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Analysis of DNA methylation variation in wheat genetic background after alien chromatin introduction based on methylation-sensitive amplification polymorphism

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During the process of alien germplasm introduced into wheat genome by chromosome engineering, extensive genetic variations of genome structure and gene expression in recipient could be induced. In this study, we performed GISH (genome *in situ* hybridization) and AFLP (amplified fragment length polymorphism) on wheat-rye chromosome translocation lines and their parents to detect the identity in genomic structure of different translocation lines. The results showed that the genome primary structure variations were not obviously detected in different translocation lines except the same 1RS chromosome translocation. Methylation sensitive amplification polymorphism (MSAP) analyses on genomic DNA showed that the ratios of fully-methylated sites were significantly increased in translocation lines (CN12, 20.15%; CN17, 20.91%; CN18, 22.42%), but the ratios of hemimethylated sites were significantly lowered (CN12, 21.41%; CN17, 23.43%; CN18, 22.42%), whereas 16.37% were fully-methylated and 25.44% were hemimethylated in case of their wheat parent. Twenty-nine classes of methylation patterns were identified in a comparative assay of cytosine methylation patterns between wheat-rye translocation lines and their wheat parent, including 13 hypermethylation patterns (33.74%), 9 demethylation patterns (22.76%) and 7 uncertain patterns (4.07%). In further sequence analysis, the alterations of methylation pattern affected both repetitive DNA sequences, such as retrotransposons and tandem repetitive sequences, and low-copy DNA.

Triticum aestivum, *Secale cereale*, chromosome translocation lines, epigenetics, DNA methylation, methylation sensitive amplification polymorphism

Epigenetic inheritance refers to all modifications to genes expression other than changes in DNA sequence itself. This epigenetic modification pattern can be inherited through mitosis and/or meiosis[1,2]. The methylation of DNA is one of the main epigenetic modification phenomenons in eukaryotic cells. In higher plant genome, about 20%—50% cytosines are methylated, among which about 90% methylated sites lie in 'CpG' dinucleotide or 'CpNpG' trinucleotide[3−5]. DNA methylation plays an essential role in many basic research and applied research fields of life sciences and is closely related to gene expression, embryonic development, cell differentiation, genomic imprinting, sex chromosome, cell memory and so on[1,4−6]. The role of DNA methylation in life process has become a focus of recent biology sciences. Recent studies on interspecific hybridization of *Arabidopsis*[3], cotton[7], *Spartina*[8] and rice[9,10] have

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shown that introduction of alien germplasm can evoke the remarkable variety of DNA methylation extent and pattern in recipient genome and bring wide variations in hybrid progeny transcriptome which differ from parents. Moreover, this cooperative mutation pattern between genome DNA methylation and genome transcription can be transfered effectively to offspring by sexual or asexual approach and exhibit different phenotypic traits in offspring[4,8,9,11].

During our wheat improvement process, various superior alien germplasms from relatives of wheat were introduced into its own genome and a lot of new wheat germplasms were created. Some wheat new germplasms holding alien germplasm represented a series of fresh characters which were not ever detected in their parents[12−14]. Further research showed that just using the genetic effects aroused by introgression of alien germplasm into wheat genetic background to explain the selection of outstanding character seemed to be far from being enough[12,13]. We believe that application of epigenetics research methodology maybe provide a new research idea to interpret the genetic variation mechanism of these new characters. In this paper, a set of 1RS/1BL translocation sister lines separated at advanced generation and their parents were used to examine the methylation pattern at 'CCGG/GGCC' sites with methylation-sensitive amplification polymorphism (MSAP) technique[15]. The methylation difference and inheritance patterns were analyzed between sister lines and their partners. The results of present study will be not only helpful to understanding the distribution law and existence characteristics of DNA methylation variations which were induced in the process of alien germplasm introduced into wheat genome, but also can provide a theoretical basis for further fundamental research and breeding application.

1 Materials and methods

1.1 Plant material

Three wheat-rye chromosome translocation lines (Chuannong12 (CN12), Chuan-nong17 (CN17), Chuan-nong18 (CN18)) and their agronomic parents (a common wheat (*Triticum aestivum*) cultivar 'MY11' and a rye (*Secale cereale*) inbred line 'L155') were included in the present study. The translocation lines, which were the main grown cultivars in the southwest of China and were released in 2002, 2003 and 2004, respectively, contained the 1RS/1BL translocation chromosome and exhibited heritable, novel morphological characteristics in multiple traits in comparison with their parents[12,16]. All samples used in this work were maintained in our laboratory, and had been previously characterized using various cellular and molecular analyses[12,16].

1.2 DNA isolation

Mature seeds were surface-sterilized with 75% ethanol and were placed in 15 cm Petri dishes on two layers of soaked filter paper. After germination, plant seedlings were grown in a incubator at (25±0.5)℃ under 16 h of artificial daylight and 8 h of darkness for 10 d. Then, the seedling genomic DNA was isolated from leaf tissue using cetyltrimethyl-ammonium bromide (CTAB) procedure according to Murray and Thompson[17]. The genomic DNA was checked for quality and quantity by agarose gel electrophoresis (0.8% agarose) and fluorimetry (BioSpec-mini, Shimadzu, Japan).

1.3 Preparation of mitotic chromosomes

Chromosome samples were prepared according to Ren[12] with minor modifications. In brief, seed germination was conducted in a growth chamber held at (22±0.5)℃ and root tips were harvested at 1.5−2.0 cm in length and incubated in ice water at 0℃ for 24 h. After ice water treatment, the root tips were incubated in freshly prepared Carnoy's fixative I (1 glacial acetic acid∶3 ethanol) for 30 min and hydrolyzed in 0.2 mol/L HCl for 6 min at 60℃. Chromosome slides were prepared by macerating the tips on slides in 40% acetic acid and squashing under coverslip followed by removal of coverslip after freezing with liquid nitrogen. Slides with good quality were used for GISH analysis.

1.4 Genomic *in situ* hybridization for rye chromatin detection

The total genomic DNA from *Secale cereale* inbred line 'L155' was labeled with DIG-dUTP (Roche, Germany) by nick translation, being used as a probe for GISH on the metaphase spreads of wheat-rye chromosome translocation line with wheat total genomic DNA as blocking DNA (1∶50). GISH was carried out as described by Zhang et al.[18]. The slides were examined with an Olympus BX-51 fluorescence microscope (Olympus, Japan). FISH images of suitable metaphases were acquired and stored with a cooled charge-couple device (Spot Diagnostic, America) equipped with the version

4.0.8 Spot Rtke soft (Spot Diagnostic, America).

1.5 Molecular identity of 1RS/1BL translocation chromosome

According to the method of Froidmont[19] and Chai et al.[20], the low molecular weight glutenin gene present on the short arm of wheat chromosome 1B (locus: *Glu-B3*) and the ω-secalin gene present on the short arm of rye chromosome 1R (locus: *SEC-1b*) were amplified as a molecular marker for 1RS/1BL translocation line rapid detection, using primer combinations O11B3/O11B5 and ω-sec-P1/ω-sec-P2, respectively.

