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DNA methylation polymorphism in flue-cured tobacco and candidate markers for TMV resistance*

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Abstract: DNA methylation plays an important role in the epigenetic regulation of gene expression during plant growth, development and polyploidization. However, there is still no distinct evidence in tobacco regarding the distribution of the methylation pattern and whether it contributes to qualitative characteristics. We studied the levels and patterns of methylation-polymorphism at CCGG sites in 48 accessions of allotetraploid flue-cured tobacco, *Nicotiana tabacum*, using a methylation-sensitive amplified polymorphism (MSAP) technique. The results showed that methylation existed at a high level among tobacco accessions, among which 49.3% sites were methylated and 69.9% allelic sites were polymorphic. A cluster analysis revealed distinct patterns of geography-specific groups. In addition, three polymorphic sites significantly related to TMV-resistance were explored. This suggests that tobacco breeders should pay more attention to epigenetic traits.

Key words: Methylation-sensitive amplified polymorphism (MSAP); Epigenetic modification; Tobacco mosaic virus (TMV)-resistance; Flue-cured tobacco

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1 Introduction

Plants usually adapt to complex and changeable environmental stresses through reversible epigenetic modifications to avoid unnecessary excessive genetic rearrangements and population diversification. The spectrum of external and internal influences experienced during the life span may lead to the generation of specific changes in gene expression that can be epigenetically fixed and passed to progeny to form epigenetic memories (Boyko and Kovalchuk, 2008). These changes are important for plants with regard to polyploidization and ecological adaptation (Lee and Chen, 2001; Liu and Wendel, 2003).

DNA methylation is one of the most important epigenetic modification strategies, which is performed by DNA methyltransferases that catalyze the transfer of a methyl group from S-adenosyl-l-methionine to cytosine bases in DNA, and is especially limited to symmetrical CG and CHG sites (where H is A, C or T) in plants (Bird, 2002). It can inhibit gene expression and plays a crucial role in defending against foreign or mobile DNA elements and in maintaining specific patterns of gene expression during plant growth, development and reproduction (Rassoulzadegan *et al*., 2006). In maize, a significant decrease in DNA methylation is observed in roots upon cold treatment (Steward *et al*., 2002) and in the *P1-wr* gene under a different pigment phenotype of kernel pericarp (Sekhon *et al*., 2007). In tobacco, a glycerophosphodiesterase-like protein

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encoding gene (*NtGPDL*) and a pathogen-responsive gene (*NtAlix1*) are demethylated and expressed in response to aluminum stress (Choi *et al*., 2007) and tobacco mosaic virus (TMV) infection (Wada *et al*., 2004), respectively.

