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Full Length Research Paper

Methylation sensitive-sequence related amplified polymorphism (MS-SRAP) marker system and its application to *de novo* methylation detection in *Brassica napus*

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DNA methylation is one of the most important epigenetic processes that participates in the organization of chromatin structure and plays an important role in regulating gene expression. Sequence-related amplified polymorphism (SRAP) is a simple but an efficient gene amplification marker system for both mapping and gene tagging in plants. Combined methylation sensitive restriction enzyme digested genomic DNA with SRAP and methylation sensitive-sequence related amplified polymorphism (MS-SRAP) marker system was developed to investigate *de novo* DNA methylation in the plant genome. To investigate the efficiency of this new marker system, DNA samples of 5 different tissues from 3 individuals of *Brassica napus* W10 (DH line) were employed for *de novo* DNA methylation analysis. Results indicated that approximately 0.2% reproducible polymorphic fragments were found among the five different tissues. Sequencing results demonstrated that some of these polymorphic fragments were matched well with the chloroplast encoding genes, photosynthetic related genes and the genes encoding protein with unknown functions in Genbank. Therefore, MS-SRAP marker system is a simple but efficient system for detecting gene *de novo* methylation.

Key words: *Brassica napu*,*de novo* methylation,sequence related amplified polymorphism, methylation sensitive.

INTRODUCTION

Epigenetics can be defined as a study of heritable changes that modulate regional chromatin organization without altering the corresponding DNA sequence. Methylation of the C5 position of cytosine residue which (an asymmertric site, where H is A, C or T) sequences is the most common covalent modification in higher plants. The presence of 5-methylcytosine in genomic DNA is the result of enzymatic activity of the 5C DNA methyltransferases, that is, maintenance and *de novo* methyltransferases. It is thought that one of the mechanisms of *de novo* DNA methylation is guided by small interfering RNA (siRNA) (Chan et al., 2004). In contrast to *de novo* DNA methylation, plants have also developed a glycosylase-based mechanism for removing DNA methylation (Kinoshita et al., 2004; Gong et al., 2002). This epigenetic process participates in the organization of chromatin structure and plays an important role in regulating gene expression during normal plant growth (Finnegan et al., 1996; Richards, 1997).

A methylated promoter is usually associated with a close configuration and is no longer accessible to transcription factors, resulting in disabling of the activity of the

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Abbreviations: SRAP, Sequence-related amplified polymorphism; **MS-SRAP,** methylation sensitive-sequence related amplified polymorphism; **siRNA,** small interfering RNA; **PCR,** polymerase chain reaction; **YL,** young leaves; **ML,** mature leaves; **SB,** small buds; **MB,** mature buds; **F,** bagged flowers; **RNAPs,** RNA polymerase; **PEP,** plastid encoded RNAP polymerase; **NEP,** nucleus-encoded RNAP.

promoter; while un-methylated promoter displays an open chromatin structure and is accessible to transcription factors and other co-activators to initiate gene transcription. However, recent reports indicate that a DNA *de novo* methylation could spread from the coding region to the other regions such as the promoter regions both in plants and mammals, and this can also lead to an inhibition of gene transcriptin (Hisano et al., 2003; Singal and vanWert, 2001; Thomas et al., 1996). Therefore, studies on the status of DNA methylation level in the genome, especially *de novo* DNA methylation, become more and more meaningful for investigating the gene spatial expression pattern during development.

Some techniques have been developed for analyzing global and gene specific DNA methylation. For a global analysis of methylated hot-spots or CpG islands in the genome, several methods such as methylation sensitive-sequence related amplified polymorphism (MS-AFLP) (Yamamoto et al., 2001) and CpG island microarray (Gitan et al., 2002; Shi et al., 2003) have been created. But each system has its own advantages and disadvantages. For example, MS-AFLP, that is, methylation restriction enzyme is used to digest genomic DNA followed by polymerase chain reaction (PCR) amplification, and is now widely used for genomic DNA methylation detection due to its high multiplexing ratio. However, the disadvantage of this method is not only on the complexity but also on less gene fragments amplified. CpG island microarray is a high throughput technology, but considerably expensive, which prevents it to be widely used. Therefore, a strong demand for cheap and convenient marker system is required for better uncovering the *de novo* DNA methylation or demethylation genome widely.

