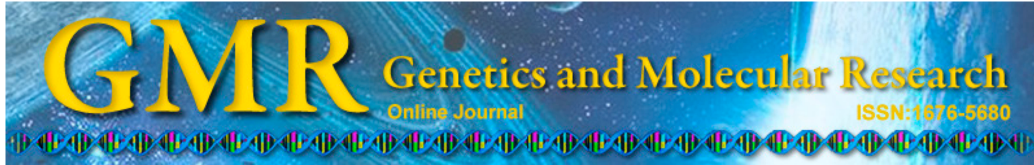

Analysis of DNA methylation patterns and levels in maize hybrids and their parents

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ABSTRACT. Heterosis is the superior performance of heterozygous individuals and has been widely exploited in plant breeding, although the underlying regulatory mechanisms still remain largely elusive. To understand the molecular basis of heterosis in maize, in this study, roots and leaves at the seedling stage and embryos and endosperm tissues 15 days after fertilization of 2 elite hybrids and their parental lines were used to estimate the levels and patterns of cytosine methylation by the methylation-sensitive amplification polymorphism method. The relative total methylation levels were lower in all the tissues of all hybrids than their corresponding mid-parent values, and the number of demethylation events was higher in the hybrids. These results implied that the decreasing trend and demethylation in hybrids relative to their parents may enable the derepression and possibly expression of many genes that were associated with the phenotypic variation in hybrids. To further analyze the observed methylation pattern changes, a total of 63 differentially displayed DNA fragments were successfully sequenced. Basic Local Alignment Search Tool analysis showed that 11

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fragments shared similarity with known functional proteins in maize or other plant species, including metabolism, transposon/retrotransposon, development, stress response, and signal transduction, which indicated that these genes might play a significant role in maize hybrid vigor.

Key words: Heterosis; DNA methylation; Hybrid; Maize; Methylation-sensitive amplification polymorphism

INTRODUCTION

Heterosis is the superiority of hybrids over the average parental performance with respect to various characteristics, such as speed of development and maturity, grain yield, resistance to insect pests and environmental stress, and many other changes in desirable agronomic traits (Hochholdinger and Hoecker, 2007; Chen, 2010). Although this phenomenon has been widely exploited to increase agronomic production for well over a century and brought great economic or societal benefits, the underlying molecular mechanisms remain a matter of debate and are less understood despite the research that has been done in the last century. For example, 3 main explanatory hypotheses have been postulated: the dominance, over-dominance, and epistasis hypotheses (Hochholdinger and Hoecker, 2007; Birchler et al., 2003, 2010; Jahnke et al., 2010). However, these hypotheses are largely conceptual and do not explain the molecular basis of heterosis (Hochholdinger and Hoecker, 2007; He et al., 2011). Recently, genetic quantitative trait locus and high-throughput transcript profiling have been used to analyze the heterotic phenotypes and indicate that a large number of genes may be associated with heterosis in all possible modes of action (Frascaroli et al., 2007; Uzarowska et al., 2007; Radoev et al., 2008; Meyer et al., 2010).

Recent progress demonstrated that variation at the DNA sequence level, epigenetic regulation of chromatin structure, is essential to interpret genetic information and determine the phenotype (Groszmann et al., 2011; Shen et al., 2012). Cytosine DNA methylation is an important epigenetic modification that plays significant roles in various cellular activities, including the orchestration of gene expression across plant development, maintenance of the overall genomic integrity, control of genomic imprinting, and formation and perpetuation of heterochromatin (Bourc'his and Bestor, 2004; Rangwala and Richards, 2004; Tariq and Paszkowski, 2004; Rapp and Wendel, 2005). Therefore, disturbed DNA methylation patterns may lead to functional consequences for the organisms with this epigenetic code (Martienssen and Colot, 2001; Tariq and Paszkowski, 2004). Of the various approaches to study global methylation, the methylation-sensitive amplification polymorphism (MSAP) technique has demonstrated the consistency and reproducibility that are required to detect global cytosine methylation patterns and levels in several plant species, such as *Arabidopsis thaliana* (Cervera et al., 2002), *Sorghum bicolor* L. (Zhang et al., 2007), *Hordeum brevisubulatum* (Li et al., 2008), *Codonopsis lanceolata* (Guo et al., 2007), and maize (Zhao et al., 2007). Moreover, it has been used in various studies, all of which obtained useful results in areas such as biotic and abiotic stresses (Wang et al., 2011), development (Zhang et al., 2007), and tissue culture (Kou et al., 2011; Wang et al., 2013). In addition, hybrid vigor has been studied at the DNA methylation level using this technique. For example, using the MSAP method, Zhao et al. (2007) reported that a great majority of cytosine methylation sites manifested faithful epigenetic inheritance between 3 sets of reciprocal maize hybrids and their inbred parents. Although the research on

