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Full Length Research Paper

Inheritance of DNA methylation in DH and its backcrossed lines of *Brassica napus*

Zhen Guo[1], Liping Zeng[1], Maoteng Li[2], Yan Long[1]* and Jinling Meng[1]

[1]National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China.
[2]College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China.

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Nineteen (19) pairs of methylation sensitive amplified polymorphism (MSAP) primers were used for analysing DNA methylation in two DH lines and their two back-crossed generations, namely BC1 and BC2. Constitutive DNA methylation was stable across three generations and inherited changes in it were of six types. 60 fragments were chosen for sequencing and then, were analysed using the Blast database and the strength of their expression was assessed. Two types of genes, those related to retro-transposons and those involved in other functions, were identified but the degree of expression of those genes was lower in the progeny than in the parents.

Key words: DNA methylation, methylation sensitive amplified polymorphism (MSAP), backcross line, *Brassica napus.*

INTRODUCTION

DNA methylation is a major co-modification of chromatin that does not entail any change in the nucleotide sequence and is referred to as epigenetic modification. DNA methylation can be transmitted through both mitosis and meiosis; once inherited, it is stable across generations (Kakutani 2002; Henderson and Jacobsen, 2007), is widely distributed within the genome of eukaryotes encompassing fungi, plants and animals including humans (Finnegan et al., 1998; Field et al., 2004; Taby and Issa, 2010) and plays an important role in controlling pheno-types, cancer and nutrition. For example, Kucharski et al. (2008) found that in honeybees, DNA methylation, which is used for storing epigenetic information, can be altered by nutrition and that, the flexibility of epigenetic modifications underpins profound shifts in the course of development, with significant implications for reproduction and behaviour. In plants, DNA methylation affects many traits and many epi-alleles have been discovered to control such traits such as seed development in maize and *Arabidopsis* and fruit ripening In tomato (King et al., 2010). Early studies have found about 6 to 24% of methylation sites (5 mC) in plants (Chen and Li, 2004). Salmon et al. (2008) found that, in different accessions of *Brassica oleracea* (C), DNA methylation ranged from 52 to 60%. In *Brassica rapa* (A), tissue-specific monoallelic *de novo* methylation within the 5' promoter region of *SP11* gene, which specifies the male component of sporophytic self-incompatibility at the *S* locus, was found to determine the dominance interaction that results in self-incompatibility in the phenotype (Shiba et al., 2006). In *Brassica napus* (AC), rapid alterations of gene expression and cytosine methylation were detected in newly synthesized allopolyploids and transcriptomic changes (4.1%) were found to be consistent with changes in DNA methylation (6.8%) in synthetic *B. napus* (Xu et al., 2009). Changes in DNA methylation were also shown to be brought about by the environment; in *B. napus*, these changes affected seed germination (Lu et al., 2006) and response to salt stress (Lu et al., 2007).

Introgressed lines have been widely used as experimental material in genetic analysis and molecular breeding, for example in fine mapping, QTL cloning and molecular-maker-assisted selection (MAS) once a target gene is found (Eshed and Zamir, 1995; Ramsay et al., 1996; Jiang et al., 2003; Tan et al., 2004; Yoon et al.,

*Corresponding author. E-mail: longren0807@gmail.com. Tel: 86-27-87286871.Fax: 86-27-87280016.

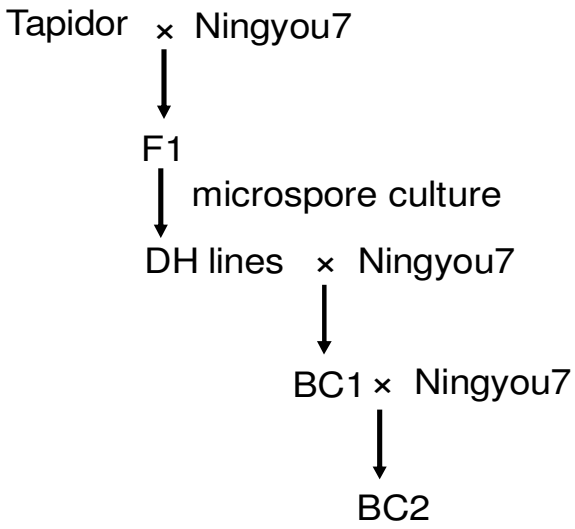

Figure 1. The process of breeding of the plant materials used in the study.