Tobacco is one of the most widely-grown

Table 1 Tobacco accessions used for MSAP analysis

ID	Accession	Geographic origin	ID	Accession	Geographic origin	TMV resistance test
1	Qiandongnan retention	Guizhou ,China	37	Oxford 2007	America	---
2	Pianpianhuang	Guizhou, China	38	ReamsM-1	America	---
3	Aizihuang	Guizhou, China	39	RG11	America	---
4	Zimei	Guizhou, China	40	RG 8	America	---
5	Baiyanshi	Guizhou, China	41	special 400	America	---
6	Nanjiang 3	Guizhou, China	42	V2	America	---
7	Jiucaiping 2	Guizhou, China	43	Va444	America	---
8	Wuchun	Guizhou, China	44	Vesta 64	America	---
9	Qianxi 1	Guizhou, China	45	Dixie Bright 101	America	---
10	Jinyan 1	Guizhou, China	46	Hicks(Broad leaf)	America	---
11	Dafang retention	Guizhou, China	47	K326	America	---
12	Bina	Guizhou, China	48	Coker 176	America	R
13	Caohai 1	Guizhou, China	49	Coker86	America	R
14	Guanghuang 21	Guangdong, China	50	GY-22	Guizhou,China	R
15	Guidan 1	Guangxi, China	51	GY-41	Guizhou,China	R
16	Honghuadajinyuan	Yun nan, China	52	GY-89	Guizhou,China	R
17	Renminliudui	Yun nan, China	53	GY-107	Guizhou,China	R
18	Yunyan 87	Yun nan, China	54	GY-151	Guizhou,China	R
19	Liaoyan7910	Liaoning, China	55	GY-162	Guizhou,China	R
20	Mudan 78-7	Jilin, China	56	GY-204	Guizhou,China	R
21	Qinyuanhuang	Henan, China	57	GY-A10	Guizhou,China	R
22	Qianjinhuang	Henan, China	58	K394	America	S
23	Jingyehuang	Henan, China	59	NC89	America	S
24	Xujin 1	Henan, China	60	GY-1	Guizhou,China	S
25	Jintai7618	Shanxi, China	61	GY-3	Guizhou,China	S
26	Xiaohuangjin 1025	Shandong, China	62	GY-7	Guizhou,China	S
27	Zhongyan 14	Shandong, China	63	GY-12	Guizhou,China	S
28	Zhongyan 90	Shandong, China	64	GY-32	Guizhou,China	S
29	T.T.6	Taiwan, China	65	GY-49	Guizhou,China	S
30	Kutsaga E1	Zimbabwe	66	GY-50	Guizhou,China	S
31	PVH06	Brazil	67	GY-52	Guizhou,China	S
32	T.I.245	South America	68	GY-97	Guizhou,China	S
33	Meck	America	69	GY-A1	Guizhou,China	S
34	Nc37NF	America	70	GY-A8	Guizhou,China	S
35	Nc82	America	71	GY-76	Guizhou,China	S
36	Oxford 1	America	---	---	---	---

Note: "S" indicates TMV-susceptible; "R" indicates TMV-resistant. "---" is blank space.

non-food crop plants in the world and is a major economic force in almost 100 countries. Most cultivars belong to the species *Nicotiana tabacum* L. in the family *Solanaceae*. Flue-cured tobacco is a natural allotetraploid that presents wide environmental adaptability as a result of many years of extensive artificial cross-breeding which would increase its complexity through epigenetic modification and regulation. There have been no previous reports on the pattern of DNA methylation in *N. tabacum* cultivars or on its relationship with the genotype or ecotype. In this study we analyzed the DNA methylation pattern by a methylation-sensitive amplified polymorphism (MSAP) technique in 48 tobacco accessions that were selected to represent different geographic origins and assessed phenotype-related markers. The results should provide important insights for further studies on tobacco epigenetic polymorphism.

2 Materials and Methods

2.1 Plant materials and DNA extraction

A total of 61 accessions of flue-cured tobacco in genus *N. tabacum* L. were used for a methylation-sensitive amplified polymorphism (MSAP) analysis in this study. They included cultivars from America, Brazil, Zimbabwe and China, and 24 accessions were used for a simultaneous analysis of tobacco mosaic virus (TMV)-resistance (Table 1). Fresh tobacco seeds of all cultivars were selected and incubated in a Petri dish with wet gauze at 25 ℃. Genomic DNA was extracted from seeds germinated for 7 days using a DNA extraction kit (Takara, Japan). The entire genomic DNA was diluted to 100 ng·μl^{-1} and stored at -20 ℃. Care was taken to collect samples at the same developmental stage to reflect genotype- or ecotype-specific variation in methylation patterns.

2.2 Methylation-sensitive amplified polymorphism analysis

About 400 ng of genomic DNA was digested by *Hap*II-*EcoR*I or *Msp*I- *EcoR*I (Takara, Japan) in a total volume of 10 μl at 37 ℃ overnight. The reaction was stopped by heating the mixture to 80 ℃ for 10 min, and then adaptor ligation was carried out. Adaptors were prepared by mixing oligonucleotide pairs at equal concentrations of 100 μM. The mixtures were heated to 95℃ for 5 min and allowed to anneal by cooling slowly. Next, 1.5 U of T4 DNA ligase (Takara, Japan), 50 pmol of each adaptor, and 1×T4 ligase buffer were incubated at 16 ℃ overnight with the digestion product, with a total reaction volume of 20 μl.

