

Genome-wide high-resolution mapping of DNA methylation identifies epigenetic variation across different salt stress in Maize (*Zea mays* L.)

Lifang Sun · Xingfen Miao · Jin Cui · Jie Deng · Xia Wang · Yufeng Wang · Yifei Zhang · Shuren Gao · Kejun Yang

Received: 1 August 2017 / Accepted: 16 November 2017 / Published online: 11 January 2018
© Springer Science+Business Media B.V., part of Springer Nature 2018

Abstract DNA methylation is a vital epigenetic modification for the regulation of plant response to environmental stresses. In order to investigate changes in DNA methylation under salt stress, the levels of cytosine methylation in maize leaves at the seedling stage were estimated using the Methylated DNA Immunoprecipitation Sequencing (MeDIP-seq) method. The profiling of the DNA methylation results showed that a total of 163.27 million raw reads were obtained, with an average 33.06% of which were

uniquely mapped to a specific region in the maize genome. Cytosine methylation mainly occurred in CG, CHG, and CHH (H = A, T, or G) sites, and the CG contexts were lower than CHG and CHH contexts. The distribution of highly methylated regions (HMRs) mainly focused on the upstream 2k, intron, and downstream 2k, and the HMR distribution in these elements of YH 200 was higher than control (YH 0) and other samples. In addition, a total of 4402 differential methylated region (DMR)-associated genes were observed between stress samples and control, in which more hypomethylation-related genes were present than hypermethylation-related ones under 100 and 200 mmol L⁻¹ NaCl stress. Meanwhile, these DMR-associated genes were found to be involved in many biological functions by gene ontology (GO) analysis, such as cellular processes, metabolic processes, and signal transduction. Real-time qRT-PCR results showed that the expression of some methylated genes was consistent with the results of MeDIP-seq, while others showed an opposite trend, indicating that DNA-methylated regions did not uniformly affect the transcription of the corresponding genes. These experimental results are expected to improve our understanding of salt tolerance in maize.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10681-017-2076-0>) contains supplementary material, which is available to authorized users.

L. Sun · K. Yang
Postdoctoral Research Station for Crop Science of Heilongjiang Bayi Agricultural University, Daqing 163319, China

L. Sun · X. Miao · J. Deng · X. Wang · Y. Wang · Y. Zhang · S. Gao (✉) · K. Yang (✉)
Key Laboratory of Modern Agricultural Cultivation and Crop Germplasm Improvement of Heilongjiang Province, Agronomy College of Heilongjiang Bayi Agricultural University, Daqing 163319, China
e-mail: gaoshr107@126.com

K. Yang
e-mail: byndykj@163.com

J. Cui
Department of Plant Science, The Pennsylvania State University, University Park, PA 16802, USA

Keywords Maize (*Zea mays* L.) · Salt stress · DNA methylation · MeDIP-seq · Gene expression

Introduction

DNA methylation is one of the most important biological forms of epigenetic modification, which plays a significant role in many biological processes, such as gene regulation (Meng et al. 2016), plant growth and development (Yang et al. 2015), and response to abiotic and biotic stress (Dowen et al. 2012; Garg et al. 2015). In plants, DNA methylation is present in three forms, CG, CHG, and CHH, which are maintained by methyltransferase 1 (MET1) and chromomethylase 3 (Khan et al. 2016; Yaari et al. 2015).

