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

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Abstract

In order to better understand the molecular basis of heterosis in maize, the methylation-sensitive amplification polymorphism method was used to estimate patterns of cytosine methylation in seedling roots and leaves and 15-d post-fertilization embryo and endosperm tissues of hybrids and their parental lines Zheng58 and Chang7-2. In all tissues, total relative methylation levels in the hybrids were lower than corresponding mid-parent values, with a higher number of demethylation events inferred for the hybrids. The trend of reduced methylation and increased demethylation in the hybrids relative to their parents may allow de-repression and possibly the expression of various genes associated with a hybrid phenotypic variation. To further investigate observed methylation pattern changes, we sequenced 50 differentially displayed DNA fragments. The *BLAST* analysis revealed that 13 fragments shared similarity with known functional proteins in maize or other plant species including proteins related to metabolism, transposons/retrotransposons, development, stress response, and signal transduction. The genes associated with these proteins may thus contribute significantly to maize hybrid vigour.

Additional key words: heterosis, methylation-sensitive amplification polymorphism, *Zea mays*.

Introduction

Heterosis refers to the superior performance of hybrids over their parents with respect to various agronomic traits, such as accelerated development, increased grain yield, and improved resistance to abiotic and biotic stresses (Shull 1908, 1952, Hochholdinger and Hoecker 2007, Chen 2010). Although much research has been performed during the previous century, the molecular mechanisms underlying heterosis remain poorly understood and a subject of debate. Three major genetic mechanisms have been proposed to explain heterosis, namely dominance, over-dominance, and epistasis (Bruce 1910, Hochholdinger and Hoecker 2007, Birchler *et al*. 2010), but these hypotheses are largely conceptual and have not been completely elucidated (He *et al*. 2011). Numerous studies investigating the genetic basis of heterosis were conducted during the 20th century, including quantitative trait loci and high-throughput transcript profiling. Such studies have revealed the probable association of many genes in all possible modes of action with heterosis (Frascaroli *et al*. 2007, Radoev *et al*. 2008).

Recent research has demonstrated that variation at the DNA sequence and epigenetic regulation of chromatin structure is essential for proper interpretation of genetic information and phenotypic determination (Groszmann *et al*. 2010, Yang *et al*. 2010, Shen *et al*. 2012). Cytosine DNA methylation is an important chromatin modification playing a significant role in various cellular activities including orchestration of gene expression during plant development, maintenance of overall genomic integrity, control of genomic imprinting, and formation and perpetuation of heterochromatin (Rapp and Wendel 2005,

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Abbreviations: C - Chang7-2, MSAP - methylation sensitive amplification polymorphism, Z - Zheng58.
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Greaves *et al*. 2012, Candaele *et al*. 2014). The disturbance of DNA methylation patterns may consequently have functional consequences in organisms with this epigenetic code (Kumari *et al*. 2013, Dyachenko *et al*. 2014). Compared to other approaches used to study global methylation, for example bisulfite genomic sequencing, the methylation-sensitive amplification polymorphism (MSAP) technique may underestimate actual methylation levels (Lu *et al*. 2008), but it has lower cost and has been demonstrated to be a highly efficient and powerful tool for detecting cytosine methylations in plant genomes, such as *Arabidopsis thaliana* (You *et al*. 2012), *Paspalum notatum* (Rodriguez *et al*. 2012), cotton (Osabe *et al*. 2014), *Sorghum bicolor* (Wang *et al.* 2010, Zhang *et al*. 2011), *Phoenix dactylifera* (Fang and Chao 2007), *Hordeum brevisubulatum* (Li *et al*. 2008), *Codonopsis lanceolata* (Guo *et al*. 2007), wheat (Zhang *et al*. 2008), and maize (Shan *et al*. 2013). The MSAP method has also been applied in studies associated with various topics including biotic and abiotic stresses (Wang *et al*. 2011, Karan *et al*. 2012), development (Meng *et al*. 2012), varying ploidy (Li *et al*. 2011), and tissue cultures (Wang *et al*. 2013). Zhao *et al*. (2007) used MSAP to demonstrate that most cytosine methylation sites display faithful epigenetic inheritance between three sets of reciprocal maize hybrids and their inbred parents. In this study, the MSAP technique was used to compare differences in cytosine methylation patterns in a set of reciprocal maize hybrids and their parents based on roots and leaves of three-leaf stage seedlings and 15-d post-pollination embryo and endosperm tissues. We isolated and sequenced 68 differentially displayed DNA fragments detected by MSAP profiling. Of these fragments, 13 shared similarity with known functional proteins in maize or other plant species. The possible roles of these proteins in developmental regulation were consequently examined.

