Tissues	Methylation level detected by MSAP	Methylation level detected by HPLC
Radicel	16.8%	20.6%
Hypocotyls	19.7%	28.3%
Cotyledon	20.2%	31.7%

39 methylation-sensitive fragments were differentially amplified from radicel, hypocotyls and cotyledon. These tissue-specific methylation sites fell into 15 types (T01 to T15 in Table 4), among which type T07 had the largest number of bands. Twelve fragments belonging to this type were present after digestion with H and M in radicel but not found in hypocotyls and cotyledon (Table 4), indicating that hypocotyls and cotyledon were completely methylated while radicel was unmethylated at these sites.

2.4 Isolation, validation and sequence analysis of differentially methylated fragments

To obtain more information on the sequences that

Table 4 Tissue-specific cytosine methylation in radicel, hypocotyls and cotyledon

Types	Radicel[a)]		Hypocotyls		Cotyledon		Number of sites
	H	M	H	M	H	M	
T01	+	+	+	+	+	−	2
T02	+	+	+	+	−	−	4
T03	+	+	+	−	−	−	2
T04	+	+	+	−	+	−	2
T05	+	+	−	+	−	+	2
T06	+	+	−	+	−	−	3
T07	+	+	−	−	−	−	12
T08	+	−	+	−	−	−	1
T09	+	−	−	−	+	−	1
T10	−	+	−	−	+	−	1
T11	−	+	−	−	−	+	1
T12	−	−	−	−	+	+	2
T13	−	−	+	−	+	−	3
T14	−	−	−	+	−	+	2
T15	−	−	−	−	−	+	1

a) H, pattern after digestion with *Eco*R I/*Hpa* II; M, pattern after digestion with *Eco*R I/*Msp* I; "+", presence of a band; "−", absence of a band.

are differentially amplified in H and M digestion, we isolated, verified and sequenced eleven fragments that were differentially methylated in specific tissue or at certain stages of germination. These fragments included: M1, M2, M3 and M4 (bands only appearing in dry seeds); M5, M6, M7 and M8 (bands only appearing in germinating seeds); M9 (bands only appearing in radicel); M10 (bands only appearing in hypocotyls); M11 (bands only appearing in cotyledon). Two cloned fragments (M5 and M10) representing typical methylations occurring at certain stages of germination or in specific tissues were used as probes to hybridize with genomic DNA from different sources in order to verify their detection in MSAP analysis. Southern hybridization with probe M5 revealed that a smaller fragment was present in dry seed in H lane, while a larger band appeared in other 3 lanes (Fig. 3(a)), this was in good agreement with MSAP pattern (pattern D2 in Table 2). In the hybridization result of probe M10, all three tissues (cotyledon, hypocotyls and radicel) had a larger band in H lane, but only cotyledon and radicel had a smaller fragment in M lane, this also agreed with the expectation based on the assumption that hypocotyls was fully methylated at both cytosines, with the disappearance of a smaller bands (Fig. 3(b), see also in Table 4 for type T11).

Eleven fragments cloned were sequenced and then

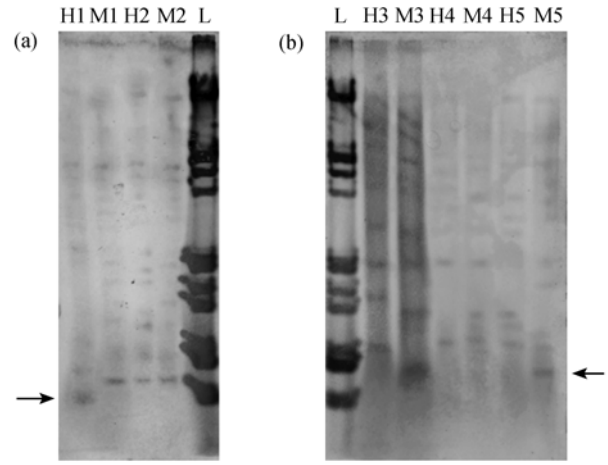

Fig. 3. Southern analysis to confirm the methylation pattern. (a) Hybridization pattern using probe M5, arrow indicating the specific fragment; (b) Hybridization pattern using probe M10, arrow showing the polymorphic bands. H, *Eco*R I/*Hpa* II digestion; M, *Eco*R I/ *Msp* I digestion; L, DNA ladder of λ/*Eco*R I+*Hin*d III; 1, dry seeds; 2, germinating seeds; 3, cotyledon; 4, hypocotyls; 5, radicel.

subjected to blast search. The results showed that all of the analyzed sequences were relatively short in length (26—174 bp), and only one (M8) displayed an internal 5′-CCGG-3′ site. Consequently, the detection of a fragment appears to be resulted from the lack of methylation of the cytosine to which the isoschizomer is sensitive. Six of the 11 sequenced bands were highly homologous to particular gene coding regions. One of the remaining five bands was homologous to a pseudogene, and the other four shared high similarities with non-coding regions. Thus, these differentially methylated sequences seemed to be equally distributed between coding and non-coding regions (Table 5).