heterosis in maize has been performed using the MSAP method, few reports have focused on the large-scale cultivation of maize varieties.

In this paper, the MSAP technique was used on leaves and roots at the seedling stage and embryos and endosperm tissues 15 days after pollination to investigate the cytosine methylation status of the maize genome and the differences in the patterns and levels between 2 elite hybrids and their parents. A total of 63 differentially displayed DNA fragments detected by MSAP profiling were isolated and sequenced, but only 11 fragments shared similarity with known functional proteins in maize or other plant species. Their functions or roles might help us to understand the molecular mechanisms of hybrid vigor.

MATERIAL AND METHODS

Plant materials

Two elite hybrids (Xianyu335 and Jida101), their parent lines [PH6WC (W), PH4CV (V), MH251 (M), P125 (P)], and their anti-cross hybrids [PH4CV x PH6WC (VW); P125 x MH251 (PM)] were used in this study. Xianyu335 came from the USA varieties, and the Jida101 hybrid was bred in our laboratory. All the inbred lines were manually maintained by strict self-pollination for many generations, and the hybrids were made by careful pollination.

DNA extraction

Genomic DNA was isolated from leaves and roots at seedling stage and embryos and endosperm tissues 15 days after pollination. The DNA was purified by phenol extractions, and the quality and quantity were checked by agarose gel electrophoresis and spectrophotometric measurement. In order to analyze the uniformity or variation of methylation alterations among different hybrid individuals, genomic DNA was isolated from the same stage.

MSAP analysis of DNA methylation

The MSAP analysis method was performed as reported by Xiong et al. (1999). Two combinations of restriction enzymes were used by mixing *Eco*RI with each of the isoschizomers *Hpa*II and *Msp*I, which recognize the same sequence (CCGG) and cut with differential sensitivity to the DNA methylation of the internal or external cytosine. The restriction enzymes *Eco*RI, *Hpa*II, and *Msp*I were purchased from Takara Biotech in Japan. Subsequently, one adaptor pair (*Hpa*II/*Msp*I adaptor and *Eco*RI adaptor) was used in ligation reactions. One pair of pre-selective primers and 64 pairs of selective primers were used for amplification (**Table S1**).

A total of 5 μL ligated sample, diluted 10-fold with sterilized distilled water, was used for the pre-amplification reactions. The polymerase chain reaction (PCR) conditions were: 25 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min. Selective amplification reactions were done with 5 μL of the pre-amplified cDNA that had been diluted 20-fold using the following touchdown PCR conditions: 94°C for 2 min; 12 cycles of 94°C for 30 s, 65°C for 1 min (-0.7°C per cycle), and 72°C for 1 min; and 23 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min.

The resulting products of selective amplification were denatured and separated on a 4.5% polyacrylamide gel that was run at 60 W and 50°C until the bromophenol blue reached the bottom of the gel, and the bands were visualized by silver staining. The gel was put in a

clean and ventilated area to dry completely for further data analysis.

