Methylation-sensitive amplified polymorphism-based genome-wide analysis of cytosine methylation profiles in *Nicotiana tabacum* cultivars

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ABSTRACT. This study aimed to investigate cytosine methylation profiles in different tobacco (*Nicotiana tabacum*) cultivars grown in China. Methylation-sensitive amplified polymorphism was used to analyze genome-wide global methylation profiles in four tobacco cultivars (Yunyan 85, NC89, K326, and Yunyan 87). Amplicons with methylated C motifs were cloned by reamplified polymerase chain reaction, sequenced, and analyzed. The results show that geographical location had a greater effect on methylation patterns in the tobacco genome than did sampling time. Analysis of the CG dinucleotide distribution in methylation-sensitive polymorphic restriction fragments suggested that a CpG dinucleotide cluster-enriched area is a possible site of cytosine methylation in the tobacco genome. The sequence alignments of the *Nia1* gene (that encodes nitrate reductase) in Yunyan 87 in different regions indicate that a C-T transition might be responsible for the

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tobacco phenotype. T-C nucleotide replacement might also be responsible for the tobacco phenotype and may be influenced by geographical location.

Key words: Methylation-sensitive amplified polymorphism; Nia1; Epigenetics; Methylation; Tobacco (*Nicotiana tabacum*); Phenotype

INTRODUCTION

Epigenetics involves mechanisms that alter gene expression without changing DNA sequences, and can be inherited by mitosis or meiosis (Guimil and Dunand, 2006; Henderson and Jacobsen, 2007). DNA methylation is one of the most important processes in epigenetics, in addition to histone modifications and small regulatory RNAs. Methylation-sensitive amplified polymorphism (MSAP) has been widely used to study epigenetic information in plants (Noyer et al., 2005; Sha et al., 2005; Li et al., 2007), particularly in non-model species in which genome assembly information is not available. In the case of model organisms with genome assemblies, whole-genome bisulfite sequencing is the best option for reduced-representation libraries.

Amplified fragment length polymorphism (AFLP) is a molecular marker technique that can be combined with restriction fragment length polymorphism and polymerase chain reactions (PCRs) to analyze DNA (Vos et al., 1995). Two restriction endonucleases (*Eco*RI and *Mse*I) are usually used in AFLP to slice the genome in order to form restriction endonuclease-sliced fragments with different ends. The technology has been widely used to analyze the relationships between germplasm resources in rice (*Oryza*, Chandel et al., 2010), cotton (*Gossypium*, Zhang et al., 2005), corn (*Zea mays* L., Frascaroli et al., 2013), barley (*Hordeum brevisubulatum*, Shan et al., 2012), peanuts (*Arachis hypogaea*, Jiang et al., 2007), rye (*Secale cereale* L., Falke et al., 2008), and tobacco (*Nicotiana tabacum*, Bahulikar et al., 2004). However, few studies have investigated the relationship between epigenetic factors, yield, and quality.

MSAP was first reported to be capable of detecting methylation differences in 1997 in dimorphic fungi (Reyna-López et al., 1997) and can detect polymorphisms based on the methylation status of whole genomes. MSAP has been successfully applied to study methylation-based polymorphisms in rice (*Oryza*, Xiong et al., 1999), pepper (*Capsicum annuum*, Portis et al., 2004), corn (*Zea mays* L., Zhao et al., 2007; Lu et al., 2008), tomato (*Solanum lycopersicum*, Mason et al., 2008), and banana (*Musa* x *paradisiaca*, Baurens et al., 2003). However, few studies have been conducted on C-methylation-based polymorphisms in tobacco.

In this study, MSAP was used to analyze epigenetic information, specifically cytosine methylation, in tobacco, including the extent, patterns, and site preferences of this process. Methylated polymorphic fragments were reamplified, and the characteristics of their distributions were analyzed to determine the potential predominance of CpG islands. The sequence characteristics of the *Nia1* gene (that encodes nitrate reductase, NR) were also analyzed, and the possible effects of geographical factors on tobacco characteristics are discussed.