1.6 Amplified fragment length polymorphism (AFLP) analysis

The AFLP procedure was performed according to the protocol of Vos et al.[21], with minor modifications. Five hundred nanogram of genomic DNA was digested at 37℃ for 16 h. The restriction reaction contained 5 U *Eco*R I (Fermentas, Lithuanian) and 5 U *Mse* I (Fermentas, Lithuanian) in a final volume of 15 μL. Two different adaptors were ligated to the DNA after digestion, by adding to each final digestion 5 μL of a mix containing 3 pmol/L *Eco*R I adaptor (Table 1), 30 pmol/L *Mse* I adaptor (Table 1), 0.5 mmol/L ATP and 0.5 U T_4 DNA ligase (TaKaRa, Japan). The ligation was incubated overnight at 16℃. The digested-ligated DNA was diluted 1∶5 with 80 μL double-distilled water.

The preselective amplification by polymerase chain reaction (PCR) was carried out by using a single selective base at the 3′ end of each of the *Eco*R I and *Mse* I primers (Table 1). Each reaction contained 75 ng *Eco*R I-A primer, 75 ng of *Mse* I-C primer, 0.2 mmol/L of each dNTPs, 1×*Taq* DNA polymerase buffer (TaKaRa, Japan), 0.5 U *Taq* DNA polymerase (TaKaRa, Japan), and 5 μL diluted restrictionligation products in a final volume of 25 μL. The preselective amplifications were performed in a thermal cycler (I-Cycler, Bio-RAD, America) using the following program: after extension at 72℃ for 2 min, amplification was performed with 20 cycles of 0.5 min at 94℃, 1 min at 56℃, and 1 min at 72℃, followed by a final extension at 72℃ for 5 min. Ten microliters of this PCR were electrophoresed on 1.5% agarose gels and stained with ethidium bromide to verify the success of the preselective amplification (a DNA smear in the size range of 100－1500 bp was expected).

After successful preamplification, the PCR products were diluted 1∶25 with double-distilled water. Selective amplification was performed with *Eco*R I and *Mse* I selective primers. The sequence of the selective primers was similar to that of the preselective primers, with the addition of two variable nucleotides at the 3′ end (Table 1). The selective amplification reaction contained 30 ng *Eco*R I-ANN primer, 30 ng *Mse* I-CNN primer, 0.2 mmol/L each dNTPs, 1×*Taq* DNA polymerase buffer (TaKaRa, Japan), 0.5 unit of Taq DNA polymerase

Table 1 Sequences of AFLP and MSAP adapters and primers used in this study

Adaptors	
*Eco*R I-adaptors-F	5′-CTCGTAGACTGCGTACC-3′
*Eco*R I-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 Ⅰ-**A**	5′-GACTGCGTACCAATTC**A**-3′
Mse Ⅰ-**C**	5′-GACGATGAGTCCTGAGTAA**C**-3′
Hpa Ⅱ/*Msp* Ⅰ-**T**	5′-ATCATGAGTCCTGCTCGG**T**-3′
Selective primer combinations used in AFLP	
*Eco*R Ⅰ-**AAC**+*Mse* Ⅰ-**CAG**	*Eco*R Ⅰ-**ACT**+*Mse* Ⅰ-**CTA**
*Eco*R Ⅰ-**AGG**+*Mse* Ⅰ-**CTT**	*Eco*R Ⅰ-**AAC**+*Mse* Ⅰ-**CTA**
*Eco*R Ⅰ-**ACA**+*Mse* Ⅰ-**CTG**	*Eco*R Ⅰ-**AAG**+*Mse* Ⅰ-**CTG**
*Eco*R Ⅰ-**ACG**+*Mse* Ⅰ-**CGT**	*Eco*R Ⅰ-**ACG**+*Mse* Ⅰ-**CAA**
Selective primer combinations used in MSAP	
*Eco*R Ⅰ-**ACA**+*Hpa* Ⅱ/*Msp* Ⅰ-**TCA**	*Eco*R Ⅰ-**ACA**+*Hpa* Ⅱ/*Msp* Ⅰ-**TTC**
*Eco*R Ⅰ-**ACC**+*Hpa* Ⅱ/*Msp* Ⅰ-**TGC**	*Eco*R Ⅰ-**ACC**+*Hpa* Ⅱ/*Msp* Ⅰ-**TTC**
*Eco*R Ⅰ-**ACG**+*Hpa* Ⅱ/*Msp* Ⅰ-**TCCA**	*Eco*R Ⅰ-**AAG**+*Hpa* Ⅱ/*Msp* Ⅰ-**TCGA**
*Eco*R Ⅰ-**ACA**+*Hpa* Ⅱ/*Msp* Ⅰ-**TCGA**	*Eco*R Ⅰ-**ACT**+*Hpa* Ⅱ/*Msp* Ⅰ-**TCGA**

(TaKaRa, Japan) and 5 μL of diluted pre-amplified products in a final volume of 20 μL. The selective amplification was carried out with classical AFLP cycling parameters[21].

At the end of the selective PCR amplification, samples were denatured by adding an equal volume of formamide-buffer (100% formamide, 10 mmol/L EDTA, pH 8.0, 0.05% bromophenol blue and 0.05% xylene cyanol) and heating for 3 min at 94℃. Aliquots (5 μL) of each sample were separated on 6% denaturing polyacrylamide gels (acrylamide∶bisacrylamide = 19∶1, containing 8 mol/L urea) running at 60 W for 2 h in 1×TBE buffer. After silver staining[22], the gels were dried at room temperature and photographed.

1.7 Methylation-sensitive amplification polymorphism (MSAP) analysis

The modified protocol substituted for *Mse* I as the frequent cutter isoschizomers *Hpa* II and *Msp* I (Fermentas, Lithuanian), which recognized the same tetranucleotide sequence 5′-CCGG-3′ but with different sensitivities to methylation at cytosine. The rare cutter *Eco*R I was unchanged. Preselective amplification was accomplished by using *Eco*R I-A and *Hpa*II/*Msp*I-T primers (Table 1). Selective amplification was performed using *Eco*R I-ANN primers and *Hpa* II/*Msp* I- TNN(N) primers (Table 1). Reaction components and conditions were exactly the same as described earlier for the AFLP analysis. The adapter and the basic primer sequences for the *Hpa* II/*Msp* I were the same as those provided in the protocol according to Xing et al.[23]. All of the adaptor and primer sequences were synthesized by the nucleic acid synthesis unit at the Invitrogen Company (U.S.).

