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Variation of genomic DNA methylation in the nitrate reductase gene of sibling tobacco (*Nicotiana tabacum*) cultivars

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ABSTRACT. To better understand genomic DNA methylation in sibling plant cultivars, methylation-sensitive amplification polymorphism analysis was used to investigate two sibling tobacco cultivars, Yunyan85 and Yunyan87, and their two parents, K326 and Yunyan No. 2. Differences in the degree of genomic DNA methylation were found among the four tobacco cultivars. Compared with parents, the two sibling cultivars had fewer methylated sites. Twenty-nine methylation-sensitive amplification polymorphism fragments that exhibited methylation alteration in the four tobacco cultivars were recovered and sequenced. BLAST (nucleotide BLAST) searches showed that two of the 29 sequences have 99% similarity with nucleotides 1442-1694 of the *nia-1* gene and the other 27 sequences contain GC, CAAT or TATA box. The nitrate reductase genes from Yunyan87, K326 and Yunyan No. 2 were found to be identical; however, the third intron of the nitrate reductase gene from Yunyan85 was different compared to the third introns of Yunyan87, K326 and Yunyan No. 2. We conclude that methylation alteration of promoter regions could be responsible for the different phenotypes in tobacco and that introns of the nitrate reductase

gene can vary as a result of intra-species crossing in tobacco.

Key words: Tobacco; Sibling cultivars; Methylation; NR gene; Intron variation

INTRODUCTION

It is well known that methylation is involved in epigenetic processes (Weinhold, 2006). In the higher plant genome, about 20-50% of cytosines are methylated, among which about 90% methylated sites lie in 'CpG' dinucleotides or 'CpNpG' trinucleotides (Madlung et al., 2002; Chan et al., 2005; Xiao et al., 2006). DNA methylation plays an important role in gene expression (Assaad et al., 1993; Matzke and Matzke, 1998; Akimoto et al., 2007). The cytosine methylation of tobacco (*Nicotiana tabacum*) 18S, 5.8S and 26S rRNA genes in leaf calli and in regenerated plants and their progeny have been studied (Koukalova et al., 2005). The DNA methylation patterns in transgenic tobacco plants have been extensively investigated (Fojtova et al., 2003; Oh et al., 2009). These previous studies have given us direct evidence that genomic DNA methylation has a close relationship with gene expression. During plant breeding processes, some sibling cultivars have been developed. These sibling cultivars are the best models for research on the relationship between DNA methylation and gene expression. Characterizing the methylation alteration of sibling plant cultivars will provide a better understanding of the gene expression phenotype, because they often have a similar genetic background and genomic structure (Zhang et al., 2008). Yang et al. (2011) have reported the different genomic DNA methylation pattern between two sibling tobacco cultivars with similar genomic structure. The GC methylation has already been discovered in the nitrate reductase (NR) gene.

NR is an NAD(P)H-dependent enzyme and it is responsible for the conversion of nitrate to nitrite, which is considered to be the rate-limiting step in nitrogen assimilation. NR has been shown to be the subject of multifactorial control at the level of both gene expression and posttranslational modification (Kaiser and Huber, 1994; Kaiser et al., 1999). Analysis of NR genes in plants showed that their expressions were primarily regulated at the transcriptional level (Campbell, 1999). Some genes for nitrate reductase have been cloned from fungi (Fu and Marzluf, 1987; Okamoto et al., 1991), algae (Falcão et al., 2010) and higher plants (Choi et al., 1989; Vaucheret et al., 1989; Wilkinson and Crawford, 1991; Wu et al., 1995) and their evolutionary relationships were investigated (Zhou and Kleinhofs, 1996). The NR genes from fungi, algae and higher plants show substantial sequence similarity, particularly within functional domains, and large variations in GC content at the third codon position and in intron number (Zhou and Kleinhofs, 1996). The intron positions are different between fungi and plants, but conserved within these groups (Zhou and Kleinhofs, 1996). Although NR genetics, biochemistry and molecular biology have been intensively investigated, little is known about the NR gene variation between sibling tobacco cultivars.

