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Full Length Research Paper

Analysis of DNA methylation variation in sibling tobacco (*Nicotiana tabacum*) cultivars

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Amplified fragment length polymorphism (AFLP) and methylation-sensitive amplification polymorphism (MSAP) analysis were used to investigate the genome of two sibling tobacco cultivars, Yunyan85 and Yunyan87, their parent K326 and the other tobacco cultivar NC89. AFLP analysis indicated that, the genome primary structure variations were not obviously detected among Yunyan85 and Yunyan87 and K326. However, the methylation extent had different alteration between the sibling tobacco cultivars relative to their parent; it rose to 42.97 and 36.55% in Yunyan85 and Yunyan87, respectively. Both the full and semi-methylation modifications on the 'CCGG/GGCC' site were significantly increased in Yunyan85 and Yunyan87, however, the full-methylation modification on the 'CCGG/GGCC' site in Yunyan85 was significantly higher than the one in Yunyan87. Among the polymorphic methylated sites, most of them (53.70%) were hypermethylation sites, 35 (16.20%) sites were demethylation modification and 62 (28.70%) sites were uncertain methylation modification. The sequences involved in methylation alteration mainly included promoter regions. The results in this study have provided a better understanding of gene expression phenotype.

Key words: *Nicotiana tabacum*, methylation, amplified fragment length polymorphism (AFLP), methylation-sensitive amplification polymorphism (MSAP).

INTRODUCTION

Central dogma has told us that the genetic code heredi-table changes are inevitably related with the changes in DNA sequences. However, researchers have already realized that both DNA sequence and DNA structure are inherited. That is, gene expression is determined not only by the nucleotide sequence of DNA but also by chromo-somal structures including interaction of DNA with one another and also with proteins, RNA molecules and other macromolecules (Tchurikov, 2005). This case has already been well known as "epigenetics". The term "epigenetics" has evolved to include the process that alters gene activity without changing the DNA sequence and leads to modifications that can be transmitted to daughter cells (Weinhold, 2006). It has been well known that methy-lation, acetylation, phosphorylation, ubiguitylation and sumolyation are all involved in epigenetic processes (Weinhold, 2006). Until now, the best known epigenetic process is DNA methylation, partly because it has been the easiest to study with existing technology. In higher plant genome, about 20 to 50% cytosines are methylated, among which about 90% methylated sites lie in 'CpG' dinucleotide or 'CpNpG' trinucleotide (Madlung et al., 2002; Chan et al., 2005; Xiao et al., 2006). DNA methy-lation plays an essential role in many basic research and applied research fields of life sciences and is closely related to gene expression (Assad et al., 1993; Matzke and Matzke, 1998; Akimoto et al., 2007), germ-cell development (Sasaki and Matsui, 2008), embryonic development (Dyban and Dyban, 2006), cell differen-tiation (Koukalova et al., 2005; Sjakste and Sjakse, 2007; Li et al., 2007), genomic imprinting (Platonov and Isaev, 2006; Takeda and Paszkowski, 2006), sex expression (Janoušek et al., 1996) and so on. The cytosine methy-lation of tobacco 18S, 5.8S and 26S rRNA genes in leaf

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Abbreviations: AFLP, Amplified fragment length polymorphism; **MSAP,** methylation-sensitive amplification polymorphism; **PCR,** polymerase chain reaction; **PH,** polymorphism site; **PD,** demethylation polymorphism; **PU,** uncertain polymorphism; **MZ,** monozygotic.

Table 1. Sequences of AFLP and MSAP adaptors primers used in this study.