Recently, a new marker system, sequence-related amplified polymorphism (SRAP), was developed by combining two different arbitrary primers containing AATT with CCGG core sequence, respectively (Li and Quiros, 2001). This rationale is based on the fact that exons are normally GC-rich regions, whereas AT-rich regions are normally found on promoters or introns. Approximately, 45% of the gel-isolated bands matched with known functional genes in the database of GenBank after genomic DNA was employed with PCR using this marker system and with gel separation (Li and Quiros, 2001).

In the present study, a new marker system, MS-SRAP, was developed which combined a digestion of genomic DNA using methylation sensitive restriction enzyme with the SRAP marker system. Tissue specific *de novo* methylation/de-methylation of few functional genes was revealed based on this maker system.

MATERIALS AND METHODS

Plant material and DNA extraction

Seeds of a DH line, *Brassica napus* cv W10, derived from Westar were planted in the greenhouse and used for sampling different tissues in this experiment only because an efficient gene transformation system has been developed(unpublished data). At the flowering time, tissues with the same age were sampled from different parts, that is, young leaves (YL), mature leaves (ML), small buds (SB), mature buds (MB) and bagged flowers (F) of three individuals and used for DNA extraction, respectively. Total DNA was isolated following the method of Horn and Rafalski (1992). DNA concentrations were determined with a Beckman DU640 UV/V spectrophotometer.

Arbitrary primers

Arbitrary primers were designed according to the concept of Li and Quiros (2001) and Hu and Vick (2003) (Table 1). Each of the arbitrary primers containing a core region of AATT or CCGG sequence, 3 selective nucleotides at the 3' end and the filler sequences at the 5' end, was synthesized by Sangon Company (Shanghai, China).

PCR amplifications

Five micro gram total genomic DNA from the 5 different tissues were digested with 10 U *Hpa*II restriction enzyme in a 20 µl reaction volume overnight at 37°C. After incubation, the digested products were dissolved using water to a final concentration of 50 ng/µl for PCR amplification, respectively. PCR reaction was carried out in a 15 µl volume containing 2 µl of 50 ng/µl digested DNA sample, 50 ng of each forward and reverse arbitrary primers, 200 µM of each dNTP, 1.2 U *Taq* polymerase (Promega, Madison, WI, USA) and 1×·*Taq* polymerase buffer supplied with the enzyme. The PCR reactions were done as described by Hu and Vick (2003). The amplicons were separated by denaturing acrylamide gels and detected by silver staining.

Sequencing analysis

Each polymorphic band reflecting different methylation status at the same locus among the five different tissues were excised from the acrylamide gel and transferred into a 1.5 ml tube containing 20 µl sterile ddH_2O, respectively. After boiling for 5 min in the water, the recovered DNA was dissolved and then 2 µl of them were used as the PCR templates for re-amplification with the corresponding primer pair, respectively. The re-amplified products with the expected size were inserted into pEGMT-easy vector (Promega) and sequenced by Gene Company Limited (Shanghai, China).

RESULTS AND DISCUSSION

Polymorphism

In the present study, 170 different primer combinations were performed for PCR amplification among the five different tissues. On an average, each PCR reaction can generate as much as 50 scorable fragments after separation on polyacrylamide gel. Approximately, 0.2% polymorphic fragments, which can be fully reproducible when same DNA samples were employed in independent experiment, were found among the five different tissues in our experiment. Considering methylation of the C5 position of cytosine residue which can be by methylation

Table 1. Arbitrary primers used for MS-SRAP PCR amplification in this experiment.

Primer	Code No.	Oligonucleotide sequence
Forward	me1	TgAgTCCAAACCggATA
	me2	TgAgTCCAAACCggAGC
	me3	TgAgTCCAAACCggAAT
	me4	TgAgTCCAAACCggACC
	me5	TgAgTCCAAACCggAAG
	me6	TgAgTCCAAACCggTAG
	me7	TgAgTCCAAACCggTTG
	me8	TgAgTCCAAACCggTGT
	me9	TgAgTCCAAACCggTCA
	me10	TgAgTCCAAACCggTAC
Reverse	em1	gACTgCgTACgAATTAAT
	em2	gACTgCgTACgAATTTgC
	em3	gACTgCgTACgAATTgAC
	em4	gACTgCgTACgAATTTgA
	em5	gACTgCgTACgAATTAAC
	em6	gACTgCgTACgAATTgCA
	em7	gACTgCgTACgAATTATg
	em8	gACTgCgTACgAATTAgC
	em9	gACTgCgTACgAATTACg
	em10	gACTgCgTACgAATTTAg
	em11	gACTgCgTACgAATTTCg
	em12	gACTgCgTACgAATTgTC
	em13	gACTgCgTACgAATTGgT
	em14	gACTgCgTACgAATTCAg
	em15	gACTgCgTACgAATTCTg
	em16	gACTgCgTACgAATTCgg
	em17	ggggCCgCgggAATTCgAT