Materials and methods

An elite hybrid maize (*Zea mays* L.) line Zhengdan 958 (widely planted in northeastern China) and its parental lines Zheng58 (Z) and Chang7-2 (C) were used in the present study. Zhengdan 958 and its inbred parental lines were developed by the Henan Academy of Agricultural Sciences of China in 2000, and have been maintained by careful hand-pollination for many generations. The expanded leaves and roots of three-leaf stage seedlings as well as embryo and endosperm tissues collected 15 d post-pollination of hybrid and parental lines were used in this study.

Genomic DNA was isolated by the modified cetyl trimethyl amonium bromide (CTAB) method (Kidwell *et al*. 1992) from four tissues (leaves, roots, embryo, and endosperm) of hybrid and parental lines. The isolated DNA was purified by phenol extraction, and then checked for quality and quantity by agarose gel electrophoresis and spectrometric measurement.

A MSAP analysis was performed using previously reported protocols (Salmon *et al*. 2005, Zhao *et al*. 2007) which are based on the amplified fragment length polymorphism (AFLP) technique (Xu *et al*. 2000). Two combinations of restriction enzymes (*Takara*, Tokyo, Japan) were used: the restriction enzyme *Eco*RI that is blocked by CG DNA methylation in specific sequence contexts was mixed separately with each of the two isoschizomers *Hpa*II and *Msp*I which recognize the same sequence (CCGG) but cut with differential sensitivity to DNA methylation of internal or external cytosine. Two different adaptors, *Hpa*II/*Msp*I or *Eco*RI, were used in subsequent ligation reactions. One pair of pre-selective primers and 64 pairs of selective primers were used for amplification (Table 1 Suppl.).

A total of 5 mm^3 of each ligated sample, diluted 10-fold with sterilized distilled water, was used for pre-selective amplification reactions. PCR reaction conditions consisted of 25 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min. Selective amplification reactions were carried out on 5 mm^3 of the pre-amplified DNA that had been diluted 20-fold using the following touchdown PCR conditions: 94 °C for 2 min followed by 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 a subsequent 23 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min.

Products of the selective amplification were denatured and separated on a 4.5 % (m/v) polyacrylamide gel run at 60 W and 50 °C until the bromphenol blue dye reached the bottom of the gel. The separated bands were visualized by silver staining. For further data analysis, the gel was placed in a clean and ventilated area and allowed to dry completely.

Fragments with molecular masses of 100 - 600 bp were subjected to statistical analysis. The number of different methylation patterns displayed by each genotype was counted based on the differential sensitivity of *Hpa*II and *Msp*I to cytosine methylation. When the external cytosine of a recognition site is hemi-methylated, the site can be cleaved by *Hpa*II, but not by *Msp*I, yielding a single band. In the case of full methylation (methylation of both strands) of the internal cytosine, a band can conversely be detected in the *Eco*RI + *Msp*I digest, but not in the *Eco*RI + *Hpa*II digest. In addition, according to previous researches reported, different cytosine methylation patterns were observed between hybrids and their parental lines and divided into four major classes**,** including class A (the same band in both a hybrid and its parents), class B (a band present in the hybrid but not in both parents means demethylation in the hybrid compared

with its parents), class C (the band presence in both parents but not in the hybrid means hyper-methylation in the hybrid corresponding to its parents), class D (different methylated sites in the hybrid compared with its parents (Zhao *et al.* 2008, Joel and Zhang 2011). Based on these guidelines, methylation site changes were comparatively analyzed between hybrids and parents.

The silver-stained MSAP gels were examined for bands corresponding to altered methylation states in the hybrids relative to their parents. Bands of interest were moistened with distilled water, extracted from the gel, dissolved in 30 mm^3 of double distilled H_2O and incubated at 37 °C for 12 h. The excised bands were re-amplified with the appropriate selective primer combinations. The sizes of the PCR products were checked by agarose gel electrophoresis. The PCR products were extracted from the agarose gel and cloned into a *pGM-T* cloning vector (*Tiangen*, Beijing China). Successful clones were screened by a standard ampicillin selection and identified by PCR. The DNA segments of positive clones were sequenced by the *HuaDa* gene technology company (*BGI*; Beijing, China). The *DNAMAN* sequence analysis software (*v. 4.1*) was used for the sequence analysis and editing, and the *BLASTN* and *BLASTX* programs on the *NCBI* website were used for the sequence homology analysis.