Adaptor	Sequence
*Eco*R I-adaptors-F	5′CTCGTAGACTGCGTACC3′
*Eco*R I-adaptors-R	5′AATTGGTACGCAGTC3′
Mse I-adaptors-F	5′GACGATGAGTCCTGAG3′
Mse I-adaptors-R	5′TACTCAGGACTCAT3′
*Hpa*II/*Msp*I-adaptors-F	5′GATCATGAGTCCTGCT3′
*Hpa*II/*Msp*I-adaptors-R	5′CGAGCAGGACTCATGA3′
Preselective primers	
*Eco*RI-A	5′GACTGCGTACCAATTCA3′
*Mse*I-C	5′GACGATGAGTCCTGAGTAAC3′
*Hpa*II/*Msp*I-T	5′ATCATGAGTCCTGCTCGGT3′
Selective primer combinations used in AFLP	
*Eco*RII-ACA+*Mse*I-CAA	*Eco*RI-AAG+*Mse*I-CTT
*Eco*RI-AAG+*Mse*I-CAA	*Eco*RI-ACA+*Mse*I-CCT
*Eco*RI-ACA+*Mse*I-CTA	*Eco*RI-ACG+*Mse*I-CAA
*Eco*RI-AAG+*Mse*I-CAG	
Selective primer combinations used in MSAP	
*Eco*RI-ACA+*Hpa*II/*Msp*I-TCAA	*Eco*RI-AAC+*Hpa*II/*Msp*I-TCTG
*Eco*RI-AAC+*Hpa*II/*Msp*I-TCTC	*Eco*RI-AAG+*Hpa*II/*Msp*I-TCAA
*Eco*RI-AAC+*Hpa*II/*Msp*I-TCTT	*Eco*RI-AAC+*Hpa*II/*Msp*I-TCTA
*Eco*RI-AAC+*Hpa*II/*Msp*I-TCAT	*Eco*RI-AAG+*Hpa*II/*Msp*I-TCTA

calli and in regenerated plants and their progeny were studied (Koukalova et al., 2005). The DNA methylation patterns in trangsgenic tobacco plants have been extensively investigated (Fojtova et al., 2003; Kim et al., 2007; Oh et al., 2009). Few studies have paid their attentions to the DNA menthylation of sibling tobacco (*Nicotiana tabacum*) cultivars. Characterizing the methylation alteration of sibling tobacco cultivars will provide a better understanding of gene expression phenotype. In the present study, two sibling tobacco cultivars and their parents were used to investigate the methylation pattern at 'CCGG/GGCC' site with methylation-sensitive amplification polymorphism (MSAP) technique (Zhang et al., 2008).

MATERIALS AND METHODS

Plant materials

Two sibling tobacco cultivars, Yunyan 85 and Yunyan87 and their parent, tobacco cultivar K326, were used. The pedigree of the sibling tobacco cultivars was described by Tan et al. (1997) and Li et al. (2001). In addition, another tobacco cultivar NC89 was also used. These tobacco cultivars were planted in Ya'an, Sichuan, China and their leaves were collected on 20 August 2009. The genomic DNA was isolated from leaf tissue according to Tang et al. (2008).

Amplified fragment length polymorphism (AFLP) and methylation-sensitive amplification polymorphism (MSAP) analysis

EcoR I and *Mse* I were used for restriction reaction in amplified fragment length polymorphism (AFLP) analysis and *Hpa*II/*Msp*I and *EcoR* I were used for methylation-sensitive amplification polymorphism (MSAP) analysis. The AFLP and MSAP procedures were performed according to Zhang et al. (2008). The *EcoR* I, *Mse* I and *Hpa*II/*Msp*I adaptor, the preselective primers and the selective primer combinations are listed in Table 1.

In both AFLP and MSAP procedures, repeats were carried out and patterns resulting from two independent digestions were compared for each sample. In addition, for both AFLP and MSAP gels, the upper and the lower part of the gel, resolution was not satisfactory and were not used for band scoring. Only stable and repeatable patterns were retained for analysis.

Isolation and sequencing of polymorphic methylated fragment

The polymorphic MSAP fragments were isolated from polyacrylamide gels, reamplified by polymerase chain reaction (PCR) and sequenced. The procedures were performed according to Zhang et al. (2008).

RESULTS

The genetic diversity of the tobacco cultivars

Using 7 different AFLP selective primer combinations, 258 bands were obtained. The polymorphic amplified sites were not observed among Yunyan85, Yuanyan87 and K326 (Figure 1). However, the different AFLP bands were observed between NC89 and the other three tobacco cultivars (Figure 1). These results indicated that, the distinct variations of the genome primary structure of the sibling tobacco cultivars and heir parent have not occurred.