sensitive restriction enzyme, *Hap*II is only one portion in the genome of *B. napus*. This indicates that the percentage of the *de novo* methylation/de-methylation on the genome scale must be over 0.2%. After being separated, 18 polymorphic fragments were revealed in this experiment. According to the appearance of different tissues, these loci were mainly divided into four different patterns (Table 2). Nine of them are only present in bagged flowers which are named as F specific type (Figure 1A); five are present both in the YL and ML but absent in other tissues which are called leaf specific type (Figure 1B); and the other two types, which scarcely appeared, were called ML specific type and ML and F specific type. In these two types, polymorphic fragment is present in mature leaves and both mature leaves and bagged flowers, respectively. Because the different DNA samples from different tissues have actually the same DNA constitute, the polymorphic fragments then might reflect a different methylation status of the DNAs from different tissues if PCR templates were fully digested with methylation sensitive restriction enzyme. As for these 18 different methylated/de-methylated loci revealed in the different tissues of *B. napus*, only the gene coding sequences could be used for analyzing the relationship between gene expression and DNA metylation/de-methylation, because the non-coding sequences cannot be assigned to the specific region upstream of a coding gene if the whole genome sequence is still unavailable such as in *B. napus*.

Fortunately, as revealed by Li and Quiros (2001) that at least 40% of polymorphic fragments are representing a coding sequence, it is also likely that about 40% of these 18 polymorphic fragments are coding sequences. Additionally, DNA methylation can spread from promoter region to the coding region or from coding region to the promoter region, and then *de novo* genes' methylation among these polymorphic fragments is possibly associated with the silencing of gene expression. Thereafter, it may provide an opportunity to investigate the biological roles that gene *de novo* methylation played during development. For example, the important case detected in this experiment may imply that silencing of genes expression via epigenetic modification is common and is so much essential for embryo development; by

Table 2. Characteristics of the polymorphic bands amplified from five tissues of *B. napus* at the flowering stage using 15 primer combinations.

Combination No.	Fragment size (bp)	Specific Type	Homologous analysis (identity)	Function (predicted function)
me1×em4	99	F	AT5G54190 (76%)	Similar to protochlorophyllide reductase B
me1×em10	206	F	AT3G07580 (75%)	Expressed protein
me2×em8	105	F	/	/
me2×em9	237	F	/	/
me4×em6	155	F	/	/
me4×em8	98	F	/	/
me8×em14	88	F	/	/
me9×em5	107	F	/	/
me10×em12	184	F	/	/
me2×em10	132	L	ATCG00190 (93%)	Chloroplast DNA-dependent RNA polymerase B subunit
me2×em17	143	L	ATCG01000 (90%)	Expressed protein
me2×em11	169	L	ATCG00950 (99%)	Chloroplast-encoded 23S ribosomal RNA
me2×em16	221	L	ATCG00680 (89%)	Encodes for CP47, subunit of the photosystem II reaction center
me7×em4	119	L	/	/
me1×em3	150	ML and F	/	/
me8×em14	185	ML and F	AT4G32620 (84%)	Expressed protein
me8×em14	280	ML and F	/	/
me8×em14	127	ML	AT1G70770 (80%)	Expressed protein