MATERIAL AND METHODS

Plant materials

Four tobacco cultivars were used: Yunyan 85, Yunyan 87, K326, and NC89. These cultivars were planted in Ya'an, Sichuan, China. Two other Yunyan 87 plants were planted in Yaojia and Xingwen, Sichuan, China. The leaves of all four tobacco cultivars were collected on July 14,

2009. The leaves of three more Yunyan 87 plants used in the study were collected from Guanyuan, Luzhou, and Yibin in Sichuan, China on August 15, 2009 and August 15, 2010.

DNA extraction

Genomic DNA was isolated from leaf tissues according to Tang et al. (2008).

AFLP and MSAP analysis

The *Mse*I, *Hpa*II/*Msp*I, and *Eco*RI adaptors, pre-selective primers, and selective primer combinations are listed in Table 1 (Vos et al., 1995; Xiong et al., 1999). Repeats were conducted for the AFLP and MSAP procedures. The upper and lower parts of the AFLP and MSAP gels were not used for band scoring, because the bands were not satisfactory; only repeatable and stable patterns were used for the analysis. During the MSAP, bands of two parallel digestions for each sample (*Eco*RI + *Hpa*II and *Eco*RI + *Msp*I) were compared. The AFLP and MSAP procedures were performed according to Zhang et al. (2008).

Table 1. Sequences of amplified fragment length polymorphism (AFLP) and methylation-sensitive amplified polymorphism (MSAP) adaptors, and pre-selective and selective primers.

Primers	Sequnce
Adaptors	
*Eco*RI-adaptors-F	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI-adaptors-R	5'-AATTGGTACGCAGTC-3'
*Mse*I-adaptors-F	5'-GACGATGAGTCCTGAG-3'
*Mse*I-adaptors-R	5'-TACTCAGGACTCAT-3'
*Hpa*II/*Msp*I-adaptors-F	5'-GATCATGAGTCCTGCT-3'
*Hpa*II/*Msp*I-adaptors-R	5'-CGAGCAGGACTCATGA-3'
Preselective primers	
*Eco*R-A	5'-GACTGCGTACCAATTCA-3'
Mse-C	5'-GACGATGAGTCCTGAGTAAC-3'
Hpa/Msp-T	5'-ATCATGAGTCCTGCTCGGT-3'
Selective primer combinations used in AFLP	
*Eco*RI-AAC + *Mse*I-CAC	*Eco*RI-AAC + *Mse*I-CTC
*Eco*RI-AAC + *Mse*I-CTG	*Eco*RI-AAG + *Mse*I-CTA
*Eco*RI-AAG + *Mse*I-CTG	*Eco*RI-AAG + *Mse*I-CAG
*Eco*RI-AAG + *Mse*I-CAC	*Eco*RI-ACA + *Mse*I-CAG
*Eco*RI-ACA + *Mse*I-CTA	*Eco*RI-ACA + *Mse*I-CTC
*Eco*RI-ACA + *Mse*I-CTG	
Selective primer combinations used in MSAP	
*Eco*RI-ACA + *Hpa*II/*Msp*I-**TCAA**	*Eco*RI-AAC + *Hpa*II/*Msp*I-**TCTA**
*Eco*RI-AAC + *Hpa*II/*Msp*I-**TCTC**	*Eco*RI-AAC + *Hpa*II/*Msp*I-**TCTG**
*Eco*RI-AAC + *Hpa*II/*Msp*I-**TCTT**	*Eco*RI-AAG + *Hpa*II/*Msp*I-**TCAA**
*Eco*RI-AAG + *Hpa*II/*Msp*I-**TCTA**	*Eco*RI-ACA + *Hpa*II/*Msp*I-**TCTA**

Bold letters indicate selective nucleotides.

Isolation and sequencing of polymorphic methylated fragments

The polymorphic MSAP amplicons were sliced from polyacrylamide gels using blades, reamplified by PCR, and sequenced. The procedures were performed according to Tang et al. (2008).

