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Analysis of the leaf methylomes of parents and their hybrids provides new insight into hybrid vigor in *Populus deltoides*

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Abstract

Background: Plants with heterosis/hybrid vigor perform better than their parents in many traits. However, the biological mechanisms underlying heterosis remain unclear. To investigate the significance of DNA methylation to heterosis, a comprehensive analysis of whole-genome DNA methylome profiles of *Populus deltoides* cl.'55/65' and '10/17' parental lines and their intraspecific F1 hybrids lines was performed using methylated DNA immunoprecipitation (MeDIP) and high-throughput sequencing.

Results: Here, a total of 486.27 million reads were mapped to the reference genome of *Populus trichocarpa*, with an average unique mapping rate of 57.8%. The parents with similar genetic background had distinct DNA methylation levels. F1 hybrids with hybrid vigor possessed non-additive DNA methylation level (their levels were higher than mid-parent values). The DNA methylation levels in promoter and repetitive sequences and transposable element of better-parent F1 hybrids and parents and lower-parent F1 hybrids were different. Compared with the maternal parent, better-parent F1 hybrids had fewer hypermethylated genes and more hypomethylated ones. Compared with the paternal parent and lower-parent L1, better-parent F1 hybrids had more hypermethylated genes and fewer hypomethylated ones. The differentially methylated genes between better-parent F1 hybrids, the parents and lower-parent F1 hybrids were enriched in the categories metabolic processes, response to stress, binding, and catalytic activity, development, and involved in hormone biosynthesis, signaling pathway.

Conclusions: The methylation patterns of the parents both partially and dynamically passed onto their hybrids, and F1 hybrids has a non-additive methylation level. A multidimensional process is involved in the formation of heterosis.

Background

Heterosis/hybrid vigor is the phenomenon in which progeny are superior to their parents (with distinct genetic backgrounds) in many traits, such as biomass, growth rate, adaptability, fertility, and resistance [1-5]. Since interspecific hybrid tobacco with hybrid vigor was produced in the 1760s by crossing *Nicotiana rustica* with *N. paniculata* [6], heterosis has often been exploited in

crop and tree breeding. However, the genetic basis of heterosis is still far from being understood and is still a controversial subject [1,7,8]. Three classic hypotheses, i.e., dominance, overdominance, and epistasis, were proposed as genetic explanations for heterosis. In the dominance hypothesis, the inferior parental alleles in the hybrids are complemented by the superior or dominant alleles from the other parent. In the overdominance hypothesis, heterosis arises from allelic interactions within each of many genetic loci. An alternate model, epistasis, postulates that interactions between different parental genes in hybrids lead to heterosis. Although numerous examples support each of these hypotheses,

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they only partially explain the genetic basis of heterosis [1,3,9].

Genome-wide analyses of heterosis have revealed altered gene expression profiles in F1 hybrids compared with their parents, as well as non-additive patterns of gene expression [2,10], including studies in maize (*Zea mays* L.) [11-14], rice (*Oryza sativa* L.) [15,16], *Arabidopsis thaliana* [17], wheat (*Triticum aestivum* L.) [18], *Larix kaempferi* (Lamb.) Carr [19], and *Populus tremula* [20]. Recently, heterosis was observed in hybrids derived from parents with similar genetic backgrounds. Such parents with highly similar genomic features had distinct epigenomes [21-23], and epialleles that arise from epigenetic modification were also identified. Epi-alleles cause allelic variation and altered gene expression activity, which are essential to the architecture of plant heterosis [24]. One type of epigenetic regulation, DNA methylation, primarily serves as an epigenetic silencing mechanism and predominantly occurs in transposons and other repetitive DNA elements [5,25-29] and has been explored in model plants and crops, such as maize, rice, cotton (*Gossypium herbaceum* L.), and *A. thaliana*.

The genus *Populus* (poplar) includes species that are important for the health of ecosystems and are vital to the timber, paper, and biofuel industries. Poplars are also used as a model woody plant species and models of interest for epigenetic studies [30,31]. Variations in DNA methylation between genotypes and tissues and in response to drought, as well as the relationship between gene-body DNA methylation and tissue-specific gene expression, have been reported [31-34].

During the last century, many poplar varieties with enhanced growth or adaptability have been generated using inter- or intraspecific hybridization approaches, which take advantage of the presence of heterosis in poplars. Although investigations of the molecular basis of heterosis in poplar have been undertaken via genetic mapping and gene expression profiling, the global patterns of epigenetic modification such as DNA methylation have not been determined, and whether DNA methylation plays a role in the architecture of heterosis is still unclear. In this study, *P. deltoides* cl.'55/65' was maternal parent which has straight bole, round crown, fast growth, high resistance to *Anoplophora glabripennis* and strong rooting ability, and *P. deltoides* cl.'10/17' was paternal parent which fast-growth and high stress resistance. This cross-combination is multigeneration convergent cross. Intraspecific F1 hybrids of *P. deltoides* with significant hybrid vigor or lower-parental performance were examined. Methylated DNA immunoprecipitation, combined with a high-throughput sequencing (MeDIP-Seq) approach were applied to analyze the genome-wide DNA methylation landscapes in *Populus deltoides* parental lines and F1 hybrids lines. The results showed that better-parent F1 hybrids have higher methylation levels than the average of

the parents, suggesting that non-additive level of DNA methylation is related to heterosis/hybrid vigor. The hypermethylated genes of better-parent F1 hybrids relative to the parents and lower-parent F1 hybrids were enriched in the processes of metabolism and development, which may be highly relevant to heterosis.

Methods

Plant materials and growth conditions

Two *P. deltoides* intraspecific parental lines, *P. deltoides* cl.'55/65' (Salicaceae, *Populus*, Section *Aigeiros*) and *P. deltoides* cl.'10/17' (Salicaceae, *Populus*, Section *Aigeiros*) and their intraspecific hybrids, designated here as H₁, H₂, H₃, L₁ and L₂, were used in this study. All F1 Hybrids was generated by the same intraspecific cross-combination of *P. deltoides* cl.'55/65' as maternal parent and *P. deltoides* cl.'10/17' as paternal parent. *P. deltoides* cl.'55/65' was primitively generated from the inbred seeds of excellent individual plants in former Yugoslavia and introduced into China in 1981. *P. deltoides* cl.'10/17' was generated by intraspecific crossing *P. deltoides* Bartr. cv.'Shanhaiguanensis' (which was primitively generated from the inbred seeds of excellent individual plants and introduced into China in 1900) with *P. deltoides* Bartr. cl.'Harvard' (I-63/51) (which was primitively generated from the inbred seeds of excellent individual plants in Mississippi Delta and introduced into China in 1972).

Hybrids were generated by hand pollination. All seeds were grown in a greenhouse at the Chinese Academy of Forestry (the authority responsible is the Chinese Academy of Forestry, Beijing, China) in January, 2002. One-year-old seedlings were made into cuttings to accelerate cloning, which were planted in the greenhouse in January, 2003 and transplanted to Yuquan mountain nursery (the authority responsible is the Chinese Academy of Forestry, Beijing, China) in May, 2003. No specific permits were required for these locations. The locations are not privately owned in any way, and the field studies did not involve endangered or protected species. A total of 149 F1 hybrids were introduced into Jiaozuo Research Institute of Forestry (Henan province, China) in 2003 and 2004. Of these, 18 F1 hybrids that had good performance in tree height and Diameter at breast height (DBH) were selected over the course of the two-year seedling test. Parents and their 18 F1 hybrids were planted in Xifeng village, Wuzhi Country, Jiaozuo city in Henan province in 2005 and then transplanted to Yangcheng, Wuzhi Country, Jiaozuo city of Henan province (35°8' N, 113°17' E), in 2007. No specific permits were required for these locations. The location is not privately owned in any way, and the field studies did not involve endangered or protected species. This site has an annual average precipitation of 625.4 mm, with an annual average temperature of 15.2°C (ranging from 14.3°C to 43.6°C), an accumulated temperature above 0°C

of 4,633°C, and a frostless period of 224 days per year. The average relative humidity and annual sunshine duration are 61% and 2,434 hours, respectively. The experimental field had an average soil pH of 6.8 and was irrigated. This trial was designed in randomized complete blocks, with four blocks and eight trees per treatment (planting spacing of 3 m × 5 m). After 5 years of growth, three F₁ hybrids (H₁, H₂, and H₃) which exhibited the highest tree heights and largest DBHs and two F₁ hybrids (L₁ and L₂) that showed the lowest tree heights and DBHs were selected.

Since DNA methylation differences among tissues are obvious in Poplar [34] and leaves are important to plant growth and development, after five years of growth, the leaves at the top of main trunk were collected at the vigorous stage (9:30-10:30 am on August 10, 2011). Three trees (three leaves per tree) per replication were sampled, thus, twelve trees and 36 leaves were sampled for every line. Samples for every parent and F₁ hybrid were pooled and stored in liquid nitrogen prior to DNA extraction.

Evaluation of heterosis

Since planting (in 2007), two important economic traits, tree height and DBH were continuously measured. Considering heterosis over higher parent was important for poplar breeding, after five years of growth, heterosis over higher parent was calculated as $H = (F_1 - P_s) / P_s \times 100\%$, where H is the amount of heterosis, F₁ is the trait value measured in the hybrid, and P_s is the trait value measured in the higher parent [35].