Fragments with molecular weights of 100-600 bp were subjected to statistical analysis. According to the differential sensitivity of *Hpa*II and *Msp*I to cytosine methylation, the number of different methylation patterns was counted in each genotype. Hemi-methylation of the external cytosine is recognized by *Hpa*II and can be detected as one band, but *Msp*I does not recognize it. Conversely, full methylation (methylation of both strands) of the internal cytosine can be detected in *Eco*RI + *Msp*I digests, but *Hpa*II cannot cleave this recognition site. However, it is worth noting that *Hpa*II and *Msp*I cannot cleave the full methylation of the external cytosine or both cytosines and hemi-methylation of the internal cytosine. Therefore, according to the MSAP profiles, the methylation levels will be underestimated, and the total cytosine methylation loci was the relative total methylation. In addition, if an amplification band from a particular enzyme digestion was present in the hybrid but not in both parents, the methylation pattern was categorized as demethylation, while an amplification band that was present in both parents but absent in the hybrid was categorized as the hypermethylation pattern. Therefore, changes in the methylation sites of hybrids were critically analyzed by comparison with the sites of the parents.

Cloning and sequencing of MSAP fragments

These altered methylation bands in a hybrid relative to its parents were obtained from the silver-stained MSAP gels. The bands of interest were wetted with distilled water, extracted from the gel, dissolved in 30 μL ddH$_2$O, and incubated for 12 h at 37°C for further investigation. These bands were re-amplified with the appropriate selective primer combinations. Sizes of the PCR products were checked by agarose gel electrophoresis. The PCR products were extracted from the agarose gel and cloned into the pGM-T cloning vector (Tiangen, China). Successful clones were screened with standard ampicillin selection and identified by PCR. The DNA segments of positive clones were sequenced by the BGI Company (China). The DNAMAN sequence Analysis software (version 4.1) was used for sequence analysis and editing. The nucleotide Basic Local Alignment Search Tool (BLASTn) and translated DNA BLAST (BLASTX) programs from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) were used to analyze the homology of the sequences.

RESULTS

Differences in cytosine methylation levels of embryo and endosperm tissue from maize hybrids and their parental inbred lines

The MSAP profiles of hybrids and their parents were generated from leaves and roots at the seedling stage and embryos and endosperm tissues 15 days after pollination. A total of 64 selective primer combinations were used, and fragments of 209 roots, 475 leaves, 764 embryos, and 507 endosperms were amplified from the maize hybrids and their inbred parental lines (Table 1). The number of relative total cytosine methylation sites, hemi-methylation of the external cytosine, and full methylation of the internal cytosine were calculated. Among the maize lines analyzed, the relative total methylation levels of these tissues were in the following ranges: 66.44-69.18% (root), 68.26-70.51% (leaves), 18.49-27.67% (embryo), and 11.2-21.21% (endosperm). These values included 18.52-22.11% (root), 18.06-21.42% (leaves),

Table 1. Relative levels of cytosine methylation at CCGG sites in different tissues of hybrids and their parent lines.

