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Analysis of target sequences of DDM1s in *Brassica rapa* by MSAP

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Abstract

DNA methylation is an important epigenetic modification regulating gene expression and transposon silencing. Although epigenetic regulation is involved in some agricultural traits, there has been relatively little research on epigenetic modifications of genes in *Brassica rapa*, which includes many important vegetables. In *Brassica rapa*, ortholog of DDM1, a chromatin remodeling factor required for maintenance of DNA methylation, has been characterized and DNA hypomethylated knock-down plants by RNAi (*ddm1*-RNAi plants) have been generated. In this study, we investigated differences of DNA methylation status at the genome-wide level between a wild-type plant (WT) and a *ddm1*-RNAi plant by methylation sensitive amplification polymorphism (MSAP) analysis. MSAP analysis detected changes of DNA methylation of many repetitive sequences in the *ddm1*-RNAi plant. Search for body methylated regions in the WT plant revealed no difference in gene body methylation levels between the WT plant and the *ddm1*-RNAi plant. These results indicate that repetitive sequences are preferentially methylated by DDM1 genes in *B. rapa*.

Keywords: DNA methylation; MSAP; *Brassica rapa*; repetitive sequence

Introduction

DNA methylation is an important epigenetic mark for regulation of gene expression and heterochromatin silencing. In plants, cytosines in all contexts, including CG, CHG, and CHH (H is A, T, or C), are methylated. In *Arabidopsis thaliana*, DNA methylations in CG contexts are maintained by METHYLTRANSFERASE1 (MET1), and those in non-CG contexts are maintained by CHROMOMETHYLASE 3 (CMT3). A Dnmt3 class DNA methyltransferase, i.e., DOMAINS REARRANGED METHYLASE 2 (DRM2), is *de novo* methyltransferase (Chan et al. 2005, Law and Jacobsen 2010). In plants, *de novo* methylation is triggered by a 24-nt small RNA mediated pathway, called RNA-directed DNA methylation (RdDM) (Matzke et al. 2009). Methylated cytosines are not randomly distributed but mainly found in condensed chromosomal regions, called heterochromatin. Recent epigenomic research has revealed that DNA methylation is rich in pericentromeric heterochromatin, which consists of repetitive sequences and transposable elements. These regions are abundant in loci corresponding to siRNAs (Cokus et al. 2008, Lister et al. 2008).

In addition to DNA methyltransferases, a chromatin remodeling factor, DDM1 (decrease in DNA methylation 1), also regulates DNA methylation. DDM1 is essential for maintenance of DNA methylation, and in a *ddm1* mutant of *A. thaliana*, overall reduction of DNA methylation and reactivation of transposable elements have been observed (Miura et al. 2001, Singer et al. 2001, Tsukahara et al. 2009). Although *ddm1* mutants show slight morphological change in the first generation, notable morphological abnormalities have been observed after repeated self-pollination for several generations (Kakutani et al. 1996, Kakutani 1997). The abnormalities in the *ddm1* mutants are not linked to the *DDM1* gene (Kakutani et al. 1999), and due to activation of endogenous transposons or misregulation of expression of genes near transposons (Soppe et al. 2001, Saze and Kakutani 2007, Fujimoto et al. 2008a, Tsukahara et al. 2009).