Isolation of the NR gene, ornithine decarboxylase, and ribulose decarboxylase

Five primers pairs were designed according to the sequence for amplifying the NR gene,

based on the genome sequence of *Nia1* (GenBank accession No. X14058.1) and the role of the NR gene in quality differences between different tobacco cultivars; they were Nia1-1 (5'-TACTGG TGTTGGTGCTTTTGGTC-3', 5'-TCAGGGGAGATGGATTGTTTGTC-3'), Nia1-2 (5'-GCCACTCCT CCGAAAATAAAT-3', 5'-CTGTCTGATTCTTGGGTGGTG-3'), Nia1-3 (5'-GTTTCCAGTACGAATGA TAAT-3', 5'-GTGATCTAGTGTGCAAACTTG-3'), Nia1-4 (5'-CAAGTTTGCACACTAGATCAC-3', 5'-ATCTTGACAACCAACTCGAAG-3'), and Nia1-5 (5'-TTGGTGAACTCCTAACTACTG-3', 5'-AAG ACACATGAGCACAATGTT-3'). The genomic DNA of Yunyan 87 was used as the template for PCR amplification. The methylated polymorphic restriction fragments were excised, cloned, and sequenced according to Zhang et al. (2008), and at least five clones were randomly selected for the sequencing of each fragment.

Analysis

Bands along the same lines as polyacrylamide gels were counted by scale. Monomorphic and polymorphic sites were summed, and the percentage was calculated. Sequences were analyzed using DNAMAN software (version 5.2.2; Lynnon Biosoft) and the GENSCAN Web Server (http://genes.mit.edu/GENSCAN.html). CpG island characteristics were analyzed according to the definition of Gardiner and Frommer (1987): GC content ≥ 30%, observed CpG/expected CpG ≥ 0.60, CpG island length ≥ 50 bp, and CpG distance ≥ 100 bp.

RESULTS AND DISCUSSION

Test of DNA integrity and detection of AFLP and MSAP pre-amplification effects

Gel electrophoresis of genomic DNA (0.5% agarose) showed that only one bright band was near the sample loading slot, indicating that the isolated DNA was not sheared and was suitable for restriction fragment-based fingerprinting.

The agarose gel electrophoresis map of the AFLP and MSAP pre-amplified products (Figure 1) shows that the smear dispersed between 100 and 750 bp. Therefore, the well-distributed and continuous fragments met the requirements for selective amplification.

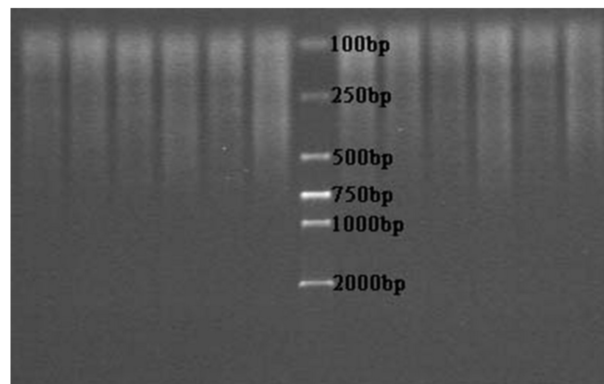

Figure 1. Agarose gel electrophoresis map of pre-amplified products.1-6 represent pre-amplification products; 100-2000 bp represents the size of the DNA marker ladder; A indicates the pre-amplification map for methylation-sensitive amplified polymorphism (MSAP), and B indicates the pre-amplification map for amplified fragment length polymorphism (AFLP).

AFLP and MSAP analysis

AFLP has been used previously to analyze the relationships between tobacco germplasm resources (Marché et al., 2001; Julio et al., 2006; Zhang et al., 2008). The mapping and tagging of the underlying genes using AFLP and MSAP markers in plant genomes has mainly been limited to stress tolerance (Labra et al., 2002; Lu et al., 2007; Orellana et al., 2010), and few studies have investigated the relationship between yield, quality, and epigenetic modifications. Eleven different AFLP-selective primer combinations were used in this study, and 335 bands were obtained. Analysis of the AFLP fingerprints of the NC89, K326, Yunyan 85, and Yunyan 87 plants grown in Ya'an, and the two Yunyan 87 plants grown in Yaojia and Xingwen indicated the absence of polymorphic sites for non-methyl-sensitive restriction enzymes (Figure 2). The AFLP profiles indicated a paucity of polymorphisms at the primary sequence level. Two categories were observed in the polyacrylamide electrophoresis gel patterns: 1) Monomorphic loci: the appearance of identical bands; 2) Polymorphic loci: presence-absence polymorphism (dominant markers). The same 11 AFLP primer combinations were used to amplify Yunyan 87 plants collected in 2009 and 2010 (each sample had three repeats), and 242 sites were legible and produced reproducible bands (Table 2); only one monomorphic locus was detected in these sites (Figure 3). The difference between the Yunyan 87 plants collected in 2009 and 2010 was only 0.39% (34.71% [84] for 2009 and 35.10% [85] for 2010), whereas the highest difference in the number of monomorphic sites between Yunyan 87 plants in different areas was 8.26% (12.40% [30] for Guangyuan and 20.66% [50] for Luzhou).