MeDIP-Seq

Genomic DNA was isolated from each sample using a DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France). The DNA integrity was verified by agarose gel electrophoresis. The DNA was quantified using a Qubit Fluorometer and a Quant-iT™ dsDNA BRAssay Kit (Life Technologies, USA).

The MeDIP process was almost identical to the method of Pomraning et al [36]. Before carrying out MeDIP, genomic DNA was sheared to 350-450 bp fragments with a Bioruptor (Sonics, Newtown, USA, VC130PB), and the fragments were recovered using a Qiaquick PCR Purification Kit (Qiagen, Courtaboeuf, France). The fragments were end-repaired, phosphorylated, and A-tailed. The fragments were then ligated to Illumina sequencing adapters [37]. The sheared DNA was diluted in 450 µl of TE buffer, denatured in a 100°C heat block for 10 min, and snap-cooled on ice for 5 min. Immunoprecipitation buffer (100 mM Na-Phosphate pH 7.0, 1.4 M NaCl, 0.5% TritonX-100) and 1 µl of 5meC antibody (Diagenode, Liège, Belgium #Mab-5MECYT-100, 1 µg/µl) were added to the DNA solution followed by incubation for 2 h on an orbital rotator at 4°C. Bound DNA was precipitated with sheep

anti-mouse IgG Dynabeads (M-280, Invitrogen, California, USA), washed three times with immunoprecipitation buffer for 10 min at room temperature with shaking, resuspended in 250 µl proteinase K digestion buffer (5 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 0.05% SDS) with 7 µl of 10 mg/ml proteinase K, and incubated for 3 h on an end-over-end rotator at 50°C to digest the antibodies and release the 5meC-containing DNA. Methylated DNA was extracted by phenol-chloroform extraction followed by ethanol precipitation. The DNA pellets were resuspended in 50 µl TE buffer and stored at -20°C.

The immunoprecipitated DNA was used to generate a DNA colony template library using the Fasteris procedure (Fasteris, Plan-les-Ouates, Switzerland). The DNA samples were quantified using a 2100 Bioanalyzer (Agilent, USA) and a StepOnePlus Real-Time PCR System (ABI, California, USA). Illumina sequencing was performed in a HiSeq-2000 system (Illumina, San Diego, CA, USA).

Bioinformatics processing and statistical analysis

MeDIP-Seq reads were aligned to the *Populus trichocarpa* v2.2 reference genome (<http://www.phytozome.net/poplar.php>, February 2012). The alignments were carried out with SOAP aligner (BGI, version 2.01) [38], allowing up to two mismatches for successful mapping. The mapped rate (the ratio of the number of mapped reads to that of original reads), and the uniquely mapped rate (the ratio of the number of uniquely mapped reads to that of original reads) were calculated. The coverage depth was calculated as the coverage times of specific loci by sequencing reads. The genome coverage was calculated as the proportion of eligible base numbers in the entire genome. In the distribution analysis of the MeDIP-Seq sequencing reads in a chromosome, each chromosome was scanned with windows of 100 kb, the reads coverage depth per window was calculated, and the reads were standardized with the following formula: reads number of specific 100 kb windows * 1,000,000/number of uniquely mapped reads. The methylation coverage of CG/CHG/CHH contexts was calculated as the proportion of CG/CHG/CHH site over certain coverage depth in all CG/CHG/CHH sites from as determined by MeDIP-Seq.

Peak summit coordinates were generated using model-based analysis of ChIP-Seq (MACS; version 1.4.0 beta) [39]. The summit files were then used for further analysis (total peaks number, peak mean length, peak median length, peak total length, and peak covered size in the genome).

To detect differentially methylated gene between the two samples, the peak summits of two samples were merged, and the normalized reads number of each sample the merged region was determined. The false positive reads were removed using a chi-square test. For genes

that overlapped with a merged region, if the reads number of sample 2 in this region was more than that of sample 1, then the gene was designated as hypermethylated during the Sample 1 versus Sample 2 comparison, while if the opposite situation occurred, the gene was considered to be hypomethylated.

Gene Ontology (GO) analysis was performed to obtain the functional classifications of differentially methylated genes using the TermFinder tool (<http://search.cpan.org/~sherlock/GO-TermFinder-0.86/>). P-values were multiple test corrected to reduce false positive rates. GO terms with adjusted P-values of <0.05 were considered to be significant.

The known genes were submitted to the KEGG Automatic Annotation Server (<http://www.genome.jp/kegg/pathway.html>) for pathway analysis. A hypergeometric test was performed to identify the significantly enriched pathways in differentially methylated genes compared with the whole genome. Pathways with Q-values ≤ 0.05 were considered to be significant.

Results

Heterosis performance

Analysis of Variance (ANOVA) of the height and DBH of trees of various ages (from one to five years old) for the parents and F1 hybrids was performed. The results showed that the tree height and DBH in each age of parents and F1 hybrids were significantly different (Figure 1, Additional file 1: Table S1). The tree heights and DBHs of H₁, H₂, and H₃ at each age were higher than those of the parents (except for the annual tree height of H₂). The tree heights of H₁, H₂, and H₃ at five-year were significantly greater than those of the parents. The tree heights of L₁ and L₂ at five-year were lower than those of the parents, and the DBH of L₁ and L₂ at five-year were both significantly lower than those of the parents. Since heterosis over higher parent is important for poplar breeding, we estimated the heterosis over higher parent values (Table 1). Hybrids H₁, H₂, and H₃ exhibited heterosis over higher parent for tree height (7.81% for H₁, 12.55% for H₂, and 11.09% for H₃) and DBH (1.26% for H₁, 1.49% for H₂, and 0.72% for H₃), while hybrids L₁ and L₂ possessed negative heterosis over higher parent for tree height (-5.77% for L₁ and -7.59% for L₂) and DBH (-20.92% for L₁ and -21.82% for L₂).

Mapping of MeDIP-Seq reads to the reference genome

MeDIP-Seq libraries were constructed from DNA extracted from the parents and their hybrids and subjected to high-throughput Solexa sequencing (Illumina, San Diego, CA, USA). A total of 670 million reads were produced from the P₁, P₂, H₁, H₂, H₃, L₁, and L₂ lines. The reads were mapped to the *P. trichocarpa* v2.2 reference genome (<http://www.phytozome.net/poplar.php>).

A total of 486.27 million reads could be mapped in this process. The mapped rates for the seven lines were 83.4%, 74.2%, 82.4%, 65.1%, 65.9%, 63.5%, and 71.7%, respectively (average of 72.31%). The uniquely mapped rates were 66.4%, 56.8%, 65.6%, 55.3%, 52.0%, 53.0%, and 55.6%, respectively (average of 57.8%; Table 2). To facilitate the access and use of the *P. deltoides* methylome sequencing data, the raw data in the FASTQ format was deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database with accession number SRP034728.

Comparison of methylation status among parents and F1 hybrid genomes

In this study, the leaf methylomes of the parents and F1 hybrids were investigated. The distribution of MeDIP-Seq reads on the 19 scaffolds (each scaffold represents a putative chromosome) of *Populus* was shown in Figure 2. Distinct DNA methylomes were observed among the parents and F1 hybrids. Among the 19 chromosomes, nine (I, IV, VI, VII, XI, XII, XVI, XVIII, and XIX) had greater methylation coverage in the middle parts of chromosomes, which may comprise the centromeric regions [34]. Moreover, three chromosomes (II, VIII, and XIII) had greater methylation coverage in distal parts of the chromosomes, while four (III, IX, X, and XV) had greater methylation coverage in proximal parts of the chromosomes.

Different types of methylation coverage were also observed in the parents and F1 hybrids (Figure 3). In plant, DNA methylation is often found in all sequence, namely, the symmetric CG and CHG contexts (H represents A, T, and C) and asymmetric CHH contexts [40]. We calculated the methylation coverage of CG/CHG/CHH contexts. We found that remarkable methylation occurred in each cytosine context in the genomes of parents and F1 hybrids. Moreover, the methylation coverage in CG, CHH, and CHG displayed clear differences between parents P₁ and P₂. The methylation coverage in the three sequence contexts for maternal parent P₁ was 17.24%, 19.24%, and 18.83%, respectively, while that for paternal parent P₂ was 12.61%, 13.35%, and 12.92%, respectively (Figure 3). For better-parent hybrids, the methylation coverage of H₁ was 16.97% (CG), 18.79% (CHG), and 18.44% (CHH), respectively. H₂ and H₃ had comparable values to H₁, with an average of 16.06%, 17.40%, and 17.33% for CG, CHG, and CHH contexts, respectively (Figure 3). These results indicate that the methylation levels of all better-parent hybrids were between those of the two parents (less than P₁ and more than P₂), while the values were higher than the average levels of the parents (14.94%, 16.31%, and 15.88%, respectively). For lower-parent hybrids, L₂ displayed an average methylation coverage of 16.35% for the three contexts, which was less than that of P₁ but