2006). Introgressed lines are usually constructed by backcrossing (Eshed and Zamir, 1995) and candidates for further research are usually chosen from screened genotypes. Besides, for gene transferred from a donor parent to a recipient parent, introgressed lines also incorporate epigenetic modifications. Liu Bao and his group carried out a series of experiments using *Zizania latifolia* Griseb., a wild grass from the tribe *Oryzeae*, as a donor parent and cultivated rice (*Oryza sativa* L.) as a recipient parent (Liu et al., 1999) to investigate the inheritance pattern of DNA methylation in introgressed lines and found that, introgression of alien DNA into a plant genome can induce extensive alterations in DNA methylation and transcription of both protein-coding genes and transposon/retro-transposon segments (Liu et al., 2004; Dong et al., 2006). It is therefore, likely that many changes found in the progeny of backcrossed lines could ultimately find their way into introgressed lines.

The status of variation of DNA methylation can be ascertained by a number of methods including southern blotting using methylation-revealing isoschizomers and bisulphite sequencing (Osborn et al., 2003). MSAP is another method, which is not only rapid but also efficient that, it gives high throughput and is widely used for detecting DNA methylation status of an entire genome (Duan et al., 2009) as well as for comparing the status of DNA methylation in different cultivars, tissues and organs (Xiong et al., 1999; Liu et al., 2004; Chen and Chen, 2008; Lu et al., 2008). For example, Xiong et al. (1999) used MSAP to analyse the differences in DNA between an F1 hybird and its parents in rice and found that, although the DNA methylation status had no significant influence on heterosis overall, different loci with methylated DNA were significantly correlated with heterosis. However, little information is available on naturally occurring intraspecific methylation polymerphism. In general, the MSAP method uses the isoschizomers HpaII/MspI to detect the methylation status of CCGG in plant genomes. CNG methylation is also an important modification of 5 mC in *Arabidopsis*; CNG methylation accounts for approximately 23% of the 5 mC content (Xiong et al., 1999; Liu et al., 2004; Chen and Chen, 2008; Lu et al., 2008).

The objectives of the present work were: (1) to characterize the extent of variability in methylation at the genome level in different backcross generations; (2) to find the types of genes exhibiting DNA methylation polymorphism and (3) to confirm the status of expression of methylated genes. We found a high proportion (approximately 66%) of constitutive methylation in MSAP-methylated fragments and six types of variation in DNA methylation in DH and its back-crossed generations. When 60 randomly selected fragments were sequenced and then, analysed using Blast genomic database and gene transcriptome data, it was found that, DNA methylation involved retro-transposon-related genes and other functional genes in the genome of different backcross generations.

MATERIALS AND METHODS

Plant material

Two original parents, two DH lines of TN DH population in *B. napus* (Qiu et al., 2006) and two backcrossed generations (BC1 and BC2) were chosen for the analysis (Figure 1). Each line and the parents were represented by three individual plants as replications. All lines were planted under normal controlled conditions. Genomic DNA was isolated from the fifth leaf (so that all leaves were at the same