Pre-amplification was conducted in a total volume of 25 μl with 2 μl ligation product, 1×PCR buffer, 200 μM dNTPs, 0.5 μM each of the pre-selective primers and 1U DNA polymerase. The reaction procedure was 94℃ for 5 min, followed by 21 cycles of 94℃ for 30 s, 56℃ for 1 min, 72℃ for 1 min, and then 72℃ for 10 min and holding at 12℃. Selective amplification was carried out in a system similar to that described above with diluted DNA substrate of pre-amplification product and selective primers. The PCR parameters included an initial hold at 94℃ for 5 min; 13 touchdown cycles (94℃ for 30 s, 65 ℃ for 30 s, and 72 ℃ for 1 min) during which the

Table 2 Primers and adaptors used in this study

Items	Sequence (5'-3')
Adaptors	
*Hap*II/*Msp*I adaptor-F	GAT CAT GAG TCC TGC T
*Hap*II/*Msp*I adaptor-R	CGA GCA GGA CTC ATG A
*EcoR*I adaptor-F	CTC GTA GAC TGC GTA CC
*EcoR*I adaptor-R	AAT TGG TAC GCA GTC TAC
Preselective primers	
*EcoR*I	GTA GAC TGC GTA CCA ATT CA
*Hap*II/*Msp*I	ATC ATG AGT CCT GCT CGG T
Selective primers	
H/M+TAA	*Hap*II/*Msp*I +AA
H/M +TCC	*Hap*II/*Msp*I +CC
H/M +TTC	*Hap*II/*Msp*I +TC
E+ATC	*EcoR*I +TC
E+AGA	*EcoR*I +GA
E +ACG	*EcoR*I +CG
E+ATG	*EcoR*I +TG
E+AGC	*EcoR*I +GC
E+ACC	*EcoR*I +CC
E +ACA	*EcoR*I +CA
E+ACT	*EcoR*I +CT
E+AGG	*EcoR*I +GG
E +AGT	*EcoR*I +GT

annealing temperature was decremented 0.7℃ each cycle; and 23 cycles of 94℃ for 30 s, 56℃ for 30 s, and 72℃ for 1 min; then 72℃ for 10 min; and a final hold at 12℃. All primers and adaptors used are listed in Table 2.

The selective PCR product was mixed with a one-fifth volume of 6×loading buffer and heated at 95℃ for 10 min, and then quickly transferred to ice. A final 5 μl denatured mixture was electrophoresed on 6% denaturing polyacrylamide gel and silver-stained. The gel was then analyzed on a gel documentation system with Quantity One software (Bio-Rad, USA). The repeatability of the MSAP pattern was tested by analyzing the same DNA in triplicate.

2.3 TMV-resistance investigation

Twenty tobacco lines were selected for a TMV-resistance analysis (Accession IDs 48 to 71) among all of the accessions that originated from self-bred tobacco lines. The TMV strain was obtained by macerating leaf tissue of infected tobacco seedlings and diluting the sap in 20 mM sodium phosphate buffer (pH 7.0) and carborundum. Viral inoculations were then carried out by rubbing the TMV suspension onto 4- to 5-week-old tobacco leaves with a sponge. Mock inoculations were performed as a control by rubbing tobacco leaves with phosphate buffer and carborundum alone. Plants were scored for the development of a resistance or susceptible response to TMV from 3 to 20 days after infection.

2.4 Data analysis

*Msp*I and *Hap*II show different sensitivities in the outer cytosine methylation of the CCGG recognition sequence. When bands for one accession were present in one of the isoschizomer lanes, this was considered "methylated", "hemimethylated" for *Hap*II and "full methylated" for MspI, while "not methylated" referred to bands present in both lanes, and "unknown" referred to bands absent from both lanes. A site was considered to be "methylation polymorphic" if it was methylated for some accessions and not methylated for others accessions (Keyte *et al*., 2006) (Fig. 1).