To validate the methylation polymorphisms patterns uncovered by MSAP, Southern blotting was performed using portions of representative cloned fragments as probes. The probes were labeled with digoxygenin-11-dUTP using a random primer DNA labeling system (*Roche*, Mannheim, Germany). A total of 100 μg of genomic DNA of each genotype was digested separately using the restriction enzyme combinations *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I. The two digests of each sample were fractionated on 0.8 % (m/v) agarose gels and then transferred to *Hybond* N^+ membranes (*GE Healthcare*, Little Chalfont, UK) by capillary transfer with an alkali solution as described by Sambrook *et al*. (1989). Hybridization and detection were carried out using a detection starter kit II (*Roche*).

Results and discussion

In recent years, various studies have uncovered evidence that the fidelity of epigenetic inheritance may vary in plant hybrids and may be accompanied by extensive modifications in DNA methylation (He *et al*. 2010, Sanetomo and Hosaka 2011). The results of these studies suggest that a detailed investigation of epigenetic regulation of critical loci in hybrid genomes may lead to a better understanding of the mechanisms underlying hybrid vigour. In addition, heterosis is manifested at the early seedling stage in hybrids, and the pollination stage is closely related to grain yield (Joel and Zhang 2001), thus MSAP profiles were generated for Zhengdan 958 hybrids and their parents from the following tissues and developmental stages: leaf and root tissues at the three-leaf seedling stage, and embryo and endosperm tissues at the 15-d post-pollination stage.

A total of 64 selective primer combinations were used to compare the status of cytosine methylation in different tissues of hybrids and their parents, resulting in the generation of 4 099 fragments, and the number of non-methylated sites, hemi-methylated external cytosines, and fully methylated internal cytosines were calculated (Table 1). Based on the MSAP profiles, the DNA cytosine methylation in the reciprocal hybrids was very similar in accordance with the findings of previous studies (Riddle and Richards 2005, Zhao *et al*. 2007, He

Table 1. Relative cytosine methylation [%] at the CCGG sites among different tissues in hybrids and their parent lines (MP - mid-parent, Z - Zheng58, C - Chang7-2, R - root, L - leaf, Em - embryo, En - endosperm; * - values significantly lower than in the corresponding mid-parents at $P \leq 0.05$.

	Non-methylated CCGG sites				Methylated CCGG sites total				hemi-methylated sites				fully-methylated sites			
	R	L	Em	En	R	L	Em	En	R	L	Em	En	R	L	Em	En
Z	100	214	583	435	217	480	190	98	59	142	53	27	158	338	137	71
(♀)	(31.55)	(30.84)	(75.42)	(81.61)	(68.45)	(69.16)	(24.58)	(18.39)	(18.61)	(20.46)	(6.86)	(5.06)	(49.84)	(48.70)	(17.72)	(13.33)
C	104	243	553	416	208	522	235	117	58	147	66	30	150	375	143	87
(♂)	(33.33)	(31.93)	(72.57)	(78.05)	(66.67)	(68.07)	(30.84)	(21.95)	(18.59)	(19.32)	(8.66)	(5.63)	(48.08)	(48.75)	(18.77)	(16.32)
MP	102	229	568	426	213	498	213	108	59	145	60	29	154	353	140	79
	(32.38)	(31.50)	(73.96)	(79.92)	(67.62)	(68.50)	(27.73)	(20.26)	(18.73)	(19.94)	(7.81)	(5.44)	(48.89)	(48.56)	(18.23)	(14.82)
Z×C	107	242	653	493	219	520	159*	55	59	151	58	18	160	369	101*	37
	(32.82)	(31.76)	(80.72)	(80.11)	(67.18)	(68.24)	(19.65)	(19.89)	(18.10)	(19.82)	(7.17)	(3.28)	(49.08)	(48.42)	(12.48)	(16.61)
C×Z	107	238	667	490	206	523	146*	61*	59	160	77	32	147	457	69*	29*
	(34.19)	(31.27)	(82.55)	(88.93)	(65.81)	(68.73)	(18.07)	(11.07)	(18.85)	(21.02)	(9.53)	(5.81)	(46.96)	(47.71)	(8.54)	(5.26)