Inbreds and hybrids	Non-methylated CCGG sites (%)				Methylated CCGG sites (%)											
					Total				Hemi-methylated sites (%)				Full-methylated sites			
	Root	Leaf	Embryo	Endosperm	Root	Leaf	Embryo	Endosperm	Root	Leaf	Embryo	Endosperm	Root	Leaf	Embryo	Endosperm
PH6WC (♀)	101 (31.17%)	226 (31.04%)	515 (72.33%)	389 (81.38%)	223 (68.83%)	502 (68.96%)	197 (27.67%)	89 (18.62%)	60 (18.52%)	138 (18.96%)	43 (6.04%)	17 (3.56%)	163 (40.31%)	364 (50.00%)	154 (21.63%)	72 (15.06%)
PH4CV (♂)	97 (32.44%)	202 (29.71%)	548 (77.51%)	410 (85.24%)	202 (67.56%)	478 (70.29%)	159 (22.49%)	71 (14.76%)	63 (21.07%)	135 (19.85%)	39 (5.52%)	17 (3.53%)	139 (46.49%)	343 (50.44%)	120 (16.97%)	54 (11.23%)
MP	99 (31.73%)	214 (30.40%)	532 (74.92%)	400 (83.33%)	213 (68.27%)	490 (69.60%)	178 (26.07%)	80 (16.67%)	62 (19.87%)	137 (19.39%)	41 (5.77%)	17 (3.54%)	151 (48.40%)	353 (50.21%)	137 (19.30%)	63 (13.13%)
W x V	105 (31.91%)	46 (31.74%)	617 (81.51%)	439 (87.80%)	224 (68.09%)	529 (68.26%)	140 (18.49%)	61 (12.20%)	63 (19.15%)	140 (18.06%)	47 (6.21%)	22 (4.40%)	161 (48.94%)	389 (50.20%)	93 (12.28%)	39 (7.80%)
V x W	104 (33.23%)	246 (31.66%)	600 (79.68%)	442 (88.40%)	209 (66.77%)	531 (68.34%)	153 (20.32%)	58 (11.60%)	64 (20.45%)	141 (18.15%)	64 (8.50%)	22 (4.40%)	145 (46.32%)	390 (50.19%)	89 (11.82%)	36 (7.20%)
MH251 (♀)	94 (30.72%)	202 (29.49%)	546 (73.29%)	381 (80.21%)	212 (69.18%)	483 (70.51%)	199 (26.71%)	94 (19.79%)	62 (20.26%)	146 (21.31%)	60 (8.05%)	25 (5.26%)	150 (48.94%)	337 (49.20%)	139 (18.66%)	69 (14.53%)
P125 (♂)	94 (32.98%)	222 (31.01%)	570 (76.92%)	397 (78.79%)	191 (67.02%)	494 (68.99%)	171 (23.08%)	84 (21.21%)	63 (22.11%)	142 (19.83%)	52 (7.02%)	26 (5.41%)	128 (44.91%)	352 (49.16%)	119 (16.06%)	58 (15.80%)
MP	94 (31.76%)	212 (30.24%)	558 (75.10%)	389 (81.38%)	202 (68.24%)	489 (69.76%)	185 (24.90%)	89 (18.62%)	63 (21.28%)	144 (20.54%)	56 (7.54%)	25 (5.23%)	139 (46.96%)	345 (49.22%)	129 (17.36%)	64 (13.39%)
M x P	99 (31.53%)	233 (30.82%)	643 (80.27%)	437 (87.75%)	215 (68.47%)	523 (69.18%)	158 (19.73%)	61 (12.25%)	63 (20.06%)	160 (21.16%)	64 (8.00%)	26 (5.22%)	152 (48.41%)	357 (48.02%)	94 (11.73%)	35 (7.03%)
P x M	98 (33.56%)	229 (30.66%)	644 (80.60%)	444 (88.8%)	194 (66.44%)	518 (69.34%)	155 (19.40%)	56 (11.20%)	63 (21.58%)	160 (21.42%)	67 (8.39%)	22 (4.4.0%)	131 (44.86%)	358 (47.92%)	88 (11.01%)	34 (6.80%)

MP = mid-parent; W = PH6WC; V = PH4CV; M = MH251; P = P125.

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5.52-8.50% (embryo), and 3.53-5.41% (endosperm) hemi-methylation of the external cytosine and 40.31-48.94% (root), 47.92-50.44% (leaves), 11.01-21.63% (embryo), and 6.80-15.80% (endosperm) full methylation of the internal cytosine at the CCGG sites. Compared with their corresponding mid-parent values, the relative total methylation levels of the hybrids were lower in most tissues except the roots from PM. For hemi-methylation of the external cytosine, different hybrids showed various levels (increased or decreased) compared to their corresponding mid-parent values. However, the full-methylation levels in most of the hybrids were lower than their corresponding mid-parent values. In addition, the number of cytosine methylation loci in roots and leaves was 3 times greater than that in the embryo and endosperm tissues.

