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IDENTIFICATION OF EXOTIC GENETIC COMPONENTS AND DNA METHYLATION PATTERN ANALYSIS OF THREE COTTON INTROGRESSION LINES FROM *Gossypium bickii*

© 2011 Shou-Pu He, Jun-Ling Sun, Chao Zhang, Xiong-Ming Du*

Key Laboratory of Cotton Genetic Improvement, Ministry of Agriculture; Cotton Research Institute, Chinese Academy of Agricultural Sciences, Anyang, Henan, 455004, China

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The impact of alien DNA fragments on plant genome has been studied in many species. However, little is known about the introgression lines of *Gossypium*. To study the consequences of introgression in *Gossypium*, we investigated ~2000 genomic and ~800 epigenetic sites in three typical cotton introgression lines, as well as their cultivar (*Gossypium hirsutum*) and wild parents (*Gossypium bickii*), by amplified fragment length polymorphism (AFLP) and methylation-sensitive amplified polymorphism (MSAP). The results demonstrate that an average of 0.5% of exotic DNA segments from wild cotton is transmitted into the genome of each introgression line, with the addition of other forms of genetic variation. In total, an average of 0.7% of genetic variation sites is identified in introgression lines. Simultaneously, the overall cytosine methylation level in each introgression line is very close to that of the upland cotton parent (an average of 22.6%). Further dividing patterns reveal that both hypomethylation and hypermethylation occurred in introgression lines in comparison with the upland cotton parent. Sequencing of nine methylation polymorphism fragments showed that most (7 of 9) of the methylation alternations occurred in the noncoding sequences. The molecular evidence of introgression from wild cotton into introgression lines in our study is identified by AFLP. Moreover, the causes of petal variation in introgression lines are discussed.

***Keywords: Gossypium hirsutum*, *Gossypium bickii*, introgression, AFLP, MSAP.**

Interspecific hybridization occurs widely during the evolution history of a plant [1]. In breeding, interspecific hybridization can transfer adaptive traits into cultivar varieties and create novel genotypes/phenotypes [2]. Therefore, interspecific crossing and backcrossing have been widely used in the history of plant breeding. Despite many difficulties existing in interspecific breeding (such as cross-incompatibility, F_1 sterility, etc.), a large number of elite germplasm with exotic traits has been created and selected by this approach for decades [3].

Introgression was first mentioned and discussed by Anderson [4]. This is a phenomenon accompanied by interspecific hybridization occurring in most natural species [5]. In practical applications, introgression is used to describe the exchange of genes from donor species to target cultivars [2]. Therefore, cultivars selected from the progeny of interspecific hybridization are called "introgression lines." At present, the germplasm of cotton contains a large number of introgression lines created by several important cotton-improving programs in the past decades. For instance, Pee Dee lines are a series of upland cotton with excellent fiber quality and high yield, which introgressed consanguinity of *Gossypium arbroeum*, *G. thurberi*, and *G. barbadense* [6]. Moreover, other cotton wild relatives are also used as donors for transferring useful traits in breeding [7]. In China, the study of interspecific hybridization of cotton has been conducted by several research institutes since the 1970s. In the following decades, a large quantity of varieties with excellent exotic properties was selected by breeders. Most of these varieties were later used as germplasm for further breeding programs.

DNA methylation is widely spread in all species, especially in high plants, and over 30% of cytosine residues are methylated [8, 9]. Most of the critical fundamental metabolic processes and phenotype variations are proven to be closely relevant with DNA methylation [10]. In high plants, gain and loss of DNA methylation at transposon sites is considered as a switch to control the activation process of transposons in plants [11]. In addition, DNA methylation shows the dynamic status in the different development stages of the plant. Ruiz-Garcia et al. [13] reported an increasing trend in DNA methylation from different stages of organs in *Arabidopsis*. This evidence implies that DNA methylation plays a critical role in the entire plant development.

Although the great improving effects of interspecific hybridization have been proven in many other species, leading to it being used as a common breeding method for many years [14–18], its mechanism is not well

* E-mail: duxm@cricaas.com.cn

Petal comparison of three ZYH introgression lines and their parents.

understood. In several recent studies, researchers attempted to assess the extensive genetic and epigenetic alterations in the initial stage of genome merging in plants by amplified fragment length polymorphism (AFLP) [19] and methylation-sensitive amplified polymorphism (MSAP) [20]. The results showed that almost all of the studied species are under genome-wide genetic and epigenetic regulation [21–25]. On the other hand, some studies also tried to explain introgression phenomena basing on molecular approaches. Exotic genome components were successfully detected in the introgression lines of coffee [26], rice [23], and cucumber [27] by AFLP technology. Consequently, all studies above demonstrated that AFLP and MSAP are feasible and efficient in analyzing genetic and epigenetic phenomena in plants.