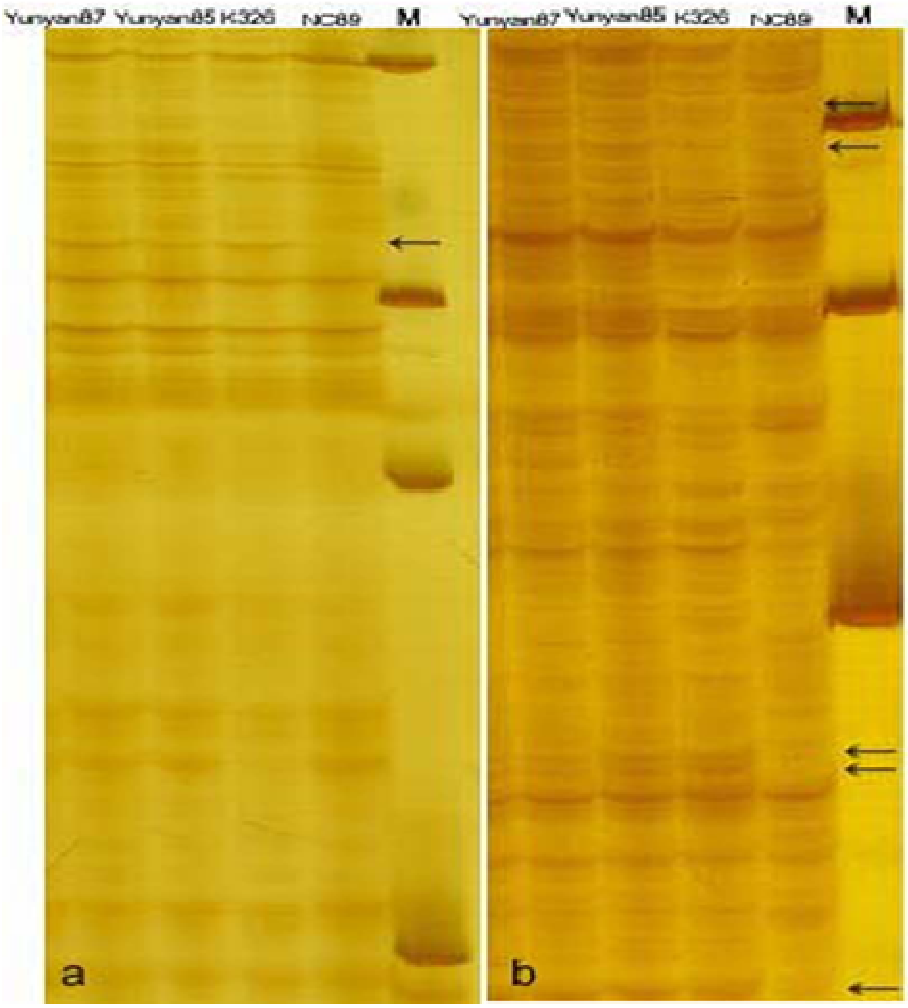

Figure 1. AFLP fingerprints of genomic DNA of tobacco cultivars NC89, K326, Yunyan85 and Yunyan87. a, b selective amplification results with different selective primer combinations; M, DNA marker; Arrows indicate the polymorphic amplified sites between NC89 and the other three tobacco cultivars.