Cytosine methylation patterns in the hybrids and parental inbred lines

Different cytosine methylation patterns were observed between hybrids and their parental lines. The methylation fragments were divided into 4 major groups (Zhao et al., 2008; Sakthivel et al., 2010) (Figure 1, Table 2, and **Table S2**). The same bands that were detected in both hybrids and their parents were called monomorphic bands, which were grouped into class A bands (196 roots, 395 leaves, 501 embryos, and 364 endosperms were included in this group); class B bands included the decreased levels of methylation or demethylation pattern in hybrids relative to its parents; class C bands included the increased levels of methylation or hypermethylation pattern in hybrids compared to its parents; and class D bands included the different methylated sites between 2 parents and different methylation patterns of hybrids from 1 parent. Moreover, according to the inheritance and alteration of cytosine methylation from parent to offspring, the 4 classes were subgrouped into 31 different groups as shown in **Table S2**. Comparative analysis revealed that the number of demethylated loci (class B) in all tissues of all hybrids was significantly higher than the number of hypermethylated loci (class C) (Table 2), which indicated that the inheritance and variation (demethylation) of DNA methylation from parents to progeny was predominant in stages of early development and grain formation. However, the percentage of class D bands was the highest in embryo and endosperm tissues; the reason for this requires further research.

Sequences underlying DNA methylation alterations in maize hybrids

To analyze the various methylation patterns, a total of 99 fragments (18 from roots, 22 from leaves, 31 from embryos, and 28 from endosperms) were extracted from the polyacrylamide gel and re-amplified using the original selective primers. Finally, a total of 63 positive clones (11 from roots, 14 from leaves, 23 from embryos, and 15 from endosperms) were sequenced. BLASTX analysis revealed that 11 fragments were highly homologous to genes with known functions, which could be classified into several groups based on the putative protein encoded, including gene regulation (No. 1), transposon/retrotransposon (Nos. 2 and 7), development (Nos. 4, 9 and 10), stress response (No. 11), and signal transduction (Nos. 3, 5, 6 and 8) (Table 3). Another 23 fragments showed homology to maize expressed sequence tags with unknown functions, and 29 clones did not match anything in the database (**Table S3**). Moreover, the sequence analysis indicated that these successfully sequenced fragment termini have the CCGG site; additionally, 32 fragments (5 from roots, 4 from leaves, 15 from embryos, and 8 from endosperms) have one or more internal CCGG sites. This indicated that the relative total methylation levels were underestimated by the MSAP technique (**Table S3**).

