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Study on the relationship between genetic variation of DNA methylation and heterosis in soybean leaves

Yuanqian Wang · Kaixin Zhang · Lifang Sun · Xiao Han · Sujie Fan · Xueying Li · Yiwei Qu · Dan Yao · Piwu Wang · Jun Zhang

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Abstract In order to well understand the molecular basis of heterosis in soybean, the methylation-sensitive amplification polymorphism (MSAP) method based on capillary electrophoresis was used to estimate levels and patterns of cytosine methylation in 15-day post-emergence leaves of four parental lines [Jilin 47 (no. 19), EXP (no. 12), Jilin 38 (no. 3) and Yi 3 (no. 6)] and 12 hybrids [Jilin 38 × Yi 3(3 × 6), Jilin 38 × EXP(3 × 12), Jilin 38 × Jilin 47(3 × 19), Yi 3 × Jilin 38(6 × 3), Yi 3 × EXP(6 × 12), Yi 3 × Jilin 47(6 × 19), EXP × Jilin 38(12 × 3), EXP × Yi 3(12 × 6), EXP × Jilin 47(12 × 19), Jilin 47 × Jilin 38(19 × 3), Jilin 47 × Yi 3(19 × 6), Jilin 47 × EXP(19 × 12)]. In addition, 12 traits of the hybrids and their parents were also analyzed to understand the relationship between DNA methylation variation and heterosis. MSAP results showed that the total relative methylation level of all hybrids was lower than the corresponding middle parent value, indicating that the methylation degree was decreasing. And may express a variety of genes related to the phenotypic variation of hybridization. Moreover, the hemi-methylation levels of Jilin 38 × Jilin 47 and Yi 3 × Jilin 47 hybrids and full-methylation levels of EXP × Yi 3 and EXP × Jilin 47 hybrids was significant higher than the corresponding mid-parent values. In addition, the heredity of methylation from parents in hybrids is more than the variations, in which there were four types appeared great higher: A1, B4, B8, and D2. Furthermore, the results of relationship between genetic variation in DNA methylation and heterosis showed that the hypo-methylation had a promoting effect to increase node number, and the hype-methylation of hybrids was helpful to add to stem thick. Our results may provide new insights into well understanding the molecular mechanisms of heterosis at the epigenetic level in soybean.

Keywords Soybean (*Glycine max*) · Heterosis · DNA methylation · MSAP · Capillary electrophoresis

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Y. Wang · X. Han · S. Fan · X. Li · Y. Qu · D. Yao · P. Wang · J. Zhang (✉)
College of Agronomy, Jilin Agricultural University, Changchun 130118, China
e-mail: zhangjun@jlau.edu.cn

K. Zhang · P. Wang
College of Agronomy, Northeast Agricultural University, Harbin 150030, China

L. Sun · P. Wang
Key Laboratory of Modern Agricultural Cultivation and Crop Germplasm Improvement of Heilongjiang Province, Agronomy College of Heilongjiang Bayi Agricultural University, Daqing 163319, China

Introduction

In the post-genomics era, cytosine DNA methylation has become one of the most important hotspots to research chromatin modification. Recent research has demonstrated that the disturbance of DNA methylation patterns may consequently have functional consequences in organisms with this epigenetic code (Dyachenko et al. 2014; Kumari et al. 2013). Moreover, numerous studies found that cytosine DNA methylation plays a significant role in various cellular activities, including plant response to the environmental stresses (Wang et al. 2016a), embryonic development (Zhang et al. 2016), cell differentiation (Hassan-Zadeh et al. 2017), inactivation of chromatin (Keown et al. 2017), and plant growth and development (Yang et al. 2015).

Heterosis is an extremely common phenomenon that refers to the superior average performance of hybrids over their parents with respect to various agronomic traits, first submitted by Shull (1908, 1952). Although this phenomenon has been widely exploited to increase agronomic production with ensuing economic and societal benefits for well over a century, the molecular mechanisms underlying heterosis remain poorly understood and mainly focused on dominance or over-dominance hypothesis (Birchler et al. 2010; Hochholdinger and Hoecker 2007). Recently, numerous studies have obtained lots of valuable research results about the relationship between DNA methylation and heterosis (Sun et al. 2015; Kawanabe et al. 2016). In addition, some papers also reported that the DNA methylation pattern of F_1 offspring experienced big changes or adjustments to coordinate the expression of heterogeneous genes derived from parents, then made some genes efficiently transcript (Wang et al. 2016a, b; Li et al. 2013).

With the rapid development of biotechnology, there are many techniques to detect the DNA methylation level, such as bisulfite sequencing (Hernandez et al. 2013), HPLC (Cappetta et al. 2015), and Bisulfite Genomic Sequencing (Garg et al. 2015). However, these methods may accomplish higher costs or lower detection efficiency of DNA methylation sites. The methylation-sensitive amplification polymorphism (MSAP) technique is base on digestion with methylation-sensitive restriction endonucleases followed by amplification of restriction fragments and has been applied in various topics, including biotic and abiotic stress (Luo et al. 2016; Wang et al. 2016a), development (Wang et al. 2016b), differentiation of ecotypes (Xia et al. 2016) and somaclonal variability (Baránek et al. 2016), which can not only reduce costs but also generate broader coverage to discover key methylated sites. In addition, this method has also been used in many plant genomes and obtained lots of valuable results, such as *Arabidopsis thaliana* (Li et al. 2015), wheat (Venetsky et al. 2015), cotton (Wang et al. 2016a), *Sorghum bicolor* (Zhang et al. 2011), rice (Xia et al. 2016), and maize (Sun et al. 2015). Although the MSAP method has been investigated in many common crops, few studies have focused on heterosis in soybean.