Genomic DNA methylation extent in the tobacco cultivars

*Hpa*II will not cut if either of the outer or the inner cytosine of the 'CCGG/GGCC' site is fully (double-strand) methylated, whereas, *Msp*I will not cut if the external cytosine is fully or hemi-(single-strand) methylated (Reyan-Lopez et al., 1997). The methylation states of the cytosine at 'CCGG/GGCC' site would lead to a differential cleavage by two isoschizomers and thus, to the appearance of different MSAP fragments in the sequencing gel. Because of the different responses to different methylation statuses, the band pattern from PCR amplification can reflect the methylation status at the certain site. Thus, according to presence or absence of polymorphic methylated fragment, methylation patterns of genomic DNA from different samples can be divided into three types with the 'CpG' methylation status of the 'CCGG/GGCC' sites as follows: Class A, present for both enzymes (*Hpa* II+/*Msp* I+), which means no methylated cytosine on double strand DNA or inner methylated cytosine on single strand DNA ('CCGG/GGCC', no-methylation); class B, absent for *Hpa* II but present for *Msp* I (*Hpa* II–/*Msp* I+), which means methylated inner cytosine on double strand DNA ('$C^{5m}CGG/GG^{5m}CC$', full-methylation); class C, present for *Hpa* II but absent for *Msp* I (*Hpa* II+/*Msp* I–), which means methylated outer cytosine on single strand DNA ('$^{5m}CCGG/GGCC$', hemi-methylation).

The eight pairs of *Eco*R I+*Hpa* II/ *Msp* I selective primer combinations have produced legible and reproducible fragments at 249 sites. Of these 249 sites, 76 (30.52%), 78 (31.33%), 107 (42.97%) and 91 (36.55%) methylated sites were detected in NC89, K326, Yuanyan 85 and Yuanyan87, respectively (Table 2). This result indicated that, apparent differences of the genomic DNA methylation extent existed among the tobacco cultivars used in

Table 2. Number of bands amplified using eight MSAP selective primer combinations in the tobacco cultivars.

Primer combination	Amplification site	NC89		K326		Yunyan85		Yunyan87	
		Methylated site		Methylated site		Methylated site		Methylated site	
		Fully	Hemi	Fully	Hemi	Fully	Hemi	Fully	Hemi
E-ACA+H/M-TCAA	27	3	5	10	0	7	6	4	3
E-AAC+H/M-TCTC	22	4	3	0	5	7	6	1	5
E-AAC+H/M-TCTT	30	9	0	3	4	7	5	8	7
E-AAC+H/M-TCAT	25	6	6	4	8	10	0	1	7
E-AAC+H/M-TCTG	47	2	9	7	5	6	13	9	10
E-AAG+H/M-TCAA	48	6	4	2	11	14	12	10	8
E-AAC+H/M-TCTA	22	5	4	6	4	3	3	4	1
E-AAG+H/M-TCTA	28	6	4	5	4	3	5	4	9
Total	249	41	35	37	41	57	50	41	50
		76		78		107		91	
Rate of methylated site (%)		16.47	14.06	14.86	16.47	22.89	20.08	16.47	20.08
		30.52		31.33		42.97		36.55	

this study. Further analysis exhibited that, the number of fully-methylated sites were 41 (16.47%), 37 (14.86%), 57 (22.89%) and 41 (16.47%) in NC89, K326, Yuanyan 85 and Yuanyan87, respectively and the corresponding hemi-methylated sites were 35 (14.06%), 41 (16.47%), 50 (20.08%) and 50 (20.08%) (Table 2). When compared with K326, both the fully-methylated and the semi-methylated sites in Yuanyan 85 and Yuanyan87 were increased. Between Yuanyan 85 and Yuanyan87, the hemi-methylated sites were identical; however, the fully-methylated sites were different.

