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Full Length Research Paper

The effects of reciprocal cross on inheritance of DNA methylation in cotton (*Gossypium hirsutum*)

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DNA methylation plays an important role for regulation of gene expression. To study the inheritance of DNA methylation, we selected two F_1 plant population by reciprocal cross with two cotton lines *Zongcaixuan No.1* and *HY428*, and analyzed the variations of DNA methylation levels and patterns in F_1 generations by methylation sensitive amplified fragment length polymorphism (MSAP) technique with 54 primer combinations. The results show that most cytosine methylated patterns are conservatively inherited from parents. The numbers of variant sites are less in F_1 generation. According to the number of sites individually inherited from female and male parents, the different choice of female and male parents made a big distinction between the sites inherited from female parent and the sites inherited from the male parent. For reciprocal cross F113 and F132, the number for sites of cytosine methylation patterns inherited from the female parent are far more than that from the male parent, which may be closely related to cytoplasmic inheritance.

Key words: Methylation sensitive amplified fragment length polymorphism (MSAP), DNA methylation, cytoplasmic inheritance, reciprocal cross, cotton.

INTRODUCTION

In plants, cytosine methylation usually happens in both CpG and CpNpG sequences, which can be reserved through cycles of DNA replication. It plays an important role in cell differentiation, genomic imprinting, gene silencing, etc. (Jaligot et al., 2008; Zeng et al., 2011). Cytosine methylation widely exists in diverse eukaryotic organisms, but the cytosine methylation level is different because of distinction in plant species. Generally, higher plants have a markedly higher level of covalent modification of their DNA, with 20 to 40% of all cytosine residues in the nuclear DNA being methylated (Suzuki and Bird, 2010; Inagaki and Kakutani, 2010). In plants changes of DNA methylation can be triggered by major genomic events, such as environmental stresses, polyploidization and hybridization (Zhong et al., 2009; Dalakouras et al., 2010; Pan et al., 2009; Mason et al., 2008; Thanananta et al., 2006; Li et al., 2010). These changes can trigger the transcriptional variations for genes, and newly acquired epigenetic states of transcriptional gene can be readily transmitted to the progeny through meiosis, which can cause heritable phenotypic modifications in the absence of changes in DNA sequence (Guo et al., 2011).

Hybridization is an important traditional way of breeding new cultivars and it plays a significant role in plant evolution. Recently, most studies show that hybridization can cause the variations of DNA methylation pattern and level for hybrids, which is closely related to the performance of heterosis (Li et al., 2010; Zhao et al., 2009; Sakthivel er al., 2010). However, the researches on the relationship between inheritance of DNA methylation and cytoplasmic inheritance by reciprocal cross are less. Methylation-sensitive amplified polymorphism (MSAP)

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Abbreviation: MSAP, Methylation sensitive amplified fragment length polymorphism.

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Table 1. Primer combinations of selective amplification and adapter in MSAP.

Sample	Enzymes	
	***EcoRI* (E) (5′ to 3′)**	***HpaII*/*MspI* (H-M) (5′ to 3′)**
Adaptors	CTCGTAGACTGCGTACC AATTGGTACGCAGTC	GATCATGAGTCCTGCT CGAGCAGGACTCATGA
Pre-amplification primers	GACTGCGTACCAATTCA	ATCATGAGTCCTGCTCGGT
Selective amplification primers	GACTGCGTACCAATTCACT(E1) GACTGCGTACCAATTCATC(E2) GACTGCGTACCAATTCACC(E3) GACTGCGTACCAATTCACG(E4) GACTGCGTACCAATTCAGG(E5) GACTGCGTACCAATTCAGA(E6) GACTGCGTACCAATTCAGT(E7) GACTGCGTACCAATTCAGC(E8)	ATCATGAGTCCTGCTCGGTGC(H1) ATCATGAGTCCTGCTCGGTGA(H2) ATCATGAGTCCTGCTCGGTAG(H3) ATCATGAGTCCTGCTCGGTTC(H4) ATCATGAGTCCTGCTCGGTTG(H5) ATCATGAGTCCTGCTCGGTAC(H6)

is a convenient protocol for detecting the cytosine methylation site. It has been used to investigate variations of DNA methylation in many kinds of plant species (Mason et al., 2008; Chen et al., 2009; Hanai et al., 2010).