In this paper, five samples, including a set of typical cotton introgression lines with *Gossypium bickii* consanguinity, and their parents were studied. The introgression lines possess the typical phenotype of *G. bickii*. There are two main purposes of this study: a) to determine whether exotic DNA components are integrated into the genome of these introgression lines and b) to estimate the genetic and epigenetic variations in cotton introgression lines and discuss their possible impacts.

EXPERIMENTAL

Plant material sampling and DNA extraction. Three cotton introgression lines with *G. bickii* consanguinity called "*hirsutum-bickii* red flower line" (HBRL), including Zhongyihong 2 (Z2), 8 (Z8), and 9 (Z9), which were bred and selected by Liang from 1989 to 1994 [28, 29], and their original parents, cultivar parent "Keyi181" (K181; *G. hirsutum,* also used as a backcross parent) and wild parent *G. bickii*, were used in this experiment. Distinguished from common upland cotton, the specific phenotype of these introgression lines was a pink petal with a purple spot inside, which looked more like the petal of their wild parent (Figure).

All five cotton germplasms were maintained by strictly selfing and then planted in the greenhouse of the Cotton Research Institute, Chinese Agricultural Academy of Sciences in Anyang China in 2008. The young seedlings were sampled for DNA extraction by using the Cetyl trimethylammonium bromid (CTAB) method [30].

AFLP analysis. As a highly sensitive technology, AFLP provided the richest polymorphism in fingerprinting analysis compared to other techniques [18, 31]. Standard AFLP analysis was performed to assess the genetic variations of introgression lines during introgression as compared to their parents by the protocol described by Vos [19] with minor modifications for higher yield of PCR production. The purified DNA was digested in 37°C for 3 h (longer than the original procedure) with excessive amounts of restriction enzymes (since cotton has a more complex genome) for obtaining completely digested templates.

Ultimately, the denatured PCR products were separated on an 8% denaturing polyacrylamide gel for 1.5 h at 75 W. The gels were stained by the silver stain method, and the band patterns were transformed to a "1, 0" matrix for statistics analysis ("1" indicates the presence of a band and "0" indicates the absence of a band).

MSAP analysis. We used the MSAP protocol described by Xu et al. [32]. The reaction components and procedures were exactly the same as for the AFLP analysis above. Technically, the only difference between AFLP and MSAP is the substitution of a pair of isoschizomers, HpaII and MspI, for EcoRI.

MSAP fragment sequencing. Distinct and repeatable MSAP fragments were chosen for sequencing. Fragments were carefully cut out of the gel by a knife and then "smashed" into tiny splinters in a 1.5 ml centrifugal tube. 20 (l of double-distilled water were added to the tube and the mixture was heated in boiling water for 10 min. 5 (l of the solution in the tube were used as a template to reamplify the target fragments using primer combinations. The fragments were separated in a 0.8% agarose gel, extracted, and ligated into the T-vector (Promega). The cloned DNA segments were sequenced with vector primers by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. Analysis of the similarity of sequenced fragments was performed at the National Center for Biotechnology Information using the BLASTN program.

Table 1. Genetic variations in introgression lines based on AFLP

K181	*Gossypium bickii*	Introgression lines	Z2	Z8	Z9
+	+	+	1134	1130	1128
+	–	+	877	880	882
–	+	+	5	16	12
–	–	+	1	5	2
+	+	–	0	2	1

Note: Here and in Table 2 "+"– indicates the presence of a band; "–" – indicates the absence of a band.

Table 2. Classification of the CCGG site status by different band patterns displayed on the gel

Classification	Site status	HpaII	MspI	E + H	E + M
Class I	CCGG GGCC	Active	Active	+	+
Class II	^{m}CCGG GGCC	Active	Inactive	+	–
Class III	C^{m}CGG GG^{m}CC	Inactive	Active	–	+
Not distinguishable	^{m}CCGG^{m} C^{m}CGG GGC^{m}C GG^{m}C^{m}C	Inactive	Inactive	–	–

Note: E + H – indicates that the DNA was digested by EcoRI + HpaII; E + M – indicates that DNA was digested by EcoRI + MspI.