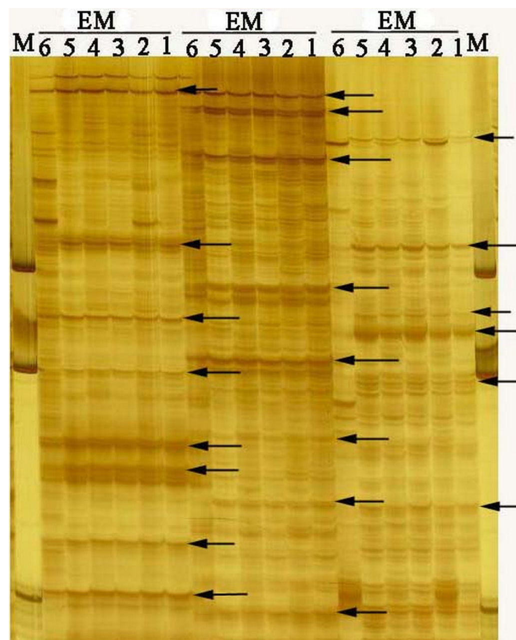

Figure 2. Polyacrylamide gel electrophoresis map of amplified fragment length polymorphism (AFLP) products from samples collected in 2009.1-6 represent amplification products for NC89, K326, Yunyan85, Yunyan 87 (Ya'an), Yunyan 87 (Xingwen), and Yunyan 87 (Yaojia), respectively; the black arrow indicates AFLP monomorphic sites; M represents the DNA marker; EM denotes *Eco*RI + *Mse*I restriction enzyme digestion production.

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Table 2. Number of bands amplified using 11 amplified fragment length polymorphism (AFLP) selective primer combinations in tobacco cultivars.

Samples	GY(09)	GY(10)	LZ(09)	LZ(10)	YB(09)	YB(10)
Monomorphism	151	118	180	117	194	105
Percentage	62.40%	48.76%	74.38%	48.35%	80.17%	43.39%
Samples	GY(09) + LZ(09) + YB(09)	GY(10) + LZ(10) + YB(10)	GY(09) + GY(10)	LZ(09) + LZ(10)	YB(09) + YB(10)	
Monomorphism	84	85	30	50	38	
Percentage	34.71%	35.10%	12.40%	20.66%	15.70%	

GY(09) indicates Yunyan 87 cultivars collected in Guangyun, SiChuan, China in 2009; GY(10) indicates Yunyan 87 plants collected in Guangyun, SiChuan, China in 2010.