In both AFLP and MSAP procedures, replicates were performed to avoid technical bias, and patterns resulting from two independent digestions were compared for each sample. Moreover, with both AFLP and 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.

1.8 Isolation and sequencing of polymorphic methylated fragment

To gain insight into the nature of the differentially methylated fragments, the polymorphic MSAP fragments were isolated from polyacrylamide gels, reamplified by PCR, and sequenced. To accomplish this, the polymorphic bands were excised from the gel, and the DNA was extracted by boiling in 100 μL of water for 5 min. The eluted DNA was used as template for PCR following the cycling conditions of the selective amplification. The PCR products were separated on a 2% agarose gel, and DNA fragements of the appropriate size were extracted from the gel using the Sangon PCR Purification Kit (China). The target bands were ligated to the pMD18 T-vector (TaKaRa, Japan) and then transferred into *Escherichia coli* strain DH5α competent cells by heat shock. The positive clones were sequenced using universal M13 primers and the nucleotide sequences were deposited in the GenBank Database.

2 Results

2.1 Existence and impact of rye germplasm in wheat genetic background

Through the process of monosomic addition, fragmentation and integration, a lot of new wheat germplasms holding alien germplasm were created. Some of these translocation lines demonstrated many new characters, such as large spike, many grains, superior plant type, leaf stay-green after anthesis and high resistance, etc., which were not existent in their parents (Figure 1(a) and (b)). By combining the PCR assay resulting in the 1.1-kb fragment from the ω-secalin gene on 1RS and the PCR assay resulting in a 0.6-kb fragment from the *Glu-B3* gene on 1BS, CN12, CN17 and CN18 could be clearly identified to carry 1RS/1BL translocation chromosome (Figure 1(c)). The total genomic DNA of rye (L155) was labeled with DIG-dUTP and hybridized to mitotic metaphase spreads of wheat-rye chromosome translocation lines (CN12, CN17 and CN18) with total genomic of wheat (MY11) DNA as blocking DNA. As illustrated in Figure 1(d)－(f), the yellow hybridization signals, which were evenly distributed and with no background interference, were mainly observed uniformly in short arm of two satellite chromosomes, suggesting that these two chromosome short arm were 1RS chromosome. From integrated molecular and cellular evidences, it was known that CN12, CN17 and CN18 were wheat-rye chromosome translocation lines including 1 RS chromosomes.

2.2 Analysis of genome structure alterations in wheat-rye translocation lines

To assess the frequency of genetic changes associated

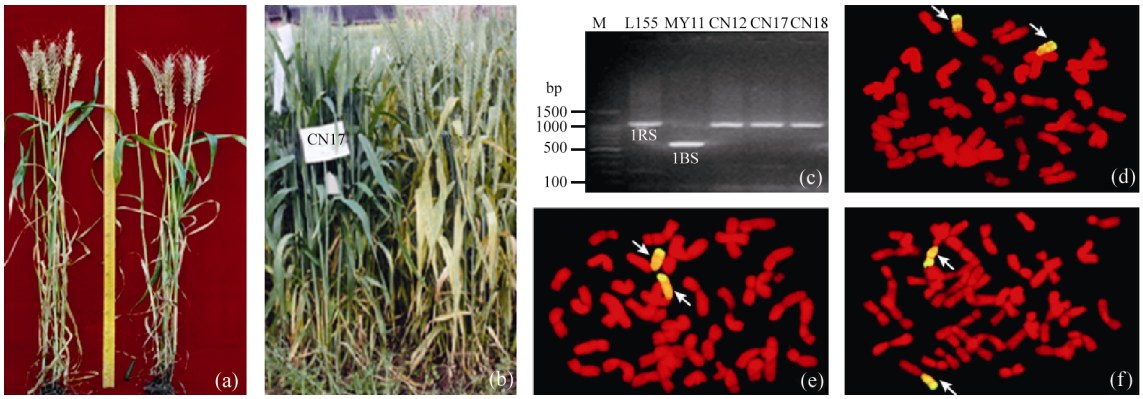

Figure 1 Some new characters demonstrating in wheat-rye translocations and existence of rye chromatin in wheat genetic background. (a) Leaf stay-green after anthesis; (b) high resistance to stripe rust; (c) polymerase chain reaction detection of 1RS/1BL translocation with the codominant marker; (d)—(f), results of genomic *in situ* hybridization on CN12, CN17 and CN18 spreads using genomic DNA of rye as probe, respectively. The hybridization signals distributing on short arm of two rye satellite chromosomes were shown by arrows.

with introduction of alien germplasm into wheat genome, a set of wheat-rye translocation sister lines and their parents were analyzed by AFLP. Using 8 different selective primer combinations, 367 bands were obtained. Of these, the polymorphic amplified sites were not detected among the translocation sister lines (CN12, CN17 and CN18), which were carrying the 1RS/1BL translocation chromosome introduced from the same rye parent. These results showed that the introduction of alien germplasm did not trigger the distinct variation of the genome primary structure of wheat recipient in CN12, CN17 and CN18 (Figure 2).

2.3 Analysis of genomic DNA methylation extent in wheat-rye translocation lines

Hpa II and *Msp* I are a pair of isoschizomers that recognize the same restriction site, 'CCGG/GGCC', but have different sensitivity to the methylation statuses of cyto-