In this study, the MSAP technique based on capillary electrophoresis was used to compare differences in cytosine methylation patterns and levels of 12 reciprocal soybean hybrids and their parents based on leaves of 15-day emergence. A total of 1239 fragments were detected in each sample on average, and the DNA cytosine methylated level of all reciprocal hybrids was remarkably lower than mid-parent heterosis (MPH). In addition, correlation coefficients between 12 traits and 16 subgroups methylation pattern were calculated to further analyze the relationship between DNA methylation variation and heterosis. These results obtained in our study would provide more theoretical basis for soybean genetic breeding.

Materials and methods

Plant materials

To determine the molecular mechanisms of heterosis at the epigenetic level in soybean, a total of four cultivars [Jilin 47 (no. 19) and Jilin 48 (no. 3) were cultivated by Jilin Academy of Agricultural Sciences in China; EXP (no. 12) and Yi 3 (no. 6) were imported from abroad] were used here. Meanwhile, the four cultivars were designated as females or males in accordance with complete diallel cross to generate 12 F_1 hybrids in 2013, including Jilin 38 × Yi 3(3 × 6), Jilin 38 × EXP(3 × 12), Jilin 38 × Jilin 47(3 × 19), Yi 3 × Jilin 38(6 × 3), Yi 3 × EXP(6 × 12), Yi 3 × Jilin 47(6 × 19), EXP × Jilin 38(12 × 3), EXP × Yi 3(12 × 6), EXP × Jilin 47(12 × 19), Jilin 47 × Jilin 38(19 × 3), Jilin 47 × Yi 3(19 × 6), Jilin

47 × EXP(19 × 12). In 2014 spring, four parents and 12 hybrids were all sown in the Jilin Agricultural University fields, with 2 m long and 2 rows per plot with 3 replicates.

Agronomic and quality traits analysis

A total of five plants every plot were randomly taken out from the parents and hybrids harvested in 2014 autumn to analyze the agronomic traits, which included plant height (cm), node number, branch number, height of low pod (cm), pod number per plant, grain weight per plant (g), insect food grain rate (%), hundred-grain weight (g), grain number per plant, stems thick (mm). Moreover, the quality of traits, protein (%) and fat (%), were analyzed by Near Infrared Spectroscope (Model N 500, BUCHI, Swiss). Then, the estimation of heterosis was obtained by calculating the mid-parent heterosis (MPH) and over-parent heterosis (BPH). However, it was noteworthy that the insect grain rate should be taken negative over-parent heterosis.

Genomic DNA isolation

After 15-day emergence, the leaves of 16 accessions were collected with 3 replicates. Then the DNA of 48 samples was extracted respectively by a modified CTAB method (Kidwell and Osborn 1992). The DNA was purified by phenol extractions, and checked for quality and quantity by agarose gel electrophoresis and spectrometric measurement (Supplementary file 1A). In order to analysis the uniformity or variation of methylation alterations among different individuals, genomic DNA was isolated from the same stage.

MSAP analysis of DNA methylation

The methylation sensitive amplified polymorphism (MSAP) analysis method was performed as reported (Sun et al. 2015; Salmon et al. 2005). Two combinations of restriction enzymes were used by mixing *Eco*RI with each of the isoschizomers, *Hpa*II and *Msp*I, which can recognize the same sequence (5′-CCGG) and cut with differential sensitivity to DNA methylation of internal or external cytosine. *Hpa*II can recognize the hemi-methylated external cytosine sites, while *Msp*I can recognize full-methylated internal cytosine sites. Therefore, if *Hpa*II can cut while *Msp*I cannot cut for same sequence (5′-CCGG), recorded (1, 0), on the contrary we recorded (0, 1); if *Hpa*II and *Msp*I can cut at the same sites, recorded (1, 1). In another case, when the hemi-methylated external cytosine sites, or the full-methylated cytosine sites throughout the inside and outside existed, *Hpa*II and *Msp*I had no bands were showed, recorded (0,0). Because this situation is more complex, uncertain, this type of methylation band information was ignored.

A total of 50 µl reaction liquid was digested in 37 °C incubator for 2 h, which contains 5 µl T4 10× reaction buffer, 10 ng BSA, 2 U *Eco*RI, 2 U *Hpa*II/*Msp*I, 150 ng DNA, and ddH_2O. The effect of digestion was detected by the electrophoresis (Supplementary file 1B). Subsequently, one pair adaptor (*Hpa*II/*Msp*I adaptor and *EcoR*I adaptor) were used in ligation reactions, which was consisted of 1 µl *Eco*RI adaptor, 1 µl H/M adaptor, and 0.1 µl T4 ligase incubated in 16 °C for 4 h. A total of one pair pre-selective primers and 20 pairs of selective primers were used for amplification and the sequences of adaptor and primers were all list in Supplementary file 2. The restriction enzymes *EcoR*I, *Hpa*II and *Msp*I were purchased from the Takara Biotech companies in Japan.