The statistical software NTSYSpc, version 2.10p (Applied Biostatistics, USA), was used for an unweighted pair grouping method of arithmetic averages (UPGMA) cluster analysis based on Jaccard's genetic similarity matrix and a principal component analysis (PCA) of the correlation matrix of the methylation polymorphic sites among all accessions.

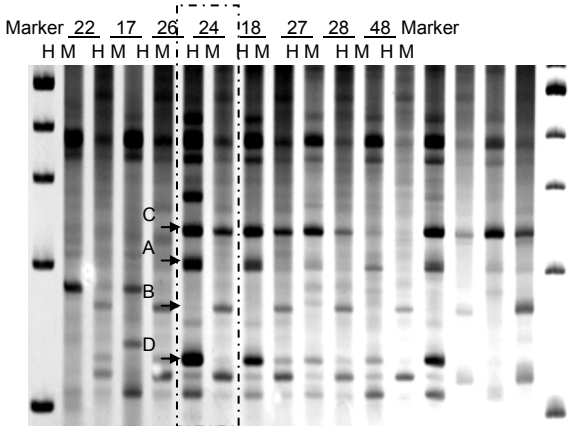

Figure 1 Example of cytosine methylation patterns in the tobacco MSAP analysis.
The tobacco accession ID and the endonuclease used are shown at the top and the types of methylation for accession 26 are labeled with arrows. A: hemimethylated site; B: full methylated site; C: not methylated site; D: polymorphic allelic site; H and M refer to digestion with *Hap*II-*EcoR*I and *Msp*I-*EcoR*I respectively; Marker: DNA ladder of 100-500 bp.

All of the MSAP bands were used for a correlation analysis with TMV-resistance. To calculate the association of MSAP bands with TMV-resistance, a general linear regression model was used for association analyses, as implemented in TASSEL software [http://www.maizegenetics.net/ bioinformatics] (Bradbury *et al*., 2007).

3 Results

3.1 Methylation polymorphism

All 30 combinations of selective primers were tested on five tobacco accessions (IDs 48, 49, 58, 59 and 71) to identify the ideal conditions for the experiment, in which 16 combinations were selected with distinct and scorable amplified bands in the gel, while the others, which yielded either very few bands or resulted in smearing, were not used in further tests. A total of 9741 H/M sites were amplified for 48 accessions with these 16 pairs of selective primers, of which 49.3% were methylated, including 2484 hemimethylated sites and 2317 full methylated sites. Among these methylated sites, 402 were allelic sites, of which 281 (69.9%) were polymorphic. The pairs E+AGT and H/M +TCC had a maximum polymorphic ratio of 92.6%, which was more than twice that

of the pairs E+ATC and H/M +TTC, with a minimum polymorphic ratio of 40.9%. Overall, there were slightly more hemimethylated H/M sites than full methylated sites (Table 3).

3.2 Cluster analysis

Jaccard's genetic similarity values based on pairwise comparisons among the 48 *Nicotiana* accessions were used for a cluster analysis. The dendrogram had coefficients ranging from 0.61 to 0.77, and the 48 accessions were clustered into three large groups and a single accession (ID 10; Jinyan 1) at a similarity level of 65% (Fig. 2a). The single accession Jinyan 1 collected from the Jinsha region of Guizhou province was closely related to group I, which consisted of three American cultivars (V2, Va444 and Vesta 64) and 13 accessions collected from other regions in Guizhou. Group III included 15 cultivars (accessions 14 to 28) from other provinces in China. The remaining accessions in group IV including "T.T.6" from Taiwan and cultivars from foreign countries (accessions 30 to 48). Some accessions with distinct genetic relationships were not clustered closely together in the MSAP phylogenetic analysis. For example, "Qianjinhuang" is a selected seedling from "Jingyehuang"; "Zhongyan 90" is a filial generation of "Jingyehuang"; "Renminliudui" and "Nanjiang 3" were both selected seedlings from "Honghuadajinyuan"; "Oxford 2007", "RG11 " and "RG 8" have the same parent "K399"; and "Yunyan 87" and "RG 8" have the same parent "K326". In addition, many Chinese flue-cured tobacco cultivars were bred or selected from foreign tobacco races, whereas members of these families were clustered into different groups or subgroups. We then performed a PCA analysis to further reveal geographic or genetic relationships. The results showed the same geographic relationship as the dendrogram (Fig. 2b). Thus, the cytosine methylation pattern of tobacco shows a strong ecotype profile through epigenetic memory.