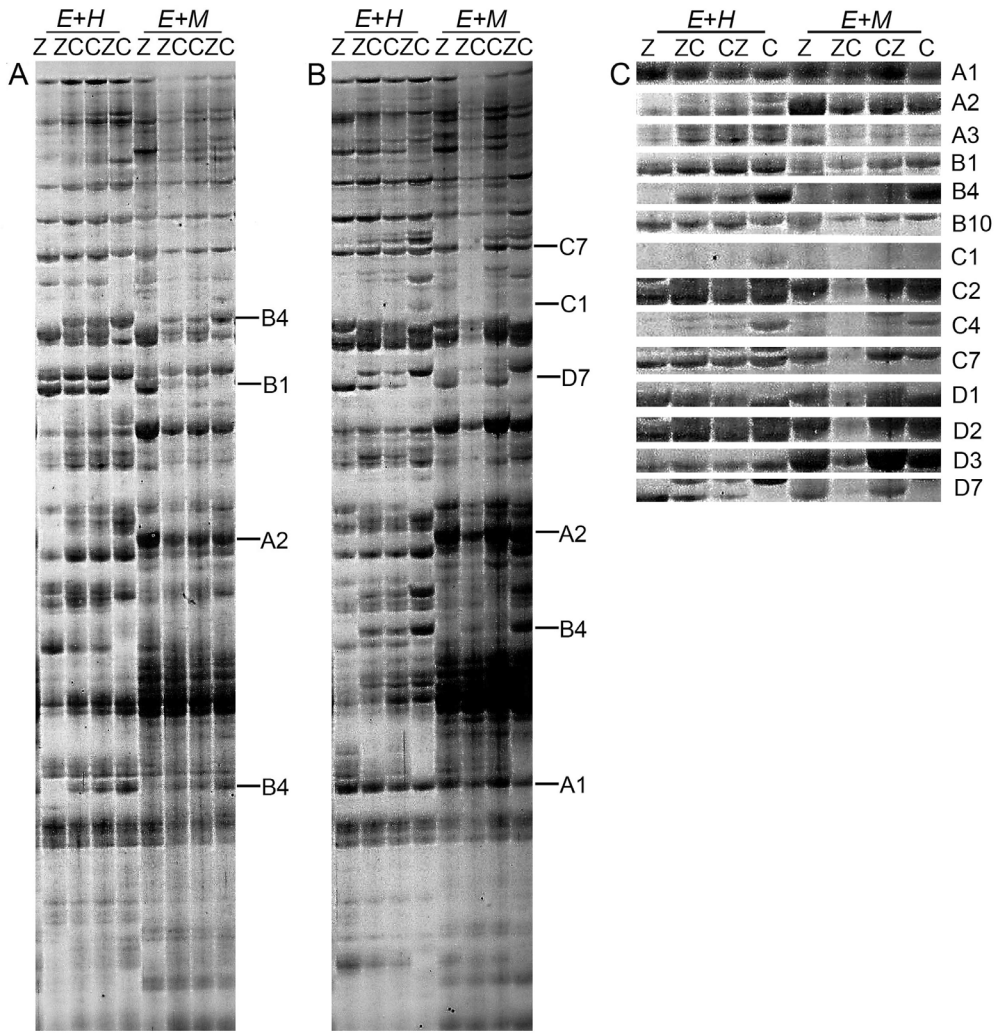

Fig. 1. Examples of MSAP profiles showing the various types of cytosine methylation patterns in hybrids and their parental lines. Two polyacrylamide gels in which the embryos were used to amplify with a pair of selective primers are shown: *A* - E6+HM3 and *B* - E6+HM4. *C* - The patterns of differentially displayed bands are shown by MSAP analysis. Z - Zheng58 ♀, ZC - Zheng58 × Chang7-2, CZ - Chang7-2 × Zheng58, C - Chang7-2 ♂; some of the methylation patterns are marked with the *arrows; E+H* and *E+M* are the *Eco*R I/*Hap*II and *Eco*R I/*Msp* I restriction enzyme combinations.

et al. 2010). In addition, the total relative methylation levels of the reciprocal hybrids in all tissues except for the C × Z three-leaf stage (leaf) were lower than the corresponding mid-parent values. In the case of hemi-methylation, reciprocal hybrids in all tissues showed various levels, most of which were lower than the corresponding mid-parent values, but none of the changes was statistically significant. However, for the full methylation in embryo and endosperm tissues, the reciprocal hybrids displayed significantly lower values (in the range of 5.26 to 12.48 %) than their corresponding mid-parent (ranging from 14.82 to 18.23 %) except for the Z × C endosperm. Li *et al.* (2013) revealed that an increased gene expression in hybrids is associated with an overall low DNA methylation.