In the present study, two sibling tobacco cultivars and their parents were first used to investigate the methylation pattern at the 'CCGG/GGCC' site. The sequences involved in methylation contained the NR gene sequence. Thereafter, the NR genes were cloned from the two sibling tobacco cultivars and their parents, and the variation in the NR gene between the two sibling tobacco cultivars was determined; the result are discussed.

MATERIAL AND METHODS

Plant materials

Two sibling tobacco cultivars, Yunyan85 and Yunyan87, and their parents, tobacco cultivar K326 and Yunyan No. 2, were used. The pedigree of the two sibling tobacco cultivars was described by Tan et al. (1997) and Li et al. (2001). The two sibling cultivars have been proven to have highly similar genomic structure (Yang et al., 2011).

DNA extraction

Tobacco cultivars Yunyan85, Yunyan87, K326 and Yunyan No. 2 were planted in Yibin, Sichuan, China, and their leaves were collected on July 13, 2010. Genomic DNA was isolated from leaf tissue according to Tang et al. (2008).

Methylation-sensitive amplification polymorphism (MSAP) analysis

*Hpa*II/*Msp*I and *Eco*RI restriction enzymes were used for MSAP analysis. The MSAP procedures were performed according to Zhang et al. (2008). The *Eco*RI and *Hpa*II/*Msp*I adaptors, the pre-selective primers, and the selective primer combinations are listed in Table 1. In MSAP procedures, repeats were carried out and patterns resulting from two independent digestions were compared for each sample. In addition, for MSAP gels, the upper part and the lower part of the gel, where resolution was not satisfactory, were not used for band scoring. Only stable and repeatable patterns were retained for analysis.

Table 1. Sequences of methylation-sensitive amplification polymorphism (MSAP) adaptors and pre-selective and selective primers used in this study.

Adaptors	
*Eco*RI-adaptors-F	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI-adaptors-R	5'-AATTGGTACGCAGTC-3'
*Hpa*II/*Msp*I-adaptors-F	5'-GATCATGAGTCCTGCT-3'
*Hpa*II/*Msp*I-adaptors-R	5'-CGAGCAGGACTCATGA-3'
Pre-selective primers	
*Eco*RI-**A**	5'-GACTGCGTACCAATTC**A**-3'
*Hpa*II/*Msp*I-**T**	5'-ATCATGAGTCCTGCTCGG**T**-3'
Selective primer combination	
*Eco*RI-**AAG**+*Hpa*II/*Msp*I-**TCAG**	*Eco*RI-**AAC**+*Hpa*II/*Msp*I-**TCTG**
*Eco*RI-**AAG**+*Hpa*II/*Msp*I-**TCTG**	*Eco*RI-**AAG**+*Hpa*II/*Msp*I-**TCAA**
*Eco*RI-**AAC**+*Hpa*II/*Msp*I-**TCTT**	*Eco*RI-**ACA**+*Hpa*II/*Msp*I-**TCAA**
*Eco*RI-**AAC**+*Hpa*II/*Msp*I-**TCAT**	

The bold letters indicate the selective nucleotides.

Isolation and sequencing of polymorphic methylated fragment

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

Isolation of the NR gene

Four sets of primer pairs, Nia1-1 (5'-GCCACTCCTCCGAAAATAAAT-3'; 5'-CTGTCTGATTCTTGGGTGGTG-3'), Nia1-2 (5'-GTTTCCAGTACGAATGATAAT-3'; 5'-GTGATCTAGTGTGCAAACTTG-3'), Nia1-3 (5'-CAAGTTTGCACACTAGATCAC-3'; 5'-ATCTTGACAACCAACTCGAAG-3'), and Nia1-4 (5'-TTGGTGAACTCCTAACTACTG-3'; 5'-AAGACACATGAGCACAATGTT-3'), were designed according to the sequence of nitrate reductase gene *nia-1* (GenBank accession No. X14058), which was cloned from *N. tabacum*. The genomic DNA of the four tobacco cultivars was used as template for PCR amplification. The LA Taq polymerase (TaKaRa) was used in the PCR amplification. The PCR cycling included 94°C for 5 min, followed by 35 cycles of 94°C for 40 s, 60°C for 1 min and 72°C for 2 min, and then a final extension step at 72°C for 10 min. PCR products were separated on 1% agarose gels. The target fragments were recovered, cloned and sequenced according to Tang et al. (2008). For each target fragment, at least three clones were randomly selected out for sequencing. The sequences cloned from each tobacco cultivar were then ligated into a complete gene sequence. Sequences were analyzed using the DNAMAN software (version 5.2.2; Lynnon Biosoft) and Clustal X (Version 1.81). In addition, the complete gene sequences were analyzed using GENSCAN Web Server (http://genes.mit.edu/GENSCAN.html).