A total of 5 µl of each ligated sample, diluted 5-fold with sterilized distilled water, was used for the pre-amplification reactions. The PCR reactions conditions were: 94 °C for 45 s, 56 °C for 45 s and 72 °C for 1 min for 30 cycles. The pre-amplified products were displayed in the Supplementary file 1C. 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 5 min, 94 °C for 30 s, 65 °C for 30 s (− 1 °C per cycle) and 72 °C for 1 min for 10 cycles, followed by 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min for 35 cycles. In order to confirm the successful of the capillary electrophoresis, the products of selective amplification was also detected by agarose gel electrophoresis (Supplementary file 1D).

Capillary electrophoresis analysis

Products of the selective amplification were denatured and analyzed on the Applied Biosystems 3730 XL Genetic Analyzer (Thermo Fisher, US) equipped with a 50 cm, 96 capillary electrophoresis. Reactions were carried out in 96-well reaction plates (Applied Bio-

Systems), which contained 1 μl diluted sample, 8.5 μl Hi-Di^{TM} formamide (Applied Bio-Systems) and 0.5 μl gene scan marker. The samples were denatured 3 min at 95 °C, then injected for 5 s at 3 V and electrophoresed for 50 min in Performance Optimized Polymer (POP-7^{TM}) at 60 °C. After that, ABI Foundation Data Collection Software version 3.0 was used to collect data, and analyzed by the GeneMapper version 4.0 (Applied Bio-Systems).

Results and discussion

Heterosis analysis of 12 hybrids

In order to analysis the heterosis, 12 traits of hybrids and parents were detected to analyze mid-parent heterosis (MPH) and over-parent heterosis (BPH). The results showed that different traits in different combinations displayed different heterosis. The MPH value of 12 traits was showed in Table 1, in which the MPH value of low pod height, insect rate, and fat were negative in all combinations; protein content in Jilin 38 × Jilin 47(3 × 19) hybrid offspring displayed negative; and the other traits in all combinations were positive. In addition, the MPH value of pod number, grain weight and number of per plant was more than 100%, in which grain weight in EXP × Yi 3(12 × 6) hybrid offspring displayed significant higher, reached 534.44%. In summary, we concluded that a total of four hybrids [EXP × Yi 3(12 × 6), Jilin 38 × Jilin 47(3 × 19), Yi 3 × EXP(6 × 12), and Yi 3 × Jilin 38(6 × 3)] have significant MPH value according to the Table 1.

Moreover, the BPH value of 12 traits was showed in Table 2, in which 3 traits (low pod height, insect rate, and fat content) of BPH were negative; five traits (branch no., pod no., grain no., grain weight, and stems thick) of BPH in all combinations were positive; and there was both negative and positive in the other traits. A total of four traits (branch no., pod no., grain no., and grain weight) of BPH in some combinations were more than 100%, in which grain weight in EXP × Yi 3 (12 × 6) hybrid offspring displayed significant higher, reached 497.84%. Concluded above results, four hybrids [EXP × Yi 3(12 × 6), Jilin 38 × Jilin 47(3 × 19), Yi 3 × EXP(6 × 12), and Yi 3 × Jilin 47(6 × 19)] have significant BPH.

MSAP analysis in cytosine methylation levels, patterns among hybrids and their parental lines

Recently, various studies have evidenced that the epigenetic inheritance may vary in plant hybrids, and may be accompanied by extensive modifications in DNA methylation (Sun et al. 2015; Sanetomo and Hosaka 2011; He et al. 2010). The results of these studies suggest that detailed investigation of epigenetic regulation of critical loci in hybrid genomes may lead to a better understanding of the mechanisms underlying hybrid vigor. In addition, heterosis is manifested at the early seedling stage in hybrids (Joel and Zhang 2001), thus MSAP profiles were generated for 12 hybrids and their parents from leaves after 15-day post-emergence.

Differences in cytosine methylation levels between the hybrids and their parents

A total of 20 selective primer combinations were used to compare the status of cytosine methylation in 12 hybrids and their parents. The results showed that approximately 1239 fragments were detected in each sample on average, and the number of non-methylated sites, hemi-methylated external cytosines and full-methylated internal cytosines were calculated (Table 3) according to the results of capillary electrophoresis (Fig. 1). Base on the MSAP profiles, total relative methylation levels of all samples were 41.33–58.89%, of which 16.9–25.43% involved external cytosine hemi-methylation, and 21.35–35.97% corresponded to full-methylated internal cytosines in 5′-CCGG recognition sites. In addition, the DNA cytosine methylated level of all reciprocal hybrids were remarkably lower than MPH, which indicated that the methylation level of hybrids was significantly decreased corresponding to parental lines, and this result was in accordance with the findings of previous studies (Li et al. 2013; Zhao et al. 2007; Zhang et al. 2007). Many studies have also reported that DNA demethylation can reactivate gene expression (Zhu et al. 2015; Hsieh et al. 2009). Thus, we can conclude that the trend of decreased methylation in the hybrids compared with their parents may enable de-repression and possibly expression of many genes associated with phenotypic variation observed in the hybrids.