In addition, we found that external hemi-methylation, internal full methylation, and total relative methylation percentages were higher in the leaf and root tissues than in the embryo and endosperm, regardless of the parent or hybrid involved. Moreover, in our study, the average total relative cytosine methylation of 67.03 and 68.55 % observed in the roots and leaves was higher than the

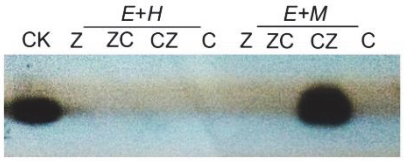

Fig. 2 An example of methylation validation patterns by DNA gel blot analysis on maize intraspecific hybrids relative to their inbred parental lines. The hybridization pattern using probe No. 12. The abbreviations as in Fig. 1.

Table 2. The homologous analysis of cloned fragments differentially methylated using *BLAST*-searching. Z - Zheng58, C - Chang7-2, ZC - Zheng58 × Chang7-2, CZ - Chang7-2 × Zheng58.

No.	Primers	Size [bp]	Patterns	Accumulation	Homology	Protein	E value
1	E6/H3	321	B12	embryo (ZC)	AF466646	transposase (Z195D10.1) gene (*Zea mays*)	4e-52
2	E6/H7	279	B3	embryo (ZC)	AY728476	chalcone synthase C2-ldf-ll gene (*Zea mays*)	4e-13
3	E6/H7	144	C4	embryo (ZC/CZ)	AY574035	rust resistance protein rp3-1 gene (*Zea mays*)	2e-24
4	E6/H8	188	B2	embryo (ZC)	AY574035	rust resistance protein rp3-1 gene (*Zea mays*)	3e-16
5	E7/H1	128	C5	embryo (ZC/CZ)	NM-001154659	transport inhibitor response protein (*Zea mays*)	3e-54
6	E7/H5	262	B2	embryo (CZ)	AY530951	growth-regulating factor (*Zea mays*)	2e-67
7	E7/H6	339	C2	embryo (ZC/CZ)	AY574035	rust resistance protein rp3-1 gene (*Zea mays*)	1e-83
8	E7/H6	176	B12	embryo (ZC)	FJ968747	hsp organizing protein (*Dactylis glomerata*)	4e-29
9	E7/H8	298	C7	embryo (ZC)	AB557931	cadmium selective transporter for low entry to shoot from Nipponbare (*Oryza sativa*)	2e-24
10	E6/H4	143	B12	root (CZ)	EU959517	histone H2A variant 3 mRNA (*Zea mays*)	2e-32
11	E7/H4	91	C5	root (ZC/CZ)	NM_001154086	ATP synthase delta chain mRNA (*Zea mays*)	1e-32
12	E6/H5	259	B12	leaf (CZ)	NM_001111894	G-box binding factor 1 (GBF1) (*Zea mays*)	1e-67
13	E7/H2	114	B12	leaf (ZC)	FJ909711	tranposon insertion Mu1007362 flanking (*Zea mays*)	3e-28

methylation of 22.44 - 26.15 % previously reported for maize leaves (Zhao *et al*. 2007). However, Lu *et al*. (2008) have found that both MSAP ratio and full methylation are highest in the bracteal leaf and lowest in the tassel of maize. Thus, such significant differences of DNA methylation may be due to technical reasons or differences in plant materials or different developmental time of plants used.

Zhao *et al.* (2008) divided the different cytosine methylation patterns among hybrids and their parental lines into four major classes (Table 2 Suppl., Fig. 1). These four classes are further divided into 30 subgroups based on epigenetic inheritance patterns and alteration in cytosine methylation among parents and F_1 offspring (Table 2 Suppl.). In our study, a comparative analysis revealed that the number of demethylated loci (class B) was significantly higher than the number of hyper-methylated loci (class C) in all the tissues of all the hybrids than of their parents. Many studies have also reported that cytosine methylation in a coding or promoter region can inhibit a target gene expression, whereas DNA demethylation can reactivate the gene expression (Ronemus *et al*. 1996, Lu *et al*. 2006, Hsieh *et al*. 2009). Corresponding to an overall low genomic DNA methylation, the trend of an increased demethylation in the hybrids might enable the de-repression and possibly the expression of many genes associated with the phenotypic variations observed in the hybrids.