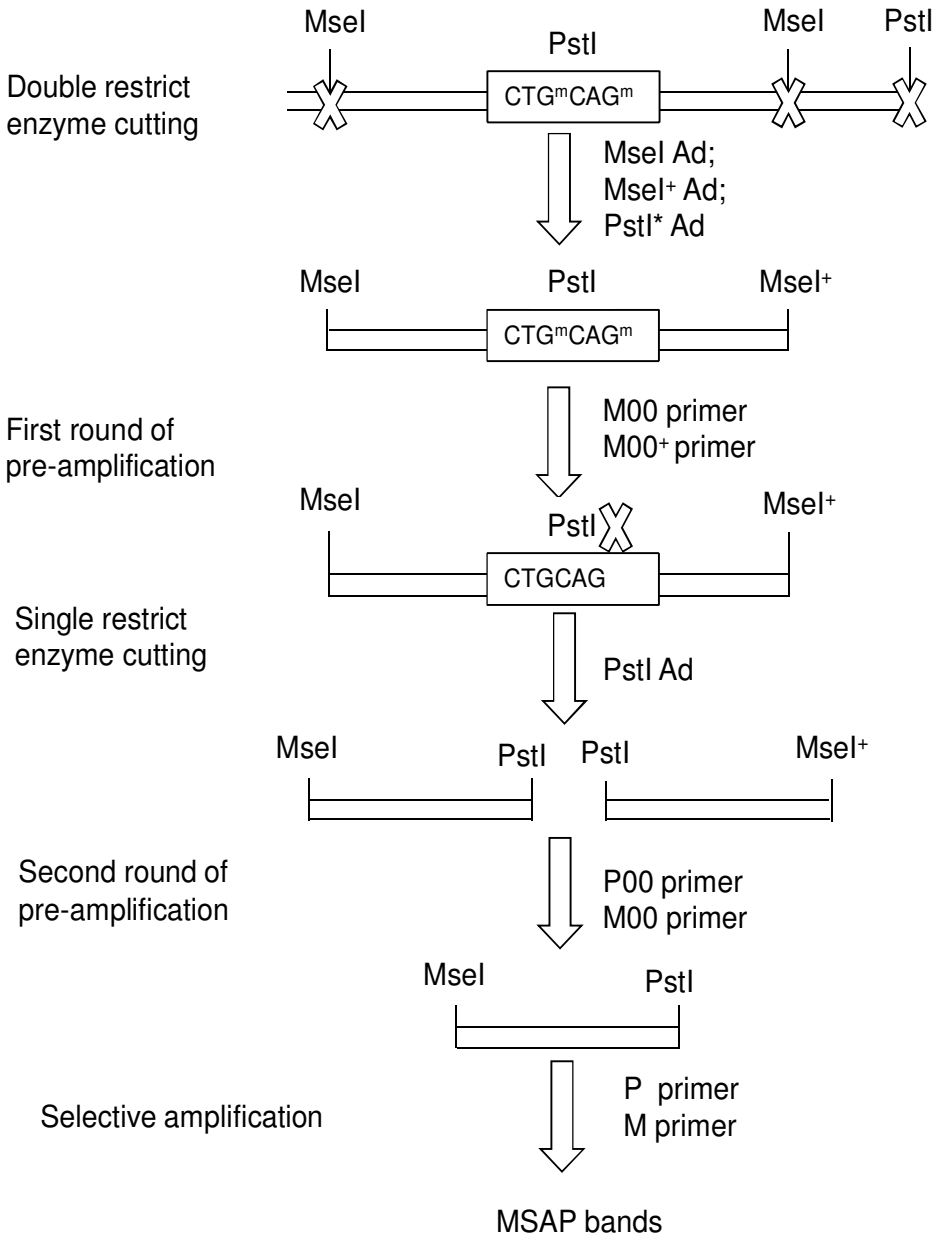

Figure 2. A flowchart showing the MSAP method.

developmental stage) of each individual plant by the CTAB method (Doyle and Doyle, 1990) and purified by repeated extractions with phenol.

MSAP analysis

The methylation sensitive amplified polymorphism (MSAP) method as adapted from Vuylsteke et al. (1999) was used. One methylation-sensitive enzyme, namely PstI, with the enzyme cutting CTGCAG site and one methylation-insensitive enzyme, namely MseI, were used as a pair (Figure 2). Altogether, one pair of a pre-selective primer and 21 pairs of selective primers were used for amplification (Table 1).

Electrophoresis, gel cut and sequencing

After the PCR amplification, the amplified product was made visible by silver staining (6% PAGE gel was used for electrophoresis and silver staining). After staining, some gels with a specific band pattern were cut and used with the pre-selective primers to repeat the PCR. After the products of the repeat PCR were confirmed as belonging to one specific band (which had the same molecular weight as the target fragment), the PCR product was electrophoresed on 1% agarose gel, cut and purified by using an agarose gel electrophoresis extraction kit (D2500-02, OMEGA Company), ligated using the TA clone vector PMD18-T (Takara Company), was transformed and a positive clone was chosen for sequencing. For each fragment, at least three positive clones were

Table 1. Adaptors and primers used for MSAP amplifications.