It used the isoschizomers HpaII and MspI as 'frequent-cutter' enzymes instead of the usual MseI according to the method of the amplified fragment length polymorphism (AFLP) technique (Vos et al., 1995). Recently, it has been successfully applied to study epigenetic variation in many plants, such as the effect of short day photoperiod on DNA methylation in rice, the DNA-methylaiton changes in wheat induced by salt stress, and variations of DNA methylation in hybrids in cotton (Zhong et al., 2009; Thanananta et al., 2006). Cytoplasmic inheritance is the descendiblity of parental characters through a non-chromosomal means. Due to the fact that the cytoplasm is usually contributed entirely by one parent, the characters encoded by genes are generally inherited from only female parent. Many genes were found in the cytoplasm, typically from the mitochondria and chloroplast (Cosmides and Tooby, 1981).

To our knowledge, research reports on the relationship between DNA methylation and cytoplasmic inheritance are few, and the effects of reciprocal cross on inheritance of DNA methylation are little known. In this study, we selected two cotton cultivars as parents: one brown cotton line with short fiber length and brown pigment in fiber, another white cotton cultivar with long fiber length and white pigment in fiber. By reciprocal cross, we compared the cytosine methylation patterns and levels between parents and mutual cross F_1 by MSAP protocol. The effects of reciprocal cross on inheritance of DNA methylation were studied, and the relationship between cytosine methylation and cytoplasmic inheritance were discussed.

MATERIALS AND METHODS

Plant materials

A brown-fiber cotton line *Zongcaixuan No.1* (P26) and a white-fiber cotton cultivar *HY408* (P55) were selected as parents, and two F_1 plant populations - F113 and F132 - were obtained by reciprocal cross in the farm of Anhui Agricultural University, P.R. China in the year 2009. The obverse cross F113 was obtained by using P26 as male parent and P55 as the female parent. Contrary to F113, for the inverse cross F132, the P26 was selected as female parent and P55 as the male parent. The parents and the reciprocal cross F_1 were cultivated in the farm of Anhui Agricultural University, P.R. China in the year 2010. At anthesis stage of cotton, fully expanded leaves at the top of the main stems were collected and stored at -70°C for use.

DNA extraction and purification

Genomic DNA was isolated from frozen leaves of two hybrids and their parents using the improved cetyltrimethylammnonium bromide (CTAB) extraction methods as described by Murray and Thompson (1980). Additionally, the RNA enzyme was added to the DNA liquor to digest residual RNA; DNA quality, purity, and its content were tested by using the 0.8% agarose gel electrophoresis and ultraviolet spectrophotometer.

Methylation-sensitive amplification polymorphism analysis

MSAP method was mainly described as follows: at first, genomic DNA of cotton was divided into two groups, for the first group, 20 units *EcoRI* (Takara, P.R.China) and 20 units *HpaII* (Takara, P.R.China) were used to digest 200 ng genomic DNA in 20 µl of reaction mixture at 37°C for 2 h. For the second group, instead of *HpaII*, the *MspI* (Takara, P.R. China) was used in combination with *EcoRI* to digest 200 ng genomic DNA with the same condition. The enzyme digestion reaction was terminated by incubation at 65°C for 10 min. Then, the ligation reaction was proceeded in a final volume of 40 µl mixture, which contains 1 unit T4 DNA ligase, 0.2 mM ATP, 5 pm *EcoRI* adapters and 50 pm *HpaII*/*MspI* adapters for additional 6 h at 20°C (Table 1). Subsequently, the ligation mixture was used