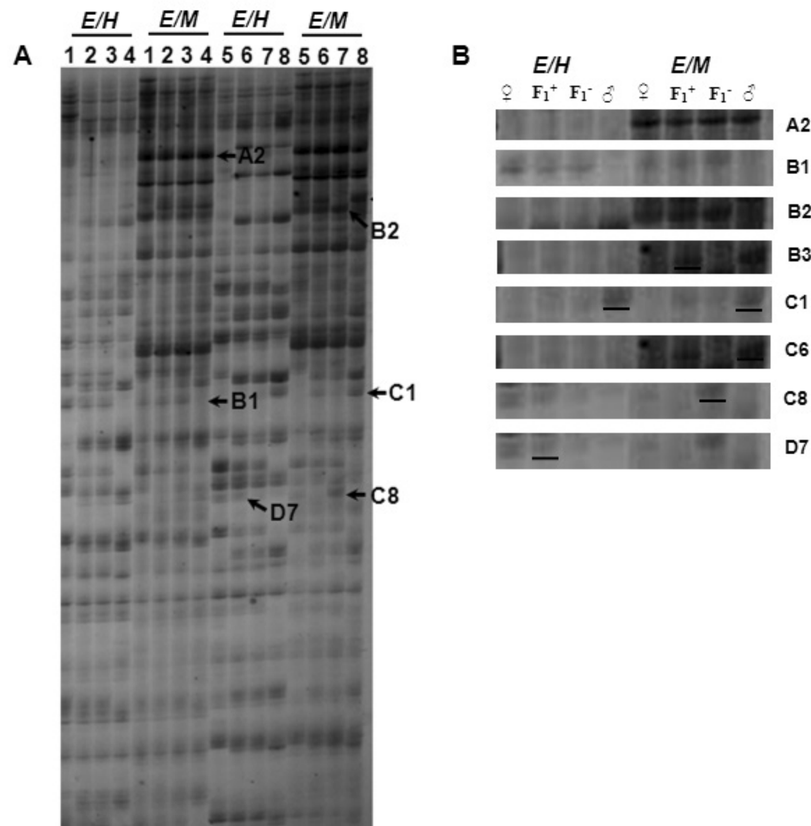

Figure 1. Examples of MSAP profiles showing the various types of cytosine methylation patterns in hybrids and their parental lines. One of the polyacrylamide gels was shown in this figure, in which the embryos were used to amplify with a pair of selective primers: E2+H/M3; the hybrids and their parents detected including: *lane 1* = MH251 (♀); *lane 2* = M×P; *lane 3* = P×M; *lane 4* = P125 (♂); *lane 5* = PH6WC (♀); *lane 6* = W×V; *lane 7* = V×W; *lane 8* = PH4CV (♂). The various patterns were marked by arrows.

DISCUSSION

It has been well documented that when 2 genetically differentiated genomes of species are brought together into a single nucleus by hybridization, the fidelity of epigenetic inheritance in the hybrid plant DNA methylation patterns may vary and accompany some extant modifications (He et al., 2010). This indicated that studying variations in DNA methylation at critical genomic loci of hybrids may help us to understand the mechanism underlying hybrid vigor. In this study, 2 elite hybrids were used to estimate the variation in DNA methylation patterns by the MASP technique. The Xianyu335 variety was planted in a large area of a northeast spring region in China and brings significant economic value. The Jida101 variety was bred in our laboratory and will be promoted for cultivation. Therefore, analyzing the relationship between the DNA cytosine methylation and heterosis using these elite hybrids might provide valuable information for understanding the molecular mechanisms of hybrid vigor.

Table 2. Cytosine methylation patterns at the CCGG sites in hybrids and their parent lines.

Pattern	Number and frequency of sites															
	W x V				V x W				M x P				P x M			
	Leaf	Root	Embryo	Endosperm	Leaf	Root	Embryo	Endosperm	Leaf	Root	Embryo	Endosperm	Leaf	Root	Embryo	Endosperm
Monomorphic loci (A2 + A3)	240	102	94	37	240	102	92	36	242	109	80	36	242	109	77	37
Demethylation loci (B)	58 (78.38%)	9 75.00%	109 (46.38%)	47 39.50%	54 (80.60%)	9 75.00%	98 (42.79%)	50 40.00%	78 (90.70%)	10 66.76%	160 (53.69%)	57 42.22%	80 (86.96%)	9 64.29%	160 (53.51%)	56 (40.88%)
Hypermethylation loci (C)	4 (5.40%)	0 0.00%	11 (4.68%)	4 3.36%	1 (1.49%)	0 0.00%	17 (7.42%)	10 8.00%	3 (3.49%)	2 13.33%	18 (6.04%)	4 2.96%	4 (4.35%)	2 14.29%	16 (5.35%)	2 (1.46%)
Differential methylation from parents (D)	12 (16.22%)	3 25.00%	115 (48.94%)	68 57.14%	12 (17.91%)	3 25.00%	114 (49.78%)	65 52.00%	5 (5.81%)	3 20.00%	120 (40.27%)	74 54.81%	8 (8.70%)	3 21.43%	123 (41.14%)	79 (57.66%)

M = MH251; P = P125; W = PH6WC; V = PH4CV; M x P = MH251 x P125; P x M = P125 x MH251; W x V = PH6WC x PH4CV; V x W = PH4CV x PH6WC.

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Table 3. Homology analysis of differentially methylated cloned fragments by Basic Local Alignment Search Tool (BLAST) analysis.