Adaptor		Pre-selective primer	
EcoRI-adapter:	5'-CTCGTAGACTGCGTACC-3'	M00:	5'-GATGAGTCCTGAGTAAC-3'
	3'-CATCTGACGCATGGTTAA-5'	M00+:	5'-GTAGACTGCGTACCTAA-3'
PstI-adapter:	5'-CTCGTAGACTGCGTACATGCA-3'	P00:	5'-GACTGCGTACATGCAG-3'
	3'-CATCTGACGCATGT-5'		
PstI-adapter*:	5'-GCATCAGTGCATGCGTGCA-3'		
	3'-GTAGTCACGTACGC-5'		
MseI-adapter:	5'-GACGATGAGTCCTGAG-3'		
	3'-TACTCAGGACTCAT-5'		
MseI-adapter+:	5'-CTCGTAGACTGCGTACC-3'		
	3'-CTGACGCATGGAT-5'		

Selective primers

M00+AA	5'-GATGAGTCCTGAGTAACAA-3'	P00+CG	5'-GACTGCGTACATGCAGCG-3'
M00+TC	5'-GATGAGTCCTGAGTAACTC-3'	P00+GT	5'-GACTGCGTACATGCAGGT-3'
M00+AT	5'-GATGAGTCCTGAGTAACAT-3'	P00+TC	5'-GACTGCGTACATGCAGTC-3'
M00+GG	5'-GATGAGTCCTGAGTAACGG-3'	P00+AAT	5'-GACTGCGTACATGCAGAAT-3'
M00+AC	5'-GATGAGTCCTGAGTAACAC-3'	P00+GGA	5'-GACTGCGTACATGCAGGGA-3'
M00+TG	5'-GATGAGTCCTGAGTAACTG-3'	P00+ATT	5'-GACTGCGTACATGCAGATT-3'
M00+GT	5'-GATGAGTCCTGAGTAACGT-3'	P00+ACG	5'-GACTGCGTACATGCAGACG-3'
M00+AG	5'-GATGAGTCCTGAGTAACAG-3'	P00+TAC	5'-GACTGCGTACATGCAGTAC-3'
M00+TA	5'-GATGAGTCCTGAGTAACTA-3'	P00+CGA	5'-GACTGCGTACATGCAGCGA-3'
M00+GA	5'-GATGAGTCCTGAGTAACGA-3'	P00+TCG	5'-GACTGCGTACATGCAGTCG-3'

chosen for sequencing.

Blast and gene expression analysis

Once all the chosen positive clones had been sequenced, each was analysed using Blastn and BlastX available at <http://ncbi.nlm.nih.gov>. After the gene types of the fragments (Table 3) had been identified by Blastn and BlastX, the gene expression status was analysed by comparison with data on sequences expressed by the genes Tapidor and Ningyou7, which was obtained in 2009 (Trick et al., 2009) (Table 3). Each sequenced fragment was used as a reference sequence to map the sequenced data of Tapidor and Ningyou7 by using the method of mapping and assembly with qualities (MAQ), which consisted of the following steps (Figure 3): (1) conversion of the reference and the read sequences to the required format: given the reference sequences in a fasta file ref.fasta, the command fasta2bfa converts the file into the BFA format. Before doing the alignment, maq needs to convert the read sequences into the BFQ. (Given the read sequences in the fastq file reads.fastq, command fastq2bfq achieves this conversion); (2) alignment of the read sequences to the reference: the map command aligns them in the BFQ format to the reference in the BFA format. If only one read file, namely reads.bfq, is provided, match command will do the single-end alignment; (3) viewing of the read alignment: the read sequences together with their positions can be extracted to a text file from out.map with the command map view; (4) use of the software tablet to view the result of the alignment. In general, the greater the number of read sequences that match the reference sequence, the greater the degree of gene expression.