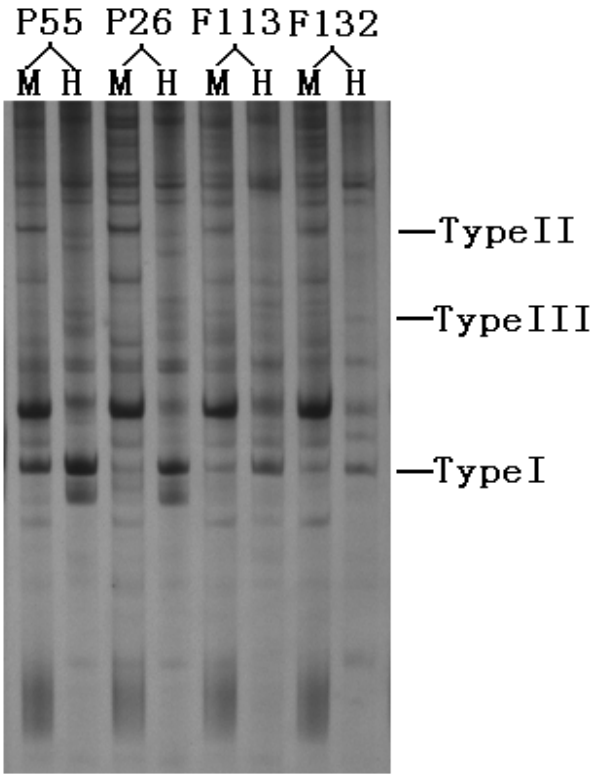

Figure 1. DNA methylation patterns of enzymes sites of *HpaII* and *MspI*. The result of MSAP amplified using primer H4/E3. M, Enzyme sites cleaved with *EcoRI* and *MspI*; H, sites cut with *EcoRI* and *HpaII*. MSAP, Methylation sensitive amplified fragment length polymorphism.

as the template for the preselected amplification reaction with *EcoRI*+A and *HpaII* / *MspI*+T primers (Table 1). The polymerase chain reactions were carried out in a 25 µl reaction mixture with 1 µl of ligation reaction mixture, 50 ng of E+A primer, 50 ng of HM +T primer, 0.5 unit Taq DNA polymerase (Biocentury transgene, P.R. China), 0.2 mM dNTP (Biocentury transgene, P.R.China) and 2.5 µl of 10× polymerase buffer (Biocentury transgene, P.R. China) for 21 cycles with 1 min denaturation at 94°C, 1 min annealing at 56°C, and 1 min extension at 72°C. The preselected amplification products were used as the template for the selective amplification reaction. The primers were the *EcoRI* and *HpaII/MspI* primers with two added selective nucleotides (Table 1). The polymerase chain reactions were carried out in total volumes of 25 µl, containing 0.3 µl of pre-amplification product, 50 ng of *EcoRI* primer, 50 ng of *HpaII/MspI* primer, 1 unit Taq polymerase, 0.5 mM dNTP and 2.5 µl of 10× PCR buffer. The PCR procedure was performed according to the standard amplified fragment polymorphism touchdown protocol (Vos et al., 1995). The products of selective amplification were mixed with 8 µl of denaturating buffer (98% formamide, 10 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol), then denatured at 95°C for 5 min and separated on 6% polyacrylamide gel in 1× 44.5 Mm Tris/Borate, 0.5 Mm EDTA, pH 8.0 (TBE) buffer at 80 watts for 1 h. Gels were stained according to the silver staining method described by Bassam et al. (1991).

Gel electrophoresis belt analysis

After staining, the bands that appeared in the electrophoretogram were detected and counted by using Genescope software of gel imaging system Biosens SC645 (Biotop, P.R. China). The scored MSAP bands were transformed into a binary character matrix, using "-" to define the absence of a band and "+" to define the presence of a band, respectively.

RESULTS

Cytosine methylation status of hybrids and their parents

In the MSAP experiment, two isoschizomers *HpaII* and *MspI* were used to recognize the 5'-CCGG-3′ sequence. Due to different sensitivity to methylation sites, *HpaII* only cuts hemimethylated sequence, whereas *MspI* digests methylated sites at the internal cytosine $C^{m}CGG$. According to the absence or presence of a band, DNA methylation patterns could be divided into four types (Figure 1). Type I was recognized when both enzymes *HpaII* and *MspI* were used. Type II was shown based on a band presence by *MspI* and absence by *HpaII.* Type III was identified when the inverse pattern of type II was observed. Type IV was identified by the absence of bands when both enzymes *HpaII* and *MspI* are inactive.