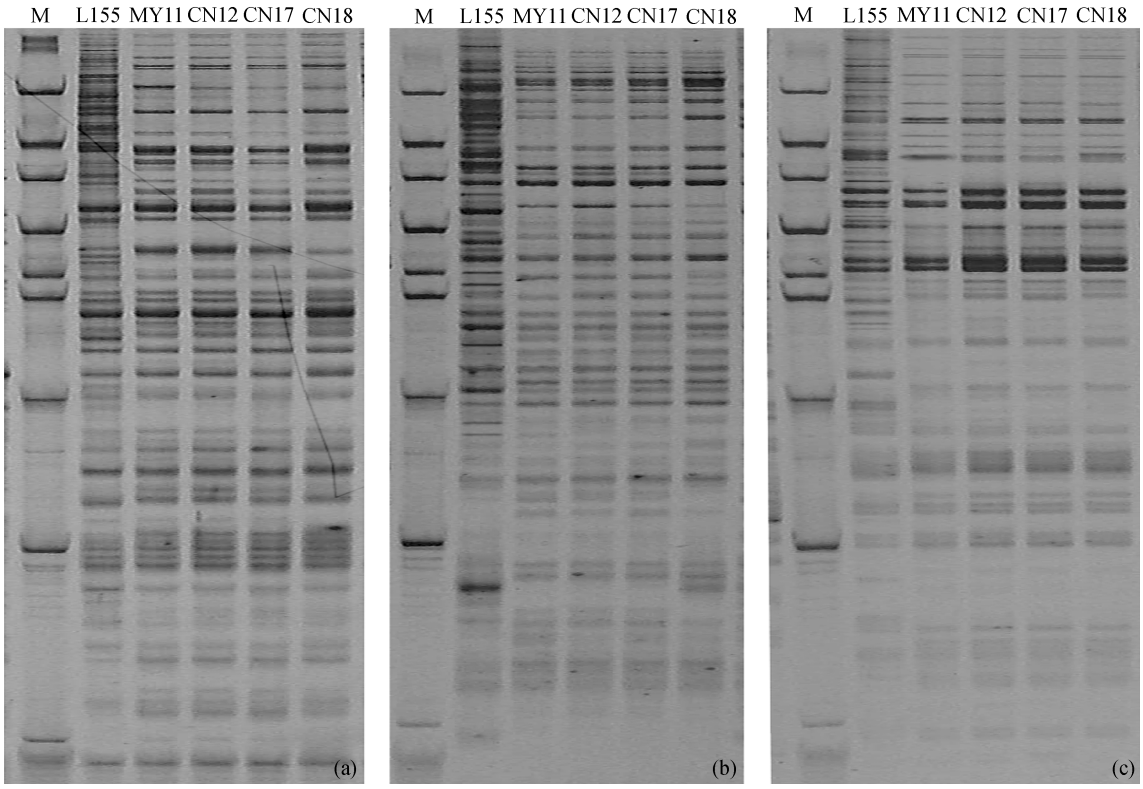

Figure 2 AFLP fingerprints of genomic DNA of wheat-rye translocations and their parents. (a)—(c) selective amplification results with different selective primer combinations

sine: *Hpa* II will not cut if either of 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[15]. Each amplified band from the enzyme combinations of either *Eco*R I+*Hpa* II or *Eco*R I+*Msp* I stands for one 'CCGG/GGCC' recognition site. Therefore, the methylation states of the cytosine at 'CCGG/GGCC' site would lead to a differential cleavage by two isoschizomers and thus to the appearance of different MSAP fragments in the sequencing gel. Because of the different responses to different methylation statuses, the band pattern from PCR amplification can reflect the methylation status at the certain site (Figure 3). Thus, according to presence or absence of polymorphic methylated fragment, methylation patterns of genomic DNA from different samples can be divided into three types with the 'CpG' methylation statuses of the 'CCGG/GGCC' sites as follows: Class A, present for both enzymes (*Hpa* II+/*Msp* I+), which means no methylated cytosine on double strand DNA or inner methylated cytosine on single strand DNA ('CCGG/GGCC', no-methylation); Class B, absent for *Hpa* II but present for *Msp* I (*Hpa* II–/*Msp* I+), which means methylated inner cytosine on double strand DNA ('$C^{5m}CGG/GG^{5m}CC$', full-methylation); Class C, present for *Hpa* II but absent for *Msp* I (*Hpa* II+/*Msp* I–), which means methylated outer cytosine on single strand

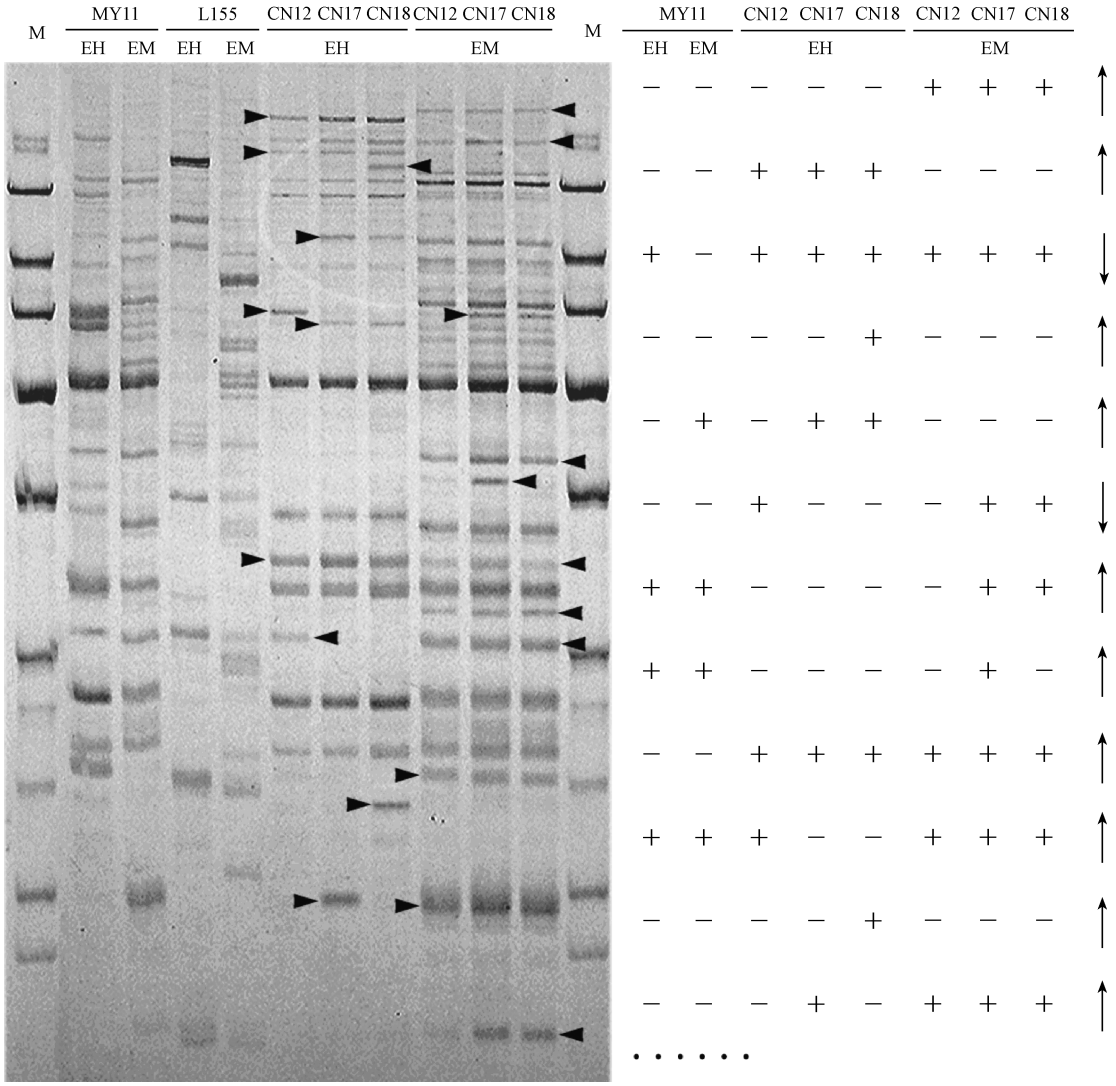

Figure 3 MSAP fingerprints of genomic DNA of the wheat-rye translocations and their parents and variation of methylation pattern. M, the DNA molecular-weight mark; EH, *EcoR* I+*Hpa* II; EM, *EcoR* I+ *Msp* I; –, band absent; +, band present; ↑, methylation extent increase; ↓, methylation extent decrease.