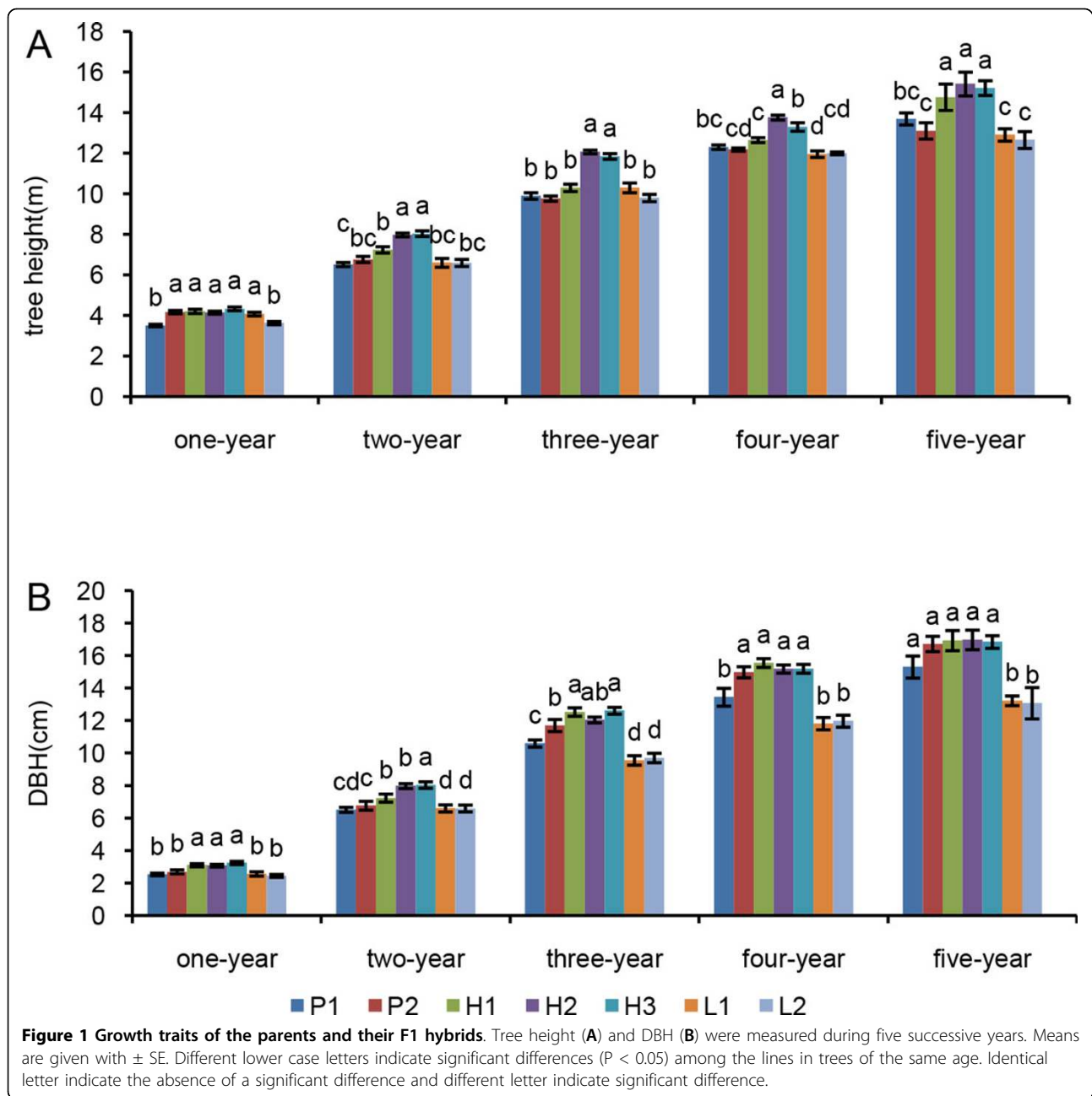


Table 1 Heterosis of F1 hybrids

Clone No.	Age (year)	heterosis over better parent %	
		Tree height	DBH
H ₁	5	7.81 \pm 0.05	1.26 \pm 0.03
H ₂	5	12.55 \pm 0.06	1.49 \pm 0.02
H ₃	5	11.09 \pm 0.04	0.72 \pm 0.01
L ₁	5	-5.77 \pm 0.03	-20.92 \pm 0.03
L ₂	5	-7.59 \pm 0.05	-21.82 \pm 0.05

Heterosis over higher parent was calculated as $H = (F1 - P_s) / P_s \times 100\%$, where H is the amount of heterosis, F1 is the trait value of the hybrid, and P_s is the trait value of the better parent.

more than that of P₂, while the values of L₁ were 10.21%, 10.21%, and 10.05%, respectively, which were less than those of both parents (Figure 3).

Mapping of MeDIP-Seq reads to genes

The distribution of MeDIP-Seq reads on various genomic features, including CpG islands, promoters, 5' untranslated regions (UTRs), 3' UTRs, coding sequences (CDS), and introns, was characterized based on methylation coverage. Promoters are defined as the 2-kb region upstream of the annotated transcription start site.

Table 2 Summary of MeDIP-Seq experimental results

line No.	length of sequence reads (bp)	Total reads	No. mapped reads ^a	Percent mapped read (%)	No. mapped base	No. unique mapped reads ^b	No. unique mapped base	Percent unique mapped read (%)
P ₁	49	97,959,184	81,729,006	83.4	4,004,721,294	65,078,019	3,188,822,931	66.4
P ₂	49	97,959,184	72,679,485	74.2	3,561,294,765	55,648,568	2,726,779,832	56.8
H ₁	49	97,959,184	80,814,131	82.4	3,959,892,419	64,231,654	3,147,351,046	65.6
H ₂	49	97,959,184	63,730,201	65.1	3,122,779,849	54,140,276	2,652,873,524	55.3
H ₃	49	97,959,184	64,510,366	65.9	3,161,007,934	50,943,321	2,496,222,729	52.0
L ₁	49	82,792,658	52,534,101	63.5	2,574,170,949	438,630,69	2,149,290,381	53.0
L ₂	49	97,959,184	70,275,601	71.7	3,443,504,449	54,474,434	2,669,247,266	55.6
Total		670,547,762	486,272,891					

The sequencing reads were mapped to the *Populus trichocarpa* v2.2 reference genome (<http://www.phytozome.net/poplar.php>), allowing for up to two mismatches per read. (a) the mapped reads indicate the reads mapped to the reference genome. (b) the uniquely mapped reads indicate the reads number mapped to the unique loci in the reference genome.

Methylation coverage in promoters of the parents and F1 hybrid was higher than that of the gene body (Figure 4). Moreover, CDS and introns of the gene body had higher methylation coverage, while the 5'- and 3'- UTRs had very low methylation coverage. We also compared the methylation coverage on various genomic features among parents, better-parent F1 hybrids, and lower-parent F1 hybrids. We found that three better-parent F1 hybrids had higher methylation coverage in promoters, 5' UTRs and 3' UTRs than those of the parents and the lower-parent hybrids. In intron, the methylation coverage of H₂ was higher than other lines, the coverage of P₁, H₁, H₃ and L₂ were similar, the coverage of P₂ and L₁ were similar (Figure 4, Additional file 2: Figure S1). H₂ had higher methylation coverage in CDS, whereas the coverage of L₂ was higher than H₁ and H₃ and the parents.

CpG islands (CGI) are a prime target for epigenetic modification. Moreover, CpG islands are more frequently found in plant genomes than in the human genome [41]. CGIs were previously thought to be unmethylated unless they were located at genomic imprinting sites or on the inactivated X chromosome [26]. Recent studies have shown that some CpG islands are methylated [42,43]. To further observe the distribution trend of DNA methylation in CGIs of *Populus*, 2,000-bp regions upstream and downstream of CGI were divided into 20 segments, and the CGI was divided into 40 segments. By counting the normalized average coverage depth, we determined that the CGIs in the parents and hybrids had methylation. H₂ had the highest level of CGI methylation, followed by the lower-parent hybrids L₁ and L₂, the paternal parent P₂, H₁ and H₃, and the maternal parent P₁ (Figure 5).

Transposable elements and repetitive sequences are frequently methylated regions. Previous reports have suggested that inferring the methylation status in transposons and repetitive sequences at the whole-genome level using classic approaches, especially microarray- and PCR-based assays, is unreliable. This problem can be overcome by

using the recently available MeDIP-Seq approach, which can be used to deduce the coverage of all major types of methylation for transposons and repetitive sequences [44]. In plants, numerous LTR-gypsy retrotransposon elements are present in the heterochromatic centromeric and pericentromeric regions. LTR-gypsy retrotransposon elements are the most abundant type of transposon element in the *Populus trichocarpa* genome [45]. We detected the enrichment of LTR-gypsy retrotransposons in the DNA-methylated fraction of the genomes of the parents and F1 hybrids. In addition, genome regions containing LTR copia, DNA/En-Spm, Low-complexity (which contains a highly non-uniform amino acid composition [46,47]), and Simple-repeat were also methylated (Figure 6). The variations in methylation in transposable elements and repetitive sequences seem dependent on each genotype. The methylation coverages of the F1 hybrids were between those of the two parents in LTR-Gypsy, simple-repeat and Low_complexity (except for L₂ in LTR-Gypsy). In LTR-Copia, H₁, H₃ and L₂ had higher methylation coverage with H₂ and L₁ had lower methylation coverage. In contrast, in rRNA and DNA/En-Spm, H₂ and L₁ had higher methylation coverage, whereas the methylation coverage of H₁ and H₃ were lower.