Variation of methylation patterns in sibling cultivars and their parent

Through comparing MSAP amplified fingerprints among K326, Yunyan85 and Yunyan87, the polymorphism sites were observed. (Figure 2) and these methylation sites were classed into two major types (Table 3), monomorphism and polymorphism site. Three monomorphism methylated sites were observed among the two sibling cultivars and their parent, which comprised 1.39% methylaion site detected and only hemi-methylated sites were observed. The other 213 methylated sites comprising 98.61% methylated sites exhibited polymorphism among the three cultivars (Table 3). According to the extent of methylation variation in 'CCGG/GGCC' site, the polymorphic methylation sites were further divided into three types: (1) Hypermethylation polymorphism site (PH), the extent of cytosine methylation of this site in sibling tobacco cultivars was stronger than their parent; (2) demethylation polymorphism site (PD), the extent of cytosine methylation of this site in sibling tobacco cultivars was weaker than their parent; (3) uncertain polymorphism site (PU), the extent of cytosine methylation of this site could not be accurately qualitative compared among the three tobacco cultivars. The number of PH, PD and PU sites were 116 (53.70%), 35 (16.20%) and 62 (28.70%), respectively. There were 21 kinds of hypermethylation polymorphic sites (PH1-PH21), 14 kinds of demethylation polymorphism sites (PD1-PD14) and 13 kinds of uncertain polymorphism sites (PU1-PU13). Among the polymorphic methylation sites, 7 sites (PH3, PH10, PH13, PD4, PD5, PD13 and PD14) exhibited consistent cytosine methylation between Yunyan85 and Yuanyan87, but different from their parent K326. The other polymorphic methylation sites displayed different cytosine methylation among Yunyan85, Yuanyan87 and K326.

Sequencing of polymorphic MSAP fragments

Sixteen MSAP fragments that exhibited methylation alteration in Yunyan85, Yuanyan87 and K326 were recovered and sequenced. The BLAST (Nucleotide blast) searches showed that, these sequences involved in methylation alteration included promoter regions, chloroplast DNA, mRNA sequences in *N. tabacum*, bacteria artificial chromosome (BAC) clone in *Capsicum* annuum and *N. tabacum nia-1* gene for nitrate reductase (Table 4).

DISCUSSION

Recent epigenetic research showed that, DNA methylation is one of the major mechanisms which control gene expression. The samples with identical maternals and parental sets of chromosomes are the best models for the research on the relationship between DNA methylation and gene expression. For example, particularly, DNA methylation could lead to discordance of monozygotic (MZ) twins (Singh et al. 2002). Some other reports have also indicated that, epigenetics was associated with MZ twin