Moreover, the differences of DNA cytosine methylation levels in the reciprocal hybrids were

Table 1 The MPH of agronomic and qualitative traits

Hybrids	Height (cm)	Node number per plant	Branch number per plant	Height of low pod (cm)	Pod number per plant	Grain number per plant	Insect rate (%)	Hundred-grain weight (g)	Grain weight per plant (g)	stems thick (mm)	Fat (%)	Protein (%)
Jilin 38 × Yi 3	4.42	4.42	180.00	− 39.08	204.38	162.13	− 56.68	17.87	208.39	64.57	− 2.55	3.58
Jilin 38 × EXP	10.57	5.36	66.67	− 40.20	100.74	288.21	− 70.67	12.10	120.74	42.91	− 1.89	3.07
Jilin 38 × Jilin 47	0.12	0.85	399.70	− 64.20	303.38	299.63	− 63.65	14.39	344.99	74.85	− 1.54	− 0.10
Yi 3 × Jilin 38	6.39	7.96	340.00	− 55.56	263.35	241.54	− 56.05	2.92	258.34	77.43	− 1.61	4.14
Yi 3 × EXP	8.34	10.68	100.00	− 45.45	282.11	310.60	− 67.11	14.74	397.14	124.34	− 7.50	10.06
Yi 3 × Jilin 47	15.12	9.26	271.32	− 52.56	234.58	235.18	− 50.25	22.33	313.62	74.20	− 4.24	9.52
EXP × Jilin 38	14.08	16.07	166.67	− 40.97	157.99	168.28	− 30.49	10.30	215.53	72.16	− 3.03	4.93
EXP × Yi 3	2.92	6.80	255.56	− 39.07	399.19	423.95	− 68.65	9.59	534.44	110.85	− 5.67	8.95
EXP × Jilin 47	18.84	15.89	200.08	− 35.50	179.31	167.90	− 19.72	15.90	228.41	86.75	− 0.05	4.22
Jilin 47 × Jilin 38	9.93	5.98	99.90	− 46.67	132.91	131.34	− 34.35	10.99	156.67	40.65	− 6.06	7.18
Jilin 47 × Yi 3	6.42	5.56	185.63	− 44.23	187.85	143.87	− 60.97	11.56	178.18	58.44	− 4.66	5.78
Jilin 47 × EXP	6.32	4.67	50.04	− 53.75	138.79	144.90	− 68.70	12.44	205.41	70.79	− 1.69	4.42

MPH mid-parent heterosis

Table 2 The BPH of agronomic and qualitative traits

Hybrids	Height (cm)	Node number per plant	Branch number per plant	Height of low pod (cm)	Pod number per plant	Grain number per plant	Insect rate (%)	Hundred-grain weight (g)	Grain weight per plant (g)	Stems thick (mm)	Fat (%)	Protein (%)
Jilin 38 × Yi 3	− 6.39	− 3.28	75.00	− 49.17	178.83	148.43	− 64.21	7.39	162.46	59.27	− 10.12	0.80
Jilin 38 × EXP	− 2.41	− 3.28	0.00	− 51.04	97.08	286.21	− 77.32	− 0.75	78.92	37.34	− 9.51	− 1.04
Jilin 38 × Jilin 47	− 2.98	− 3.28	233.30	− 68.13	248.91	273.17	− 64.96	6.31	285.10	73.11	− 1.99	− 0.33
Yi 3 × Jilin 38	− 4.62	0.00	175.00	− 62.92	232.85	223.69	− 63.68	− 6.23	204.97	71.72	− 9.25	1.34
Yi 3 × EXP	6.44	9.62	80.00	− 46.73	256.06	287.24	− 69.79	11.15	368.46	122.71	− 7.50	8.55
Yi 3 × Jilin 47	0.38	5.36	225.00	− 63.90	214.04	229.96	− 57.62	19.73	305.48	66.97	− 11.30	6.33
EXP × Jilin 38	0.68	6.56	60.00	− 51.67	153.28	166.90	− 46.25	− 2.34	155.75	65.45	− 10.55	0.74
EXP × Yi 3	1.12	5.77	220.00	− 40.50	365.15	394.14	− 71.20	6.16	497.84	109.32	− 5.67	7.45
EXP × Jilin 47	2.07	10.71	140.00	− 51.71	145.45	148.97	− 36.21	9.96	203.75	77.76	− 7.41	− 0.16
Jilin 47 × Jilin 38	6.53	1.64	33.33	− 52.52	101.46	116.03	− 36.73	3.16	122.13	39.25	− 6.50	6.93
Jilin 47 × Yi 3	− 7.20	1.79	150.00	− 57.56	170.18	140.08	− 66.76	9.19	172.71	51.87	− 11.69	2.70
Jilin 47 × EXP	− 8.68	0.00	20.00	− 65.37	109.85	127.59	− 75.13	6.67	182.48	62.57	− 8.94	0.03

BPH over-parent heterosis

Table 3 Relative levels of cytosine methylation at the CCGG sites in hybrids and their parents