When plants are exposed to environmental stresses, the degree of epigenetic modification is altered, which, in turn, affects the structure of chromatin and the interaction between DNA and proteins, ultimately regulating gene expression. Garg suggested that the DNA methylation levels of three rice cultivars (IR64, stress-sensitive; Nagina 22, drought-tolerant; Pokkali, salinity-tolerant) were significantly different under drought and salinity stress (Garg et al. 2015). The salt-tolerant rice variety Pokkali was remarkable in its ability to quickly relax DNA methylation in response to salt stress, whereas in the salt-sensitive rice variety IR29, the evidence for such reduction was not statistically supported (Ferreira et al. 2015). In addition, the analysis of *OsMYB91*, a R2R3 MYB transcription factor in rice, revealed that DNA demethylation in the promoter region of this gene could be increased under salt stress, leading to increased expression of the gene (Zhu et al. 2015). These results suggest that DNA modification could contribute to genetic variation and environmental adaptability. In recent years, besides model plants *Arabidopsis thaliana* (Xu et al. 2015) and rice (Garg et al. 2015; Ferreira et al. 2015; Zhu et al. 2015), many other plant species have been used to study DNA methylation under abiotic stress, such as cotton (Wang et al. 2016a, b), sorghum (Wang et al. 2010), soybean (Song et al. 2012), tomato (Huang et al. 2016), and *Populus trichocarpa* (Liang et al. 2014), which suggested that DNA methylation plays an important role in regulating plant adaptation to environmental stress. Maize is one of the most important food crops in our world. However, it is moderately sensitive to salt stress (Farooq et al. 2015), and an increase in soil salinization levels could limit its yield. Therefore, selecting salt-tolerant inbred lines of maize to study the genomic modification by DNA methylation might

help us understand the underlying molecular mechanism of salt-stress tolerance.

With the development of second-generation sequencing technology, Methylated DNA Immunoprecipitation Sequencing (MeDIP-seq) has been extensively used in many fields. It has led to the discovery of a series of key results in areas such as human diseases (Bell et al. 2016), bovine muscle tissue formation (Huang et al. 2014), mouse embryonic cell development (Ficz et al. 2011), male sterility in rice (Hu et al. 2015), and hybrid vigor in *Populus deltoids* (Gao et al. 2014). MeDIP-seq technology is based on the principle of antibody enrichment of the whole genome methylation and its subsequent detection, which can be accomplished at significantly lower costs than those associated with the bisulfite genomic sequencing technique, which is known as the “gold standard” for methylation detection. It can also generate broader coverage to discover key methylated sites. However, investigating the molecular mechanism of salt stress tolerance in maize is a novel application for this method.

In this study, we used MeDIP-seq to investigate the methylation level and obtain the differential methylated regions (DMRs) across different concentrations of salt stress (YH100, YH200, and YH250) and control (YH0), in a salt stress tolerant inbred line that was screened in our lab. A total of 4402 DMR-associated genes were identified, which are involved in many biological activities, such as cellular and metabolic processes, signal transduction, and catalytic activity. In addition, the expression levels of 7 DMR-methylated genes were assessed to analyze the relationship between transcriptional expression level and the DNA methylation level, using quantitative reverse transcription polymerase chain reaction (qRT-PCR). These results could provide new insights into understanding the molecular mechanisms of salt tolerance at the epigenetic level in maize, in addition to guidelines for further research.

Materials and methods

Plant samples and growth condition

A salt-tolerant inbred line, Hei maize, was screened from 96 inbred lines using salt stress, several years ago in our lab (Fu et al. 2011). It was used in this study to investigate the salt tolerance mechanism in maize. All

the seeds were sterilized in 0.1% NaClO (v/v) and then germinated at 28 °C in an incubator for about 3 days. The germinated seeds were sown in plastic pots measuring 20 cm in diameter and 22 cm in height (six plants per pot), containing 2.5 kg of vermiculite. All the pots were placed in a greenhouse (25 ± 1.5 °C during the day and 20 ± 1.5 °C at night) with a photoperiod of 14:10 h (light/dark). These pots were watered using 1/2 concentration of the Hoagland nutrient solution once a day.

Salt stress treatment

When the seedlings were 3 weeks old, 16 pots were selected from a large number of pots and randomly divided into four sets (four pots per set), in which the seedlings were growing uniformly. One set was used as a control, and the other sets were treated with 100, 200, and 250 mmol L⁻¹ NaCl, (named YH0, YH100, YH200, and YH250, respectively). Each pot was considered as a single replicate, and therefore, there were four replicates per set. Stress treatments were performed once a day with 1/2 Hoagland nutrient solution containing the appropriate concentrations of salt. Control plants were watered with just the 1/2 Hoagland nutrient solution. All pots were watered thoroughly and the experiment lasted for 7 days. Then, the four replicates of per set were mixed for DNA and RNA isolation.