RESULTS

Changes in DNA methylation pattern in DH line and its offspring

Altogether, 19 pairs of primers were selected for the MSAP analysis. Data from two treatments (DH/BC1/BC2) were combined. Of the 394 bands that were scored, 262 showed no polymorphism with the two original parents, two DH lines and plants from their BC1 and BC2 generations. These non-polymorphic bands meant that, the DNA methylation status was constitutive and varied little across the generations. Another 132 polymorphic bands showed six types of DNA methylation that was different from that seen in the DH parent line and in plants from BC1 and BC2 generations (Figure 4). Among the bands, types I, III and V showed that, a gene of the DH parent was methylated but the methylation status was lost in the offspring of its backcross in the corresponding

Table 3. DNA methylation status in DH and backcross generation and results of Blast mapping of fragments types and of gene expression.

Gene type	Marker name	DH1	BC1	BC2	DH2	BC1	BC2	Gene name	Read	Feature
None										
	P2M7-N-455	+	+	+	+	+	+	None	0	0
	P1M9-N-220	+	-	+	+	-	-	None	19	0
	P8M7-N-430	+	+	+	+	+	+	None	0	0
	P2M7-N-455	+	+	+	+	+	+	None	0	0
	P2M7-N-455	+	+	+	+	+	+	None	0	0
	P4M4-N-300	+	+	-	+	-	-	None	1	0
	P8M1-T-90	+	-	-	-	-	+	None	2	0
	P10M8-N-150	+	+	+	+	+	+	None	8	0
	P2M7-T-90	+	-	-	+	-	-	None	0	0
	P8M24-N-380	+	+	-	+	+	-	None	0	0
	P4M2-T-250	+	+	+	+	+	+	None	1	0
	P10M43-N-200	+	+	-	+	+	-	None	0	0
	P13M16-N-350	-	-	+	+	+	-	None	39	0
	P10M43-T-180	-	+	+	-	+	+	None	0	0
	P8M24-T-360	+	+	-	+	+	-	None	1	0
	P13M06-N-130	+	+	-	+	+	-	None	0	0
Retro-related										
	P1M9-N-150	+	+	+	+	+	+	Non-LTR retroelement reverse transcriptase-like protein	0	0
	P8M3-N-480	+	+	+	+	+	+	Retrotransposon-athila protein-like	0	0
	P4M4-N-370	+	+	-	-	-	-	Putative non-LTR reverse transcriptase	0	0
	P4M4-T-380	+	+	+	+	+	+	Similar to retrotransposon proteins	0	0
	P8M3-N-130	+	-	-	+	-	-	Non-LTR retroelement reverse transcriptase	0	0
	P1M9-N-130	+	+	+	+	-	+	Transposon protein	0	0
	P1M9-N-350	+	-	+	-	-	-	Retroelement pol polyprotein-like	0	0
	P10M1-N-380	+	+	-	+	-	+	Putative retrotransposon athila-like protein	0	0
	P8M1-N-210	+	+	-	-	-	+	Putative non-LTR retroelement reverse transcriptase	0	0
	P6M3-T-210	-	+	+	+	+	+	Copia-like polyprotein	0	0
	P8M1-N-160	+	-	-	-	-	+	Putative non-LTR retroelement reverse transcriptase	0	0
	P8M1-T-350	+	+	+	-	-	+	Retrotransposon like protein	0	0
	P8M8-N-280	+	+	+	+	+	+	Putative non-LTR retroelement reverse transcriptase	0	0
	P8M8-T-500	-	+	+	-	+	+	Reverse transcriptase, putative	0	0
	P5M2-T-380	-	+	-	-	+	+	Putative non-LTR retroelement reverse transcriptase	0	0

Table 3. cont.