Methylated peaks analysis

To avoid the false positive results generated from MeDIP-Seq, model-based analysis of ChIP-Seq (MACS) was used to obtain methylation peak summits. In this analysis, the total number of peaks of the better-parent hybrids was higher than that of the parents, while that of the lower-parent hybrids was lower than that of the parents. The parents had distinct DNA methylation peak coverage. The number of peak coverage of the better-parent hybrid H₁ was more than that of the parents, and those of the other two better-parent hybrids (H₂ and H₃) were higher than the average levels of the parents. Moreover, the number of peak coverages of both

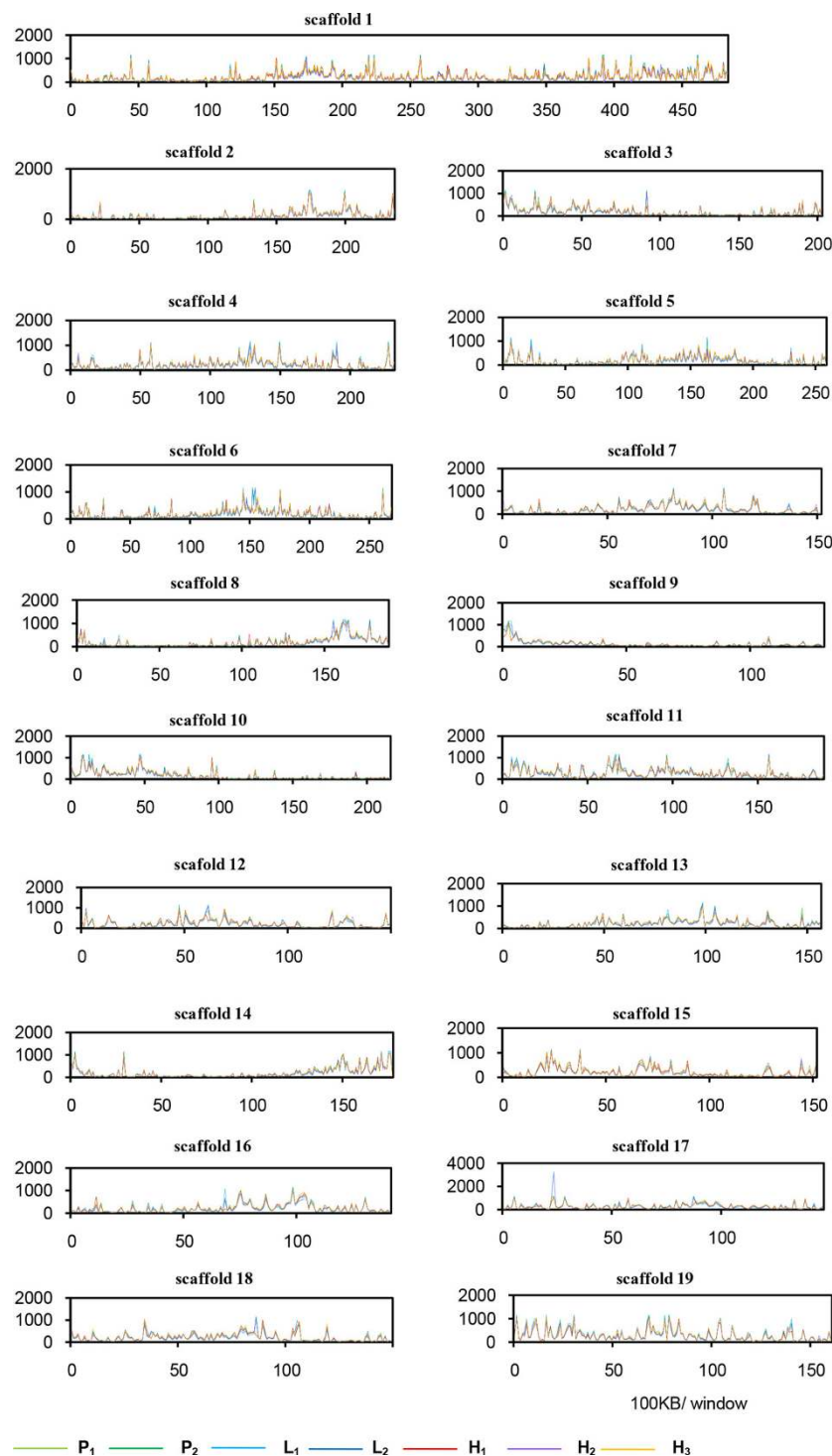
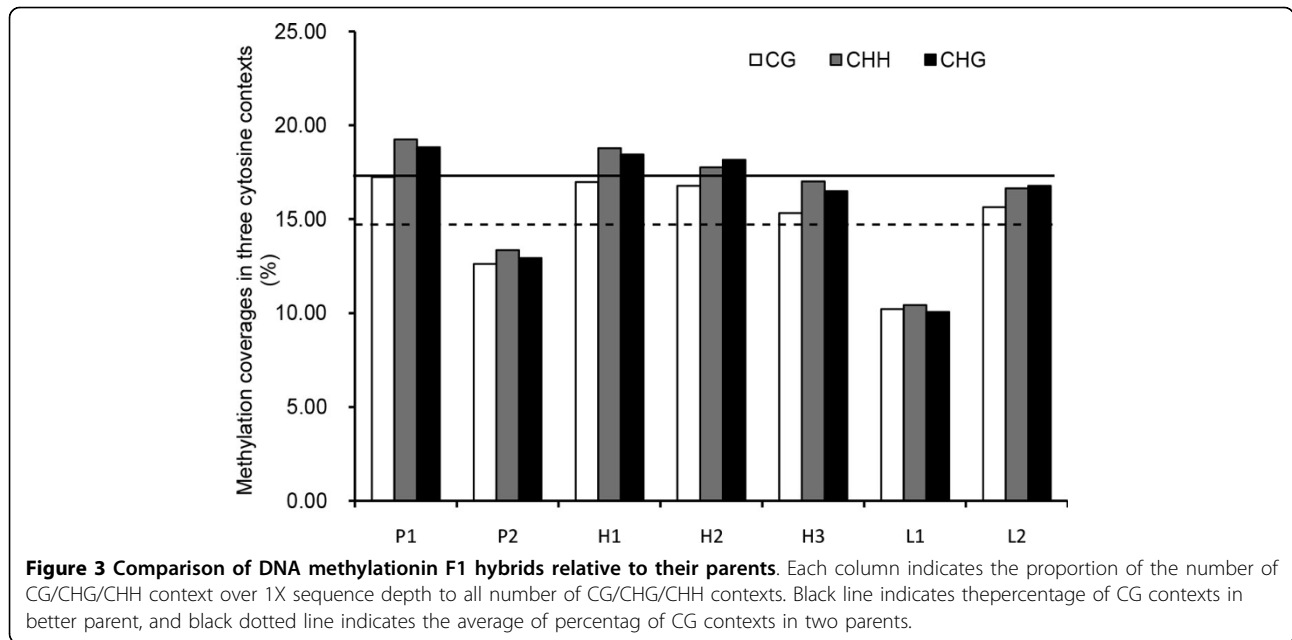


Figure 2 Chromosome-level view of methylation in parents and F1 hybrids. MeDIP-Seq reads were plotted in 100-kb windows along each chromosome. One line is shown for each line.

lower-parent hybrids was lower than those of the low parents (Table 3).

We further mapped peaks to various genomic features. Table 4 shows the peak number contained in each

genomic feature. The peak number in the promoter regions was greater than that of the gene body. In the gene body, CDS had a higher peak number than introns and UTRs. The peak numbers in the promoter, 5'UTR,