Genomic DNA isolation

In order to comparatively analyze the methylation alterations between different salt concentrations and control, a single pooled genomic DNA sample was isolated from ten different plants of four pots (biological replicates) in one set. In addition, the samples were also used for real-time qRT-PCR analysis to maintain equal experimental conditions. Genomic DNA was isolated from leaves using the modified CTAB method (Kidwell and Osborn 1992; Sun et al. 2015). The quality and quantity of DNA was assessed using 1.0% agarose gel electrophoresis and spectrometric measurement (Additional file 1a).

MeDIP library construction

Four DNA libraries were constructed using the MeDIP-seq method, namely YH0, YH100, YH200,

and YH250. The MeDIP was performed according to a previously described protocol (Li et al. 2010). A total of 4 mg genomic DNA was sheared into 100–500 bp fragments with a Bioruptor (Sonics, Newtown, USA, VC130 PB), and the terminals of DNA fragments were modified using Paired-End DNA Sample Prep kit (Illumina co.) for end-repairing, A-tailing, and ligation to Illumina sequencing adapters (Sati et al. 2012). The sheared and modified genomic DNA was denatured in a 100 °C heat block for 10 min, then immunoprecipitated using 32-mg 5'-methylcytosine mouse monoclonal antibody in 400-mL IP buffer (10 mM Tris-HCl, pH 7.5; 280 mM NaCl; 1 mM EDTA) at 4 °C for 5.5 h according to the Magnetic Methylated DNA Immunoprecipitation Kit (Diagenod, Belgium). The enriched methylated DNA was amplified using QPCR method, then excised the bands between 200 and 300 bp from the gel, purified with the QLAquick Gel Extraction Kit (Qiagen, Germany), and quantified with the Quant-iTTM dsDNA HS Assay Kit (Invitrogen, USA) using an Agilent 2100 Analyzer (Agilent Technologies, USA). Following, the DNA libraries were sequenced on the Illumina HiSeq 2000 (Illumina, CA, USA) with the Illumina Genome Analyzer II (BGI, China) to obtain paired-end 50 bp reads.

Sequencing data analysis

The raw data were obtained from the Illumina sequencing of DNA, conducted by BGI at Shenzhen, China. First, adaptor sequence (3' and 5' adaptors) contamination and low quality reads were filtered and removed (Yan et al. 2010; Huang et al. 2014). Second, clean reads were mapped to the B73 genome assembly, using SOAPaligner v2.21 (<http://soap.genomics.org.cn>) with no more than 2-bp mismatches (Li et al. 2008). Third, the uniquely mapped data were used for read distribution analysis, including the distribution on each maize chromosome and the distribution in different elements of genome (such as CpG islands, the 2k upstream region, 5'-UTR, CDS, introns, 3'-UTR, the 2k downstream region, and repeats). In addition, the distribution of CpG, CHG, and CHH sites, varying with sequencing depth, were calculated for positive strand, negative strand, and both.

Lastly, genome-wide methylation peak scanning was conducted using the Model-based analysis of ChIP-Seq (MACS) v1.4.0 (<http://liulab.dfci.harvard.edu/MACS>) (Zhang et al. 2008). Moreover, the

number and distribution of peaks in different components were recorded. In addition, to detect differentially methylated regions (DMRs) between two samples, the peaks of two samples were merged. For each candidate DMR, the normalized read number of each sample was calculated. The false positive reads were removed using Chi square statistics and FDR statistics, to get true DMRs. DMRs were divided into two groups: the hypermethylated (up) and hypomethylated (down) DMRs. If the read number of sample 2 in this region was larger than that of sample 1, then the region was designated as hypermethylated during the sample 1 versus sample 2 comparison, whereas if the opposite was true, the region was designated as hypomethylated. Moreover, DMRs with a two-fold or greater difference in read numbers at $p \leq 0.01$ were selected.