To analyze fragments associated with the various methylation patterns, 68 bands (18 from roots, 10 from leaves, 28 from embryos, and 12 from endosperm) were extracted from the polyacrylamide gel and re-amplified using the original selective primers. A total of 50 positive clones (13 from roots, 6 from leaves, 24 from embryos and 7 from endosperm) were then sequenced. The results of the *BLASTX* analysis, summarized in Table 3 Suppl., reveal that 13 fragments were homologous to known functional genes (Table 2), whereas 10 fragments showed homology to maize expressed sequence tags (ESTs) with unknown functions and 27 clones had no matches in the *GenBank* database. To further validate DNA methylation patterns that were detected by the MSAP analysis, three sequenced fragments were randomly selected as probes in the Southern blot analysis. But only one probe (No. 12) showed a smear in the autoradiograph (Fig. 2), possibly as a consequence of its high-copy nature, and the result was consistent in both cases. DNA methylation has been identified as an important epigenetic regulator of gene expression (Lister *et al.* 2008, Chen *et al.* 2010, Nakamura and Hosaka 2010, Shen *et al.* 2012). These function genes detected in our study with a cytosine

methylation variation might play an important role in heterosis. A further analysis of the functions and underlying epigenetic regulatory polymorphisms of these candidate genes may provide insights into the role of DNA methylation in heterosis.

References

Birchler, J.A., Yao, H., Chudalayandi, S., Vaiman, D., Veitia, R.A.: Heterosis. - Plant Cell **22**: 2105-2112, 2010.

Bruce, A.B.: The Mendelian theory of heredity and the augmentation of vigor. - Science **32**: 627-628, 1910.

Candaele, J., Demuynck, K., Mosoti, D., Beemster, G.T.S., Inzé, D., Nelissen, H.: Differential methylation during maize leaf growth targets developmentally regulated genes. -Plant Physiol. **164**: 1350-1364, 2014.

Chen, Z.J.: Molecular mechanisms of polyploidy and hybrid vigor. - Trends Plant Sci. **15**: 57-71, 2010.

Dyachenko, O.V., Tarlachkov, S.V., Marinitch, D.V., Shevchuk, T.V., Buryanov, Y.I.: Expression of exogenous DNA methyltransferases: application in molecular and cell biology. - Biochemistry **79**: 77-87, 2014.

Fang, J.G., Chao, C.T.: Methylation-sensitive amplification polymorphism in date palms (*Phoenix dactylifera* L.) and their off-shoots. - Plant Biol. **9**: 526–533, 2007.

Frascaroli, E., Cane, M.A., Landi, P., Pea, G., Gianfranceschi, L., Villa, M., Morgante, M., Pè, M.E.: Classical genetic and quantitative trait loci analyses of heterosis in a maize hybrid between two elite inbred lines. - Genet. J. **176**: 625-644, 2007.

Greaves, I.K., Groszmann, M., Ying, H., Taylor, J.M., Peacock, W.J., Dennis, E.S.: Trans-chromosomal methylation in *Arabidopsis* hybrids. - Proc. nat. Acad. Sci. USA **109**: 3570-3575, 2012.

Groszmann, M., Greaves, I.K., Albertyn, Z.I., Scofield, G.N., Peacock, W.J., Dennis, E.S.: Changes in 24-nt siRNA levels in *Arabidopsis* hybrids suggest an epigenetic contribution to hybrid vigor. - Proc. nat. Acad. Sci. USA **108**: 2617-2622, 2010.

Guo, W.L., Wu, R., Zhang, Y.F., Liu, X.M., Wang, H.Y., Gong, L., Zhang, Z.H., Liu, B.: Tissue culture-induced locus-specific alteration in DNA methylation and its correlation with genetic variation in *Codonopsis lanceolata* Benth. *et* Hook. f. - Plant Cell Rep. **26**: 1297-1307, 2007.

He, G., Elling, A.A., Deng, X.W.: The epigenome and plant development. - Annu. Rev. Plant Biol. **62**: 411-435, 2011.

He, G.M., Zhu, X.P., Elling, A.A., Chen, L.B., Wang, X.F., Guo, L., Liang, M.Z., He, H., Zhang, H.Y., Chen, F.F., Qi, Y.J., Chen, R.S., Deng, X.W.: Global epigenetic and transcriptional trends among two rice subspecies and their reciprocal hybrids. - Plant Cell **22**: 17-33, 2010.

Hochholdinger, F., Hoecker, N.: Towards the molecular basis of heterosis. - Trends Plant Sci. **12**: 427-432, 2007.