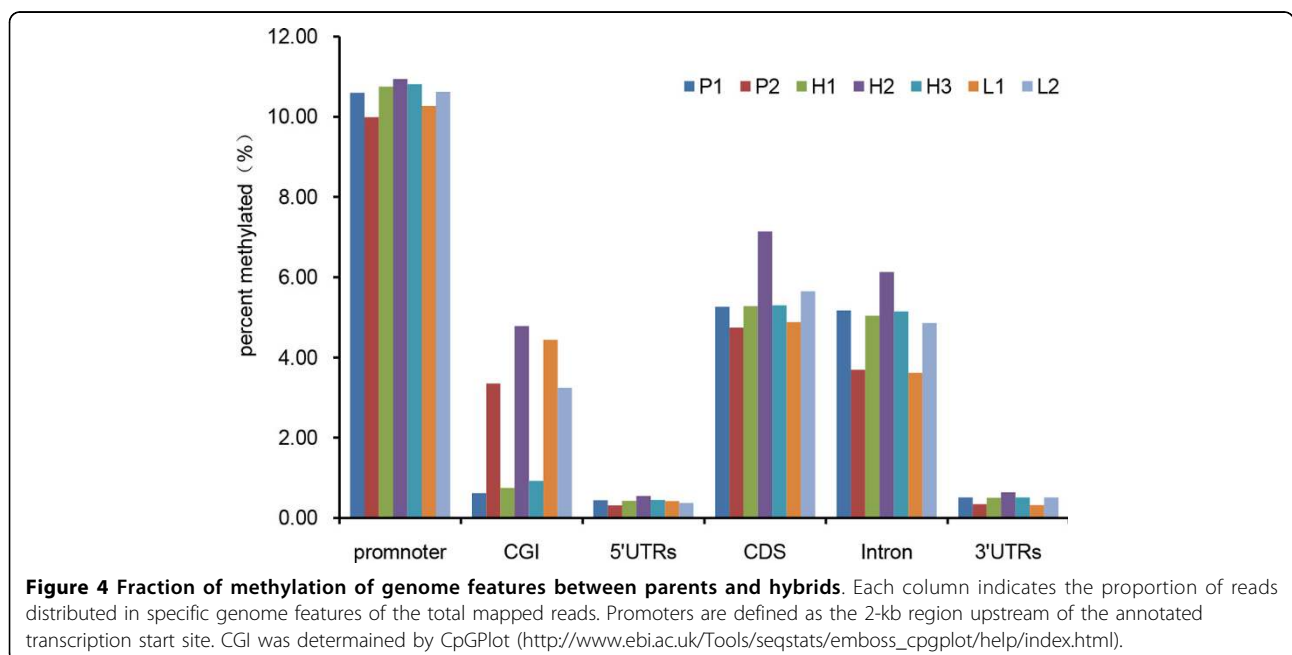


and 3'UTR of better-parent hybrid F1 plants were higher than those of the parents. In introns, the peak number of L₁ was higher than that of the other lines, while the peak numbers of P₁, H₁, and H₂ were similar, and the peak numbers of P₂, H₃, and L₂ were also similar.

Analysis of differentially methylated genes in the parental and F1 hybrid genomes

The number of differentially methylated gene among the parents and F1 hybrids is shown in Figure 7. Better-

parent hybrid H₁ had a similar number of hyper- and hypomethylated genes as the maternal parent P₁ (589 and 580, respectively). By contrast, remarkably fewer genes with hypermethylated were detected relative to hypomethylated genes in better-parent hybrid H₂ (1,338 versus 4,189 genes) and H₃ (414 versus 1,046 genes). Compared with the paternal parent P₂, the better-parent hybrids displayed higher levels of hypermethylation at protein-coding genes, as more hypermethylated than hypomethylated genes were found in H₁ (2,887 versus



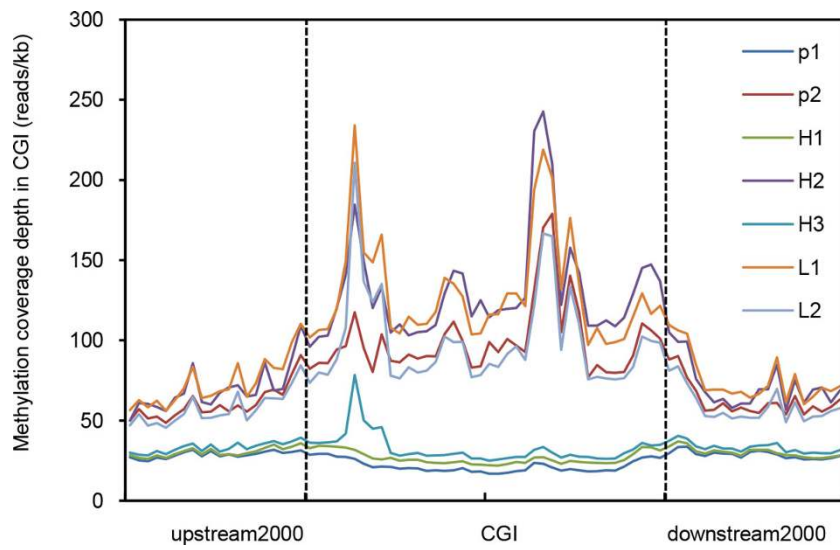


Figure 5 The trend of average coverage depth of DNA methylation in CGI. The region within the dotted line indicates CGI region.

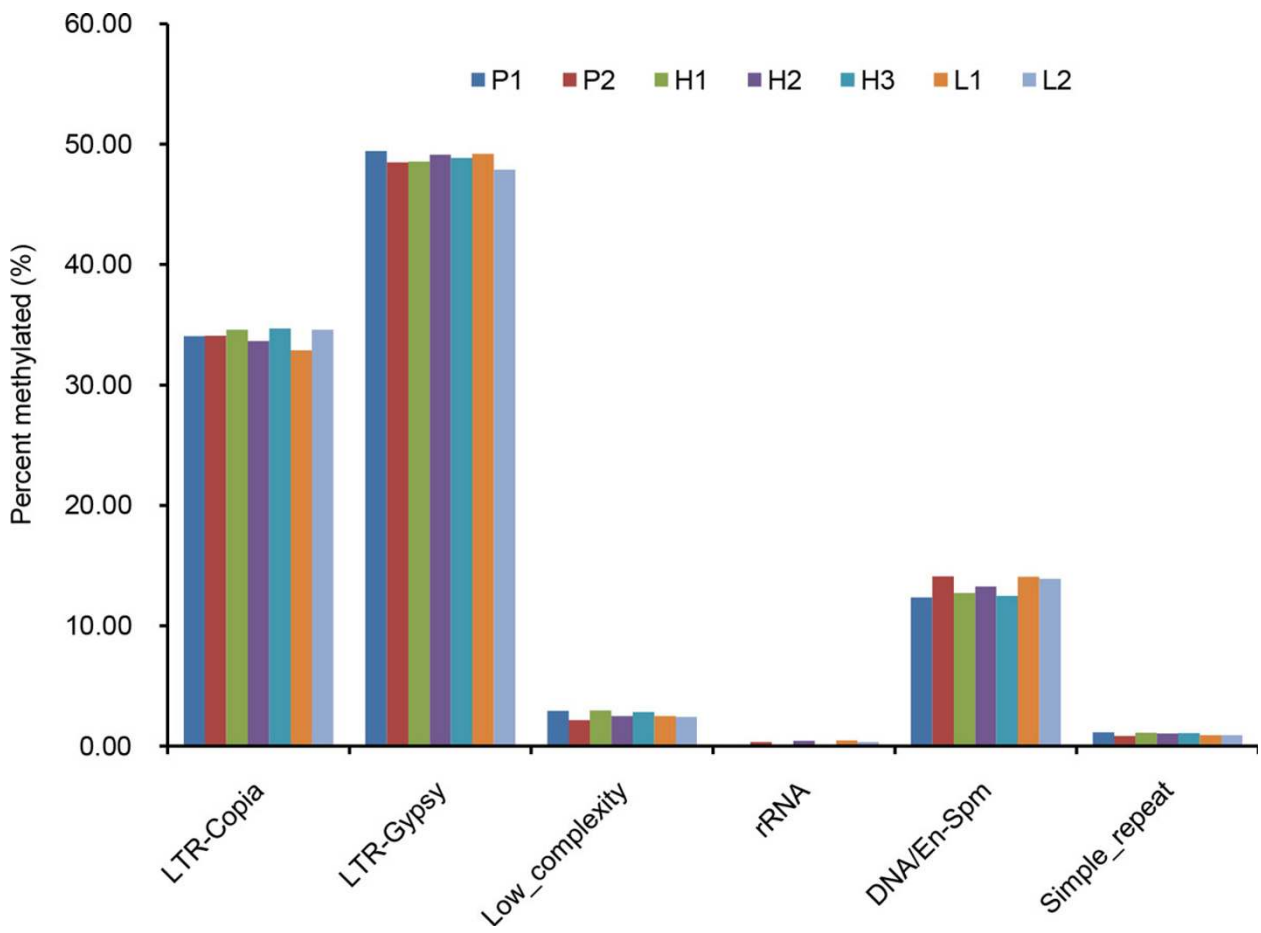


Figure 6 Fraction of methylation of repetitive sequence and transposable element between parents and hybrids. Each column indicates the proportion of reads distributed in a specific element of the total mapped reads. Information of repeat element was obtained from the RepeatMasker (<http://www.repeatmasker.org/faq.html>).