Parents and hybrids	Total CCGG sites	Non-methylated CCGG sites (%)	Methylated CCGG sites (%)		
			Total (%)	Hemi-methylated sites (%)	Fully-methylated sites (%)
Jilin 38	1351	582 (43.08)	769 (56.92)	330 (24.43)	439 (32.49)
Yi 3	1226	504 (41.11)	722 (58.89)	281 (22.92)	441 (35.97)
MPH	1289	543 (42.10)	746 (57.76)	306 (23.68)	440 (34.23)
Jilin 38 × Yi 3	1234	631 (51.13)	603 (48.87)	255 (20.66)	348 (28.20)
Yi 3 × Jilin 38	1267	656 (51.78)	611 (48.22)	264 (20.84)	347 (27.39)
Jilin 38	1351	582 (43.08)	769 (56.92)	330 (24.43)	439 (32.49)
EXP	1142	568 (49.74)	574 (50.26)	259 (22.68)	315 (27.58)
MPH	1247	575 (46.41)	672 (53.59)	295 (23.56)	377 (30.04)
Jilin 38 × EXP	1234	605 (49.03)	629 (50.97)	280 (22.69)	349 (28.28)
EXP × Jilin 38	1213	678 (55.89)	535 (44.11)	205 (16.90)	330 (27.21)
Jilin 38	1351	582 (43.08)	769 (56.92)	330 (24.43)	439 (32.49)
Jilin 47	1262	636 (50.40)	626 (49.60)	274 (21.71)	352 (27.89)
MPH	1307	609 (46.74)	698 (53.26)	302 (23.07)	396 (30.19)
Jilin 38 × Jilin 47	1220	621 (50.90)	599 (49.10)	**309 (25.33)**	290 (23.77)
Jilin 47 × Jilin 38	1273	699 (54.91)	574 (45.09)	239 (18.77)	335 (26.32)
Yi 3	1226	504 (41.11)	722 (58.89)	281 (22.92)	441 (35.97)
EXP	1142	568 (49.74)	574 (50.26)	259 (22.68)	315 (27.58)
MPH	1184	536 (45.43)	648 (54.58)	270 (22.80)	378 (31.78)
Yi 3 × EXP	1169	583 (49.87)	586 (50.13)	266 (22.75)	320 (27.37)
EXP × Yi 3	1160	560 (48.28)	600 (51.72)	214 (18.45)	*386 (33.28)*
Yi 3	1226	504 (41.11)	722 (58.89)	281 (22.92)	441 (35.97)
Jilin 47	1262	636 (50.40)	626 (49.60)	274 (21.71)	352 (27.89)
MPH	1244	570 (45.76)	674 (54.25)	278 (44.63)	397 (31.93)
Yi 3 × Jilin 47	1235	592 (47.94)	643 (52.06)	**314 (25.43)**	329 (26.64)
Jilin 47 × Yi 3	1226	600 (48.94)	626 (51.06)	254 (20.72)	372 (30.34)
Jilin 47	1262	636 (50.40)	626 (49.60)	274 (21.71)	352 (27.89)
EXP	1142	568 (49.74)	574 (50.26)	259 (22.68)	315 (27.58)
MPH	1202	602 (50.07)	600 (49.93)	267 (22.20)	334 (27.74)
Jilin 47 × EXP	1246	731 (58.67)	515 (41.33)	249 (19.98)	266 (21.35)
EXP × Jilin 47	1278	699 (54.23)	585 (45.77)	226 (17.68)	*359 (28.09)*

Bold indicates more than 25% of hemi-methylated sites

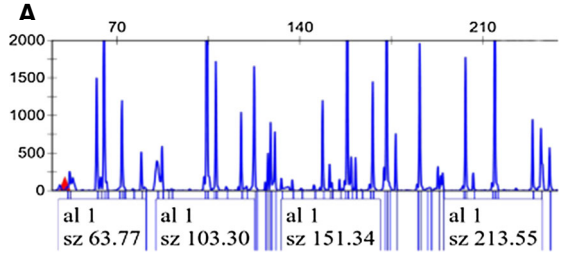
MPH mid-parent heterosis

0.65–6.86%, of which the combination Jilin 38(3) and EXP (12), Jilin 38(3) and Jilin 47(19), EXP(12) and Jilin 47(19) displayed significantly high, 6.86, 4.01, and 4.44%, respectively, while other combinations were very low. Such significant differences of DNA methylation levels in different hybrids may be due to differences in plant materials.

Cytosine methylation patterns between the hybrids and their parents

To further analysis cytosine methylation variation, the different cytosine methylation patterns between hybrids and their parental lines were observed, and then divided into four major classes (Table 4) according to previous reported (Sun et al. 2015; Zhao et al.