RESULTS

Standard AFLP analysis

About 2000 sites were generated by 108 primer pair combinations in each of the three introgression lines. Nevertheless, only an average of 0.5% of the sites of each introgression line were putatively derived from *G. bickii*. This value is close to the one derived from the study of rice introgression lines (< 0.1%) [23]. Table 1 reveals that five sites introgressed into the Z2 line from *G. bickii*, 16 sites into Z8, and 12 sites into Z9, with an average of 0.5% in each introgression line. Moreover, number of missing fragments in the introgression lines of Z2, Z8, and Z9 were 0, 2, and 1, respectively. Novel fragments in the introgression lines of Z2, Z8, and Z9 were 1, 5, and 2, respectively. This showed that the exotic fragments from *G. bickii* introgressed the most into line Z8, then into line Z9, and the lowest in line Z2. In summary, with reference to K181, an average of 0.7% of genetic variation occurred in each introgression line. However, Z8 was more genetically unstable than the other two lines.

MSAP analysis

Both HpaII and MspI recognize the same sequence "5-CCGG-3" but display differential sensitivity to cytosine methylation. HpaII is inactive when any of the two cytosines is fully methylated but cuts the hemi-methylated sequence, whereas MspI cuts 5-C^{5m}CGG-3 but not 5-^{5m}CCGG-3 (see Table 2). Therefore, it is possible to distinguish the different status of DNA methylation at the same CCGG site by the distinct band patterns displayed on a gel.

Forty primer combinations were used in the MSAP analysis. About 800 sites were generated in K181 and introgression lines. Because of the different sensitivity of the two isoschizomers at the CCGG sequence, we could not only distinguish whether the CCGG site was methylated or not, but also to determine the methylation status of this site. Although the DNA methylation level was confirmed by MSAP to be lower than the absolute value [33], Dong et al. [25] suggested that using this technology comparison of the relative DNA methylation levels could be reliable and efficient. According to the methylation level formula: ClassII + ClassIII/ClassI + ClassII + + ClassIII, the total DNA methylation level in K181,

Table 3. Number of sites in the introgression lines and their original parent based on MSAP

Description	Classification	K181	*Gossypium bickii*	Z2	Z8	Z9
Unmethylated sites	Class I	480 (77.5%)	390 (75.3%)	480 (77.3%)	482 (77.6%)	479 (77.3%)
Hemi-methylated sites	Class II	18 (2.9%)	16 (3.1%)	20 (3.2%)	18 (2.9%)	18 (2.9%)
Fully methylated sites	Class III	121 (19.6%)	112 (21.6%)	121 (19.5%)	121 (19.5%)	123 (19.8%)
Total methylated sites	Class II + Class III	139 (22.5%)	128 (24.7%)	141 (22.7%)	139 (22.4%)	141 (22.7%)
Total sites		619 (100%)	518 (100%)	621 (100%)	621 (100%)	620 (100%)

G. bickii, Z2, Z8, and Z9 was 22.5, 24.7, 22.7, 22.5, and 22.7%, respectively (Table 3). Interestingly, further results indicated that the percentage of fully methylated sites was much larger (~6 times) than the hemi-methylated sites in all five samples. Taken together, both the total methylation level and the subtype of methylation level were stable between the introgression lines and their upland cotton parents. Only *G. bickii* presented a slightly higher total methylation level.