Table 3 Statistics of peak summits

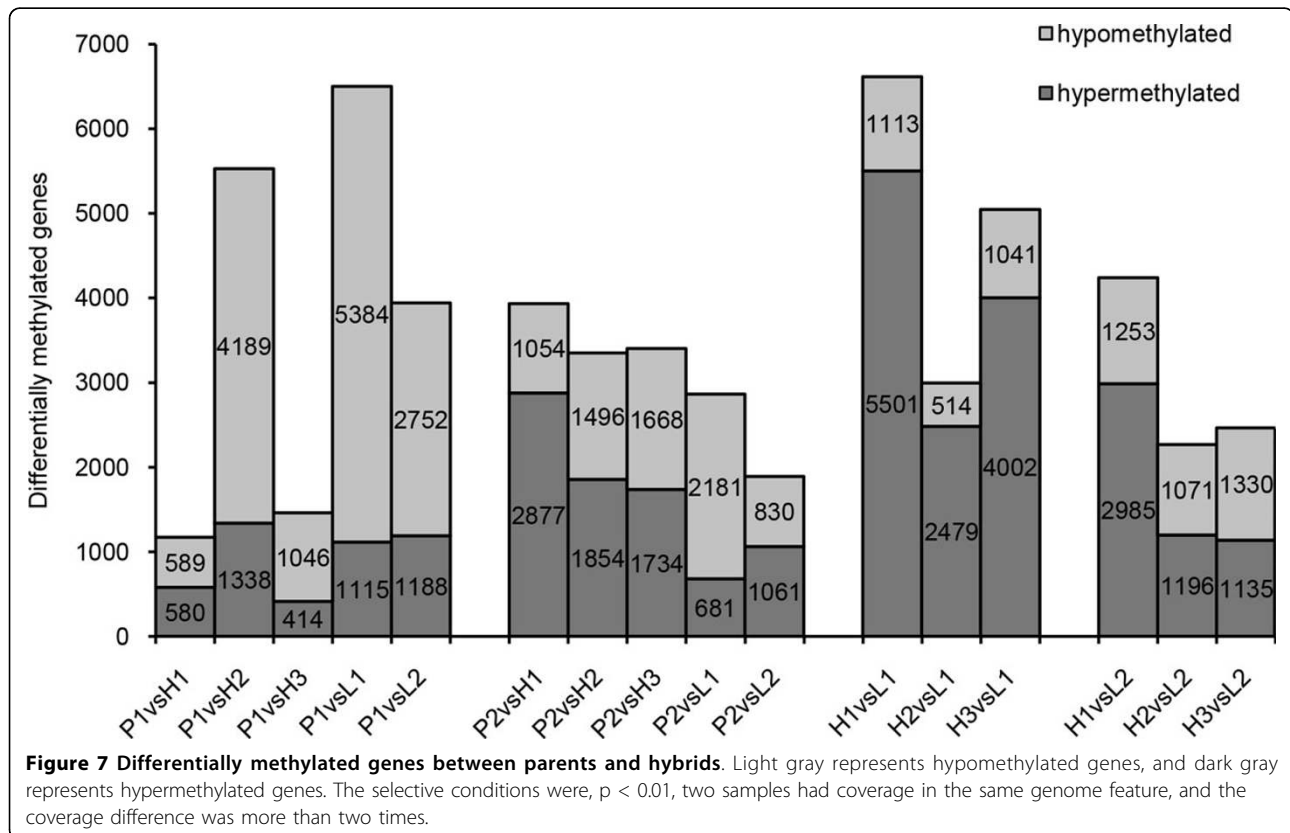
Clone No.	Total peaks	Peak mean length /bp	Peak total length /bp	Peak coverage in genome (%)
P ₁	21,355	1697.33	36,246,472	8.69
P ₂	18,754	1791.66	33,600,816	8.06
H ₁	22,932	1617.75	37,098,304	8.89
H ₂	24,114	1418.43	34,203,905	8.20
H ₃	21,582	1633.20	35,247,816	8.45
L ₁	18,699	1705.54	31,891,918	7.65
L ₂	17,711	1861.72	32,972,998	7.90

Peak summits were generated using Model-based analysis of ChIP-Seq (MACS; version 1.4.0 beta).

Table 4 Number of peaks in genome features

Genome feature	P ₁	P ₂	H ₁	H ₂	H ₃	L ₁	L ₂
Promoter	2841(13.3%)	2487(13.3%)	3031(13.2%)	2998(12.4%)	2905(13.5%)	2514(13.4%)	2427(13.7%)
5'UTR	588(2.7%)	375(2.0%)	659(2.9%)	732(3.0%)	590(2.7%)	755(4.0%)	347(1.9%)
CDS	2392(11.2%)	1999(10.7%)	2561(11.2%)	2899(12.0%)	2318(10.7%)	2524(13.5%)	1845(10.4%)
Intron	1768(8.3%)	1441(7.7%)	1840(8.0%)	1941(8.0%)	1650(7.7%)	1661(8.9%)	1383(7.8%)
3'UTR	339(2.6%)	276(1.5%)	345(1.5%)	327(1.4%)	330(1.5%)	320(1.7%)	284(1.6%)

Number of peaks in Promoter, 5'UTR, CDS, Intron and 3'UTR were calculated. The percentage indicates the ratio of the number of peaks in each feature to total peaks.



1,054 genes), H₂ (1,854 versus 1,496 genes), and H₃ (1,734 versus 1,668 genes). However, there were less hypermethylated than hypomethylated genes in hybrid L₁ (681 versus 2,181 genes). When better-parent and lower-parent F₁ hybrids were compared, we found higher levels of methylation in the better-parent hybrids than in L₁, as inferred by the larger number of hypermethylated genes than hypomethylated genes in H₁ (5,551 versus 1,113 genes), H₂ (2,479 versus 514 genes), and H₃ (4,002 versus 1,041 genes). Compared with L₂, more genes with hypermethylation than hypomethylated genes were detected in H₁ (2,985 versus 1,253 genes) and H₂ (1,196 versus 1,071 genes), whereas H₃ showed higher levels of methylation, with a greater number of hypomethylated genes (1,135 hypermethylated versus 1,330 hypomethylated).

For all of the differentially methylated genes identified, we performed Gene Ontology (GO) functional category analysis to determine whether these genes were enriched for certain pathway or network (Additional file 2: Figure S2). The results showed that the differentially methylated genes between better-parent hybrid H₁ and maternal parent P₁ were enriched in 28 biological functional categories, and ten additional enriched functional categories (biological adhesion, cell proliferation, locomotion, reproductive process, extracellular region, extracellular region part, enzyme regulator activity, molecular transducer activity, protein binding, and transcription factor activity) were also found for genes identified in the H₁-P₂ comparison. Compared with maternal parent P₁, better-parent hybrid H₂ possessed more hypermethylated genes enriched in 35 functional categories, such as biological adhesion, cell proliferation, protein binding, and transcription factor activity. The differentially methylated genes between H₂ and paternal parent P₂ were enriched in 33 functional categories (e.g., pigmentation). The differentially methylated genes between better-parent hybrid H₃ and maternal parent P₁ were enriched in 31 functional categories, and two additional categories (cell proliferation and molecular transducer activity) were found to have enriched differentially methylated genes between H₃ and P₂. As a whole, the majority of hypermethylated genes between three better-parent hybrids and both parents tended to fall into seven functional categories, including metabolic processes, cellular, response to stress, cell, cell part, binding, and catalytic activity.

To further investigate the differentially methylated genes between better-parent F₁ hybrids and the parents, we analyzed hypermethylated genes enriched in specific functional categories in H₁, H₂, and H₃ versus the two parents (Figure 8A). 20 and 199 genes showed hypermethylation in all three better-parent F₁ hybrids compared with P₁ and P₂, respectively. Among these genes, 97 fell into seven major GO functional categories, namely metabolic process (31), cellular metabolic process (21), primary metabolic

process (17), small molecule metabolic process (10), nitrogen compound metabolic process (7), developmental process (6), and anatomical structure development (5). Notably, four genes (POPTR_0008s18650, POPTR_0010s02290, POPTR_0017s03190, and POPTR_0010s19920) with hypermethylation were detected in all better-parent hybrid-parent comparisons (Table 5). Among these, POPTR_0010s02290 encodes a predicted GTP-binding protein with GTPase activity and protein binding functions, and POPTR_0010s19920 encodes 3-dehydrosphinganine reductase, which participates in metabolic processes and oxido-reductase activity. The genes were submitted to the KEGG Pathway (<http://www.genome.jp/kegg/pathway.html>) database to obtain their KEGG orthology annotations. POPTR_0010s19920 was found to participate in sphingolipid metabolism (ko00600). No annotation was retrieved for POPTR_0008s18650 or POPTR_0017s03190, suggesting that these genes have unknown functions.

The differentially methylated genes between better- and lower-parent F₁ hybrids were also analyzed (Figure 8B). The number of hypermethylated genes of better-parent F₁ hybrids versus lower-parent hybrids L₁ and L₂ were 523 and 132, respectively. For these genes, hypermethylation predominantly occurred in the promoter (171 genes compared with L₁ and 27 genes compared with L₂) and gene body (296 genes compared with L₁ and 80 genes compared with L₂), while less hypermethylation occurred in the 5' UTR (28 genes compared with L₁ and 15 genes compared with L₂) and the 3' UTR (15 genes compared with L₁ and 10 genes compared with L₂). A total of 40 hypermethylated genes were found in all three better-parent F₁ hybrids compared with lower-parent hybrids L₁ and L₂, ten of these genes could be annotated (Table 5). The GO functional categories of the 10 genes mainly involve metabolic process (4), primary metabolic process (2), cellular metabolic process (3), signaling (3), small molecule metabolic process (2), anatomical structure development (2), and biological regulation (2). These genes were then submitted to the KEGG Pathway database, yielding pathway information about four genes as follows: (1) POPTR_0012s07360 is calcium-dependent protein kinase gene involved in plant-pathogen interactions (ko04626); (2) POPTR_0015s09720 belongs to cytochrome P450 CYP4/CYP19/CYP26 subfamilies involved in steroid hormone biosynthesis (ko00140); (3) POPTR_0019s09910 encodes a molybdopterin synthase catalytic subunit involved in multiple processes such as metabolism, metabolism of cofactors, vitamin and folate biosynthesis (ko00790), genetic information processing, folding, sorting, and degradation, and the sulfur relay system (ko04122); and (4) POPTR_0008s18420 encodes an erbb2-interacting protein involved in a NOD-like receptor signaling pathway (ko04621).