Gene ontology (GO) annotation analysis

In order to identify the functional ontologies that are associated with the DMR-associated genes and to estimate the enrichment of the functional categories across the samples, an enrichment analysis based on Gene Ontology (<http://www.geneontology.org>) annotation was performed using TermFinder tool (<http://search.cpan.org/~sherlock/GO-TermFinder-0.86>), with $p < 0.05$.

Transcriptional analysis of selected genes by qRT-PCR

In order to analyze the relationship between gene expression and DNA methylation, real-time qRT-PCR was performed. The total RNA was extracted from leaves belonging to the four treatments using the RNAiso Reagent kit (Takara, Japan) (Sun et al. 2012). The quality and the integrity of RNA were measured by 1.0% agarose gel electrophoresis and spectrometric measurement (Additional file 1b).

Following a previously established protocol (Manickavelu et al. 2007), cDNA syntheses were performed with modifications, whenever necessary. A total of 1.5- μg total RNA was mixed with 0.5- μl RNase inhibitor (40 U/ μl), 2- μl oligo (dT)₁₈ primer (50 μM), 4- μl 5 \times M-MLV buffer, 4- μl dNTPs (2.5 mM each), 1- μl M-MLV reverse transcriptase (200 U/ μl), and RNase-free water to yield a final volume of 20 μl for making the first-strand cDNA synthesis reaction

solution. Next, the reaction solution was incubated at 42 °C for 1 h, and 70 °C for 15 min. Immediately after that, the reaction solution was placed on ice for 2 min. All reagents were purchased from Takara Bio Inc., Japan. The quality of cDNA was assessed using 1.0% agarose gel electrophoresis (Additional file 1c).

Real-Time qRT-PCR was carried out using a standard SYBR[®] Premix Ex Taq[™] (Takara, Dalian, China) on Bio-Rad CFX96 Real-Time System (Bio-Rad, USA, Hercules, CA). CFX Manager System software version 2.0 was used for analysis, according to the manufacturer's instructions. Maize *ACTIN* was used as the endogenous control gene. The primers were designed and synthesized according to the gene sequences in maize sequence database (<http://www.maizesequence.org/index.html>) (Additional file 2). To exclude the occurrence of primer and non-specific PCR products, the melt-curve data were obtained using the CFX Manager System software (version 2.0) (Additional file 3). The amount of transcript levels for each gene was calculated using the $2^{-\Delta\Delta C_T}$ method.

Results and analysis

Plant growth status under salt stress

The post-treatment growth status of plants is shown in Fig. 1. Compared with the control, the plant height gradually decreased with increasing salt concentration. Moreover, the plants showed normal growth under 100 and 200 mmol L⁻¹ NaCl stress, whereas at 250 mmol L⁻¹ NaCl stress, a small proportion of the leaves turned yellow.

Global mapping analysis of DNA methylation

For global mapping of DNA methylation in maize, a total of 163,265,308 MeDIP-seq raw reads from each sample were generated. Of the total reads, 93.27, 93.19, 92.97, and 93.07% were mapped to the reference genome for the YH0, YH100, YH200, and YH250 samples, respectively, of which, 27.76, 34.32, 35.18, and 34.96%, respectively, were uniquely mapped to a specific region in the maize genome (Table 1).

MeDIP-seq reads were detected in most chromosomal regions in each group, although there were some



Fig. 1 Observation of Hei inbred line under different NaCl concentration. YH0 was control; YH100, YH200 and YH250 was treated by 100, 200, 250 mmol L⁻¹ NaCl, respectively