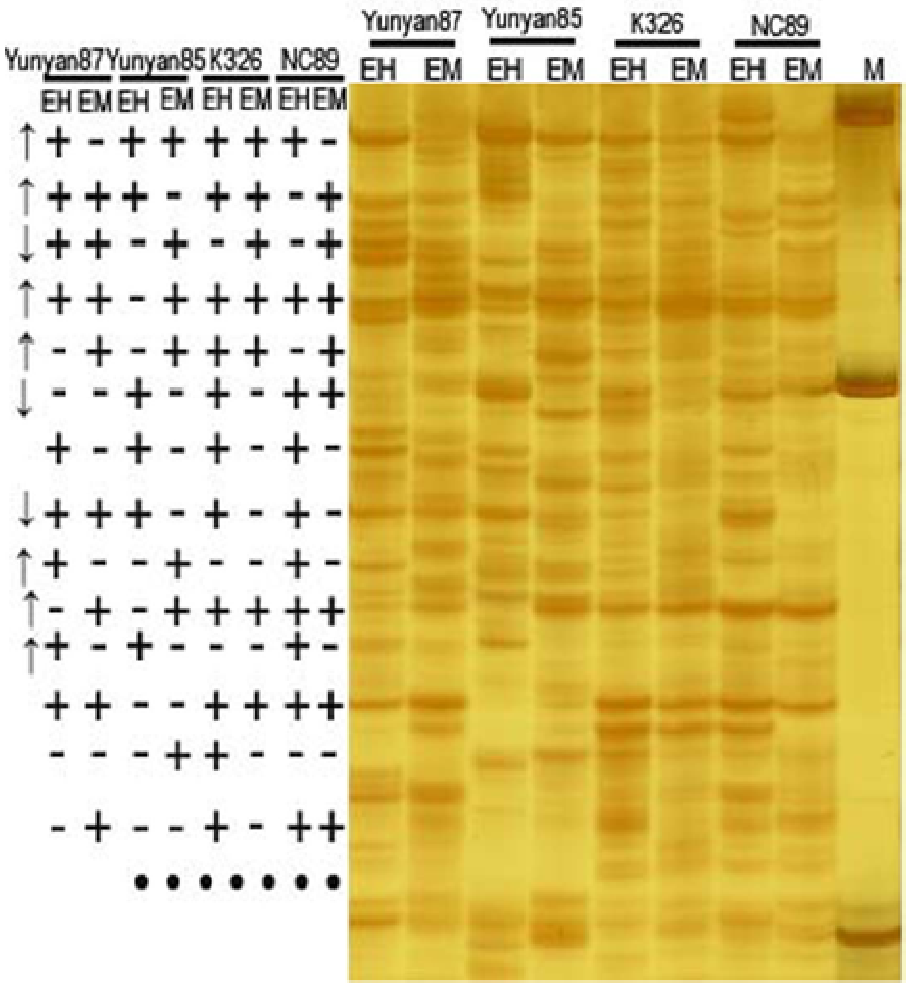

Figure 2. MSAP fingerprints of genomic DNA of tobacco cultivars NC89, K326, Yunyan85 and Yunyan87 and the variation of methylation pattern. M, DNA marker; EH, *EcoR* I+*Hpa* II; EM, *EcoR* I+ *Msp* I; –, band absent; +, band present; ↑, methylation extent increase; ↓, methylation extent decrease.

Table 3. Patterns of cytosine methylation in tobacco.

Methylation patterns		K326		Yunyan85		Yunyan87		Number of sites	Total (%)
		EM	EH	EM	EH	EM	EH		
Monomorphic sites		-	+	-	+	-	+	3	3 (1.39%)
Hypermethylation sies	PH1	-	-	+	-	-	-	15	116 (53.70%)
	PH2	-	-	-	+	-	-	15	
	PH3	-	-	-	+	-	+	5	
	PH4	-	-	+	-	-	+	9	
	PH5	-	-	-	-	-	+	11	
	PH6	-	-	-	-	+	-	5	
	PH7	-	+	+	-	-	+	5	
	PH8	-	+	+	-	-	-	2	
	PH9	-	+	-	-	+	-	4	
	PH10	-	+	+	-	+	-	1	
	PH11	+	+	-	+	+	-	5	
	PH12	+	+	+	-	+	+	4	

Table 3. Continue

	PH13	+	+	+	-	+	-	5	
	PH14	+	+	+	+	-	+	1	
	PH15	+	+	+	-	-	-	6	
	PH16	+	+	-	-	+	-	8	
	PH17	+	+	-	-	-	+	3	
	PH18	+	+	+	+	+	-	4	
	PH19	+	+	+	-	-	+	3	
	PH20	+	+	-	+	-	-	3	
	PH21	+	+	-	+	+	+	2	
Demethylation sites	PD1	+	-	+	+	+	-	2	35 (16.20%)
	PD2	+	-	+	-	+	+	4	
	PD3	+	-	-	-	+	+	5	
	PD4	+	-	-	+	-	+	1	
	PD5	+	-	+	+	+	+	4	
	PD6	+	-	-	+	-	-	3	
	PD7	+	-	+	-	-	+	1	
	PD8	+	-	-	-	-	+	2	
	PD9	-	+	+	+	-	+	2	
	PD10	-	+	-	-	-	-	6	
	PD11	-	+	-	+	+	+	1	
	PD12	-	+	-	-	+	+	1	
	PD13	-	+	+	+	+	+	1	
	PD14	-	-	+	+	+	+	2	
Uncertain sites	PU1	+	-	-	-	-	-	7	62 (28.70%)
	PU2	+	-	-	-	+	-	6	
	PU3	+	+	-	-	-	-	7	
	PU4	+	+	-	-	+	+	7	
	PU5	+	+	+	+	-	-	1	
	PU6	-	+	-	+	-	-	10	
	PU7	-	+	-	-	-	+	5	
	PU8	-	+	+	-	+	+	1	
	PU9	-	-	+	+	+	-	1	
	PU10	-	-	-	+	+	+	1	
	PU11	-	-	+	+	-	-	4	
	PU12	-	-	+	+	-	+	5	
	PU13	-	-	-	-	+	+	7	