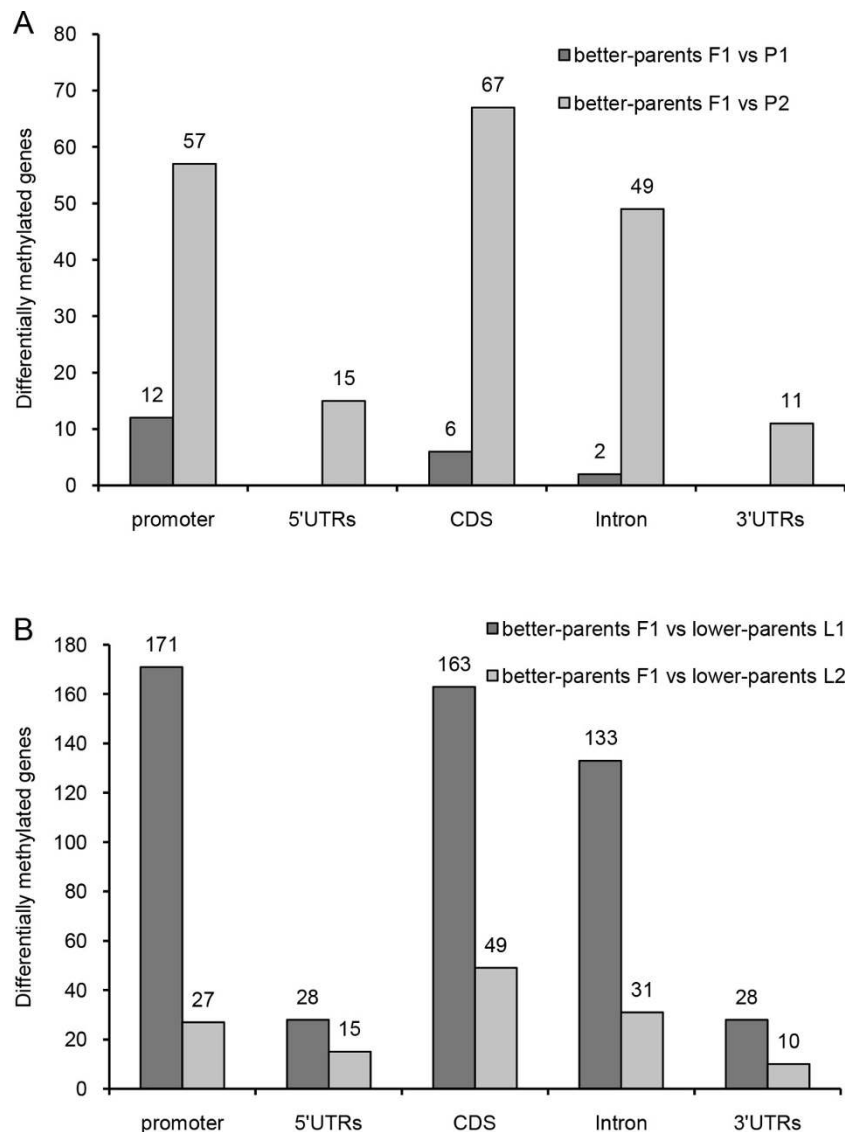


Figure 8 Comparison of differentially methylated genes in various genome features among better-parent hybrids, the parents, and lower-parent hybrids. (A) The number of differentially methylated genes between better-parent hybrids and the maternal parents were calculated (dark gray columns), and the number of differentially methylated genes between better-parent hybrids and the paternal parents are indicated in the light gray columns. (B) The number of differentially methylated genes between better-parent hybrids and below-parent hybrids L1 were calculated (dark gray columns), and the number of differentially methylated genes between better-parent hybrids and below-parent hybrids L2 are indicated in the light gray columns.

Discussion

Several classical hypotheses about heterosis are based on the differences between genomes [1], and allelic diversity may produce heterosis. However, hybrid vigor can be observed even when parents are genetically very similar [24]. Recent studies have shown that parents with similar genome sequences have distinct epigenomes, which may contribute to heterosis [5,24]. In *Populus*, hybrids with heterosis are often obtained by intrasection and interspecific hybridization, whereas hybrids obtained by intersection hybridization always have mid-parent performance,

and hybrids with growth vigor are obtained less frequently. In Section *Aigeiros*, excellent hybrids with heterosis have been produced by intraspecific hybridization; the level of heterosis increases with a decrease in genetic distance between parents and polymerization of excellent genetic composition. Super high yield varieties are often generated by convergent crossing of *P. deltoides* varieties (strains). In this study, *P. deltoides* cl. '55/65', was used as the maternal parent and *P. deltoides* cl. '10/17' was used as the paternal parent. This cross combination is a multigeneration convergent cross, and the level

Table 5 Annotations of hypermethylated genes

Gene ID	Located scaffold	Start position of the gene in the scaffold	End position of the gene in the scaffold	Length /bp	Annotation
POPTR_0010s02290	scaffold_10	2630875	2632874	2000	Predicted GTP-binding protein (ODN superfamily)
POPTR_0010s19920	scaffold_10	17678728	17680727	2000	Predicted 3-ketosphinganine reductase
POPTR_0007s08200	scaffold_7	6768492	6770491	2000	Ubiquitin-like protein
POPTR_0019s08620	scaffold_19	10148682	10150681	2000	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies
POPTR_0009s10580	scaffold_9	9068290	9070289	2000	15-hydroxyprostaglandin dehydrogenase and related dehydrogenases
POPTR_0012s07360	scaffold_12	8260585	8262584	2000	Ca ²⁺ /calmodulin-dependent protein kinase, EF-Hand protein superfamily
POPTR_0015s09720	scaffold_15	10859015	10861014	2000	Cytochrome P450 CYP2 subfamily
POPTR_0019s02490	scaffold_19	2267881	2269880	2000	Serine/threonine protein kinase
POPTR_0019s09910	scaffold_19	11534694	11536693	2000	Molybdopterin converting factor, small subunit
POPTR_0008s18420	scaffold_8	12397080	12398840	1761	Leucine-rich repeat protein
POPTR_0017s04200	scaffold_17	3227408	3228528	535	Serine/threonine protein kinase
POPTR_0019s09760	scaffold_19	11360305	11360904	600	Apoptotic ATPase

The annotation of the hypermethylated genes in better-parent hybrids compared to the parents and lower-parent hybrids. These annotations were obtained according to orthologous genes in *P. trichocarpa*. Additional files

of heterosis is outstanding. Intraspecific F1 hybrids of *P. deltoides* with significant hybrid vigor or lower-parental performance were examined, providing a unique opportunity to accurately analyze the contribution of DNA methylation to heterosis in trees. This is the first investigation of DNA methylation maps with high resolution in *P. deltoides* plants and their F1 hybrids at the genome-wide scale using high-throughput sequencing.

A total of 670.55 million reads were generated using MeDIP-Seq, 486.27 million of which could be mapped onto the reference genome of *P. trichocarpa*, the average of the uniquely mapped rates was 57.8%. The relatively low rate of mapping using genomes of closely related species as a reference suggests that species in different sections within the genus *Populus* are genetically divergent (*P. trichocarpa* belongs to sect. *Tacamahaca*, and *P. deltoides* belongs to sect. *Algeiros*). Similar observations were also documented in studies of *Populus alba* and *Populus tremula* based on single nucleotide polymorphism (SNP) analysis of the two species [48]. Our dataset of leaf methylomes shows that the parents and F1 hybrids had significant methylation in the CG/CHH/CHG contexts, with CHG and CHH methylation being more consistent, and cytosines in CG context were less methylated than those in the other two contexts. Previous studies have reported that CGs are dominant in methylome, especially in coding regions, while less frequent in general, 5meCHH is more common in repeat regions and short transposable elements [49-51]. In *populous*, CG and CHG methylation were more consistent within tissues. However, in the two targets with cytosine content < 10%, cytosines in CHH context were

methylated more frequently than those in the other two contexts [34]. The two parents had distinct methylomes reflected by different methylation coverage in the CG/CHG/CHH contexts. The methylation coverage of three better-parent F1 hybrids was higher than the average of the parental values (mid-parent value, MPV), indicating that the F1 hybrids had an altered epigenome, and the DNA methylation level was non-additive. Unlike in animal systems, where “Erase and Reset” of cytosine methylation occurs in each generation, in plants, the parental methylation states can be stably inherited by the progeny [52,53]. However, many plants species often exhibit the remodeling of parental methylation patterns in interspecific hybrids and allopolyploids [54-56]. In these scenarios, DNA methylation partly functions epigenetically and dynamically over generations, thus achieving the control and balance of gene expression under specific circumstances [27,54,57].