Recent studies have revealed some epigenetic modifications which affect agriculturally important traits (Manning et al. 2006, Hauben et al. 2009, Martin et al. 2009), suggesting the importance of epigenetics in agricultural science. However, there is little information on epigenetic regulation of gene expression in *Brassica rapa*, which includes various vegetables. In *B. rapa*, three DNA methyltransferase genes, i.e., *BrMET1a*, *BrMET1b*, and *BrCMT*, and two genes for chromatin remodeling factors, i.e., *BrDDM1a* and *BrDDM1b*, have been isolated, and hypomethylated plants with an RNAi construct of *BrDDM1* (*ddm1*-RNAi plants) have been obtained (Fujimoto et al. 2006a, 2008b). The *ddm1*-RNAi plants have been shown to have demethylation in centromeric repeats and some retrotransposons (Fujimoto et al. 2006b, 2008b). However there is little information on the regions methylated by BrDDM1s at the genome-wide level, and it is not known whether BrDDM1s play a role in DNA methylation in protein-coding genes. In the present study, we performed MSAP (methylation-sensitive amplification polymorphism) analysis to investigate target regions of BrDDM1s. MSAP is a modified method of AFLP (amplification fragment length polymorphism) analysis using *Hpa* II/*Msp* I instead of *Mse* I, in which the difference of DNA methylation at 5'-CCGG-3' sites can be detected as polymorphism (Xiong et al. 1999). *Hpa* II and *Msp* I are isoschizomers which recognize CCGG sites, but their respective sensitivities to DNA methylation differ. *Hpa* II cannot digest CCGG sites when either cytosine is fully methylated, while *Msp* I can digest C5mCGG but not 5mCCGG. Differences in DNA methylation between a hybrid and its parental lines in rice (Xiong et al. 1999), among different developmental stages in *A. thaliana* (Ruiz-Garcia et al. 2005), and between an artificial amphiploid and their parent species (Beaulieu et al. 2009) have been revealed by MSAP. In the present study, searching for sequences whose DNA methylation is regulated by BrDDM1a and/or BrDDM1b, we found that the BrDDM1 genes regulate DNA methylation of repetitive sequences such as transposons. By contrast, gene body methylation was not changed in a *ddm1*-RNAi plant, suggesting that DDM1s play a role in DNA methylation in repetitive sequences preferentially.

Materials and methods

Plant materials

An F_1 cultivar of *Brassica rapa,* 'Osome' (Takii Seed Co. Kyoto, Japan), was used as a wild-type (WT) plant, and a *ddm1*-RNAi plant, which has been transformed with an RNAi construct of the common sequence between *BrDDM1a* and *BrDDM1b* (Fujimoto et al. 2006a), and self-pollinated T_1 plants were also used. Genomic DNAs for MSAP analysis were isolated from leaves by the CTAB (cetyl trimethyl ammonium bromide) method (Murray and Thompson 1980).

MSAP analysis

Methylation sensitive amplification polymorphism (MSAP) analysis was performed following Xiong *et al*. (1999). For MSAP analysis, genomic DNA isolated from the WT plant and a T_0-2 plant of the *ddm1*-RNAi plant, in which DNA methylation status was lowest among T_0 plants produced by Fujimoto et al. (2006a), were used. DNAs (100 ng) were digested with *Hpa* II/*Eco* RI or *Msp* I/*Eco* RI (TaKaRa Bio, Shiga, Japan). Oligonucleotides used in the MSAP analysis are shown in Supplemental Table 1. Polymerase chain reaction (PCR) products were electrophoresed in 5% polyacrylamide gel containing 8.5 M urea. After the electrophoresis, DNA fragments were stained by the silver staining method. MSAP analysis was repeated twice using the same primer pair, and reproducible polymorphic bands were counted.

Characterization of polymorphic bands

Bands showing polymorphism were cut from polyacrylamide gel and heated with 50 µl of PCR buffer at 65°C for 2 hours. DNAs were reamplified by PCR using HM+0/Eco+0 primers listed in Supplemental Table 1. The PCR condition was 94°C for 1 minute, followed by 45 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, and final extension at 72°C for 3 minutes. Reamplified PCR products were cloned using pGEM-T Easy System I (Promega) and the nucleotide sequences were determined with a CEQ 2000XL DNA Analyzer (Beckman Coulter, USA). Sequence data were analyzed using Sequencher (Gene Codes Corporation, USA), and BLAST search of DDBJ was performed (http://blast.ddbj.nig.ac.jp/top-j.html).

To characterize the flanking sequences, suppression PCR was performed following Siebert *et al.* (1995). Amplified PCR products were cloned and sequenced.