3 Discussion

There are two major classes of methods used for detecting DNA methylation in plant genome. One class of methods is to detect the global levels of methylated cytosines. HPLC is one such method, the basic procedure of which is to degrade genomic DNA into nucleotides by nuclease P1, then separate and quantify the percentage of different forms of cytosines (methylated or unmethylated) by HPLC analysis[18]. The other class of methods is to examine the methylation of cytosines in specific gene sequence. MSAP is one such technique, the principle of which is the same as AFLP, except that the "frequent cutter" enzyme *Mse* I is replaced by isoschizomers *Hpa* II and *Msp* I that display differential sensitivity to DNA methylation. Each DNA samples is

Table 5 Sequence analysis of differentially methylated fragments

Fragment	Size	Band pattern[b)]	Sequence homology
M1	152	B1	CP000116[c)] (196587—196614)[d)] *Thiobacillus denitrificans* ATCC 25259. Features in this part of subject sequence: thioredoxin family protein, putative.
M2	44	B1	XM_505961 (3072—3095) *Yarrowia lipolytica* CLIB99, YALI0F27753g predicted mRNA
M3	80	B1	AF219992 (6124—6143) *Mus musculus* aquaporin-4 (Aqp4) gene, exons A, B, 0, X, 1 and 2 and partial cds, alternatively spliced.
M4	106	A1	BX883044 (34213—34232) *Rattus norvegicus* chromosome 20, major histocompatibility complex. Features flanking this part of subject sequence: 17067 bp at 3′ side: butyrophilin-like 7.
M5	115	D2	AP006444 (78681—78745) *Brassica napus* mitochondrial DNA. Features in this part of subject sequence: NADH dehydrogenase subunit 1.
M6	109	D2	CR734755 (381—403) *Tetraodon nigroviridis* full-length cDNA.
M7	26	D2	AF428287 (395—415) *Arabidopsis* AT3g30390/T6J22_16 mRNA, complete cds.
M8 [a)]	45	D1	AL162651 (58918—58948) Arabidopsis DNA chromosome 3, BAC clone F26K9.
M9	174	T13	AJ430548 (197—310) *Brassica napus* transposon 14G32-20 RT pseudogene for reverse transcriptase.
M10	144	T11	AY606857 (104—126) *Mus musculus* clone M46 atherosclerosis-associated DNA methylation polymorphic fragment, genomic sequence.
M11	115	T08	AL049711 (59220-59275) *Arabidopsis* DNA chromosome 3, BAC clone F4F15.

a) An internal 5′-CCGG-3′ sequence was present; b) to see band patterns in Table 2 and Table 4; c) accession number; d) homologous region were shown within bracket.

separately restricted with *Eco*R I—*Hpa* II and *Eco*R I—*Msp* I, then ligated into specific adaptors. After two rounds of PCR amplification, the resulting products are resolved on denaturing polyacrylamide gel and detected by silver nitrate[19]. Isoschizomers *Hpa* II and *Msp* I share the same recognition sequence 5′-CCGG-3′ but differ in their sensitivity to cytosine methylation. *Hpa* II is inactive if one or both cytosines are fully methylated (both strands methylated) but cuts the unmethylated or hemimethylated sites (only one DNA strand methylated), whereas *Msp* I cleaves 5′-C^{5m}CGG-3′ but not 5′-^{5m}CCGG-3′ [17]. Therefore, different banding patterns can be generated from *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I digestions when 5′-CCGG-3′ sites are cytosine methylated (Table 6). Contrarily, the methylation status at 5′-CCGG-3′ sequence can also be deduced from the different DNA fragments profile on the sequencing gel due to the differential DNA cleavage properties of the isoschizomers used.