DNA ('^{5m}CCGG/GGCC', hemi-methylation).

By using eight pairs of *EcoR* I+*Hpa* II/*Msp* I selective primer combinations for MSAP analysis (Table 1), 397 legible and reproducible amplified fragments were generated in the wheat-rye translocations and their parents, of which 128 (32.25%), 166 (41.81%), 165 (41.56%), 176 (44.33%), and 178 (44.83%) methylated sites were detected in the rye parent (L155), wheat parent (MY11) and three translocations (CN12, CN17 and CN18), respectively (Table 2). This result indicated that there were greater differences of the genomic DNA methylation extent between the rye and wheat parents, and at the same time, the absolute or relative number of the genomic DNA methylated sites detected in translocation lines were different to some degree from their wheat parent. Further analysis revealed that the number of fully-methylated sites were 65, 80, 83 and 89 for MY11 (16.37%), CN12 (20.15%), CN17 (20.91%) and CN18 (22.42%), respectively, and the corresponding hemi-methylated sites were 101 (25.44%), 85 (25.44%), 93 (23.43%) and 89 (22.42%) (Table 2). From that, it was known that fully-methylated sites were significantly increased and hemi-methylated sites were markedly decreased in the genome of translocations compared with wheat parental cultivar MY11. Data analysis has indicated that the existence of alien germplasm in wheat genetic background has evoked the obvious and multiform variation of the genomic DNA methylation extent and pattern of its recipient.

2.4 Variation of methylaion patterns in the wheat-rye translocation lines

Through comparing different MSAP amplified fingerprints between translocation lines and wheat parent (Figure 3), two major classes were identified among these methylation sites (Table 3): (i) monomorphism site, methylation pattern of the same 'CCGG/GGCC' site was consistent between wheat parent and different translocation lines; (ii) polymorphism site, methylation pattern of the same 'CCGG/GGCC' site was dissimilar between wheat parent and different translocation lines. 96 monomorphism methylated fragments were generated in the wheat-rye translocation lines, which comprised 39.02% methylation site detected, among them, fully-methylated sites were 43 (M1), and hemi-methylated sites were 53 (M2), while other 150 methylated sites, which comprised 60.98% methylated sites detected, showed polymorphism among the translocation lines and the wheat parent (Table 3).

According to the extent of methylation variation in 'CCGG/GGCC' site, the methylated site which showed polymorphism patterns could be further divided into three types: (i) hypermethylation polymorphism site (PH), the extent of cytosine methylation in this site was stronger than wheat parent; (ii) demethylation polymorphism site (PD), the extent of cytosine methylation in this site was weaker than wheat parent; (iii) uncertain polymorphism site (PU), the extent of cytosine methylation in this site could not be accurately qualitative compared with wheat parent. Among them, the number of PH, PD and PU sites were 83 (33.74%), 56 (22.76%) and 10 (4.07%), respectively. There were thirteen kinds (PH1－PH13) in hypermethylation polymorphic sites, in which eight types (PH6－PH13) showed methylation alterations in translocation lines but not in wheat parent. Also, nine types (PD1－PD9) could be identified in the demethylation polymorphic sites, in which only one type

Table 2 Number of bands amplified using eight MSAP selective primer combinations in the wheat-rye translocations and their parents

Primer combinations	Amplification site	L155		MY11		CN12		CN17		CN18	
		Methylated site		Methylated site		Methylated site		Methylated site		Methylated site	
		Fully	Hemi	Fully	Hemi	Fully	Hemi	Fully	Hemi	Fully	Hemi
E-ACA+H/M-TCA	43	8	6	10	6	13	5	13	5	15	5
E-ACA+H/M-TTC	57	7	2	9	11	9	12	9	13	9	13
E-ACC+H/M-TGC	52	5	16	8	23	11	16	11	15	12	15
E-ACC+H/M-TTC	62	9	15	7	16	8	16	7	16	9	13
E-ACG+H/M-TCCA	38	5	10	6	8	9	6	9	6	9	6
E-AAG+H/M-TCGA	46	8	5	10	17	8	13	8	14	8	13
E-ACA+H/M-TCGA	39	11	2	5	10	5	10	6	16	8	15
E-ACC+H/M-TCGA	60	10	9	10	10	17	7	20	8	19	9
Total	397	63	65	65	101	80	85	83	93	89	89
		128		166		165		176		178	
Rate of methylated site(%)		15.87	16.37	16.37	25.44	20.15	21.41	20.91	23.43	22.42	22.42
		32.25		41.81		41.56		44.33		44.83	

Table 3 Patterns of cytosine methylation in wheat-rye translocation lines and wheat parent[a)]

Methylation patterns			MY11		CN12		CN17		CN18		Number of sites	Total (%)
			EH	*EM*	*EH*	*EM*	*EH*	*EM*	*EH*	*EM*		
Monomorphic sites		M1	–	+	–	+	–	+	–	+	43	96 (39.02)
		M2	+	–	+	–	+	–	+	–	53	
Polymorphic sites	Hypermethylation sites	PH1	+	+	–	+	–	+	–	+	18	83 (33.74)
		PH2	+	+	+	–	+	–	+	–	5	
		PH3	+	+	+	+	+	+	+	–	1	
		PH4	+	+	+	+	–	+	–	+	1	
		PH5	+	+	+	+	+	–	+	–	1	
		PH6	–	–	–	+	–	+	–	+	21	
		PH7	–	–	+	–	+	–	+	–	20	
		PH8	–	–	–	–	+	–	+	–	8	
		PH9	–	–	+	–	+	–	+	+	3	
		PH10	–	–	–	–	–	–	–	+	2	
		PH11	–	–	–	+	–	+	+	+	1	
		PH12	–	–	–	–	+	–	–	–	1	
		PH13	–	–	+	–	–	–	–	–	1	
	Demethylation sites	PD1	+	–	+	+	+	+	+	+	17	56 (22.76)
		PD2	+	–	–	–	–	–	–	–	14	
		PD3	+	–	+	–	+	–	+	+	2	
		PD4	+	–	+	–	+	–	+	+	2	
		PD5	+	–	+	+	+	+	+	–	1	
		PD6	+	–	+	+	+	+	+	+	1	
		PD7	–	–	+	+	+	+	+	+	10	
		PD8	–	+	+	+	+	+	+	+	8	
		PD9	–	+	+	–	+	–	+	–	1	
	Uncertain sites	PU1	+	–	–	–	–	–	–	–	2	10 (4.07%)
		PU2	+	–	+	–	–	–	+	+	1	
		PU3	+	–	+	+	+	+	–	+	1	
		PU4	+	–	–	–	+	–	–	–	1	
		PU5	–	+	–	–	–	–	–	–	3	
		PU6	–	–	–	–	–	–	–	+	1	
		PU7	–	–	+	+	–	+	–	+	1	