	P5M6-N-400	+	-	+	+	-	-	Putative non-LTR retroelement reverse transcriptase	0	0
	P10M43-N-280	-	-	+	+	+	-	Putative non-LTR retroelement reverse transcriptase	1	0
	P13M25-N-430	+	-	+	+	-	-	Very similar to retrotransposon reverse transcriptase	0	0
	P5M6-N-130	+	+	-	+	+	-	Putative non-LTR retroelement reverse transcriptase	0	0
	P2M10-N-305	-	-	+	+	-	-	Copia-type polyprotein, putative	0	0
	P13M06-T-490	-	+	+	+	+	+	Reverse transcriptase, putative	12	0
	P8M24-T-390	+	+	+	-	+	+	Ty1-copia retrotransposon subclass	0	0
	P5M2-N-410	+	+	+	+	+	-	Retrotransposon protein	1	0
	P5M2-T-560	-	+	+	+	+	+	Putative retroelement pol polyprotein	0	0
Gene related										
	P8M3-N-390	-	-	-	+	-	-	Hypothetical protein	5	0
	P4M4-T-80	-	+	+	+	+	+	NADH dehydrogenase	0	0
	P4M4-N-500	+	+	+	+	+	+	Phosphatidylinositol synthase PIS1	29	0
	P4M4-N-500	+	+	+	+	+	+	Hypothetical protein	0	0
	P8M3-T-295	+	+	-	-	-	-	Hypothetical protein	0	0
	P8M7-N-420	+	+	-	+	+	-	Hypothetical protein	0	0
	P2M7-N-450	+	+	-	+	+	-	Hypothetical protein	0	0
	P10M1-T-260	+	+	+	+	+	+	Olfactory receptor	13	0
	P10M1-T-280	+	+	+	+	+	+	GCN5-related N-acetyltransferase	1	0
	P10M1-T-360	+	+	-	-	+	+	RNA polymerase beta subunit-1	153	0
	P10M1-T-360	+	+	-	-	+	+	Calcium-binding EF hand family protein	10	0
	P2M7-N-210	-	-	+	+	-	-	TPIS1 protein	12	0
	P8M24-T-420	-	+	+	-	+	+	Hypothetical protein	0	0
	P13M06-T-405	-	+	+	+	+	+	rDNA sequence	270166	0
	P8M24-N-350	+	-	-	+	+	-	Complement component receptor 1-like	0	0
	P8M24-N-460	+	+	-	+	+	-	Oxysterol-binding family protein	6	0
	P13M25-T-140	-	+	+	+	-	-	Transcription factor Sox-5	2	0
	P10M43-N-330	+	+	+	+	+	+	Hypothetical protein	0	0
	P4M2-N-260	+	+	-	-	-	+	Putative cell division membrane protein	0	0
	P8M24-N-140	+	+	+	+	+	-	Oxysterol-binding family protein	9	0

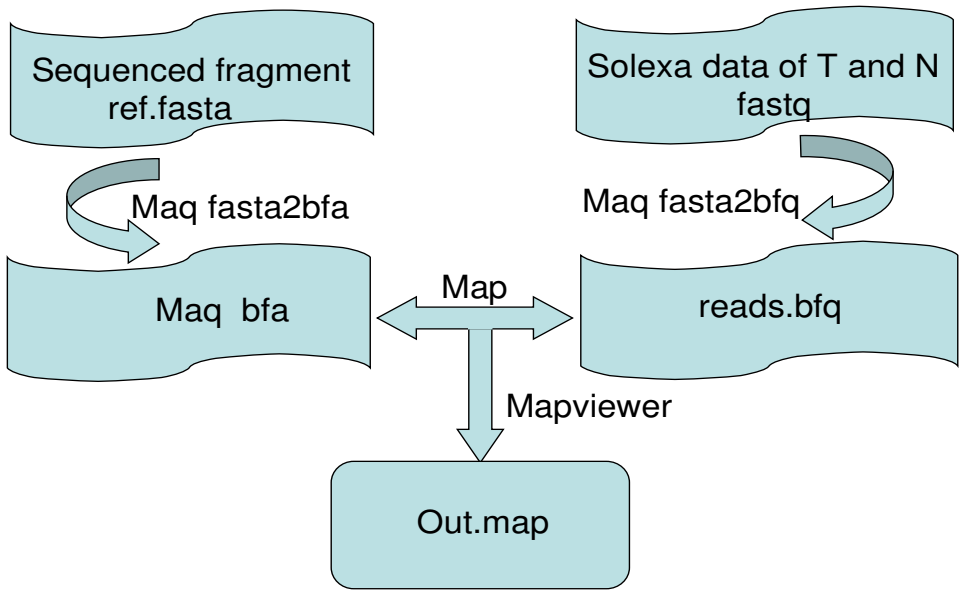

Figure 3. A flowchart of mapping of the data from the sequenced fragments to transcriptomes of Tapidor and Ningyou7.