Southern blot analysis

Genomic DNAs (2 µg) isolated from leaves of WT plants were digested with *Eco* RI (TaKaRa Bio) for analysis of copy number of *DDM1* target sequences. For analysis of DNA methylation status, genomic DNAs isolated from the WT plants, the T_0-2 plant of the *ddm1*-RNAi plants, and T_1 progeny of the T_0-2 plant were digested with *Hpa* II or *Msp* I. Used T_1 progeny were T_1-1, T_1-2, and T_1-3, which have the RNAi construct and are hypomethylated as T_0-2 (Fujimoto et al. 2006a). Digested DNAs were electrophoresed on 1.0% agarose gel and transferred onto a nylon membrane (Nitran, Whatman, UK). Probes were labeled by PCR using the PCR dig-labeling mix (Roche, Switzerland), HM+0/Eco+0 primers listed in Supplemental Table 1, and cloned polymorphic sequences as templates. After pre-hybridization at 65°C for 6 hours, the membrane was hybridized with a digoxigenin-labeled probe at 65°C for 12 hours. After hybridization, the membrane was washed twice in a solution of 0.5×SSC containing 0.1% SDS for 20 minutes at 65°C and rotated in blocking buffer containing anti-digoxigenin AP Fab fragment (Roche) at room temperature for 30 minutes. CSPD (Roche) was used as an alkaline phosphatase substrate and emitted light was exposed to FUJI MEDICAL X-RAY FILM (FUJIFILM, Tokyo, Japan).

Bisulfite sequencing

Genomic bisulfite sequencing analysis was performed as described by Paulin *et al*. (1998). Genomic DNAs (1 µg) extracted from leaves of the WT plant and the T_0-2 plant of the *ddm1*-RNAi plants were digested with *Eco* RI and *Sal* I or with *Eco* RI and *Xho* I in 200 µl of the reaction mixture. After ethanol precipitation, DNAs were dissolved in 20 µl of water. After being heated at 94°C for 10 minutes and then cooled on ice, DNAs were denatured by the addition of 2.2 µl of 3 N NaOH and incubated at 37°C for 30 minutes. The denatured DNAs were dissolved in 208 µl of urea/bisulfite solution (7.5 g of urea (Wako, Osaka, Japan) and 7.6 g of sodium metabisulfite (MERCK, Germany) dissolved in 20 ml of water, adjusted to pH 5.0) and 12 µl of 10 mM hydroquinone (SIGMA, USA) and overlaid with mineral oil. The samples were subjected to 30 cycles of 95°C for 30 seconds and 55°C for 15 minutes, followed by 55°C for 15 hours in a PCR instrument. After the reaction, DNAs were purified using the Gene Clean Kit (Q-BIOgene, USA) and eluted with 20 µl of water. For the desulfonation, 3 N NaOH was added and the DNAs were incubated at 37°C for 15 minutes. After ethanol precipitation, DNAs were eluted with 20 µl of water. PCR was performed in 50 µl of reaction mixture containing 5 µl of DNA as a template. The PCR condition was 94°C for 2 minutes followed by 40 cycles of 94°C for 20 seconds, 50°C for 30 seconds, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The primers employed are listed in Supplemantal Table 1. The PCR products were gel-purified and cloned. Ten to twelve independent clones were sequenced. We confirmed perfect conversion of unmethylated cytosines to thymines in all 8 clones of MSAPg-13 sequence.

Results

DNA methylation targets of BrDDM1s in B. rapa

To identify the DNA methylation targets of *BrDDM1s*, MSAP analysis was performed using genomic DNAs of a wild-type (WT) plant and a *ddm1*-RNAi hypomethylated plant. A total of 1913 fragments were amplified by 72 primer pairs, and 94 of their fragments (4.9%) showed different patterns between the WT plant and the *ddm1*-RNAi plant. Eighty-six fragments (91.5%) and eight fragments (8.5%) newly appeared and disappeared, respectively, in the *ddm1*-RNAi plant (Fig. 1, Table 1). Thirty-four fragments in the 94 polymorphic fragments were sequenced (Table 2). BLAST search of DDBJ revealed that 14 sequences were similar to the transposon sequences, two were parts of the rDNA region, and one was a putative centromeric repeat. The remaining 17 sequences had no homology with known sequences. To confirm the differences of DNA methylation between the WT plant and the *ddm1*-RNAi plant, Southern blot analyses using *Hpa* II and *Msp* I were performed. All the 12 examined probes revealed higher levels of cleavage by the methylation-sensitive restriction enzyme, i.e., *Hpa* II, in *ddm1*-RNAi plants than in the WT plants (Fig. 2, Table 2).