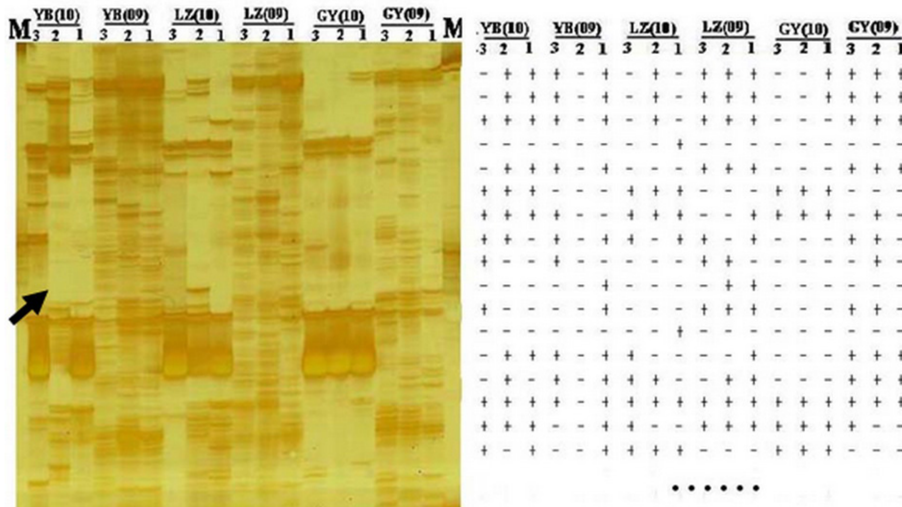

Figure 3. Electrophoresis map ofamplified fragment length polymorphism (AFLP) products and band statistics for Yunyan 87 collected from different areas in 2009 and 2010. 1, 2, and 3 represent three repeated samples of GY(09), GY(10), LZ(09), LZ(10), YB(09), and YB(10); - indicates the absence of bands; + indicates the presence of bands; M indicates a DNA marker; the black arrow indicates AFLP monomorphic sites; the primer combinations were E-AAC + *Mse*I-CTC (*Eco*RI with a selective base pair of AAC and *Mse*I with CTC), E-ACA + MseI-CTG (*Eco*RI with ACA and *Mse*I with CTG),and E-ACA + *Mse*I-CTC (*Eco*RI with ACA and *Mse*I with CTC);the Figure is part of the polyacrylamide gel electrophoresis map of the 2009 samples.

In this study, two isoschizomers (*Hpa*II and *Msp*I) that recognize the target site (CCGG) with different methylation sensitivities in eukaryotes were used to generate the MSAP. *Hpa*II can digest both non-methylated CCGG and the hemi-methylated mCCGG motif, whereas *Msp*I can cleave hemi- or fully methylated CmCGG and mCmCGG sequences, and non-methylated CCGG. In this study, two categories were analyzed, according to their polyacrylamide electrophoresis gel patterns: 1) Monomorphic loci: CCGG/GGCC methylation patterns were consistent between samples; 2) Polymorphic loci: CCGG/GGCC methylation patterns were different between samples. The eight selective primer combinations used for the parallel digests of *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I produced 251 reproducible amplicons. Of these, only one monomorphic locus was detected (Figure 4). A total of 81 (32.27%), 90 (35.86%), 97 (38.65%), and 90 (35.86%) methylated sites were detected in NC89, K326, Yunyan 85, and Yunyan 87, respectively, which were planted in Ya'an, whereas 90 (35.86%), 108 (43.03%), and 79 (31.47%) were detected in Yunyan 87 planted

in Ya'an, Yaojia, and Xingwen, respectively (Table 2). The MSAP analysis showed that the amount of methylation in different tobacco cultivars planted in the same area was almost identical (32.27, 35.86, 38.65, and 35.86%), whereas the amount of methylation in similar tobacco cultivars planted in different areas varied from 31.47 to 43.03%. These data suggest that methylation levels were influenced by regional factors. Therefore, both the AFLP and MSAP results indicate that geographical location affects fingerprint polymorphisms in the tobacco genome.