RESULTS

Genomic DNA methylation extent in sibling cultivars and their parents

Because *Hpa*II will not cut if either 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, the methylation states of the cytosine at 'CCGG/GGCC' sites would lead to a differential cleavage by the two isoschizomers. Thus, the band pattern from PCR amplification can reflect the methylation status at a certain site. The seven pairs of *Eco*RI+*Hpa*II/*Msp*I selective primer combinations produced legible and reproducible fragments at 162 sites. Of these 162 sites, 82 (50.62%), 66 (40.74%), 72 (44.44%), and 50 (30.86%) methylated sites were detected in Yunyan No. 2, K326, Yunyan85, and Yunyan87, respectively (Table 2). This result indicates that apparent differences in the extent of genomic DNA methylation existed among the tobacco cultivars used in this study. Further analysis showed that there were differences between full-methylation and hemi-methylation extent in all four tobacco cultivars (Table 2). The number of full-methylated sites were 55 (33.95%), 25 (15.43%), 35 (21.60%), and 10 (6.17%) in Yunyan No. 2, K326, Yunyan85, and Yunyan87, respectively, and the corresponding hemi-methylated sites were 27 (16.67%), 41 (25.31%), 37 (22.84%), and 40 (24.69%). In both Yunyan No. 2 and Yunyan87, significant differences existed between full-methylation and hemi-methylation. Compared to Yunyan No. 2, the full-methylated sites of Yunyan85 and Yunyan87 were decreased, whereas the hemi-methylated sites of the two cultivars were increased. Compared to K326, the full-methylated sites were increased in Yunyan85 and were greatly decreased in Yunyan87; however, the hemi-methylated sites were decreased in both sibling cultivars. Between Yunyan85 and Yunyan87, the full-methylated sites were significantly different. This result indicates that the extent of genomic DNA methylation could exhibit great differences between sibling tobacco cultivars.

Table 2. Number of bands amplified using eight methylation-sensitive amplification polymorphism (MSAP) selective primer combinations in the tobacco cultivars.

Primer combination	Amplification site	Yunyan No. 2		K326		Yunyan85		Yunyan87	
		Methylated site		Methylated site		Methylated site		Methylated site	
		Full	Hemi	Full	Hemi	Full	Hemi	Full	Hemi
E-AAC+H/M-TCTG	22	10	0	1	7	3	5	1	8
E-AAC+H/M-TCTT	26	7	6	8	7	2	4	0	7
E-AAC+H/M-TCAT	25	7	6	1	5	6	5	3	1
E-AAG+H/M-TCAA	26	8	6	6	6	6	7	1	11
E-ACA+H/M-TCAA	27	6	6	4	3	7	7	1	5
E-AAG+H/M-TCTG	18	11	1	2	4	7	3	3	6
E-AAG+H/M-TCAG	18	6	2	3	9	4	6	1	2
Total	162	55	27	25	41	35	37	10	40
		82		66		72		50	
Rate of methylated site (%)		33.95%	16.67%	15.43%	25.31%	21.60%	22.84%	6.17%	24.69%
		50.62%		40.74%		44.44%		30.86%	

Sequencing of polymorphic MSAP fragments

Twenty-nine MSAP fragments that exhibited methylation alteration in Yunyan85, Yunyan87, K326, and Yunyan No. 2 were recovered and sequenced. The BLAST (nucleotide BLAST) searches showed that two of the 29 sequences had 99% similarity with the nucleotides 1442-1694 bp of the *nia-1* gene (GenBank accession No. X14058). In addition, the two sequences were respectively cloned from K326 and Yunyan87, and they showed 100% similarity. All remaining 27 sequences were found to contain a GC, CAAT or TATA box.