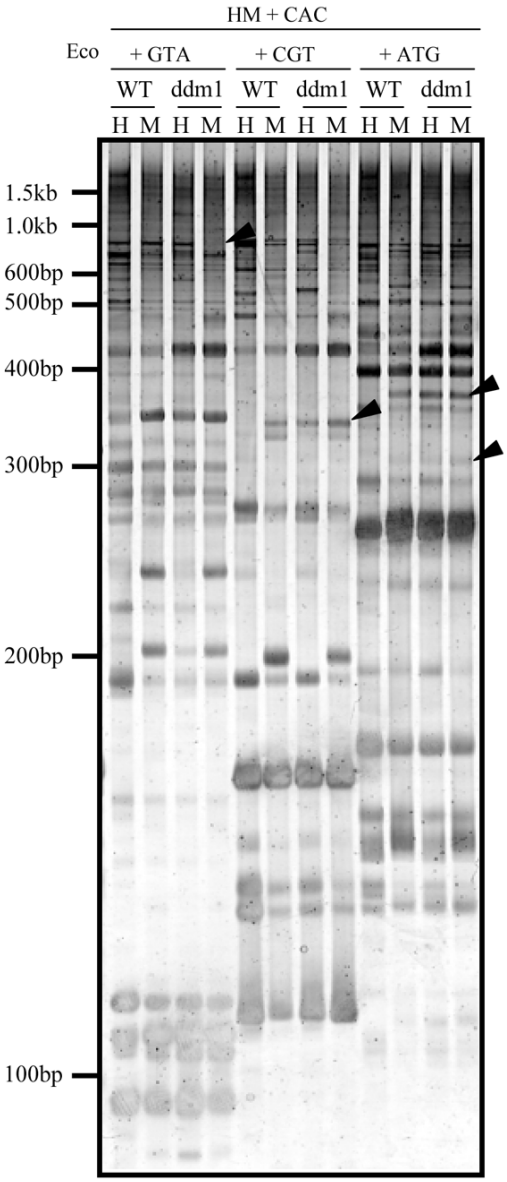

Figure 1. Polymorphic bands between a wild-type plant and a *ddm1*-RNAi plant in MSAP analysis.
Arrowheads indicate polymorphic bands between the WT plant and the *ddm1*-RNAi plant. WT, wild-type plant; ddm1, *ddm1*-RNAi plant; H, *Hpa* II/*Eco* RI digestion; M, *Msp* I/*Eco* RI digestion.

Table 1. The numbers of polymorphic bands between WT and *ddm1*-RNAi plant in MSAP

WT		RNAi		No. of sites
H	M	H	M	
-	+	+	+	76
-	-	+	-	3
-	-	+	+	7
+	-	-	-	4
+	+	-	-	3
+	+	-	+	1
Total				94

H, *Hpa* II digestion; M, *Msp* I digestion
+, band present; -,band absent

Hypomethylation status of genomic regions was confirmed in both sequences newly appearing in the *ddm1*-RNAi plant and those disappearing in the *ddm1*-RNAi plant.

Southern blot analyses using the annotated sequences newly appearing or disappearing in the *ddm1*-RNAi plant as probes showed multiple bands except for the reverse transcriptase regions, which showed 1-2 bands (Fig. 3, Table 2). Band patterns of multiple bands were categorized as multi I or multi II, multi I having many bands with a smear background and multi II having more than three bands with no smear background (Fig. 3). Seventeen probes of unknown sequences revealed ten sequences to have more than six copies, termed multi I, and seven sequences to have 1-3 copies (Table 2). Taken together, these findings indicate that BrDDM1s regulate DNA methylation of repetitive sequences in *B. rapa*.

DNA methylation in protein-coding regions

DNA methylation in genic regions was analyzed. Recent studies have revealed that actively transcribed genes are methylated at their CG sites, which is called "body methylation" (Zhang et al. 2006, Zilberman et al. 2007). To obtain body methylated sequences, we characterized some more MSAP bands and got four candidates, i.e., MSAPg-4(4), -12, -19, and -54(1). These genes were expressed in leaves of WT plants, except for MSAPg-4(4).