No.	Primers	Length (bp)	Methylation pattern	Hybrids	Sequence similarity	E value
1	E6/H5	234	C2	Root (WV)	EU963137: transcription factor APFI mRNA	3e-82
2	E7/H3	319	C5	Root (MP)	DQ002407: gypsy retrotransposon	7e-17
3	E6/H1	287	C2	Leaf (VW)	NM_001154430: ternary complex factor MIP1 mRNA	6e-105
4	E6/H1	219	B7	Embryo (MP)	AF215823: cytoplasm male sterility restorer factor gene	2e-58
5	E6/H7	162	B11	Embryo (VW)	DQ417753: B73 serine/threonine kinase protein	3e-10
6	E7/H7	319	C2	Embryo (MP)	NM_001154453: HT1 protein kinase	1e-147
7	E6/H7	210	B4	Endosperm (MP)	DQ002407: copia retrotransposon opie1	2e-45
8	E6/H8	297	C2	Endosperm (VW)	NM_001153591: STE20/SPS1-related proline-alanine-rich protein kinase	8e-47
9	E7/H1	227	B5	Endosperm (WV)	AY530951: putative growth-regulating factor	7e-15
10	E7/H7	334	C4	Endosperm (WV)	NM_001158374: B12D protein	6e-37
11	E7/H8	195	C2	Endosperm (MP)	AY574035: rust resistance protein rp3-1 gene	4e-87

MP = MH251 x P125; PM = P125 x MH251; WV = PH6WC x PH4CV; VW = PH4CV x PH6WC.

The methylation levels among different hybrids varied (increased or decreased) compared to each other. However, the relative total cytosine methylation levels in embryos were significantly higher than those in endosperm tissues in all of the hybrids and their corresponding parents. Moreover, the relative total cytosine methylation levels in embryo and endosperm tissues of hybrids were significantly lower than the 23.3% methylation levels in 9-10th leaves of maize (Zhao et al., 2007) and significantly higher than the 8.85 and 8.87% average DNA methylation rates of pollen and leaves in *Solanum demissum* (Huang et al., 2006). These differences may result from the different types of tissues that were tested or the different species combinations that were used (Riddle and Richards, 2002). In addition, the relative total cytosine methylation levels of the reciprocal hybrids for a given combination were remarkably similar, which agreed with findings in previous studies (Riddle and Richards, 2005; Huang et al., 2006; Zhao et al., 2007). However, the relative total cytosine methylation levels in hybrids deviated from the mid-parent values. Huang et al. (2006) reported that the deviation might be due to the large number of analyzed sequences or because novel gene expression patterns emerge in hybrids.

It has been widely reported that the parental methylation states in plants are not only stably inherited by their progenies but also accompany remodeling of other additive parental methylation patterns in hybrids (Lukens et al., 2006; Marfil et al., 2006). Furthermore, many studies have also reported that the cytosine methylation in coding or promoter regions can inhibit target gene expression, while DNA demethylation can lead to reactivated gene expression (Ronemus et al., 1996; Hsieh et al., 2009). In this study, the level of methylation was lower and the number of demethylation events was significantly higher in hybrids than in the parental lines. These results implied that the decreasing trend and demethylation in hybrids relative to parents may enable the derepression and possibly expression of many genes that were associated with the phenotypic variations observed in hybrids. In order to further analyze the observed methylation pattern changes, many variation bands were sequenced. A retrotransposon was detected in the MP hybrid root and endosperm. The retrotransposon, as a ubiquitous component of plant genomic DNA, plays an important role in species evolution and can insert within or near functional genes, inhibiting the expression of the adjacent genes (Kumar and Bennetzen, 1999; Kashkush et al., 2003). In addition, some other metabolic regulation and stress-response genes were also detected, which represented demethylation or hypermethyl-

ation patterns, but their functions and roles in hybrid vigor are unknown. Therefore, further analysis of the functions of these candidate genes and their underlying epigenetic regulatory polymorphisms might help us to understand the role of DNA methylation in heterosis.

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Supplementary material

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