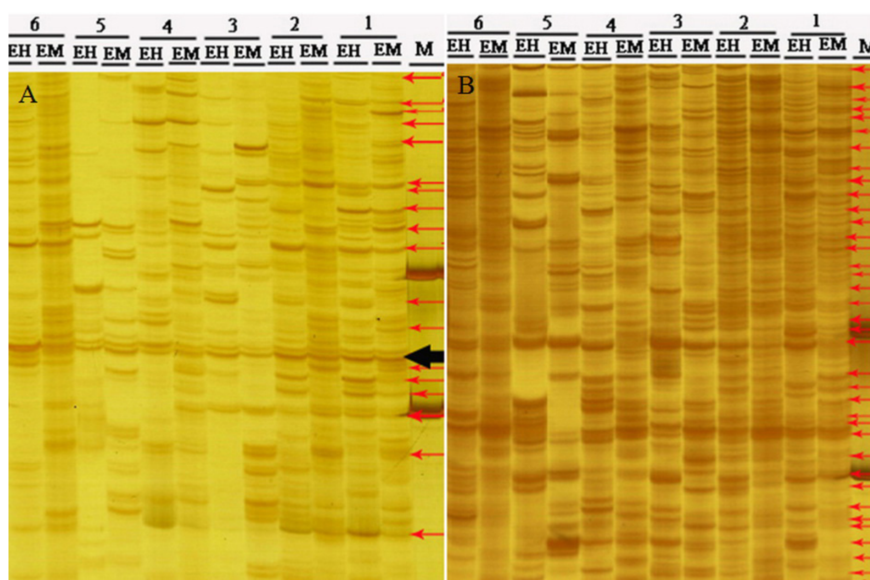

Figure 4. Schematic diagram of methylation patterns in tobacco. A indicates the amplification products of the primer combination E-AAG+ H/M-TCAA, B indicates the amplification products of the primer combination E-AAC+H/M-TCTA; the black arrow indicates the methylation monomorphic site, and the red arrows indicate methylation polymorphic sites;1-6 represent NC89, K326, Yunyan 85, Yunyan 87 (Ya'an), Yunyan 87 (Xingwen), and Yunyan 87 (Yaojia), respectively; some sites were methylation monomorphicin 1 and 2, some sites were methylation monomorphicin 5 and 6, some sites were methylation monomorphicin 4 and 5, and other sites were methylation monomorphicin 4 and 6; M indicates the DNA marker; EH indicates *Eco*RI + *Hpa*II restriction enzyme digestion production, and EM indicates *Eco*RI + *Msp*I restriction enzyme digestion production.

Sequence characterization of methylated polymorphic restriction fragments

Forty-one polymorphic methylated fragments were successfully isolated and sequenced, and the results of a BLAST analysis showed that homologous sequences were found for 35 bands. Nucleotide BLAST searches showed that 3 of the 35 sequences were 90% similar to *Nia1* nucleotides (GenBank accession No. X14058).

Cytosine methylation is a common form of DNA modification that plays a role in the silencing of transposons and repeats in the genomes of vertebrates, plants, and fungi (Zilberman et al., 2007). In the genomes of higher plants, approximately 20 to 50% of methylation sites are located in cytosine residues, and 90% of methylated sites are located in CpG or CpNpG (Chan et al., 2005). In the current study, 25 typical CpG islands were isolated from 41 methylation fragments, and the highest values of the GC content, the observed CpG/expected CpG ratio, and CpG island

length were 53.89%, 1.52, and 195 bp, respectively (Table 3). The results show that CpG islands are abundant in methylated gene fragments. The sequence alignments of the NR gene in Yunyan 87 cultivars collected from different regions also showed that C-T changes occurred in CG-rich regions. Results from the current and previous studies suggest that CpG dinucleotide cluster-enriched areas of the genome are possible sites of DNA cytosine methylation.

Table 3. CpG dinucleotide distribution characteristics of methylated polymorphic restriction fragments.

Primer		Length (bp)	Start (bp)	End (bp)	GC content (%)	Observed CpG/ expected CpG	CpG island length (bp)
*Eco*RI-ACA	*Hpa*II/*Msp*I-TCAA	278	47	233	48.59	1.07	177
		294	47	239	53.89	1.07	193
		278	47	233	49.15	1.05	177
		204	97	147	43.14	0.43	51
		204	62	149	48.86	0.76	88
*Eco*RI-AAC	*Hpa*II/*Msp*I-TCTA	217	47	108	53.23	0.74	62
		216	47	157	47.75	0.95	111
		215	47	160	50.88	1.09	114
		239	48	184	49.64	0.91	137
		230	77	174	30.61	0.89	98
		201	47	144	42.86	0.91	98
*Eco*RI-ACA	*Hpa*II/*Msp*I-TCAA	277	47	222	50.00	1.12	176
		277	47	222	48.86	1.16	176
		250	70	193	36.29	1.04	124
*Eco*RI-AAC	*Hpa*II/*Msp*I-TCTA	236	47	180	40.30	1.11	134
		257	50	200	37.09	1.03	151
		206	96	150	27.27	0.98	55
		186	49	129	28.40	0.61	81
		162	49	105	35.09	1.52	57
		276	47	221	48.57	1.08	175
		283	47	221	43.09	1.20	181
*Eco*RI-AAC	*Hpa*II/*Msp*I-TCTT	230	49	125	25.97	0.92	77
*Eco*RI-AAG	*Hpa*II/*Msp*I-TCAA	296	48	242	47.18	1.11	195