Table 1 Data generated by MeDIP-seq

| Sample | Total number of reads | Total mapped reads | Total unique mapped reads | Percentage of mapped reads in total (%) | Percentage of unique mapped reads in total (%) |
|--------|-----------------------|--------------------|---------------------------|---|--|
| YH0 | 163,265,308 | 152,269,734 | 45,323,447 | 93.27 | 27.76 |
| YH100 | 163,265,308 | 152,153,396 | 56,030,086 | 93.19 | 34.32 |
| YH200 | 163,265,308 | 151,787,608 | 57,442,596 | 92.97 | 35.18 |
| YH250 | 163,265,308 | 151,954,258 | 57,072,876 | 93.07 | 34.96 |

gaps (Additional file 4). Genome coverage was calculated as the percentage of bases mapped by genome-wide reads. Additional file 5 shows that, in addition to CpGs, 5-methylcytosine can also be found in other sequences, such as CHG and CHH. The genome coverage of CG, CHG, and CHH (H = A, T, or G) sites under different sequencing depths are also provided in Additional file 5. This result indicated that the CG contexts were lower than CHG and CHH contexts, which is consistent with the results from studies on hybrid vigor in *P. deltoids* (Gao et al. 2014). The distribution of MeDIP-seq reads in different CG density regions is shown in Additional file 6. The densities of 25–30 CpGs/1000 bp showed the highest percentage of reads in the four groups.

According to the principle of MeDIP-seq method, the distribution of MeDIP-seq reads in different genomic components was analyzed to reflect the methylation level, including CpG islands, 2k upstream region, 5'-UTR, CDS, introns, 3'-UTR, 2k downstream region, and gene body, to examine the features of genome-wide methylation patterns. The results

showed that the uniquely mapped reads were mainly present in the CpGIs, 2k downstream, gene body, intronic, and 2k upstream regions, among which the percentages of CpGIs and gene body were the highest (Table 2). The proportion of CpGIs in YH0, YH100, YH200, and YH250 was 13.31, 10.09, 9.00, and 10.09%, respectively (Table 2). We all know that the proportion of CpGIs in each sample was widely used to predict the DNA methylation level (Xiang et al. 2010). Thus, the result indicated that the methylation level of salt stress samples (YH100, YH200, and YH250) may be lower than the control (YH0). In addition, to further analyze the distribution trend of DNA methylation in maize CpGIs, regions 2k bp upstream and downstream of the CpGIs were divided into 20 segments, and the CpGIs were divided into 40 segments. The results revealed that the 2k upstream and 2k downstream regions of CpGIs showed a greater number of methylated reads, obtained by counting the normalized average coverage depth (Fig. 2a).

We also analyzed the distribution of DNA methylation in the 2 kb region upstream of the transcription

Table 2 The distribution of MeDIP-seq uniquely mapped reads in different genome components

| Types | Reads number $\times 10^6$ | | | | Total uniquely mapped reads $\times 10^6$ | | | | Proportion of reads (%) | | | |
|---------------|----------------------------|--------|--------|--------|---|--------|--------|--------|-------------------------|--------|--------|--------|
| | YH 0 | YH 100 | YH 200 | YH 250 | YH 0 | YH 100 | YH 200 | YH 250 | YH 0 | YH 100 | YH 200 | YH 250 |
| CDS | 0.49 | 0.46 | 0.50 | 0.49 | 45.32 | 56.03 | 57.44 | 57.07 | 1.07 | 0.82 | 0.88 | 0.86 |
| CpGIIsland | 6.03 | 5.6 | 5.17 | 5.76 | 45.32 | 56.03 | 57.44 | 57.07 | 13.31 | 10.09 | 9.00 | 10.09 |
| Downstream 2k | 2.52 | 2.39 | 2.60 | 2.59 | 45.32 | 56.03 | 57.44 | 57.07 | 5.58 | 4.27 | 4.53 | 4.53 |
| 5'-UTR | 0.12 | 0.11 | 0.13 | 0.13 | 45.32 | 56.03 | 57.44 | 57.07 | 0.27 | 0.20 | 0.22 | 0.23 |
| Genebody | 3.04 | 2.9 | 3.09 | 3.09 | 45.32 | 56.03 | 57.44 | 57.07 | 6.70 | 5.20 | 5.37 | 5.41 |
| Intron | 2.38 | 2.30 | 2.41 | 2.42 | 45.32 | 56.03 | 57.44 | 57.07 | 5.26 | 4.11 | 4.19 | 4.23 |
| 3'-UTR | 0.19 | 0.18 | 0.20 | 0.20 | 45.32 | 56.03 | 57.44 | 57.07 | 0.43 | 0.32 | 0.36 | 0.36 |
| Upstream 2k | 2.87 | 2.70 | 2.95 | 2.95 | 45.32 | 56.03 | 57.44 | 57.07 | 6.33 | 4.81 | 5.13 | 5.15 |