A



B

Size88	202.27		202.37	202.33	202.29	202.38		202.4		202.39	202.34		202.32		202.39	
Size89	203.32	203.4	203.33	203.35	203.35	203.35	203.45	203.39	203.45	203.35	203.29	203.18	203.31	203.45	203.37	203.48
Size90	206.3		206.25	206.33	206.27	206.37	206.38	206.34	206.3	206.29	206.26	206.21	206.19	206.3	206.32	206.43
Size91												208.3				208.41
Size92															209.56	210.03
Size93	213.56		213.46	213.55	213.45	213.45	213.55	213.55	213.64	213.55	213.47		213.45		213.45	213.55
Size94	214.55	214.45	214.55	214.55	214.56	214.55	214.55	214.55	214.55	214.55	214.55	214.46	214.47	214.55	214.56	214.65
Size95	215.55			215.57	215.6	215.6					215.46	215.27	215.41		215.69	
Size96		216.68														
Size97												221.82			221.96	

C

Size88	1	0	1	1	1	1	0	1	0	1	1	0	1	0	1	0
Size89	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Size90	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Size91	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
Size92	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Size93	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	1
Size94	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Size95	1	0	0	1	1	1	0	0	0	0	1	1	1	0	1	0
Size96	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Size97	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0

Fig. 1 Part results of selective amplified products text by capillary electrophoresis on sample 3 (enzyme (*Hpa*II) digestion). *Note* **a** Enlarged part peak map of capillary electrophoresis; **b** raw data of a selective primer; **c** Converted from raw data into 1, 0 value of corresponding primers

2008). Class A is the methylation pattern of hybrids completely inherited in their parents; class B is the methylation pattern of hybrids inherited in one of their parents, followed the Mendelian inheritance; class C is the methylation level of hybrids increased compared to their parents, called hyper-methylation pattern; class D is the methylation level of hybrids decreased compared to their parents, called hypo-methylation pattern. Four classes were further divided into 16 subgroups based on epigenetic inheritance patterns and alteration in cytosine methylation between parents and hybrids (Table 4).

A comparative analysis revealed that the number of class B was significantly higher than other classes in all hybrids comparing their parents. The second was the class A. The number of class C was the lowest, but there was a special hybrid [Jilin 38 × Jilin 47(3 × 19)] that the number of class C was higher

Table 4 Cytosine methylation patterns at the CCGG sites between hybrid F1 and their parents

Class	Band Pattern Displayed in Capillary electrophoresis					
	Maternal parent		Paternal parent		Hybrid (F1)	
	H	M	H	M	H	M
A1	−	+	−	+	−	+
A2	+	−	+	−	+	−
Total for monomorphic loci						
B1	+	+	−	+	−	+
B2	−	+	+	+	−	+
B3	+	−	−	−	+	−
B4	−	−	+	−	−	−
B5	−	−	+	−	+	−
B6	+	+	−	−	+	+
B7	+	+	−	+	+	+
B8	+	−	−	−	−	−
Total						
C1	+	−	+	−	−	−
C2	−	+	−	+	−	−
Total						
D1	−	−	−	+	+	+
D2	−	−	−	−	+	−
D3	−	+	−	+	+	+
D4	−	+	−	−	+	+

Class	Number and frequency of sites											
	Jilin 38 × Yi 3	Yi 3 × Jilin 38	Jilin 38 × EXP	EXP × Jilin 38	Jilin 38 × Jilin 47	Jilin 47 × Jilin 38	Yi 3 × EXP	EXP × Yi 3	Yi 3 × Jilin 47	Jilin 47 × Yi 3	EXP × Jilin 47	Jilin 47 × EXP
A1	183	190	164	165	196	202	169	178	189	185	169	166
A2	38	41	60	38	68	55	42	40	50	33	34	42
Total for monomorphic loci	221	231	224	203	264	257	211	218	239	218	203	208
B1	28	9	9	5	10	12	12	13	18	13	21	19
B2	13	13	7	9	8	42	11	24	12	29	13	12

Table 4 continued

Class	Number and frequency of sites											
	Jilin 38 × Yi 3	Yi 3 × Jilin 38	Jilin 38 × EXP	EXP × Jilin 38	Jilin 38 × Jilin 47	Jilin 47 × Jilin 38	Yi 3 × EXP	EXP × Yi 3	Yi 3 × Jilin 47	Jilin 47 × Yi 3	EXP × Jilin 47	Jilin 47 × EXP
B3	21	54	20	26	20	22	28	19	57	33	21	12
B4	144	129	153	67	146	68	129	65	125	103	69	79
B5	52	19	29	17	23	32	22	22	28	38	27	61
B6	18	43	17	49	8	10	14	39	23	15	22	7
B7	60	97	58	34	56	26	77	25	90	17	26	7
B8	131	147	65	148	78	149	59	116	79	119	114	91
Total	467	511	358	355	349	361	352	323	432	367	313	288
	77.57%	81.11%	77.32%	75.37%	72.26%	70.23%	66.04%	66.6%	81.2%	71.12%	62.48%	61.54%
C1	26	21	17	30	25	27	40	42	10	22	33	33
C2	23	15	10	10	43	36	14	8	16	20	6	11
Total	49	36	27	40	68	63	54	50	26	42	39	44
	8.14%	5.71%	5.83%	8.49%	14.08%	12.26%	10.13%	10.31%	4.89%	8.14%	7.78%	9.4%
D1	4	6	6	4	5	3	17	4	6	3	2	6
D2	59	54	61	39	57	78	90	85	56	89	131	123
D3	17	17	9	8	4	7	12	11	10	10	9	6
D4	6	6	2	25	0	2	8	12	2	5	7	1
Total	86	83	78	76	66	90	127	112	74	107	149	136
	14.29%	13.17%	16.85%	16.14%	13.66%	17.51%	23.83%	23.09%	13.91%	20.74%	29.74%	29.06%
Total for polymorphic loci	602	630	463	471	483	514	533	485	532	516	501	468
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