3.3 Candidate MSAP markers for TMV-resistance

All of the MSAP bands scored by the 7th pair of primers (Table 3) were used for a correlation analysis with TMV-resistance; three polymorphism allelic sites showed a significant correlation ($p<0.001$) and explained the phenotypic variation with values of 0.3231, 0.258 and 0.2117, respectively. Therefore, to

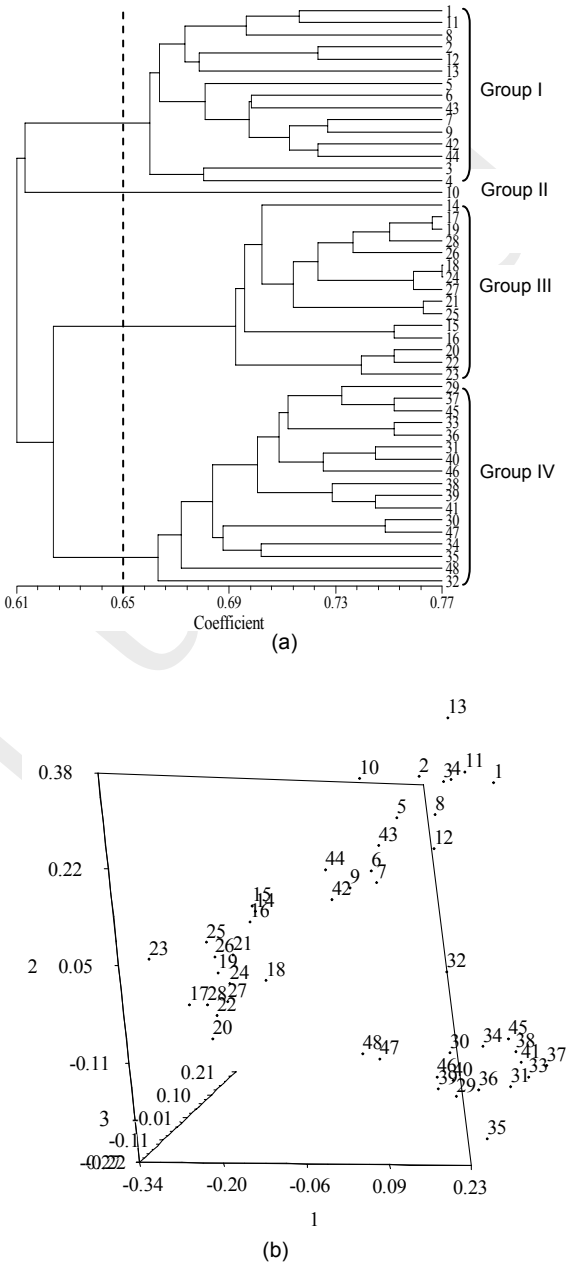

Figure 2 Clustering of tobacco accessions based on the results of MSAP of polymorphic sites.
(a) Phylogenetic tree. (b) Principal component analysis (PCA). The first 2 principal components account for 9.6% and 9.3% of the total eigenvalues. Tobacco accession IDs for three main geographical regions (1-13 from Guizhou Province of China; 14-29 from other Chinese provinces; 30-48 from abroad. For details see Table 1) are shown.