a) EH, *EcoR* I+*Hpa* II; EM, *EcoR* I+ *Msp* I; –, band absent; +, band present; M, monomorphic sites; PH, polymorphic hypermethylation sites; PD, polymorphic demethylation sites; PU, polymorphic uncertain sites.

methylation alteration was specifically detected in translocation lines but not in wheat parent. Among the polymorphic methylation sites, a total of 120 (48.78%) methylation patterns, belonging to twelve polymorphic classes (PH1, PH2, PH6, PH, PD1, PD2, PD6－PD9, PU1 and PU5), showed consistent cytosine methylation among three translocation sister lines (CN12, CN17 and CN18) but different from wheat parent (MY11). On the contrary, a total of 30 (12.20%) methylation patterns, belonging to other seventeen polymorphic classes, exhibited dissimilar cytosine methylation among three translocation sister lines.

2.5 Sequencing of polymorphic MSAP fragments

Fifteen MSAP fragments that displayed methylation alteration in translocation lines were gel isolated, reamplified, and sequenced (EF453755－EF453757, EF469547－EF469553, EF486285－EF486305) (Table 4). Of these, the blast (BlastN and BlastX) searches revealed that the alterations in methylation patterns affected both repetitive DNA sequences, such as retrotransposons and tandem repetitive sequences, and low-copy DNA.

3 Discussion

3.1 Effect of alien chromatin on its recipient genomic structure in translocation lines

During the wheat improvement process, a lot of beneficial genes contained in various alien germplasms from relatives of wheat have been introduced successfully into wheat genetic background, in which the 1RS/1BL translocation has been widely used in wheat breeding

Table 4 Sequence analysis of the polymorphic methylated fragments

Sequence	Length (bp)	Homologous sequence	Gene locus flanking	*E* value
EF453755	197	*T. turgidum* retrotransposon (AY494981)	*HMW* globulin A, protein kinase	1×10^{-74}
EF453756	634	*T. monococcum* retrotransposon (AY146588)	*GLU-A3-2*	0.0
EF453757	200	*Aegilops tauschii* transposons (AY534123)	transposons	8×10^{-17}
EF469548	326	*T. turgidum* retrotransposon (DQ871219)	*NAC* transcription factor, rhamnogalacturonate lyase	1×10^{-112}
EF469549	234	*A. speltoides* subtelomeric repeat Spelt1 sequence (AY117402)	none	1×10^{-92}
EF469552	562	*T. aestivum* ribosomal protein S11 and Rpl36 gene (EF486305)	none	0.0
EF469553	428	*T. monococcum* retrotransposon (AY485644)	*SNF2P*, putative transposase	2×10^{-148}
EF486285	358	none	none	358
EF486287	594	*A. tauschii* transposons (AY534123)	transposons	0.0
EF486292	497	*T. monococcum* retrotransposon (AY485644)	function unknown CDS, *ZCCT2*	3×10^{-30}
EF486298	393	*T. aestivum* retrotransposon (CT009735)	globulin	9×10^{-141}
EF486300	528	*T. aestivum* altered pSc119.1-1 repeat sequence (EF062311)	none	3×10^{-41}
EF486301	528	*T. monococcum* retrotransposon (AF326781)	actin, resistance protein, and nodulin-like-like protein gene	0.0

worldwide. Through the pathway of inducting chromosome translocation, we introducted the rye germplasms into wheat genome, and a lot of new elite wheat germplasms were created[12−14]. In the course of introducing foreign germplasms into wheat genetic background with sexual hybridization, the wide genetic variations of genomic structure and gene expression in recipient species could be induced. Shaked et al.[24] discovered that interspecific or intergeneric hybridization can lead to the formation of new characters which were not detected in their parents. They believed that sequence elimination or addition is one of the major and immediate responses of wheat genome to wide hybridization, that it affects a large fraction of the genome, and that it is reproducible[24]. Subsequent research in interspecific hybridization in *Arabidopsis*[3] and *Spartina*[8] and intergeneric hybridization between *T. aestivum*×*A. tauschii*[25] and *T. aestivum*×*S. cersale*[26] indicated that hybrid offspring exhibit broad genetic variations with their parents from phenotypic characteristics to gene expression and even to genomic structure. In previous study, we have reported that, similar to the situation mentioned above, a lot of new wheat germplasms holding alien germplasm demonstrated many new characters[12−14], such as large spike, many grain, superior plant type, leaf stay-green after anthesis, high resistance etc., which were not possessed in their parents (Figure 1(a) and (b)). From integrated molecular and cellular evidence it was known that rye germplasm existed as translocation chromosome in wheat genetic background and inherited steadily (Figure 1(c), (d)−(f)). With AFLP technique, we have found that the genomic structure was highly consistent among the translocation sister lines used in this paper (CN12, CN17 and CN18) (Figure 2). Compared with their parents, translocations did not exhibit other obvious variations of genomic structure except for 1RS/1BL chromosome translocation, which showed that the introduction of foreign rye germplasm did not initiate the distinct variation on genome primary structure of wheat recipient.