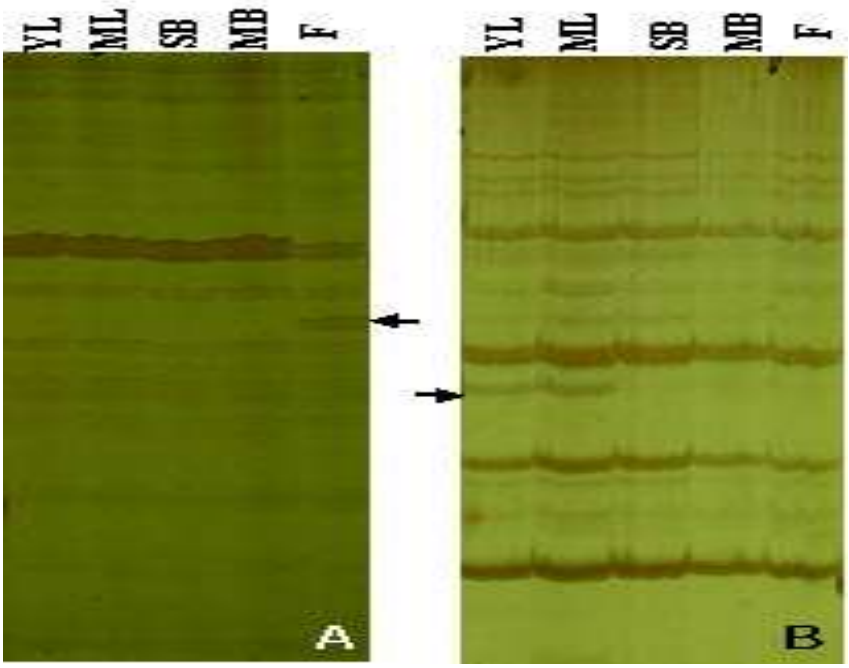

Figure 1. Two major types of *de novo* DNA methylation were found by using MS-SRAP marker system. A, Leaf specific type *de novo* DNA methylation revealed with primer pair me1 and em4. The arrow showed the polymorphic bands presented in young and mature leave tissues of *B. napus*; B, Flower specific type *de novo* DNA methylation revealed with me10 and em12. The arrow showed the polymorphic band only presented in flower tissue of *B. napus*. YL represents young leaves; ML represents mature leaves; SB represents small buds; MB represents mature buds and F means bagged flowers.

contrast, DNA *de novo* de-methylation events occurred in the reproductive tissues relative to the vegetative tissues that may reflect that the initiation of some genes' expression is possibly very essential for promoting plant to transit from vegetative to reproductive stage.

Sequencing of polymorphic fragments

Eighteen polymorphic bands from amplification with 15 different primer combinations were isolated and inserted into TA-clone vector. Two individual inserts representing each polymorphic fragment were sequenced. After a Basic Local Alignment Search Tool (BLAST) search, 8 out of 18 loci shared significant similarities to the sequences of public genes in Genbank (Table 2). Interestingly, some sequences are similar to chloroplast-encoded genes such as *rpoB* (Chloroplast DNA-dependent RNA polymerase B subunit), *psbB* (psII 47 Kda protein) and chloroplast-encoded *23S ribosomal RNA* gene.

Previous studies showed that some sequences homologous to plastid encoded genes were found to be methylated in amyloplast and resulted in a reduced expression ability (Macherel et al., 1985). Except for the case of *Chlamydomonas reinhardtii*, methylated DNA is not usually found in chloroplasts (Sager and Lane, 1972). However, whether the expression of the methylated gene homologous to chloroplast-encoded genes also have been silenced or reduced in this study, it remains unclear. Among them, *rpoB*, encoding one of the subunit of plastid encoded RNA polymerase (RNAPs), is one of the most interesting genes. The plastid genome is transcribed by two distinct RNAPs, the plastid encoded RNAP (PEP) and nucleus-encoded RNAP (NEP). Some plastid genes transcribed exclusively by PEP; some by NEP only, and the remaining parts transcribed by both of them (Eberhard et al., 2002; Cahoon et al., 2004). It is thought that NEP expressed and played a role at the early stage of plastid development, while PEP expressed at the mature stage. However, the mechanism of the dynamic relationship between the activities of PEP and NEP remains unknown.

The different methylation of *rpoB* gene discovered in this experiment from different tissues may provide a clue for further investigating the dynamic relationship between the activities of PEP and NEP. In addition to plastid encoded genes, photosynthetic related gene such as *Protochlorophyllide reductase B* gene, and the other genes responsible for encoding unknown protein were revealed as well by using this maker system. In conclusion, MS-SRAP marker system is reliable for genome-wide *de novo* gene methylation/de-methylation investigation.

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