*H HpaI*Im, *M MspI*

Table 5 Comparison of the relationship between heterosis and methylation pattern in the top six hybrids

Hybrids	MPH	BPH	Total methylation levels versus MPH	Hemi-methylation levels versus MPH	Full-methylation levels versus MPH
EXP × Yi 3	1	1	Low	Low	High
Jilin 38 × Jilin 47	2	3	Low	High	Low
Yi 3 × EXP	3	2	Low	Low	Low
Yi 3 × Jilin 38	4	5	Low	Low	Low
Yi 3 × Jilin 47	5	4	Low	High	Low
EXP × Jilin 47	6	6	Low	Low	High

than class D. Therefore, the results revealed that the inheritance of methylation (class A and class B) was higher than variation of methylation (class C and class D). Meanwhile, inheritance of methylation from one of parents (class B) was higher than both parents (class A); hypo-methylation (class D) was higher than hyper-methylation (class C). Liu and Wendel have reported that DNA methylation pattern of hybrids may be altered, but it will generally be genetic from parent to offspring when they researched on the epigenetic evolution of plant allopolyploids (Liu and Wendel 2003). Meanwhile, Wang reported that the inherited and altered methylation patterns were also present in cotton hybrid, and the inherited patterns was significantly higher than altered pattern (Wang et al. 2016a, b), which results are basically the same as our results. Therefore, our results indicated that methylation sites of hybrids inherited in the parents may be associated with some traits for plant growth and development, which was also consistent with other study (Sakthivel et al. 2010). In addition, the higher level hypo-methylation (class D) in our study may suggest that it is benefit for explaining hybrid-specific gene expression, as also indicated by Sakthivel et al. (2010).

The correlationship between heterosis and DNA methylation variation

In order to further analyze the relationship between heterosis and DNA methylation in soybean, the data of Tables 1, 2 and 3 was integrated for obtaining Table 5. The results showed that hemi-methylation level of two hybrids [Jilin 38 × Jilin 47(3 × 19), Yi 3 × Jilin 47(6 × 19)] that used 19 as father and full-methylation level of two hybrids [EXP × Yi 3(12 × 6), EXP × Jilin 47(12 × 19)] that used 12 as mother was higher than MPH (Tables 3, 5). Moreover, the four hybrids displayed great heterosis (Table 5). In addition, we also found that the total relative methylation levels of hybrids that performed significant heterosis showed a downward trend, but had different methylation patterns. Three patterns were observed: (1) hemi-methylation and full-methylation level all decreased, such as Yi 3 × EXP(6 × 12) and Yi 3 × Jilin 38(6 × 3); (2) hemi-methylation level decreased, and full-methylation level increased, such as EXP × Yi 3(12 × 6) and EXP × Jilin 47(12 × 19); (3) hemi-methylation level increased, and full-methylation level decreased, such as Jilin 38 × Jilin 47(3 × 19) and Yi 3 × Jilin 47(6 × 19).

In addition, correlation coefficients between heterosis and DNA methylation patterns were calculated (Tables 6, 7). The results of correlation analysis in MPH showed that there was only six correlation coefficients displayed significant correlation ($P < 0.05$) (Table 6), for instance, node number and the bottom pod height showed a significant negative correlation with C2 (− 0.62* and − 0.58*, respectively); and the node number displayed a significant correlation with D4 (0.69*); the hundred-grain weight performed a significant correlation with B1 (0.60*); the stem thick showed a significant correlation with C1 (0.66*), and a significant negative correlation with D1 (− 0.58*). In addition, the results of correlation analysis in BPH showed that there was only 3 correlation coefficients displayed significant correlation ($P < 0.05$) (Table 7), including the height of plants correlated to B5 (− 0.60*), the stem thick correlated to C1 (0.68*) and D1 (− 0.60*). These results in our study indicated that the hypo-methylation play an enhancement role in increasing node