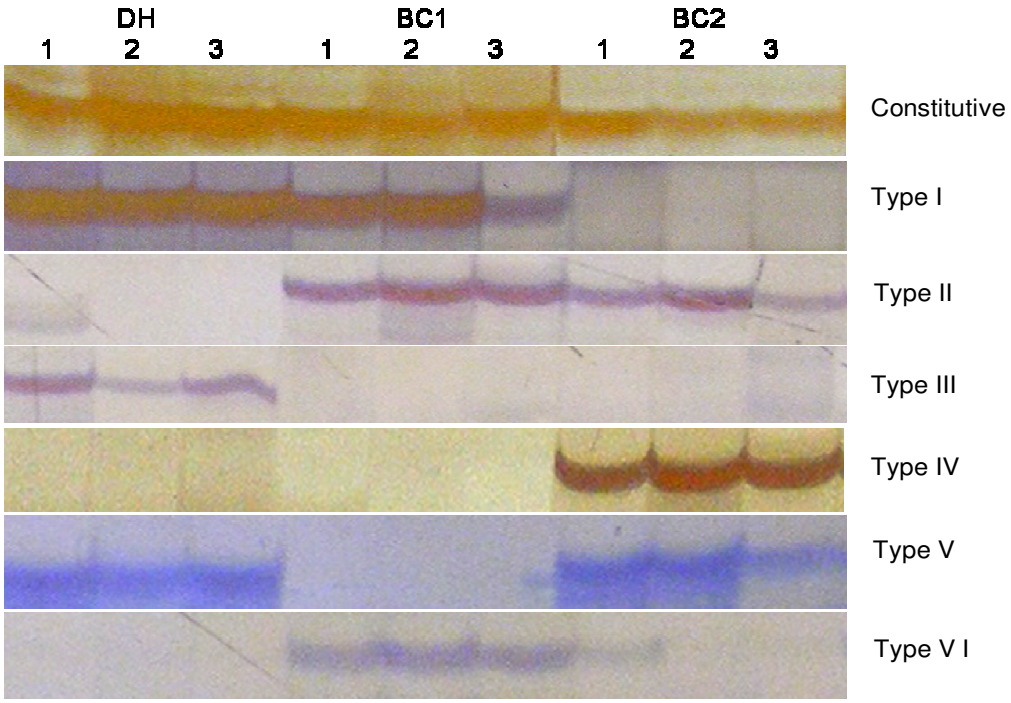

Figure 4. A model of different types of variation in DNA methylation in DH and its backcrossed progeny. Numbers 1, 2, and 3 represent replications of each plant material.

genetic region and these three types occupied 59% (Table 2). In the other three types, the DH parent showed no methylation and the offspring of its backcross did, indicating that methylation had occurred *de novo*. The presence of types II and VI points to DNA methylation in BC1 and some changes in the methylation status in type II, showed that the methylated DNA could be transmitted from BC1 and BC2, while in type VI, the DNA was un-

Table 2. Patterns of DNA methylation in DH lines and its introgression lines.

Type	DH line	BC1 generation	BC2 generation	Proportion (%)
I	+[a]	+	–[b]	32 (42/132)
II	–	+	+	27 (36/132)
III	+	–	–	19 (25/132)
IV	–	–	+	11 (14/132)
V	+	–	+	8 (11/132)
VI	–	+	–	3 (4/132)

[a] DNA methylation; [b] no methylation. The number before the slash shows the number of bands with specific transmitting features and the number after the slash shows the total number of polymorphic bands.