3.2 Genetic variation of DNA methylation extent and pattern between wheat-rye translocation lines and parents

Recent studies indicate that distant hybridization is associated with a lot of genetic and epigenetic changes. The cytosine methylation is one of the main epigenetic modification phenomenons in distant hybridization, which can regulate the transcription process of certain genes[4,8,11]. Through studies on rice[9,10,23], *Arabidopsis*[3], *Spartina*[8] and maize[27], the results showed that the extent of DNA methylation in hybrid offspring genome was obviously different from that of their parents, and the methylation pattern on different sites exhibited rich polymorphism, in which some sites presented demethylation, some sites presented hypermethylation and the others presented tissue specificity. Researchers believe that this variation of DNA methylation extent and pattern is a general phenomenon, which is closely related to the formation of a new gene regulation system for hybrid offspring, and is also necessary for related species to adapt to different growth environment[3,8,9,10,23]. In this paper, we detected the difference of genomic DNA methylation extent between wheat-rye translocation lines and their parents with MSAP tech-

nique. The test proved the proportion of methylation modification on the 'CCGG/GGCC' site in wheat genome (41.56%—44.83%) was higher than that of ray genome (32.25%) (Figure 3, Table 2), and the methylation pattern at the same 'CCGG/GGCC' site in wheat-rye translocation lines was mainly inherited from the wheat genetic background (Figure 3). Meanwhile, the methylation extent had dissimilar alteration in different wheat-rye translocation lines relative to their wheat parent: it remained unchanged basically in CN12 genome (41.56%); while it rose to 44.33% and 44.83% in CN17 and Cn18, respectively (Table 2). However, through subdividing the ratio of the different methylation modification patterns, we found that the proportion of full-methylation modification on the 'CCGG/GGCC' site was significantly increased in different translocation lines (CN12, 20.15%; CN17, 20.91%; CN18, 22.42%), while the proportion of hemi-methylation modification decreased significantly (CN12, 21.41%; CN17, 23.43%; CN18, 22.42%) compared with wheat parent (full-methylation: 16.37%; hemi-methylation: 25.44%) (Table 2). Form these data, it can be inferred that the existence of foreign germplasm in wheat-rye translocation lines induces variation of DNA methylation extent and pattern in wheat genetic background, which is in accordance with the previous studies on *Arabidopsis* allopolyploid[3], *Spartina* allopolyploid[8] and rice hybrid introgression[9,10].

Many studies have indicated that DNA methylation patterns could be inherited to offspring following cell mitosis and/or meiosis[1,2], and the dynamic change and the pattern remodeling of DNA methylation were also reported, which was particularly prevalent in interspecific crossing and allopolyploidization[3,8,9,10,23]. Results of this study showed that 39.02% of methylation patterns at the 'CCGG/GGCC' site in wheat-rye translocation lines was consistent with wheat parent, but for rye germplasm was introduced, the more variation of methylation modification pattern occurred and the rich polymorphism of DNA methylation was detected in wheat-rye translocation lines compared with wheat parent (60.98%) (Table 3). Nevertheless, high consistency was shown among the different wheat-rye translocation lines, and the proportion of polymorphic loci was only 12.20%. This result conformed to the same existing formation of rye germplasm and the consistent composition of the genome primary structure in different wheat-rye translocations proved by GISH and AFLP analysis (Figures 1(d)—(f) and 2). Twenty-nine different types of methylation modification patterns were detected, among which 13 types (33.74%) were hypermethylation modification, 9 types were demethylation modification (22.76%), and the remaining 7 categories (4.07%) methylation pattern variation could not be clearly defined (Table 3). These variations of methylation modification pattern at the same site may be closely related to activity of genomic transcription to a certain extent, thereby it could influence the variation of phenotypic characters among the different translocation lines[3,8,9,10].

3.3 Preference of methylation variation in wheat-rye translocation lines

Previous studies have proved that the methylation modification pattern of a set of certain loci including repetitive sequences, transposable elements, promoter sequence and protein coding region would be changed in the process of wide hybridization, in which transposable elements and other repetitive sequences were the main targets for methylation mutation, and these variations could affect a series of plant phenotypic characteristics such as florescence, fertility, morphology, and so on[3,10,28]. Cheng et al.[28] investigated the relationship of methylation pattern and transposition behavior in retrotransposon *Tos17*, and found that its transposition activity was negatively correlated with its DNA methylation level. They conclude that *Tos17* DNA methylation controls the transposition activity of *Tos17*, and modulates the activity of neighboring genes. Based on these analyses of *Tos17*, Cheng et al.[28] propose that another mechanism, called transcriptional interference, is involved in the control of transposable element activity, and think that this mechanism of gene regulation on DNA methylation is important to cell differentiation, organogenesis and reproductive development in the course of rice tissue culture. The researchers also confirmed the presence of transcript activation of various transposable elements, which was accompanied with demethylation of transposable elements in *Arabidopsis* allopolyploid[3] and rice-*Zizania latifolia* hybrid introgression[9,10]. Here, several polymorphic methylated fragments were isolated from wheat-rye translocation lines. The alterations in methylation patterns affected both repetitive DNA sequences, such as retrotransposons and subtelomeric repeat sequences, and single/low-copy DNA of protein coding sequences. We consider these variations of methylation modification pattern of repeat

sequences and functional genes would be related to phenotypic characteristics difference between translocation lines and parents, as well as between different translocation lines. For instance, the methylation modification pattern of the ribosomal protein subunit coding sequence (EF469552, EF486305) related to chloroplast protein synthesis in CN17 and CN18, which exhibited the character of leaf stay-green after anthesis, was dissimilar to methylation pattern in their parents and sister lines. This difference indicates that the variation of methylation may be relative to the acquirement of antisenescence property in given translocation line to a certain extent. More worthy of attention was that the *Tri-MS-5* (EF469548), a transposon sequence identified from polymorphic methylated fragments, had the same locus with *NAC*, a leaf senescence-related regulatory gene cloned from durum wheat[29]. Considering *Tri-MS-5* located at the vicinity of *NAC* regulation region, it is necessary to deeply study and analyze the relationship between the variation of *Tri-MS-5* methylation pattern and the expression activity of *NAC* gene.

3.4 Potential epigenetic mechanism of phenotypic characteristics variation in wheat-rye translocation lines induced by exogenous germplasm introduction

In normal distant hybrid offspring, genetic substance originating from different parents must stably interact in the same cell. Through epigenetic modification regulating gene expression patterns of whole genome is an important strategy to achieve this stability. In general, methylation extent and pattern in hybrid offspring were different from its hybrid parents whether in repeat sequence or in single copy sequence[3,8,9,10,28]. In this paper, a set of translocation sister lines separated at advanced generation exhibited stable agronomic traits and high genetic identity, but they also showed many new characters compared with their parents and amphidiploid, such as large spike, many grain, superior plant type, leaf stay-green after anthesis, high resistance, etc. (Figure 1(a) and (b)), which cannot be sufficiently explained just using the genetic effects exerted by introduction of alien germplasm into wheat genetic background[12,13]. In order to deepen cognition to this phenomenon, firstly, the existing formation of rye germplasm among genome of three translocation lines (CN12, CN17 and CN18) was analyzed by GISH technique to confirm the stable heredity of rye 1RS chromosome in wheat genetic bachground at the cell level (Figure 1(d)—(f)), and then the difference of genomic composition was analyzed using AFLP technique to prove the absence of obvious variation of genomic structure among translocation lines at the molecular level (Figure 2). Based on these understanding, epigenetic behavior among translocation lines was further detected with MSAP technique. The results indicated that methylation extent and pattern exhibited distinct variation between translocation lines and their parents; especially, the high level of polymorphic methylation modification pattern was found at the same site (Tables 2 and 3). Simultaneously, many transposon sequences, subtelomeric repeat sequences and single copy protein coding sequences were identified in these methylation variation site (Table 4). In view of methylation modification at different site, it is different to perform and impact. The decrease of methylation degree at certain sites is necessary to the performance of certain characters, while the increase of methylation extent at other sites may be closely related to the performance of other traits. So, the high variation of methylation modification pattern between the different translocation lines and parents was understandable.