Table 3 Cytosine-methylation polymorphism analyzed by 16 primer pairs in 48 tobacco accessions

No.	Selective primers	Methylation ratio %	Hemimethylated H/M sites	Full methylated H/M sites	Methylated allelic sites	Polymorphic allelic sites	Polymorphism ratio %
1	E+AGA-H/M+TCC	63.3	190	216	32	14	43.80
2	E+ACG-H/M+TAA	42.5	75	140	24	17	70.80
3	E+ACG-H/M+TCC	44.7	129	135	27	23	85.20
4	E+ATG-H/M+TAA	42.5	153	111	22	18	81.80
5	E+ATG-H/M+TCC	44.3	86	140	27	17	63.00
6	E+AGC-H/M+TCC	51.2	131	80	19	12	63.20
7	E+AGC-H/M+TTC	57.6	201	29	22	9	40.90
8	E+ACC-H/M+TAA	50.7	130	148	21	18	85.70
9	E+ACA-H/M+TAA	39.4	111	113	27	18	66.70
10	E+ACA-H/M+TCC	75.0	202	206	25	17	68.00
11	E+ACT-H/M+TCC	51.5	172	178	25	22	88.00
12	E+AGG-H/M+TCC	60.2	160	173	28	17	60.70
13	E+AGG-H/M+TTC	53.4	180	109	25	15	60.00
14	E+AGT-H/M+TAA	44.9	163	152	27	25	92.60
15	E+AGT-H/M+TCC	63.4	219	262	28	24	85.70
16	E+AGT-H/M+TTC	51.7	182	125	23	15	65.20
	total	49.3	2484	2317	402	281	69.90

Note: The methylation ratio indicates the percentage of methylated H/M sites among the total H/M sites, where methylation includes hemimethylation and full methylation; H and M indicate digestion with *Hap*II-*EcoR*I and *Msp*I-*EcoR*I respectively.

Table 4 Sequence annotation of MSAP fragments correlated to TMV resistance

Band ID	Size (bp)	Correlation to resistance *P*- value	Correlation to resistance Req_marker	Sequence similarity	Length similarity
P7-19	134	0.000025041	0.3231	Rice cold stress cDNA-AFLP	32
P7-13	201	0.0002281	0.2580	*Nicotiana benthamiana* cDNA	26
P7-7	261	0.00099996	0.2117	*Gossypium hirsutum* MSAP sequence	24

assess the sequence of marker sites, we isolated and sequenced selected MSAP bands. One band was similar to the *Gossypium hirsutum* MSAP sequence, and the other two sequences were similar to plant cDNA-AFLP segments (Table 4). This indicated that the sites related to TMV-resistance were protein- or enzyme-coding sequences, and methylated modification could possibly regulate gene expression and the stress response of tobacco.

4 Discussion

In this study, we analyzed the diversity of cytosine methylation at CCGG sites for allotetraploid flue-cured tobacco. The results showed that methylation polymorphism is widespread in *N. tabacum*. Among all of the H/M sites for 48 tobacco accessions, 49.3% were methylated and 69.9% of the allelic sites were polymorphic. High methylation polymorphism has also been observed in *Gossypium arboretum* L. (67%) (Keyte *et al*., 2006) and *Brassica oleracea* (95%) (Salmon *et al*., 2008), while low methylation *Arabidopsis* (Cervera *et al.*, 2002) and rice (Ashikawa, 2001). Similar results were also reported in tobacco amplified fragment length polymorphism (AFLP) and SSR analyses. For example, 46 tobacco cultivars from 18 different countries were grouped based on their geographic origins and manufacturing quality traits (Ren and Timko, 2001). Fifty-one Virginia cultivars with desirable agronomic characteristics also formed groupings based on their geographic origin (Zhang *et al*., 2006). In addition, 702 tobacco accessions collected from Central and South America were frequently clustered in a geography-specific manner using various distance coefficients (Moon *et al*., 2009). This may indicate that tobacco cultivars with narrow genetic diversity were affected by unique environmental stresses or stimuli to some extent, and then epigenetic modification developed and led to differences in the genetic relationship. Based on this hypothesis, methylation polymorphism may be useful as an epigenetic marker for certain populations and cultivars.

Additional experiments were conducted to analyze the DNA methylation pattern within TMV-resistant and TMV-susceptible groups. The results showed that three polymorphic sites were significantly correlated with TMV-resistance and their sequences were somewhat similar to existing cDNA sequences. This information may be useful for screening tobacco varieties. Further studies on this subject will be needed in the near future.

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