Early studies proposed that allelic variation is the primary cause of heterosis [58], but this notion was challenged by the observation that parents with similar genetic backgrounds can also produce hybrids with heterosis, which can arise from the diversity of epialleles. Epi-allelic changes in hybrids occur though changes in siRNA levels, trans-chromosomal methylation (TCM) or trans-chromosomal demethylation (TCdM), which fit the dominance or overdominance hypotheses and indicate that epi-alleles are essential parts of the genetic basis of heterosis. In rice hybrids, DNA methylation at many loci is inherited by non-additive inheritance [29]. Although the two rice hybrids had unequal numbers of non-additively methylated loci, in both hybrids, approximately 75% of such loci

had increased methylation levels. The increased DNA methylation was also reported in reciprocal F1 hybrids between *Arabidopsis thaliana* Landsberg erecta and C24 [5]. In this study, we found that *P. deltooides* F1 hybrids with hybrid vigor (H_1 , H_2 , and H_3) showed higher DNA methylation coverage in three contexts than the MPV. This can partially be explained by the effects of TCM. In this scenario, the better parent derived siRNA molecules associate with both alleles, maintains the methylation state of its own alleles and establishes the *de novo* methylation of lower parent hypomethylation [24], resulting in increased methylation levels in the non- or low methylation region. Therefore, the methylation levels of hybrids may exceed MPV. For lower-parent hybrids L_1 , the fact that DNA methylation coverages in three contexts are lower than parental values can be attributed to the influence of TCdM. The lower parent derived siRNA initially becomes associated across both parental alleles. This association can cause siRNA level to be present at lower levels than the threshold required for the establishment and/or maintenance of methylation, leading to hypomethylation of alleles of the lower parent allele. At the same time, with the loss of methylation, normal siRNA levels cannot be maintained (loss of siRNA), which further reduces the level of DNA methylation, as detected in the lower-parent hybrids L_1 with lower methylation levels. Thus, contrasting patterns of methylation between poplar better-parent F1 and lower-parent L_1 hybrids may result from an adjustment of methylation levels of the parents, and this difference in methylation may in turn influence and regulate the expression network of target genes, which is beneficial to the establishment of heterosis. Interestingly, one of the hybrids with negative better-parent heterosis (L_2) has methylation coverages in three contexts above the midparent value, and the variations in methylation in specific genomic features (such as intron) and in transposable elements and repetitive sequences seem dependent on each genotype. This indicates that the role of DNA methylation in heterosis is complex and multifaceted.

In addition, in some annual herb plant species, distinct epigenomes between parents can give rise to increased DNA methylation levels in the F1 hybrids and contribute to heterosis. For instance, when two rice subspecies, Nipponbare (*O. sativa* ssp *japonica*) and 93-11 (*O. sativa* ssp *indica*), were used as parents, 82.1 and 70.8% of the different methylation region (DMRs) of the genome of F1 hybrids showed high- or above high-parental DNA methylation levels, respectively [29]. When *A. thaliana* Landsberg erecta and C24 were used as parental lines, the reciprocal F1 hybrids showed increased DNA methylation levels across the entire genome, especially in the transposable elements [5].

However, other studies revealed no obviously altered or decreased methylation levels in hybrids compared

with their parents. In *Arabidopsis thaliana*, 97% of the *MspI/HpaII* recognition sites in the F1 hybrids of a Col-0 and C24 cross retained their levels of methylation [59]. The methylation levels of cotton hybrids were lower than those of the parents, and the demethylation numbers of better-parent hybrids were higher than those of the lower-parent hybrids [60]. This discrepancy may be due to the different approaches used in these two studies versus the present study. The two previous studies used a methylation-sensitive amplified polymorphism assay, which is much less sensitive than MeDIP-Seq and thus could not fully scan all methylation loci and could only partially provide the landscapes of DNA methylation.

The MACS approach can improve the spatial resolution of the aligned data and impart the robustness of the final aligned sequences based on dynamic Poisson distribution [39]. The peak coverage further illustrates that the parents had distinct DNA methylation levels, while F1 hybrids with hybrid vigor possessed elevated DNA methylation levels, and F1 hybrids with negative hybrid vigor possessed declining DNA methylation levels. In the *P. deltooides* genomes, peak data were found to be more enriched in promoters than in gene bodies, and the CDS showed more enrichment than introns or UTRs in gene bodies. The enrichment levels of various genomic features in the better-parent hybrids, parent and lower-parent hybrid were different. The growth vigor displayed in better-parent hybrids may be attributed to the increased transcriptional inactivation of CG and CHG sites and heterochromatin-mediated gene silencing, which are related to methylated enrichment. Throughout the growth and development of poplar, methylated enrichment may also suppress the expression of a proportion of genes and/or reduce spurious global transcription to enable full transcription or to initiate the expression of other suitable loci, consequently increasing hybrid vigor in the F1 hybrids; this concept deserves further investigation.

The analysis of differentially methylated genes between parents and hybrids has revealed that the hypermethylation levels of better-parent F1 hybrids were between those of the two parents, while the hypermethylation levels of the lower-parent F1 hybrids was lower than lowest value of the parents. This finding suggests that having a methylation level between that of the two parents in F1 hybrids may be more favorable for achieving better-parent heterosis, while deviating from the MPV tends to preclude the establishment of heterosis.

The analysis of GO functional categories showed that the differentially methylated genes between the better-parent F1 hybrids and the parents were enriched in metabolic processes, response to stress, and binding and catalytic activity, which indicates that heterosis in trees

may follow a comprehensive process. At the same time, compared with lower-parent F1 hybrids, the hypermethylated genes in the better-parent F1 hybrids were enriched in metabolic and development processes, such as metabolic process, cellular metabolic process, primary metabolic process, small molecule metabolic process, nitrogen compound metabolic process, developmental process, anatomical structure development, and signaling, which implied that differentially methylated genes are involved in heterosis.

Compared with the parents and lower-parent F1 hybrids, the hypermethylated genes in better-parent F1 hybrids were involved in hormone synthesis and response to stress, such as cytochrome P450, participating in the biosynthesis of hormones, defensive compounds and fatty acids, GTP-binding proteins involved in cytoskeleton organization, signal transduction, vesicle trafficking, and stress tolerance. As Ca^{2+} signal transducers, calcium-dependent protein kinases play an important role in various plant physiological process, including growth, development, defense responses, regulation of reactive oxygen species production, symbiotic interactions, guard cell turgor, osmotic, drought and salt stress, and regulation through hormones such as ABA and GA. In summary, the fact that many differentially methylated genes are involved in diverse biological pathways indicates that the inheritance of heterosis is a multidimensional process.

Conclusions

To date, studies linking epigenetics and heterosis have only been carried out in a few plant species. In this study, we identified genome-wide variations in leaf methylomes between parents and their hybrids in *P. deltoides*, a perennial forest tree species. The dataset derived from MeDIP-Seq were used to produce DNA methylation maps with high resolution of *P. deltoides* cl. '55/65' and *P. deltoides* cl. '10/17' and their five F1 hybrids. *Populus* F1 hybrids has a non-additive methylation level (higher than mid-parent values), which showed that the methylation patterns of the parents partially and dynamically passed onto their hybrids and was remodeled. In addition, the DNA methylomes of better-parent F1 hybrids were significantly different from that of lower-parent F1 hybrids, which indicates that having a methylation level between that of the two parents may be more favorable for the achievement of better-parent heterosis in F1 hybrids, while the deviation from MPV tends to preclude the establishment of heterosis. Compared with the parents and the lower-parent F1 hybrids, the hypermethylated genes in the better-parent F1 hybrids were enriched in the processes of metabolism and development, which may be highly relevant to heterosis.

Additional material

Additional file 1: Additional file 1 includes Table S1, which gives detailed information about the growth comparisons between the parents and F1 hybrids. Tree height and DBH were measured during five successive years. Means are given with \pm SE. Different letters indicate significant difference ($P < 0.05$) among the lines in trees of the same age.

Additional file 2: Additional file 2 includes Figure S1 and Figure S2. Figure S1 describes the trend of average coverage depth of DNA methylation in the intragenic region. The region within the dotted line indicates intragenic region. 2,000-bp regions upstream and downstream of intragenic are divided into 20 segments, and the intragenic are divided into 40 segments. Figure S2 provides details of GO analysis of differentially methylated genes. (A) GO analysis of differentially methylated genes between the maternal parent and better-parent F1 hybrids. Three comparison pairs (H1 versus P1, H2 versus P1, and H3 versus P1) were included in this analysis. (B) GO analysis of differentially methylated genes between the paternal parent and better-parent F1 hybrids. H1 versus P2, H2 versus P2, and H3 versus P2 were included in this analysis. (C) GO analysis of differentially methylated genes between the maternal parent and lower-parent F1 hybrids. Two comparison pairs (L1 versus P1, L2 versus P1) were included in this analysis. (D) GO analysis of differentially methylated genes between the paternal parent and lower-parent F1 hybrids. L1 versus P2 and L2 versus P2 were included in this analysis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GM and HQ carried out the experiments and performed manuscript draft writing. GM, HQ and CY performed bioinformatics and statistical analysis. DC and ZB participated in the design and coordination the study. SX designed the study and revised this manuscript. All authors read and approved the final manuscript.

Declarations

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