start sites (TSSs), gene body (from the TSS to the end of the transcript), and the transcription termination sites (TTSs). Figure 2b shows that the 2k upstream region had a high number of methylated reads. In addition, the methylation level of YH200 was similar to that of YH250, which was significantly higher than that of the control (YH0). However, the methylation level of YH100 was dramatically lower than that of the control. This result indicated that the dramatically lower methylation in promoter sequences from YH100 could cause an over expression of some genes and this way increase the tolerance to a moderate salt stress. Meanwhile, higher concentrations of salt could drive to higher methylation levels as showed by the increment in methylation in the promoter regions of YH200 and YH250.

Meanwhile, the analysis of 18 repetitive types revealed that the 5 mC methylation of LTR/Gypsy was higher in the control (YH0) than in the salt stress treatments, while LTR/Copia showed the opposite result (Additional file 7). This indicated that many more methylated Gypsy and Copia repetitive types could be associated to the expression of salt-tolerance genes. The read numbers for each class of repetitive types are listed in Additional file 7.

Distribution of highly methylated regions

The uniquely mapped reads were used to detect the highly methylated regions (HMRs), called peaks, which are methylation-rich regions. A total of 137,960, 130,483, 126,545, and 129,876 peaks were identified in YH0, YH100, YH200, and YH250, respectively (Table 3), a downward trend in the

number of DNA-methylated regions with increasing salt concentration, but this trend was truncated for YH250. Meanwhile, the number of HMRs of YH 200 was significantly lower than in the control (YH0). These results displayed that salt stress could be tolerated using hypomethylation as “a genic over expression inductor”, but this mechanism appear to work up to some salt concentration (100 or 200 mmol L⁻¹); at higher concentrations methylation might not be a tolerance mechanism in this maize inbred line, or not the unique mechanism. The detailed data of HMRs is provided in Additional files 8 through 11; the number of HMRs in length is presented in Additional file 12; the data regarding the number of CpG sites in HMRs are also given in Additional file 13.

The distribution of HMRs in different components of the genome was also obtained through the comparison of the peaks' number (Fig. 3). The results showed that the 2k upstream (YH0: 6.74%, YH100: 6.93%, YH200: 7.27%, YH250: 7.13%), intron (YH0: 4.98%, YH100: 5.16%, YH200: 5.20%, YH250: 5.19%), and 2k downstream (YH0: 5.89%, YH100: 5.90%, YH200: 6.25%, YH250: 6.23%) regions, were all highly methylated regions, which also displayed that the HMR distribution in these elements of YH 200 was higher than control (YH 0) and other samples. Summarizing above two results, we found that DNA methylation levels of YH 200 was very lower and the ratios of highly methylated regions was great higher than control, which indicated that maize was in a higher hypomethylation state and the number of HMRs sites were significantly changed under 200 mmol L⁻¹ NaCl stress.

Fig. 2 Distribution of reads around CpG Islands and gene body. **a** Distribution of reads around CpG islands; **b** Distribution of reads around gene body. The DNA methylation profile in gene region was shown by the number of reads that were mapped on the unique locus in the genome. The upstream and downstream 2 kb regions were split into 20 equal regions. CpG islands and gene body were divided into 40 equal regions. For each region, the normalized number of reads was calculated. The “Y” axis is the average of the normalized depth for each region