Hsieh, T.F., Ibarra, C.A., Silva, P., Zemach, A., Eshed-Williams, L., Fischer, R.L., Zilberman, D.: Genome-wide demethylation of *Arabidopsis* endosperm. - Sci. J. **324**: 1451-1454, 2009.

Joel, A.J., Zhang, Q.: Differential cytosine methylation in rice. - In: Book of Abstracts of 8th National Rice Biotechnology Network Meeting. Pp. 226-230. Aurangabad 2001.

Karan, R., DeLeon, T., Biradar, H., Subudhi, P.K.: Salt stress induced variation in DNA methylation pattern and its influence on gene expression in contrasting rice genotypes. - PLoS ONE 7(6): e40203. 2012.

Kidwell, K.K., Osborn, T.C.: Simple plant DNA isolation procedures. - In: Beckman, J.S., Osborn, T.C. (ed.): Plant Genomes. Methods for Genetic and Physical Mapping. Pp. 1-13. Kluwer Academic Publishers, Dordrecht 1992.

Kumari, R., Yadav, G., Sharma, V., Sharma, V., Kumar, S.: Cytosine hypomethylation at CHG and CHH sites in the pleiotropic mutants of Mendelian inheritance in *Catharanthus roseus*. - J. Genet. **92**: 499-511, 2013.

Li, A., Song, W.Q., Chen, C.B., Zhou, Y.N., Qi, L.W., Wang, C.G.: DNA methylation status is associated with the formation of heterosis in *Larix kaempferi* intraspecific hybrids. - Mol. Breed. **31**: 463-475, 2013.

Li, A., Hu, B.Q., Xue, Z.Y., Chen, L., Wang, W.X., Song, W.Q., Chen, C.B., Wang, C.G.: DNA methylation in genomes of several annual herbaceous and woody perennial plants of varying ploidy as detected by MSAP. - Plant mol. Biol. Rep. **29**: 784-793, 2011.

Li, Y., Shan, X., Liu, X., Hu, L., Guo, W., Liu, B.: Utility of the methylation-sensitive amplified polymorphism (MSAP) marker for detection of DNA methylation polymorphism and epigenetic population structure in a wild barley species (*Hordeum brevisubulatum*). - Ecol. Res. **23**: 927-930, 2008.

Lister, R., O'Malley, R.C., Tonti-Filippini, J., Gregory, B.D., Berry, C.C., Millar, A.H., Ecker, J.R.: Highly integrated single base resolution maps of the epigenome in *Arabidopsis*. - Cell **133**: 523-536, 2008.

Lu, Y., Rong, T., Cao, M.: Analysis of DNA methylation in different maize tissues. - J. Genet. Genomics **35**: 41-48, 2008.

Lu, G., Wu, X., Chen, B., Gao, G., Xu, K., Li, X.: Detection of DNA methylation changes during seed germination in rapeseed (*Brassica napus*). - Chin. sci. Bull. **51**: 182-190, 2006.

Meng, F.R., Li, Y.C., Yin, J., Liu, H., Chen, X.J., Ni, Z.F., Sun, Q.X.: Analysis of DNA methylation during the germination of wheat seeds. - Biol. Plant. **56**: 269-275, 2012.

Nakamura, S., Hosaka, K.: DNA methylation in diploid inbred lines of potatoes and its possible role in the regulation of heterosis. - Theor. appl. Genet. **120**: 205-214, 2010.

Osabe, K., Clement, J.D., Bedon, F., Pettolino, F. A., Ziolkowski, L., Llewellyn, D.J., Finnegan, E. J., Wilson I.W.: Genetic and DNA methylation changes in cotton (*Gossypium*) genotypes and tissues. - PLOS one 9(1): e86049, 2014.

Radoev, M., Becker, H.C., Ecke, W.: Genetic analysis of heterosis for yield and yield components in rapeseed (*Brassica napus* L.) by quantitative trait locus mapping. - Genetics **179**: 1547-1558, 2008.

Rapp, R.A., Wendel, J.F.: Epigenetics and plant evolution. - New Phytol. **168**: 81-91, 2005.

Riddle, N.C., Richards, E.J.: Genetic variation in epigenetic inheritance of ribosomal RNA gene methylation in *Arabidopsis*. - Plant J. **41**: 524-532, 2005.

Rodriguez, M.P., Cervigni, G.D.L., Quarin, C.L., Ortiz, J.P.A.:

Frequencies and variation in cytosine methylation patterns in diploid and tetraploid cytotypes of *Paspalum notatum*. - Biol. Plant. **56**: 276-282, 2012.