Characterization of the NR gene in the four tobacco cultivars

From each of the four tobacco cultivars, three NR gene sequences were cloned and sequenced. A total of 12 NR gene sequences were obtained. Sequence alignment analysis indicated that the nine NR gene sequences cloned from Yunyan No. 2, K326 and Yuanyan87 have 99.8-100% similarity with each other, and the three NR gene sequences cloned from Yunyan85 have 100% similarity with each other. However, the NR gene sequence of Yunyan85 exhibited differences from that of the other three cultivars. Therefore, only the NR gene sequences of K326 and Yunyan85 were deposited into GenBank, and the NR gene sequence of K326 could represent the ones of Yunyan No. 2 and Yunyan87. The NR gene sequences of K326 and Yunyan85 were designated as *K326-nia-1* (GenBank accession No. JN384019) and *Yunyan85-nia-1* (GenBank accession No. JN384020), respectively.

The lengths of *K326-nia-1* and *Yunyan85-nia-1* are 5441 and 5306 bp, respectively, and they have 96.9% similarity. Both of the two sequences have four exons and three introns (Table 3). It can be noted that in both *K326-nia-1* and *Yunyan85-nia-1* sequences, the promoter region is 235-300 bp and the transcription initiation codon is 445-447 bp; however, 5133-5135 bp is the stop codon in the *K326-nia-1* sequence, while 4998-5000 bp in the *Yunyan85-nia-1* sequence is the stop codon. The difference between the two sequences was mainly caused by the variation in the third intron of *Yunyan85-nia-1* (Table 3). The third intron of *Yunyan85-nia-1* (3019-3671 bp) is shorter than that of *K326-nia-1* (3019-3806 bp) because of nucleotide elimination (Figure 1). Between the two sequences, the main nucleotide difference also oc-

curred in the third intron (Figure 1). Besides the nucleotide changes in the third intron, A-G or C-T transitions have also occurred at another 16 nucleotide positions. However, the deduced peptide sequences of the two genes have 100% similarity.

Table 3. Structure of the nitrate reductase gene cloned from K326 and Yunyan85.

Sequence	Length (bp)	Promoter region (bp)	Transcription initiation codon (bp)	Exons (bp)	Introns (bp)	Poly(A)-signal (bp)	Stop codon (bp)
K326-nia-1	5441	235-330	445-447	445-1456 1732-1872 2786-3018 3806-5134	1457-1731 1873-2785 3019-3806	5163-5168	5133-5135
Yunyan85-nia-1	5306	235-330	445-447	445-1456 1732-1872 2786-3018 3672-5000	1457-1731 1873-2785 3019-3671	5028-5033	4998-5000