To confirm their DNA methylation status, bisulfite-sequencing analysis was carried out. A 197 bp sequence of MSAPg-4(4), which corresponds to the 10th to 11th intron of the putative serine carboxypeptidase 1 gene of *A. thaliana,* contained 42 cytosines, i.e., three in a CpG context and 39 in a non-CpG context, and two cytosines in the CpG context were methylated. In a 303 bp sequence of MSAPg-12, which corresponds to the 10th to 12th exon of a putative protein gene (homologous to At3g45045 of *A. thaliana*), containing 58 cytosines, i.e., 8 in a CpG context and 50 in a non-CpG context, two cytosines in the CpG context and one in a non-CpG context were methylated (Fig. 4B). A 287 bp flanking sequence of MSAPg-19, which corresponds to the 3rd exon to the 3rd intron of a hypothetical protein gene (homologous to At4g14850 of *A. thaliana*), contained 51 cytosines, 13 of which were of a CpG context and 38 of which were of a non-CpG context. There were nine methylated sites, all of which were of a CpG context. A 270 bp flanking sequence of MSAPg-54(1), which corresponds to putative trehalose-6-phosphate synthase, contained 51 cytosines, i.e., 14 in a CpG context and 37 in a non-CpG context. All cytosines in the CpG context were heavily methylated, and 16 cytosines in the non-CpG context were also methylated, their level of methylation being lower than that in the CpG context. DNA methylation in these four regions was confirmed not only in a CCGG context, which can be detected by MSAP analysis, but also at other CG sites. In these four sequences of the *ddm1*-RNAi plant, body methylation was not changed (Fig. 4). Regulation of DNA methylation in genic regions was contrary to that in repetitive sequences, i.e., the retrotransposon *Tto1* (Fujimoto et al. 2008c) was hypomethylated in the *ddm1*-RNAi plants in all contexts (Fig. 4). These results indicate that BrDDM1s are not involved in DNA methylation of these regions.

Discussion

DNA methylation is an important modification for epigenetic regulations of gene expression. It is involved in various

Table 1. The numbers of polymorphic bands between WT and *ddm1*-RNAi plant in MSAP

	band pattern						
	WT		RNAi				
l name	H	M	H	M	length(bp)	copy no. [a]	gene [b]
2*	-	+	+	+	209	multi I	A. thaliana retroelement pol polyprotein-like
15	-	+	+	+	323	multi II	ARATH putative retroelement pol protein
18	-	+	+	+	209	multi I	retrotransposon
26	-	+	+	+	167	multi I	retrotransposon
7(2)	-	+	+	+	199	multi I	retrotransposon-like
30	-	+	+	+	315	multi I	25S rRNA gene
1(1)	-	+	+	+	183	multi I	5S rRNA gene
45	-	+	+	+	201	multi II	transposon-like
(1)*	-	+	+	+	235	1	retrotransposon-like
55	-	+	+	+	119	multi I	retrotransposon
56	-	+	+	+	224	1 or 2	non-LTR retrotransposon reverse transcriptase
2*	-	+	+	+	189	multi II	putative retroelement pol polyprotein
3(3)	-	+	+	+	185	multi I	retrotransposon-like
5(1)	-	+	+	+	338	1	non-LTR retrotransposon reverse transcriptase
5(2)	-	+	+	+	280	multi II	ARATH putative retroelement pol protein
(2)*	-	+	+	+	240	multi I	putative centromeric repeat
(1)*	-	+	+	+	258	multi I	ARATH putative retroelement pol protein
1)*	-	+	+	+	336	multi II	-
(2)	-	+	+	+	265	1 or 2	-
1)*	-	+	+	+	175	multi II	-
12	-	+	+	+	95	multi I	-
4*	-	-	+	-	216	multi II	-
7*	+	+	-	+	238	2 or 3	-
(2)*	-	+	+	+	168	multi II	-
5(1)	-	+	+	+	323	multi II	-
5(4)	-	+	+	+	96	1 or 2	-
5(5)	-	+	+	+	90	multi I	-
)(2)	-	+	+	+	98	multi II	-
(2)*	+	+	-	-	128	1 or 2	-
(3)*	+	-	-	-	156	1 or 2	-
7(1)	-	+	+	+	290	1	-
)(1)	-	+	+	+	280	multi II	-
)(3)	-	+	+	+	143	1	-
71	-	+	+	+	353	multi I	-