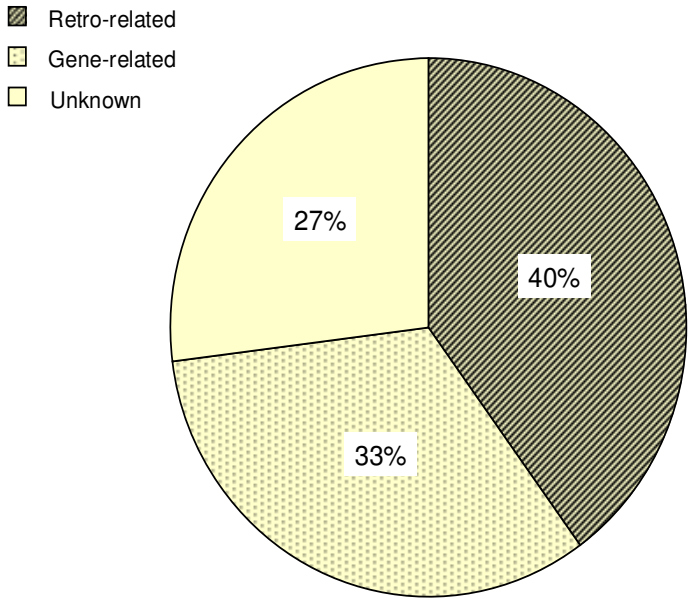

Figure 5. Proportions of the three different types of genes that responded to sequenced fragments.

methylated.

DISCUSSION

It is widely known that, fragments of genetic material from the female parent could be transferred to the male parent through chromosome recombination during backcrossing. In this study, the principle of inheritance of DNA methylation in continuous backcross generations was discovered by using TNDH lines and their backcross generations; BC1 and BC2. It was found that, methylated DNA could be transferred to the next backcross generation; with constitutive DNA methylation accounting for the bulk of the material transferred intact and that in some instances, the status of methylation underwent changes in backcross generations. This stability of constitutive DNA methylation was consistent with earlier studies in animals: Li et al. (2002) found that in fish, even when a specific allele (chromosome) derived from one species finds itself in a genetic background composed primarily of alleles from the second species (BC2 generation hybrids), it appears to conserve its methylation heritage. In plants too, Liu et al. (2004) used southern blotting to analyse DNA methylation and found that, DNA methylation status and gene transcription could be altered by introgression of alien DNA introduced by backcrossing.

In earlier reports, retro-transposon was found as an important type of movable hereditary factors widely distributed in plants and rich in DNA methylation (Lippman et al., 2004; Rabinowicz et al., 2005). In this study also, retro-transposons were found to be play an important role in modifying DNA methylation in the DH

line and the backcross generations. Of the 60 sequenced fragments, 40% were retro-transposons and accounted for the largest proportion of changed DNA methylation. Analysis of gene expression showed that, gene expression in most retro-transposons was very low, which meant that the retro-transposons were mostly without any translocated portion and once they are expressed, the target gene or phenotype showed their influence more. Yao et al. (2001) identified a gene, *MdPI*, in apetalous mutants of apples and found that, the mutation was due to a retrotransposon insertion in intron 4 and intron 6. The insertion apparently prevents normal expression of the *MdPI* gene. Kobayashi et al. (2001) found that, because of the insertion of retro-transposon in the promoter region of the regulatory gene *VvmybA1*, which controls the expression of *UFGT* gene, the phenotype of sports changes from white to red in grapes. The low expression in about 40% of the functional genes involving DNA methylation may have been due to the insertion of some retro-transposons in the target genes. Combining the two results, it can be concluded that, gene expression was suppressed not only by the insertion of retro-transposons but also by the methylation of retro-transposons.

Besides the close relationship between retro-transposons and DNA methylation in genomes of generations of backcrossing in plants, a region with modifications in DNA methylation was also found. 33% of the sequenced fragments were found to be functional genes, although, some of them coded for only hypothetical proteins. These observations are consistent with those of earlier research. In wild type *Arabidopsis*, gene body DNA methylation was found in the genome, mainly at CG sites and it is particularly noteworthy that gene body methylation is found generally in expressed genes (Zhang et al., 2006; Zilberman et al., 2007). Most of the genes identified in this study were those in which the status of DNA methylation was variable. About two-thirds of them belonged to six changeable types, a result indicating that, the expression of a gene could change in different backcross generations as the proportion of genetic background of the recipient parent continues to increase and the genetic network changes accordingly.

To conclude, two types of genes - one comprising genes related to retro-transposons and the other comprising functional genes, were found to be closely related to DNA methylation in the genome of the DH line and its backcrossed generations. This observation adds to our knowledge of genome transformation in plants and in turns, that of basic genetics and plant breeding, although, we often tend to ignore the significance of epigenetic modification and pay more attention to genetic variation available in breeding materials. We venture to suggest that researchers, especially plant breeders, pay more attention to epigenetic modification, while using backcrossing in breeding better plants.

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