discordance for common diseases (Poulsen et al., 1999; Bjornsson et al., 2004). In plant, it has been reported that the different phenotypes of sibling wheat cultivars were mainly caused by the alterations of methylation patterns (Zhang et al., 2008). In the present study, the sibling tobacco cultivars and their parent were investigated. Some agronomical traits of these tobacco cultivars such as plant height, chemical components, yield, etc. were different (Tan et al., 1997; Li et al., 2001). The distinct variations of the genome structure of the sibling tobacco cultivars and their parents were not observed (Figure 1). However, the methylation extent had different alteration between the sibling tobacco cultivars relative to their parents: it rose to 42.97 and 36.55% in Yunyan85 and Yunyan87, respectively (Table 2). Both the full-methylation and semi-methylation modifications on the 'CCGG/GGCC' site were significantly increased in Yunyan85 and Yunyan87, however, the full-methylation modification on the 'CCGG/GGCC' site in Yunyan85 was significantly higher than that in Yunyan87 (Table 2). Among the polymorphic methylated sites, most of them (53.70%) were hyper-methylation sites, 35 (16.20%) sites were demethylation modification and 62 (28.70%) sites were uncertain methylation modification (Table 3). Therefore, the different phenotypes among Yunyan85, Yuanyan87 and K326 were mainly caused by these variations of methylation modification patterns. Both the results obtained in present study and in previous studies indicated that, DNA methylation has effects on gene expression phenotype. In addition, the genome structure of the cultivar, NC89, has

Table 4. Sequence analysis of the polymorphic methylated fragments.

Sequence genbank accession no.	Length (bp)	Homologous sequence (genbank accession no.)	E value
HN153630	299	None; Contain CAAT box	None
HN153632	276	None; Contain CAAT box	None
HN153639	294	None; Contain CAAT box and GC box	None
GS926393	204	None; Contain CAAT box and TATA box	None
GS926395	204	None; Contain CAAT box	None
HN153631	216	None; Contain CAAT box and TATA box	None
HN153633	215	None; Contain CAAT box	None
GS926394	239	None; Contain CAAT box	None
HN153634	204	None; Contain CAAT box	None
HN153635	211	None; Contain CAAT box and GC box	None
HN153640	287	*Capsicum frutescens* clone BAC PEPBAC268G7 (FJ597541)	6e-67
HN153641	151	*N. tabacum* A22 mRNA for hypothetical protein (AB186042.1)	0.47
HN153642	276	*N. tabacum* nia-1 gene for nitrate reductase (X14058.1)	2e-92
HN153636	278	*N. tabacum* nia-1 gene for nitrate reductase (X14058.1)	1e-93
HN153637	278	*N. tabacum* nia-1 gene for nitrate reductase (X14058.1)	3e-95
HN153638	236	*Nicotiana sylvestris* chloroplast DNA (AB237912.1)	3e-101

displayed distinction from the other three cultivars, therefore, the DNA methylation patterns of NC89 was different from the ones of the other three cultivars, which was reasonable.

In previous studies, the methylation sequences in plants were involved in repetitive sequences, transposable elements, low-copy DNA sequences, coding and promoter sequences (Madlung et al., 2002; Chan et al., 2005; Cheng et al., 2006; Zhang et al., 2008). The main target sequences for methylation mutation were the transposable elements and other repetitive sequences and these variations could affect many plant phenotypic characteristics such as florescence, fertility, morphology, etc (Madlung et al., 2002; Dong et al., 2006; Cheng et al., 2006). Choi and Kim (2007) have investigated the phenotypic discordance between MZ twins and indicated that, epigenetic modifications might occur more frequently in heterochromatic and gene-poor regions in response to environmental signals while gene-rich regions tend to remain in an active chromatin configuration for the constitutive expression of underlying genes. In the present study, the sequences involved in methylation alteration mainly included promoter regions (Table 4). It has been proposed that, the biological function of DNA methylation is involved in gene silencing, often being associated with hypermethylation of promoter sequences (Paszkowski and Whitham, 2001; Bird, 2002). The results in the present study indicated that, methylation alteration of promoter regions may be mainly responsible for gene phenotype expression.

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