Table 6 Correlationship between DNA methylation and MPH among different traits

Patterns	Height	Pitch number per plant	Branch number per plant	Height of low pod	Pod number per plant	Grain number per plant	Insect rate	Hundred-grain weight	Grain weight per plant	stems thick	Fat	Protein
A1	− 0.35	− 0.48	0.55	− 0.51	0.26	− 0.07	0.07	− 0.02	0.06	− 0.28	− 0.20	− 0.03
A2	− 0.26	− 0.55	0.19	− 0.56	− 0.03	0.29	− 0.26	0.12	− 0.04	− 0.34	0.06	− 0.34
B1	0.05	− 0.11	− 0.12	0.24	− 0.06	− 0.31	0.06	**0.60***	− 0.06	0.02	0.19	0.02
B2	− 0.09	− 0.17	− 0.17	0.16	− 0.04	− 0.24	0.20	− 0.22	− 0.08	− 0.27	− 0.53	0.37
B3	0.24	0.19	0.47	− 0.31	0.18	0.02	0.08	0.00	0.07	0.04	− 0.09	0.32
B4	− 0.33	− 0.48	0.24	− 0.36	0.06	0.24	− 0.53	0.23	− 0.08	− 0.11	0.14	− 0.32
B5	− 0.20	− 0.43	− 0.47	0.01	− 0.41	− 0.52	− 0.25	0.28	− 0.40	− 0.34	0.18	− 0.17
B6	0.21	0.56	0.32	0.27	0.29	0.24	0.27	− 0.46	0.26	0.26	0.06	0.16
B7	0.01	0.00	0.42	− 0.33	0.28	0.33	− 0.17	0.11	0.18	0.19	− 0.03	0.10
B8	0.06	0.23	0.13	0.23	− 0.11	− 0.45	0.56	− 0.49	− 0.24	− 0.28	0.07	− 0.07
C1	− 0.20	0.23	− 0.23	0.32	0.38	0.28	− 0.02	0.23	0.51	**0.66***	− 0.29	0.24
C2	− 0.49	− **0.62***	0.35	− **0.58***	0.06	− 0.13	− 0.04	0.09	− 0.09	− 0.36	− 0.11	− 0.34
D1	− 0.10	0.05	− 0.22	− 0.17	0.24	0.38	− 0.45	0.09	0.36	− **0.58***	− 0.49	0.42
D2	0.18	0.13	− 0.39	0.23	− 0.10	− 0.18	0.08	0.10	0.02	0.25	0.08	0.14
D3	− 0.08	0.10	0.13	0.29	0.24	0.08	− 0.11	− 0.16	0.09	0.21	− 0.08	0.21
D4	0.26	**0.69***	− 0.03	0.48	0.11	0.05	0.38	− 0.26	0.19	0.32	− 0.13	0.19

Bold numbers indicate significant differences

*$P < 0.05$ represents a significant difference

Table 7 Correlationship between DNA methylation and BPH among diffenent traits

Patterns	Height	Pitch number per plant	Branch number per plant	Height of low pod	Pod number per plant	Grain number per plant	Insect rate	Hundred-grain weight	Grain weight per plant	stems thick	Fat	Protein
A1	0.06	− 0.34	0.47	− 0.36	0.18	− 0.10	0.26	0.09	0.05	− 0.22	0.30	0.28
A2	0.15	− 0.52	0.03	− 0.36	− 0.08	0.30	− 0.14	− 0.07	− 0.09	− 0.28	0.55	− 0.08
B1	− 0.27	− 0.03	− 0.01	0.04	− 0.10	− 0.34	0.04	0.56	0.00	− 0.02	− 0.17	− 0.06
B2	0.27	0.05	− 0.04	0.23	− 0.07	− 0.26	0.36	0.03	− 0.03	− 0.22	0.05	0.51
B3	0.02	0.13	0.48	− 0.35	0.22	0.05	0.07	0.12	0.10	0.02	− 0.43	0.25
B4	− 0.29	− 0.56	0.09	− 0.29	0.07	0.26	− 0.53	0.04	− 0.11	− 0.10	− 0.03	− 0.22
B5	− **0.60***	− 0.42	− 0.42	− 0.20	− 0.45	− 0.53	− 0.27	0.24	− 0.34	− 0.37	− 0.27	− 0.26
B6	0.09	0.32	0.25	0.29	0.37	0.26	0.16	− 0.45	0.20	0.24	− 0.26	0.03
B7	0.12	− 0.09	0.30	− 0.19	0.30	0.35	− 0.17	0.02	0.15	0.19	− 0.10	0.15
B8	− 0.04	0.00	− 0.04	0.14	− 0.11	− 0.46	0.56	− 0.49	− 0.28	− 0.28	− 0.19	− 0.06
C1	0.31	0.47	− 0.11	0.57	0.36	0.22	0.00	0.05	0.49	**0.68***	0.43	0.28
C2	0.03	− 0.51	0.17	− 0.40	− 0.03	− 0.16	0.15	0.03	− 0.13	− 0.29	0.51	0.04
D1	0.32	0.26	− 0.13	0.12	0.27	0.38	− 0.42	0.18	0.36	− **0.60***	0.05	0.43
D2	0.01	0.39	− 0.14	0.09	− 0.15	− 0.23	0.06	0.34	0.11	0.21	0.13	0.02
D3	− 0.14	− 0.02	0.08	0.32	0.29	0.08	− 0.16	− 0.14	0.08	0.20	− 0.43	0.13
D4	0.24	0.49	− 0.06	0.50	0.19	0.07	0.27	− 0.33	0.13	0.30	− 0.22	0.06

Bold numbers indicate significant differences

*$P < 0.05$ represents a significant difference

number in hybrid, while hyper-methylation play a promotion role for increasing stem thick in hybrid. Therefore, we can preliminary infer that there is an important relationship between DNA methylation variation of leaves and heterosis in soybean hybrid. However, to completely understand this relationship, a comprehensive analysis of the genetic and epigenetic regulation of 234 gene expression variation in hybrid should be further researched just as suggested by He et al. (2013).

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Author contributions Conceived and designed the experiments: Jun Zhang and Piwu Wang. Performed the experiments: Yuanqian, Kaixin Zhang, Xiao Han and Shujie Fan. Analyzed the data: Lifang Sun, Xueying Li and Yiwei Qu. Contributed reagents/materials/analysis tools: Dan Yao. Wrote the paper: Kaixin Zhang, Yuanqiang Wang and Lifang Sun. All authors read and approved the final version of the manuscript.

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