*, Sequences whose hypo-methylation status in the *ddm1*-RNAi plant were confirmed by Southern blot analysis
[a], Examples of multi I and multi II are shown in figure 2
[b], Highly homologous genes or clones identified by BLAST search of DDBJ (http://www.ddbj.nig.ac.jp/search/blast-j.html)
H, *Hpa* II digestion; M, *Msp* I digestion; +, band present; -,band absent

phenomena, and some agriculturally important traits are also regulated by DNA methylation (Manning et al. 2006, Hauben et al. 2009, Martin et al. 2009). In the present study, MSAP analysis was applied to investigate the sequences whose methylation is controlled by BrDDM1s at the genome-wide level in *B. rapa*. Since MSAP analysis does not require information of genome sequences, it is suitable for analyzing the difference of DNA methylation in *Brassica*, whose genome sequencing has not been completed. The sequences with low methylation levels identified in the *ddm1*-RNAi plant included transposons, repetitive sequences in centromeres, and spacer sequences of rDNA. In addition to known sequences, Southern blot analysis revealed that most unknown sequences with low methylation in the *ddm1*-RNAi plants had high copy numbers in the *B. rapa* genome, suggesting a preference of

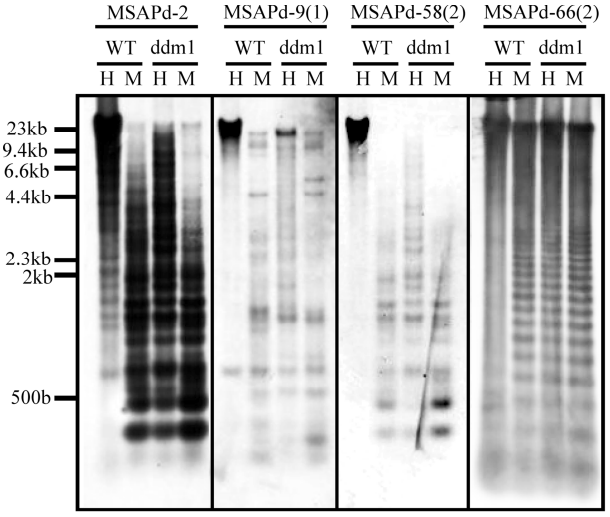

Figure 2. Detection of DNA methylation status of the target sequences of BrDDM1s in the *ddm1*-RNAi plants by Southern blot analysis.
Genomic DNA was digested by *Hpa* II(H) or *Msp* I(M) and hybridized with polymorphic bands of MSAP analysis as probes.

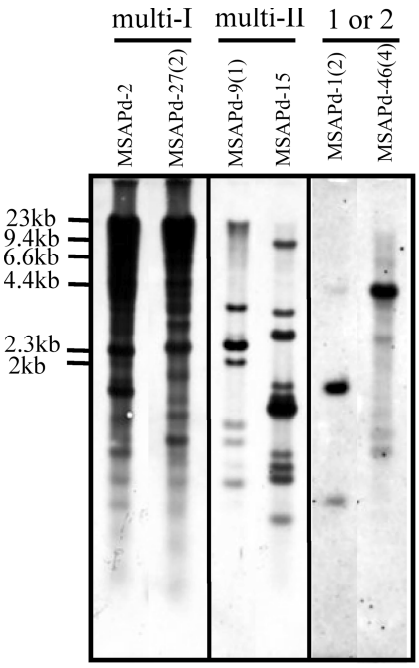

Figure 3. Detection of target sequences of BrDDM1s by Southern blot analysis.
Genomic DNA was digested by *Eco* RI and hybridized with polymorphic bands detected by MSAP analysis. The sequences were categorized into three classes by their band patterns. Multi I has many dense bands with a smear background, multi II has more than three dense bands with no smear background, and others have a few dense signals.