The consistency of genome primary structure was important for analysis of epigenetic phenomenon caused by introduction of alien germplasm. Although the molecular and cellular evidences have confirmed the relative consistency of genetic composition among translocation sister lines, because of limited resolution of detection technology, the impact brought with slight dissimilarity of genome primary structure could not be eliminated completely. However, under the same or similar detective intensity, the polymorphism of methylated sites (60.98%) was much higher than the heterogeneity of genomic composition among translocation lines, which, from another angle, suggested at least that the performance of different characters has closer relation with the genomic epigenetic variation in wheat-rye translocation lines. Considering that a series of progress were achieved in genomic methylation variation induced by wide hybridization, and DNA methylation modification was closely related to gene expression activity, we have reason to believe that the phenotypic variations in distant hybrid offspring are not only closely related to the degree of genome sequence change, but also has an inseparable relation with the variation of genomic DNA methylation extent and pattern.

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1 Saze H, Mittelsten Scheid O, Paszkowski J. Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. Nat Genet, 2003, 34(1): 65－69

2 Guimil S, Dunand C. Patterning of *Arabidopsis* epidermal cells: Epigenetic factors regulate the complex epidermal cell fate pathway. Trends Plant Sci, 2006, 11(12): 601－609

3 Madlung A, Masuelli R W, Watson B, et al. Remodeling of DNA methylation and phenotypic and transcriptional changes in synthetic *Arabidopsis* allotetraploids. Plant Physiol, 2002, 129: 733－746

4 Chan S W L, Henderson I R, Jacobsen S E. Gardening the genome: DNA methylation *Arabidopsis thaliana*. Nat Rev Genet, 2005, 6: 351－360

5 Xiao W, Custard K D, Brown R C, et al. DNA methylation is critical for *Arabidopsis* embryogenesis and seed viability. Plant Cell, 2006, 18(4): 805－814

6 Jullien P E, Kinoshita T, Ohad N, et al. Maintenance of DNA methylation during the *Arabidopsis* life cycle is essential for parental imprinting. Plant Cell, 2006, 18(6): 1360－1372

7 Adams K L, Percifield R, Wendel J F. Organ-specific silencing of duplicated genes in a newly synthesized cotton allotetraploid. Genetics, 2004, 168: 2217－2226

8 Salmon A, Ainouche M L, Wendel J F. Genetic and epigenetic consequences of recent hybridization and polyploidy in *Spartina* (Poaceae). Mol Ecol, 2005, 14: 1163－1175

9 Liu Z, Wang Y, Shen Y, et al. Extensive alterations in DNA methylation and transcription in rice caused by introgression from *Zizania latifolia*. Plant Mol Biol, 2004, 54(4): 571－582

10 Dong Z Y, Wang Y M, Zhang Z J, et al. Extent and pattern of DNA methylation alteration in rice lines derived from introgressive hybridization of rice and *Zizania latifolia* Griseb. Theor Appl Genet, 2006, 113: 196－205

11 Schob H, Grossniklaus U. The first high-resolution DNA "methylome". Cell, 2006, 126(6): 1025－1028

12 Ren Z L. Introduction of rye chromatin into wheat and its breeding behavior. Scientia Agricultura Sinica, 1991, 24(3): 18－25

13 Ren Z L. Effect of wheat genetic background on the expression of the rye gene *Lr26* for resistance to leaf rust in wheat. Acta Genetica Sinica, 1993, 20(4): 312－316

14 Ren Z L, Zhang H Q. Induction of small-segment-translocation between wheat and rye chromosomes. Sci China Ser C-Life Sci, 1997, 27(3): 258－263

15 Reyna-Lopez G E, Simpson J, Ruiz-Herrera J. Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. Mol Gen Genet, 1997, 253: 703－710

16 Tang Z X, Ren Z L, Wu F, et al. The selection of transgenic recipients from new elite wheat cultivars and study on its plant regeneration system. Agri Sci China, 2006, 5(6): 417－427

17 Murray M G, Thompson W F. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res, 1980, 8(19): 4321－4325

18 Zhang Y, Zhang S G, Qi L W, et al. Chromosome microdissection, cloning and painting of the chromosome 1 in poplar (*Populus tremula*). Silvae Genetica, 2005, 54(4-5): 211－217

19 Froidmont D. A co-dominant marker for the 1BL/1RS wheat-rye translocation via multiplex PCR. J Cereal Sci, 1998, 27: 229－232

20 Chai J F, Zhou R H, Jia J Z, et al. Development and application of a new codominant PCR marker for detecting 1BL/1RS wheat–rye chromosome translocations. Plant Breed, 2006, 125: 302－304

21 Vos P, Hogers R, Bleeker M, et al. AFLP: A new techique for DNA fingerprinting. Nucleic Acids Res, 1995, 23: 4407－4414

22 Bassam B J, Caetano-Anolles G, Gresshoff P M. Fast and sensitive silver straining of DNA in polyacrylamide gels. Anal Biochem, 1991, 196: 80－83

23 Xiong L Z, Xu C G, Maroof M A S, et al. Patterns of cytosine methylation in an elicite hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique. Mol Genet Genomics, 1999, 26: 439－446

24 Shaked H, Kashkush K, Ozkan H, et al. Sequence elimination and cytosine methylation are rapid and reproducible responses of the genome to wide hybridization and allopolyploidy in wheat. Plant Cell, 2001, 13: 1749－1759

25 He P, Friebe B R, Gill B S, et al. Allopolyploidy alters gene expression in the highly stable hexaploid wheat. Plant Mol Biol, 2003, 52: 401－414

26 Ma X F, Fang P, Gustafson J P. Polyploidization-induced genome variation in triticale. Genome, 2004, 47: 839－848

27 Zhao X X, Chai Y, Liu B. Epigenetic inheritance and variation of DNA methylation level and pattern in maize intra-specific hybrids. Plant Sci, 2007, 172: 930－938

28 Cheng C, Daigen M, Hirochika H. Epigenetic regulation of the rice retrotransposon *Tos17*. Mol Genet Genomics, 2006, 276(4): 378－390

29 Uauy C, Distelfeld A, Fahima T, et al. A *NAC* gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science, 2006, 314: 1298－1301