We have assessed the cytosines methylation rates in the rapeseed genome. In MSAP analysis, different levels of DNA methylations were observed in various tissues, with the highest in cotyledon (20.2%) and radicel the lowest (16.8%). This result was further confirmed by HPLC separation and quantification, in which the percentage of methylated cytosines variated from 20.6% in radicel to 31.7% in cotyledon. These results clearly demonstrated the suitability and reliability of

Table 6 Methylation sensitivity and restriction pattern of isoschizomers

Methylation status	Digestibility of enzymes		Restriction pattern[a)]	
	Hpa II	*Msp* I	H	M
CCGG GGCC	Active	Active	+	+
C^{5m}CGG GG^{5m}CC	Inactive	Active	–	+
^{5m}CCGG GGCC	Active	Inactive	+	–
^{5m}C^{5m}CGG GG^{5m}C^{5m}C	Inactive	Inactive	–	–

a) H, restriction pattern of *Eco*R I/*Hpa* II; M, restriction pattern of *Eco*R I/*Msp* I; "+", present of a band; "–", absent of a band.

MSAP for the evaluation of DNA methylation changes in the rapeseed genome, although the value is generally lower than that of HPLC analysis. The technique of MSAP has already been applied to DNA methylation analysis in a number of species including fungi[19], rice[20], *Arabidopsis*[21] and pepper[15]. The efficiency and reliability of MSAP were further verified by southern blot and sequence analysis in rice[20], *Arabidopsis*[21] and rapeseed in the present study. The advantages of MSAP include the simplicity, rapidness, low cost, no need of complex equipment and the additional power to clone and characterize novel methylated genes (or fragments) that would provide an effective tool to elucidate the relation between gene expression and DNA methylation. It should be noticed that, however, this technique also has three major constraints associating with resolving power. First, this method can only detect a limited spectrum of bands (50—1500 bp) on the 4%—6% sequencing gel[22]. Second, the technique can only investigate cytosines restricted to the recognition site of the isoschizomers used, and they only represent a small proportion (1/256) of the cytosines in the genome[20]. Third, it can not detect some cytosine methylations in the recognition sites given the limited resolving power of the isoschizomers. The major causes of underestimation include the occurrence of methylation at both of the cytosine residues and methylation at the external cytosine, neither of which can be detected[20].

One objective of this study was to estimate the extent and pattern of DNA methylation in rapeseed genome during seed germination. The results showed that about 15.7% of the 5′-CCGG-3′ sequences were cytosine methylated in dry seeds, with full methylation of internal cytosine occurring more often than hemimethylation of external cytosine. This value was very close to the methylation level of 16.3% in flag leaf of rice[20], but significantly differed from that of 35%—43% in seedlings of *Arabidopsis*[21]. The significant differences of DNA methylation level among various species can be resulted either from technical reasons (i.e. number of primer combinations, cycling parameters, electrophoresis time, bands visualizing method and tissue types tested) or from genetic control. It has been reported that both genetic and epigenetic mechanisms were responsible for the variation in the methylation level of the NOR region in *Arabidopsis*[23].

An interesting feature of our study was that both methylation and demethylation events were detected during seed germination, with a predominance of demethylation. It is well known that cytosine methylation plays an important role as regulator of gene expression in plant development[24], and methylation in coding regions or promoters can block the expression of these genes, while artificial demethylation will lead to the reactivation of gene expression[25]. Therefore, demethylation seems to be a necessary step toward transcriptional activation in genes controlled by this mechanism. During seed germination in rapeseed, a mass of hypomethylation events were detected, which was in good agreement with the expected large scale genes expression initiated by germination. Meanwhile, a small number of hypermethylation events were also observed, indicating that some genes are transcriptionally silent after seed germination. Thus, programmed gene expression in rapeseed appears to be controlled, at least in part, by the mechanism of DNA methylation and demethylation.

Another objective of this study was to compare the level of DNA methylation in various tissues. The result showed that a higher level of DNA methylation was detected in cotyledon than in hypocotyls, than in radicel. Significant differences in the level of cytosine methylation have already been observed among different organs in several plant species. In tomato, the level of DNA methylation was the highest in seeds, following by mature leaves and young seedlings the lowest[8]. Likewise, a higher level of DNA methylation was observed in seedlings than in flag leaves of rice[20]. Such variation of DNA methylation level is certainly expected based on current knowledge of role of methylation as one of the regulatory mechanisms of gene expression during development and differentiation[3], although the details of gene expression and regulation controlled by this mechanism is still not clear.

We also analyzed several sequences representing different methylation patterns. The blast search results showed that approximately half of the 11 fragments analyzed were homologous to particular functional genes, indicating that methylated 5′-CCGG-3′ sites were equally distributed between coding and non-coding regions. This observation is very similar to that in *Arabidopsis*[21]. By sequences alignments and database query, we have identified several putative gene fragments that may play a key role at different stage of germination or in organs formation. Further analysis of these genes in respect to their methylation status is likely to provide a better understanding of ways in which methylation of DNA is used to regulate gene

expression during seed germination.

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