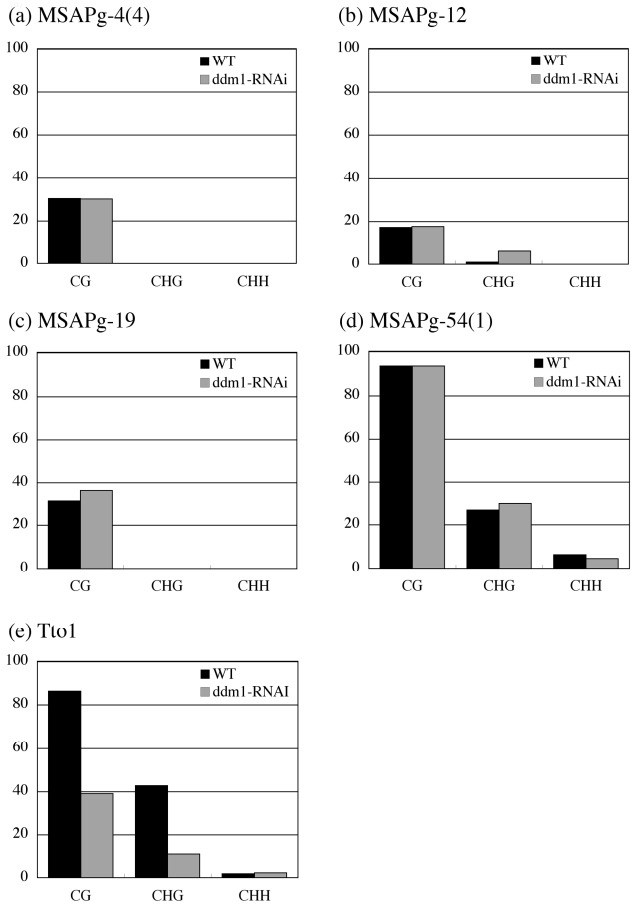

Figure 4. Differences of DNA methylation levels between a wild-type plant and a *ddm1*-RNAi plant analyzed by bisulfite sequencing.
Bar graphs show percentages of methylated cytosines in CG, CHG, and CHH context detected by bisulfite-sequencing analysis. Black and gray bars indicate the wild-type plant and the *ddm1*-RNAi plant, respectively. (a) MSAPg-4(4), (b) MSAPg-12, (c) MSAPg-19, (d) MSAPg-54(1), and (e) Tto1.

BrDDM1s for DNA methylation in repetitive sequences. However, there is a possibility that genic regions were not detected because of their relatively small ratio in methylated sequences. To determine whether BrDDM1s are involved in DNA methylation in genic regions, we analyzed DNA methylation status in WT and *ddm1*-RNAi plant within body methylated regions. We found DNA methylation in protein-coding regions in *B. rapa*, and bisulfite sequencing results showed that most of the methylated contexts were CG sites. These results were similar to the results for other plants and other organisms (Tran et al. 2005, Zemach et al. 2010, Feng et al. 2010). The gene body methylation level in the *ddm*1-RNAi plant was similar to that in the WT plant, indicating that BrDDM1s play a role in DNA methylation in repetitive sequences preferentially.

In *ddm1* mutants of *A. thaliana*, transposable elements are transcriptionally or transpositionally activated (Miura et al. 2001, Singer et al. 2001, Lippman *et al*. 2004, Tsukahara et al. 2009), and such transcriptional activation of transposable elements has also been observed in *ddm1*-RNAi plants of *B. rapa* (Fujimoto et al. 2006a). Changes of DNA methylation in transposable elements have potential to affect the transcription of protein-coding genes. Expressions of several genes were affected in the *ddm1* mutants of *A. thaliana*, and such effects on gene expression were due to the change of DNA methylation in transposable elements that were found in their flanking regions (Saze and Kakutani 2007, Soppe et al. 2001, Kinoshita et al. 2007). Disruption of gene function by

insertion of transposons in *ddm*1 mutants of *A. thaliana* has also been reported (Miura et al. 2001, Tsukahara et al. 2009). As transcription of transposable elements affects the gene expression of their flanking protein-coding genes (Kashkush et al. 2003), expression of the protein-coding genes might be changed by transcriptional activation of transposable elements in *B. rapa*. Although no change in gene body methylation in the *ddm1*-RNAi plant was detected, more detailed analysis might reveal other types of change in DNA methylation, such as *de novo* induction of DNA methylation. Further studies on whole genome transcriptome and methylome in *B. rapa* are needed to determine whether the defect of DDM1 function affects the expression of protein-coding genes directly or indirectly through hypomethylation of transposable elements.