Sequence analysis of NR genes in Yunyan 87 cultivars from different regions

Because of deamination, the 5'-ending methylation of cytosine may result in thymine and a C-T transition mutation (Hepburn et al., 1987). Unlike a cytosine to uracil mutation that is efficiently repaired, a cytosine to thymine mutation can be corrected only by mismatch system repair, which is very inefficient. The conversion from a methylated CG sequence to a TG sequence could lead to a deficiency of the CG sequence in active genes. The biological function of DNA methylation may be related to gene silencing (Paszkowski and Whitham, 2001). Previous studies have reported that DNA methylation may regulate gene expression in various ways (Severin et al., 2011). C-T changes appeared in the NR gene sequences of Yunyan 87 plants grown in Ya'an, Yaojia, and Xingwen. The changes were concentrated in CG- and CCGG-rich regions (Figure 5 and Table 4), suggesting that these variations (that mainly occurred in CpG island-enriched areas) might alter gene structures. Cell characteristics are also affected by constituent proteins, which result from specific patterns of gene expression (Bird, 2002); numerous plant proteins are related to a plant's characteristics. These data suggest that variations in cytosine methylation patterns may result in different tobacco characteristics. The sequence alignment of the NR gene also indicated that different methylated patterns might exist in the same Yunyan 87 cultivars planted in different regions. Therefore, we hypothesize that variations in tobacco quality are influenced by geographical factors.

Figure 5. Sequence alignment of the NR gene in the Yunyan87 cultivar from different regions. Red, blue, and black triangles indicate C-T changes in the gene sequence of the Yunyan 87 cultivar taken from Ya'an, Yaojia, and Xingwen, respectively. Red lines indicate CG- or GC-rich regions, black lines indicate CCGG-rich regions.

Table 4. Number of bands amplified using eight methylation-sensitive amplified polymorphism (MSAP) selective primer combinations in tobacco cultivars.

Primer combination	Amplification site	NC89		K326		yunyan85		yunyan 87 (Ya'an)		yunyan 87 (Xingwen)		yunyan 87 (Yaojia)	
		Methylated site		Methylated site		Methylated site		Methylated site		Methylated site		Methylated site	
		Full	Hemi	Full	Hemi	Full	Hemi	Full	Hemi	Full	Hemi	Full	Hemi
*Eco*RI-ACA + *Hpa*II/*Msp*I-TCAA	26	4	4	9	0	8	3	1	2	6	8	6	1
*Eco*RI-AAC + *Hpa*II/*Msp*I-TCTC	23	3	4	5	5	7	4	5	1	3	7	1	4
*Eco*RI-AAC + *Hpa*II/*Msp*I-TCTT	30	10	0	4	4	6	5	6	7	4	2	10	0
*Eco*RI-AAC + *Hpa*II/*Msp*I-TCTA	25	5	8	3	9	9	0	1	7	6	5	8	1
*Eco*RI-AAC + *Hpa*II/*Msp*I-TCTG	44	8	9	7	5	6	11	10	14	18	8	19	4
*Eco*RI-AAG + *Hpa*II/*Msp*I-TCAA	48	6	4	5	10	8	12	10	9	13	10	11	3
*Eco*RI-ACA + *Hpa*II/*Msp*I-TCTA	25	4	4	6	4	5	5	4	2	2	4	0	4
*Eco*RI-AAG + *Hpa*II/*Msp*I-TCTA	30	6	2	8	6	1	2	5	6	6	6	2	5
Total	251	46	35	47	43	50	42	42	48	58	50	57	22
		81		90		97		90		108		79	
Rate of methylated site (%)		18.32	13.91	18.72	17.13	19.92	16.73	16.73	19.12	23.10	19.92	22.71	8.76
		32.27		35.86		38.65		35.86		43.03		31.47	

In summary, different geographical factors could affect AFLP fingerprints, and variations in methylation patterns, in the tobacco genome. Different methylation patterns could influence the expression of quality-related genes through T-C C-T nucleotide replacement, resulting in the different characteristics of different tobacco cultivars. These results indicate that C-T nucleotide replacement might be responsible for the tobacco phenotype, and this expression may be affected by geographical factors. Therefore, the results of the current study suggest that molecular markers could be used to analyze polymorphisms that are affected by geographical factors, which lead to variations in cytosine-methylated patterns. In addition, tobacco assessment indices could be optimized, and the quality of tobacco improved.

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