In this study, 54 pairs of *EcoRI +HpaII/MspI* primer combinations were used to analyze the cytosine methylation status in hybrids and their parents. As shown in Tables 2 and 3, the patterns and levels of cytosine methylation are different among four plant population. For the parents, the percentages of full methylation were 16.43% for P_{26} and 17.93% for P_{55}. The ratios of hemimethylation were 6.96% for P_{26} and 10.76% for P_{55}. For two hybrids F_{113} and F_{132}, the percentage of full methylation was 15.35 and 15.66%, respectively, and the ratio of hemimethylation was 7.16 and 3.52%, individually (Table 3). There are obvious differences between obverse cross F113 and inverse cross F132.

Inheritance and variation of cytosine methylation patterns among hybrids and their parents

In order to make clear the relationship of cytosine methylation patterns between parents and hybrids, the same variation sites were observed and calculated. By comparing the cytosine methylation patterns between parents and hybrids, we defined four types of cytosine methylation inheritance origins based on the genetic resources. For the first and second types, there are six respective kinds of cytosine methylation patterns inherited from female and male parents. For the third type, there are three kinds cytosine methylation patterns inherited from both female and male parents. For the fourth type, there are 10 kinds of cytosine methylation patterns, which were dissimilar with both female and male parents. As shown in Table 4, H, M and HM were defined as patterns that the same bands were detected in parent and hybrid. V was defined as patterns that

Table 2. Bands of different patterns obtained by MSAP.

Parental lines and hybrid	Number of band			Total number of band
	Type I	Type II	Type III	
P26	760	163	69	992
P55	736	185	111	1032
F113	757	150	70	977
F132	798	155	34	987

MSAP, Methylation sensitive amplified fragment length polymorphism.

Table 3. Cytosine methylation levers among parental lines and hybrids.

Parental lines and hybrid	Percentages of non-methylated	Percentages of total methylated	Percentages of hemi-methylated	Percentages of fully methylated
P26	76.61	23.39	6.96	16.43
P55	71.31	28.69	10.76	17.93
F113	77.49	22.51	7.16	15.35
F132	80.82	19.18	3.52	15.66

different bands were observed in parent and hybrid. M was recognized according to the presence of a band produced by *MspI*, but its absence when *HpaII* was used. H was identified when the inverse pattern of M was observed. HM was identified by the absence of bands when both enzymes *HpaII* and *MspI* were used.

According to the calculated data, there are obvious differences for four types according to genetic origins. For inherited cytosine methylation patterns from the female parent, there are a total of 81 sites in F113 and F132. However, there are only 37 sites of six kinds of cytosine methylation patterns inherited from the male parent in reciprocal cross F_1. For common inherited sites from both parents, a total of 919 and 926 sites in F113 and F132 were determined respectively. For variant sites, 31 sites were detected. The results show that most cytosine methylated patterns are conservatively inherited from parents by traditional cross, and the numbers of variant sites are less in F_1 generation. Between the obverse cross F113 and inverse cross F132, the site amounts inherited from both parents are similar. However, according to the number of sites individually inherited from female and male parents, the different choice of female and male parents made a big distinction between the sites inherited from the female parent and the sites inherited from the male parent. For reciprocal cross F113 and F132, the number for sites of cytosine methylation patterns inherited from the female parent is far more than that from the male parent.

DISCUSSION

Recently, some studies indicated that the MSAP technique is highly efficient for massive detection of cytosine methylation in plant genomes. In this study, we have used this technique to study methylation of 5'-CCGG-3' sites in the cotton genome. The result shows that about 20% of the 5'-CCGG-3' sites were methylated. It was higher than that in rice and lower than that in *Arabidopsis* (Jaligot et al., 2008; Sakthivel et al., 2010), and the full methylation of internal cytosine (15.35 to 17.93%) was the main pattern of the genomic DNA methylation for cotton.