Many single nucleotide polymorphisms (SNPs) in genes responsible for genetic traits important in plant breeding have been identified. Simple and efficient techniques for SNP analysis are applicable to conventional cross-breeding (Shirasawa et al. 2006). In addition to SNPs, epigenetic modification of DNA and/or histone also contributes to variation of some traits. In hypomethylated plants, some important genes, which are silenced epigenetically in normal conditions, may be activated. In contrast to mammals, epigenetic changes are inherited in plants, and therefore they could be treated as genetic changes as well as nucleotide-based genetic changes. Moreover, information in DNA methylation could be applied to make epi-markers (Tanaka et al. 2005). Although the main targets of BrDDM1 genes are repeat sequences, a recent study in *A. thaliana* has suggested that DDM1 is also involved in regulation of genes. Saze et al. (2008) have revealed that *ddm1 ibm1* double mutants showed severe developmental abnormalities compared to *ibm1* mutants, in which IBM1 is putative histone H3K9me2 demethylase and DNA methylation levels in genes are increased by the loss of the function of IBM1 (Miura et al. 2009). Studies on Brassica epigenome may provide important information for development of plant breeding.

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Supplemental Data

Supplemental table 1. Primer pairs used in this study

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Supplemental table 1. Primer pairs used in this study

Adaptors[a]			
Eco RI-ad-F	CTCGTAGACTGCGTACC	*Eco* RI-ad-R	AATTGGTACGCAGTCTAC
Hap II/*Msp* I-ad-F	GATCATGAGTCCTGCT	*Hap* II/*Msp* I-ad-R	CGAGCAGGACTCATGA
Pre-selective promers[a]			
Eco+0	GACTGCGTACCAATTC	HM+0	ATCATGAGTCCTGCTCGG
Selective primers[a]			
Eco+CAA	GACTGCGTACCAATTC*CAA*	HM+CAA	ATCATGAGTCCTGCTCGG*CAA*
Eco+CAC	GACTGCGTACCAATTC*CAC*	HM+ CAC	ATCATGAGTCCTGCTCGG*CAC*
Eco+CAG	GACTGCGTACCAATTC*CAG*	HM+ACG	ATCATGAGTCCTGCTCGG*ACG*
Eco+CAT	GACTGCGTACCAATTC*CAT*	HM+TAG	ATCATGAGTCCTGCTCGG*TAG*
Eco+ACG	GACTGCGTACCAATTC*ACG*	HM+TGC	ATCATGAGTCCTGCTCGG*TGC*
Eco+TAC	GACTGCGTACCAATTC*TAC*	HM+GAT	ATCATGAGTCCTGCTCGG*GAT*
Eco+GTA	GACTGCGTACCAATTC*GTA*		
Eco+CGT	GACTGCGTACCAATTC*CGT*		
Eco+ATG	GACTGCGTACCAATTC*ATG*		
Eco+TGC	GACTGCGTACCAATTC*TGC*		
Eco+GCA	GACTGCGTACCAATTC*GCA*		
Eco+AGC	GACTGCGTACCAATTC*AGC*		
Primers for Bisulfite-sequencing			
MSAPg-4(4)BisF	AAAAGGGAAGTTTGAGAAGYAAAAT	MSAPg-4(4)BisR	ARTTTACRATAAAATTAAAACCAAC
MSAPg-12BisF	AATTTGATTAAAYAGGYATYGATGG	MSAPg-12BisR	CCARCACATARCACACCRCCATCATC
MSAPg-19BisF	ATGGYAGGTYTTGAGTTGGGGAGAT	MSAPg-19BisR	CTCCATACCRTTTTCCACATCCCC
MSAPg-54(1)BisF	TGTTYGAGAGGAGATAYTAAGGGGG	MSAPg-54(1)BisR	AAACTAAARTCCCRACCATTCTCTCTC
Tto1-F	AGGAAYATGGAAAGAATTAAGGAG	Tto1-R	TARACTTRCTARAATCAACATCTC

Underlined sequences indicate complementary sequences between forward (F) and reverse (R) sequences of adaptors.

Italic tri-nucleotides indicate added sequences to pre-selective primers for selective amplification.

[a] Xiong et al. (1999)