To obtain more detailed information on the inheritance and variation of the DNA methylation pattern, all MSAP sites were divided into four major types. All major types were further divided into subtypes according to different band patterns in the same site (Table 4). Type A indicated that the sites displayed monomorphism in the introgression lines and both parents. There was an average of 34.6% monomorphism sites among the three introgression lines with most unmethylated sites (A1), and the monomorphism methylation sites were only an average of 3.7%, including A2 and A3. Type B indicated that sites of the introgression lines displayed the same pattern with one of the parents, of which 11 B subtypes (B1–B11) were the same as K181 but different from *G. bickii*. Moreover, their methylation pattern included an average of 11.0% fully methylated, 1.4% hemi-methylated, and 27.8% unmethylated sites. Also there were five B subtype (B12–B16) sites in the introgression line displaying the same pattern as *G. bickii* but different from K181. Only unmethylated and fully methylated sites were detected in these subtypes, and both kinds of detectable sites in Z2 were the fewest. Moreover, in B13, B14, and B16, it is considered that the DNA methylation status was altered from K118 to *G. bickii*. However, the polymorphism of B12 and B15 could be caused by sequence introgression. Types C and D indicated that the band pattern of introgression lines was identical to neither of their parents. Based on the classification of band patterns (Table 2) in comparison with K181, hypermethylation that occurred in introgression lines was classified as type C, which accounted for 9.3%. And demethylation was classified as type D, which accounted for 1.0%. Interestingly, no hemi-methylated and fully methylated sites were detected in types C and D, respectively. In total, 11(7 + 4), 12(6 + 6), and 16(10 +6) sites had hypermethylation in Z2, Z8, and Z9, respectively. Further, 9(8 + 1), 13(8 + 5), and 13(9+4) sites had demethylation in Z2, Z8 and Z9, respectively. In total, compared with K181, the altered size of introgression lines was 20, 25, and 29 in Z2, Z8, and Z9, respectively.

MSAP fragment sequencing

Nine MSAP polymorphism fragments were isolated from the gel, reamplified, and sequenced. There were only two fragments showing significant similarities to the sequences recorded at the National Center for Biotechnology Information (Table 5). Both mRNA sequences were from *Arabidopsis thaliana*. The results indicated that most of the sequences involved in the methylation polymorphisms were located in non-coding sequences.

DISCUSSION

The DNA methylation levels of the introgression lines and the upland cotton parent were stable

In this study, an average of 22.6% CCGG sites was methylated in the cotton introgression lines and their upland cotton parent. This proportion was lower than that of the wild cotton parent (*G. bickii*) and *A. thaliana* (~35%) [34], but higher than that reported on rice (~16.0%) [20, 33]. It implied that the DNA methylation level is species-specific. On the other hand, unlike the significant increase in the methylation level (versus the rice parent) in rice introgression lines Dong et al. [25], all three introgression lines also displayed a highly stable methylation level versus their upland cotton parent. Previous studies on maize [35], rice [20, 25], and *Brassica* [36] demonstrated that DNA methylation levels could be shifted (increased/decreased) during hybridization (including interspecific hybridization) or polyploidization. One possible cause for this unexpected phenomenon was the repeated backcross process in the breeding history of our materials. Chen [37] considered that the cytosine methylation pattern alternation could happen in the very early generations and tended to be stable between advanced generations. The introgression lines used in the current study have undergone interspecific hybridization that led to drastic phenotype variations [28]. We speculated that phenotype variations could be caused by the extended genetic and epigenetic alterations. This phenom-

Table 4. Comparison of the methylation patterns between the introgression lines and their parents

Description		Site type	K181		*Gossypium bickii*		Introgression lines		Methylation status*	Z2	Z8	Z9
			H	M	H	M	H	M				
Monomorphism	Unmethylated	A1	+	+	+	+	+	+	UM	248	248	247
	Methylated	A2	–	+	–	+	–	+	FM	30	29	28
		A3	+	–	+	–	+	–	HM	1	1	1
		Subtotal								279 (34.6%)	278 (34.7%)	276 (34.5%)
Polymorphism sites inherited from one of the parents in introgression line	Introgression lines displayed the same band pattern with upland cotton parent	B1	+	+	–	+	+	+	UM	28	28	28
		B2	+	+	+	–	+	+	UM	4	4	4
		B3	+	+	–	–	+	+	UM	193	192	190
		B4	–	+	–	–	–	+	FM	73	72	74
		B5	–	+	+	+	–	+	FM	15	14	14
		B6	–	+	+	–	–	+	FM	1	1	1
		B7	+	–	–	–	+	–	HM	12	10	9
		B8	+	–	+	+	+	–	HM	1	1	1
		B9	–	–	+	+	–	–	FM	121	119	120
		B10	–	–	–	+	–	–	FM	49	47	47
		B11	–	–	+	–	–	–	FM	10	10	10
		Subtotal								507 (62.9%)	498 (62.2%)	498 (62.0%)
	Introgression lines displayed the same band pattern with wild cotton parent	$B12^D$	–	–	+	+	+	+	UM	0	2	1
		$B13^D$	–	+	+	+	+	+	UM	1	2	2
		$B14^C$	+	+	–	+	–	+	FM	3	3	3
		$B15^D$	–	–	–	+	–	+	FM	0	1	1
		$B16^C$	+	–	–	–	–	–	FM	1	3	3
		Subtotal								5 (0.6%)	11 (1.4%)	10 (1.2%)
Novel polymorphism sites in introgressed line	Hypermethylated sites in introgressed line versus upland cotton parent	C1	+	+	+	+	–	+	FM	2	2	3
		C2	+	+	+	–	–	+	FM	1	0	0
		C3	+	+	–	–	–	+	FM	2	2	4
		C4	+	+	+	+	–	–	FM	1	1	1
		C5	–	+	–	+	–	–	FM	0	0	1
		C6	+	–	+	+	–	–	FM	1	1	1
		Subtotal								7 (0.9%)	6 (0.7%)	10 (1.2%)
	Demethylated sites in introgressed line versus wild cotton parent	D1	–	+	–	+	+	+	UM	0	1	1
		D2	–	+	–	–	+	+	UM	1	2	1
		D3	+	–	–	+	+	+	UM	2	2	2
		D4	–	–	–	–	+	+	UM	2	0	2
		D5	–	–	–	+	+	+	UM	1	1	1
		D6	–	–	–	–	+	–	HM	2	2	2
		Subtotal								8 (1.0%)	8 (1.0%)	9 (1.1%)
		Total								806 (100%)	801 (100%)	803 (100%)