Hybridization is a main traditional way for breeding new cultivars. In order to gain high yield and good quality for plant, heterosis is usually used by cross. In recent years, with the in-depth studies on cytosine methylation, many researchers gradually pay attention to the relationship between DNA methylation and heterosis; they carried out a great deal of experiments in trying to illustrate the correlation between heterosis and cytosine methylation in many species such as rice, cotton, etc. (Li et al., 2010; Zhao et al., 2009; Sakthivel et al., 2010). For example, the number of demethylation loci in highly heterotic hybrids was found greater than that in lowly heterotic hybrids in cotton.

The methylation status of many genes was modified differentially in hybrid and parents. For Indian rice hybrid KRH2, in contrast to parents, the level of methylation was high during initial growth stages and reduced as the hybrid grew; a majority of cytosine methylation profiles in hybrids was transmitted from parents. These results suggest that the changes of cytosine methylation profiles and methylation status of many genes might play a role in the performance of heterosis.

Reciprocal cross is one kind of hybridization, which is usually used to detect sex linkage, maternal inheritance, or cytoplasmic inheritance. It is closely related to male parents for reciprocal cross F_1 could lead to diverse

Table 4. DNA methylation patterns between hybrids and their parents.

Genetic origin	Type	Cytosine methylation pattern						Number of site	
		Female parent		Male parent		Hybrid		F_{113}	F_{132}
		HpaII	*MspI*	*HpaII*	*MspI*	*HpaII*	*MspI*		
Female parent genetic sites	H1-1	+	–	–	–	+	–	11	6
	H1-2	–	–	+	–	–	–	2	2
	M1-1	–	+	–	–	–	+	23	16
	M1-2	–	–	–	+	–	–	1	1
	HM1-1	+	+	–	–	+	+	6	7
	HM1-2	–	–	+	+	–	–	2	4
	Sum							45	36
Male parent genetic sites	H2-1	–	–	+	–	+	–	0	0
	H2-2	+	–	–	–	–	–	1	1
	M2-1	–	–	–	+	–	+	9	8
	M2-2	–	+	–	–	–	–	0	1
	HM2-1	–	–	+	+	+	+	5	7
	HM2-2	+	+	–	–	–	–	3	2
	Sum							18	19
Common genetic sites	H3	+	–	+	–	+	–	55	27
	M3	–	+	–	+	–	+	113	126
	HM3	+	+	+	+	+	+	751	773
	Sum							919	926
Variant sites	V1	+	–	–	+	+	+	1	3
	V2	–	+	+	–	+	+	0	2
	V3	+	–	+	–	–	–	2	3
	V4	–	+	–	+	–	–	1	0
	V5	–	+	+	–	–	–	1	0
	V6	+	–	–	+	–	–	0	1
	V7	+	+	+	+	–	–	0	1
	V8	–	–	–	–	+	–	4	2
	V9	–	–	–	–	–	+	5	4
	V10	–	–	–	–	+	+	0	1
	Sum							14	17

"+", bands appeared in *EcoRI+HpaII* or *EcoRI+MspI* digested; "-", no bands in *EcoRI+HpaII* or *EcoRI+MspI* digested.

gene expression in two F_1 generations; however, the unusual expression of genes related to cytosine methylation is little known to our knowledge. In this experiment, we tried to ascertain whether the change in methylation pattern or the methylation polymorphism was present among parents and hybrids by reciprocal cross; the regulations of inheritance of DNA methylation were analyzed. As a result, an obvious difference in methylation was observed between two hybrids and their parents. Based on the whole analysis of the methylation status at 5'-CCGG-3' sites, we found that more loci were demethylated in hybrid if compared with the parents, and that most cytosine methylated patterns in hybrids are conservatively inherited from the parent. The numbers of variant sites are less in F_1 generation. According to the number of sites individually inherited from female or male parents, we found that the different choice of female and male parents made a big distinction between the sites inherited from female parent and the sites inherited from the male parent. The numbers of sites of cytosine methylation patterns inherited from the female parent are far more than that from the male parent, which suggests regular characteristics of cytoplasmic inheritance for cytosine methylation.

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