Ronemus, M.J., Galbiati, M., Ticknor, C., Chen, J., Dellaporta, S.L.: Demethylation-induced developmental pleiotropy in *Arabidopsis*. - Science **273**: 654-657, 1996.

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

Sambrook, J., Fritsch, E.F., Maniatis, T.: Commonly Used Techniques in Molecular Cloning. Molecular Cloning: a Laboratory Manual. - Cold Spring Harbor Laboratory Press, New York 1989.

Sanetomo, R., Hosaka, K.: Reciprocal differences in DNA sequence and methylation status of the pollen DNA between F1 hybrids of *Solanum tuberosum* × S. *demissum*. - Euphytica **182**: 219-229, 2011.

Shan, X.H., Wang, X.Y., Yang, G., Wu, Y., Su, S.Z., Li, S.P., Liu, H.K., Yuan, Y.P.: Analysis of the DNA methylation of maize (*Zea mays* L.) in response to cold stress based on methylation-sensitive amplified polymorphisms. - J. Plant Biol. **56**: 32-38, 2013.

Shen, H., He, H., Li, J., Chen, W., Wang, X., Guo, L., Peng, Z., He, G., Zhong, S., Qi, Y., Terzaghi ,W., Deng, X.W.: Genome-wide analysis of DNA methylation and gene expression changes in two *Arabidopsis* ecotypes and their reciprocal hybrids. - Plant Cell **24**: 875-892, 2012.

Shull, G.H.: The composition of a field of maize. - Amer. Breeders Assoc. Rep. **4**: 296-301, 1908.

Shull, G.H.: Beginnings of the heterosis concept. - In: Gowen, J.W. (ed.): Heterosis. A Record of Researches Directed Toward Explaining and Utilizing the Vigor of Hybrids. Pp. 14-48. Iowa State College Press, Ames 1952.

Wang, H., Feng Q.Z., Zhang M.S., Yang C.W., Sha W., Liu B.: Alteration of DNA methylation level and pattern in sorghum (*Sorghum bicolor* L.) pure-lines and inter-line F_1 hybrids following low-dose laser irradiation. - J. Photochem. Photobiol. B Biol. **99**: 150-153, 2010.

Wang, W.S., Zhao, X.Q., Pan, Y.J., Zhu, L.H., Fu, B.Y., Li, Z.K.: DNA methylation changes detected by methylation-sensitive amplified polymorphism in two contrasting rice genotypes under salt stress. - J. Genet. Genome **38**: 419-424, 2011.

Wang, X.R., Wu, R., Lin, X.Y., Bai, Y., Song, C.D., Yu, X.M., Xu, C.M., Zhao, N., Dong, Y.Z., Liu, B.: Tissue culture-induced genetic and epigenetic alterations in rice pure-lines, F1 hybrids and polyploids. - BMC Plant Biol. **13**: 1-12, 2013.

Xu, M.L., Li, X.Q., Korban, S.S.: AFLP-based detection of DNA methylation. - Plant mol. Biol. Rep. **18**: 361-368, 2000.

Yang, W., Jiang, D., Jiang, J., He, Y.H.: A plant-specific histone H3 lysine 4 demethylase represses the floral transition in *Arabidopsis*. - Plant J. **62**: 663-673, 2010.

You, W.H., Tyczewska, A., Spencer, M., Daxinger, L., Schmid, M.W., Grossniklaus, U., Simon, S.A., Meyers, B.C., Matzke, A., Matzke, M.: Atypical DNA methylation of genes encoding cysteine-rich peptides in *Arabidopsis thaliana*. - BMC Plant Biol. **12**: 1-15, 2012.

Zhang, M., Xu, C., Von Wettstein, D., Liu, B.: Tissue-specific differences in cytosine methylation and their association with differential gene expression in *Sorghum bicolar*. - Plant Physiol. **156**: 1955-1966, 2011.

Zhang, Y., Liu, Z.H., Liu, C., Yang, Z.J., Deng, K.J., Peng, J.H., Zhou, J.P., Li, G.R., Tang, Z.X., Ren, Z.L.: Analysis of DNA methylation variation in wheat genetic background after alien chromatin introduction based on methylation-sensitive amplification polymorphism. - Chin. sci. Bull. **53**: 58-69, 2008.

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

Zhao, Y., Yu, S., Xing, C., Fan, S., Song, M.: Analysis of DNA methylation in cotton hybrids and their parents. - Mol. Biol. **42**: 169-178, 2008.