*Indicates the DNA methylation status of introgression lines.

Note: Type A indicates that the sites display monomorphism in introgression lines and both parents; Type B indicates that sites of introgression lines display the same pattern with one of the parents; Types C and D indicate that the band pattern of introgression lines is identical to neither of their parents. The B subtypes with C or D capital letters indicate that these subtypes also show hypermethylation or hypomethylation UM – unmethylated; HM – hemi-methylated; FM – fully methylated.

Table 5. BLAST search results for sequences of DNA methylation pattern variation in introgression lines

Sequence ID	Primer combination	Fragment size, bp	Similar sequence	*E* value
Z1-164	E-AAA + H/M + TCAC	164	none	
Z2-355	E-AAA + H/M + TCAT	355	*Arabidopsis thaliana* leucine-rich repeat family protein (NM_118212) mRNA complete cds	3E-52
Z3-283	E-AAA + H/M + TCAG	283	none	
Z6-148	E-ACT + H/M + TCAC	148	none	
Z7-167	E-ACT + H/M + TCAG	167	none	
Z10-111	E-ACC + H/M + TCAT	111	none	
Z12-223	E-AAG + H/M + TCAA	223	*Arabidopsis thaliana* unknown protein (NM_100678.2) mRNA, complete cds	5E-15
Z15-203	E-AGT + H/M + TCAG	203	none	
Z16-188	E-AGC + H/M + TCAA	188	none	

enon has been confirmed in the majority of species [21, 22, 24, 25, 37]. Nevertheless, the genetic and epigenetic status tended to that of the upland cotton parent by following repeated backcross and inbreeding gradually. Another explanation could be that it is a distinct feature of cotton. Both the genetic and epigenetic status was stable in a genome-wide survey of nine sets of newly synthesized allotetrapoid and allohexaploid cotton [38].

The causes of petal variation in the introgression lines

Another interesting question was what are the causes inducing the petal variation in introgression lines. In this study, DNA fragments from *G. bickii* were identified in the genome of introgression lines by AFLP, which was the direct evidence that led to the question above. It could also be confirmed by the classic genetic analysis of the petal color and the purple spot [39]. In addition, the impacts of DNA methylation should not be ignored. Recently, more and more studies demonstrated that DNA methylation played an important role in the regulation of gene expression [11, 40]. Compared with K181, the number of genetic variation (including introgressed, missing, and novel) sites in introgression lines was 6, 23, and 15 in Z2, Z8, and Z9, respectively. Moreover, there were 20, 25, and 29 epigenetic variation sites (including sites of B12–B16, C and D types), respectively. This data implied that, in our study, both genetic and epigenetic variations were found in the upland cotton parent and introgression lines. In another study of rice introgression lines, Dong et al. [25] considered insertion of alien DNA to be one of the reasons which led to DNA methylation alternation. Liu et al. [41] also mentioned that DNA methylation could create numerous novel genetic mutations accompanied by polyploidization. Therefore, we speculate that the petal variation might be a consequence of the interaction of DNA methylation alternations with genetic variations.

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