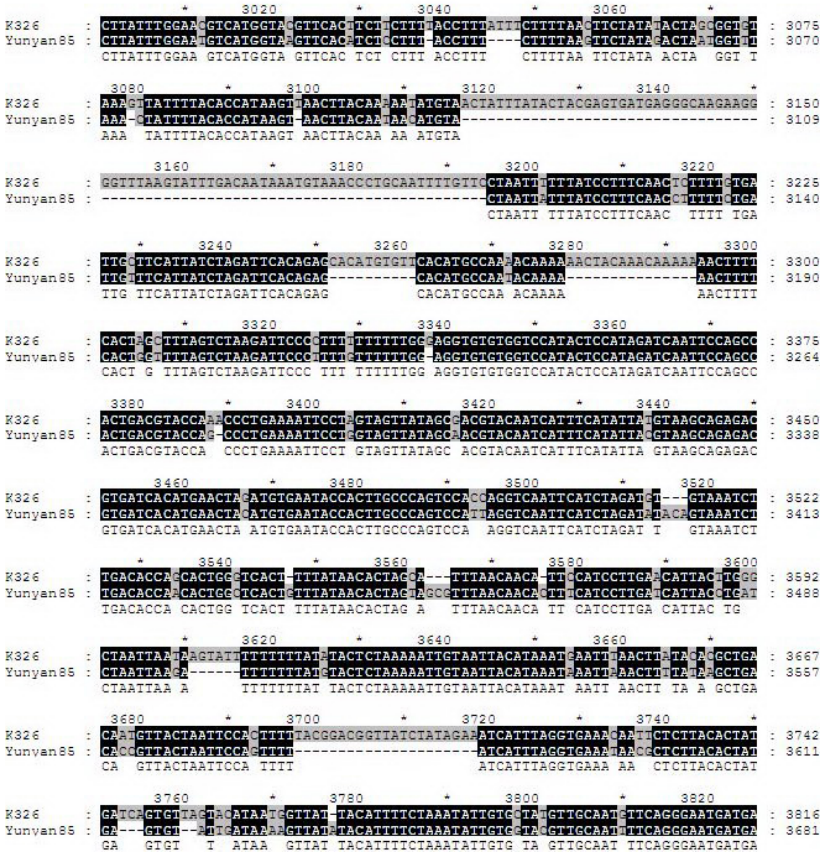

Figure 1. Alignment of the two NR genes of K326 and Yunyan85 using Clustal X. Only 3001-3816/3681 bp is shown because the differences of the two genes occurred mainly in this region. K326 and Yunyan85 indicate *K326-nia-1* and *Yunyan85-nia-1*, respectively. Note that the third intron of the NR gene of Yunyan85 is shorter than that of K326 because of nucleotide elimination.

DISCUSSION

Genomic DNA methylation in sibling tobacco cultivars

The samples with identical parents are the best models for research on the relationship between DNA methylation and gene expression. Particular DNA methylation could lead to the discordance of monozygotic (MZ) twins (Singh et al., 2002). Epigenetics has been associated with MZ twin discordance for common diseases (Poulsen et al., 1999; Bjornsson et al., 2004). Alterations in methylation patterns in sibling wheat cultivars have been discovered and these alterations may be responsible for the different phenotypes among sibling wheat cultivars (Zhang et al., 2008). In these sibling wheat cultivars, the alterations in methylation patterns mainly affected repetitive DNA sequences (Zhang et al., 2008). Many previous studies have also indicated that the main target sequences for methylation mutation are repetitive DNA sequences in hybrid plants (Shaked et al., 2001; Madlung et al., 2002, 2005). Yang et al. (2011) reported that the sibling tobacco cultivars Yunyan85 and Yunyan87 have similar primary genomic structure; however, there was a difference in methylation extent of genomic DNA between the two cultivars, and the sequences involved in the methylation alteration mainly included promoter regions. In the present study, Yunyan85 and Yunyan87 were planted at different times and in different places from that described by Yang et al. (2011). However, the results obtained in this study are very similar with the ones gotten by Yang et al. (2011), that is, the genomic DNA methylation extent exhibited great differences between the two sibling tobacco cultivars and almost all the sequences involved in methylation alteration contain a TATA, CAAT or GC box. 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). Both the results of the present study and those obtained by Yang et al. (2011) indicate that methylation alteration of promoter regions may be mainly responsible for the gene expression phenotype of tobacco, and this may not be affected by environment.

Variation in the NR gene in sibling tobacco cultivars

It has already been reported that the three introns in NR genes from *Oryza sativa* (rice), *Phaseolus vulgaris* (bean), *Lycopersicon esculentum* (tomato), and *N. tabacum* (tobacco) are located precisely at the same position (Zhou and Kleinhofs, 1996). In the present study, the intron from Yunyan85 and that from the three other cultivars are also located at the same position. That is, all four NR genes of the four tobacco cultivars used in this study have three introns and each intron started from the same position (Table 3). However, because of nucleotide elimination, the third intron of the NR gene from Yunyan85 is shorter than that from Yunyan87, K326 and Yunyan No. 2 (Figure 1). This result indicates that the intron of the NR gene in tobacco will change in crosses between the two tobacco cultivars. It is known that introns have a relationship with gene evolution (Fedorova and Fedorov, 2003). Information on the dynamics of intron evolution has come mainly from studies within species or comparisons of different species. Previous reports have, for the most part, examined the historical mutation events of introns (Liaud et al., 1992; Zhou and Kleinhofs, 1996). The variation of the third intron of the NR gene of Yunyan85 indicates that intron evolution could occur immediately after a cross between intra-plant species.

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