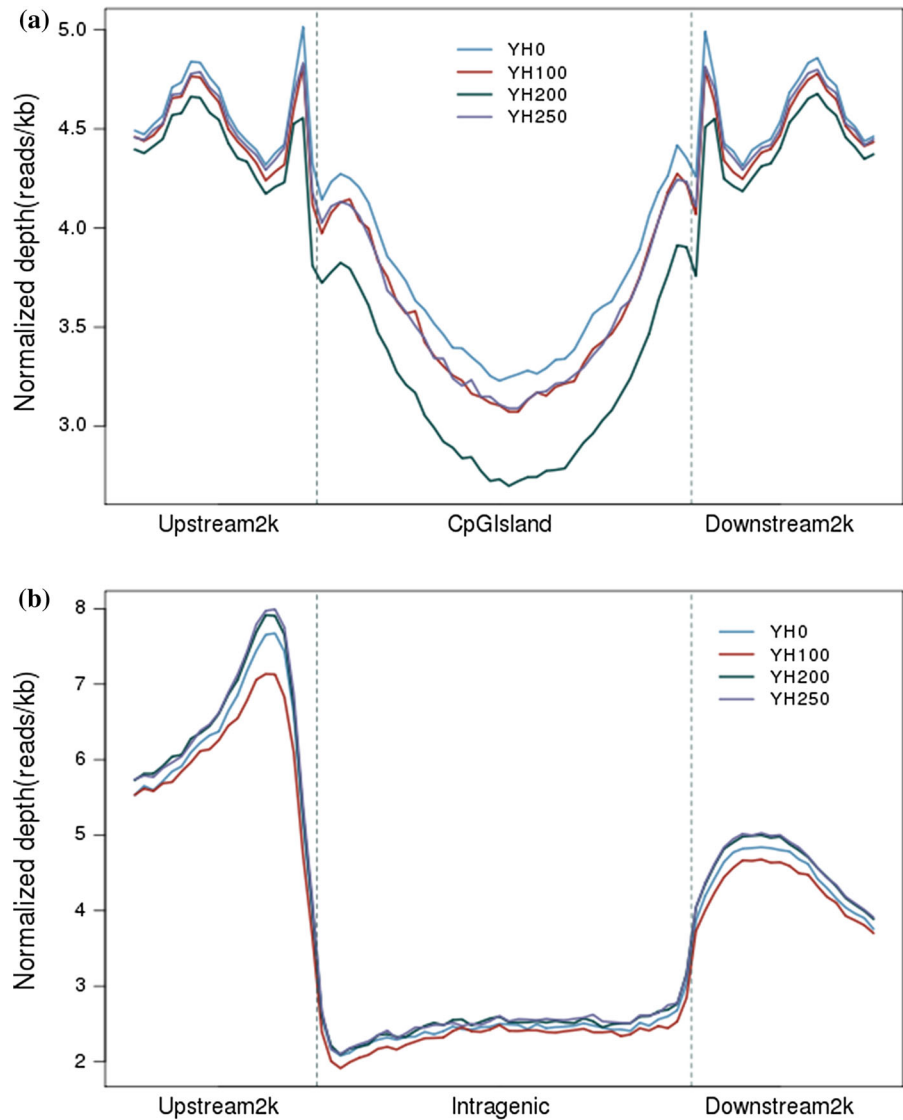


Table 3 Information of HMRs

| Sample | Total HMRs | HMR mean length | HMR total length | HMR covered size in genome (%) |
|--------|------------|-----------------|------------------|--------------------------------|
| YH0 | 137,960 | 1172.05 | 161,696,039 | 7.82 |
| YH100 | 130,483 | 1221.23 | 159,349,775 | 7.71 |
| YH200 | 126,545 | 1252.39 | 158,483,549 | 7.67 |
| YH250 | 129,876 | 1216.42 | 157,984,198 | 7.65 |

HMRs highly methylated regions

DNA methylation pattern in genes

To identify the genes associated with differentially methylated regions (DMRs) between the salt stress

treatments (YH 100, YH 200, and YH 250) and the control (YH 0), the peaks of different salt stress samples and control were merged and calculated, respectively. The number of DMRs-associated genes

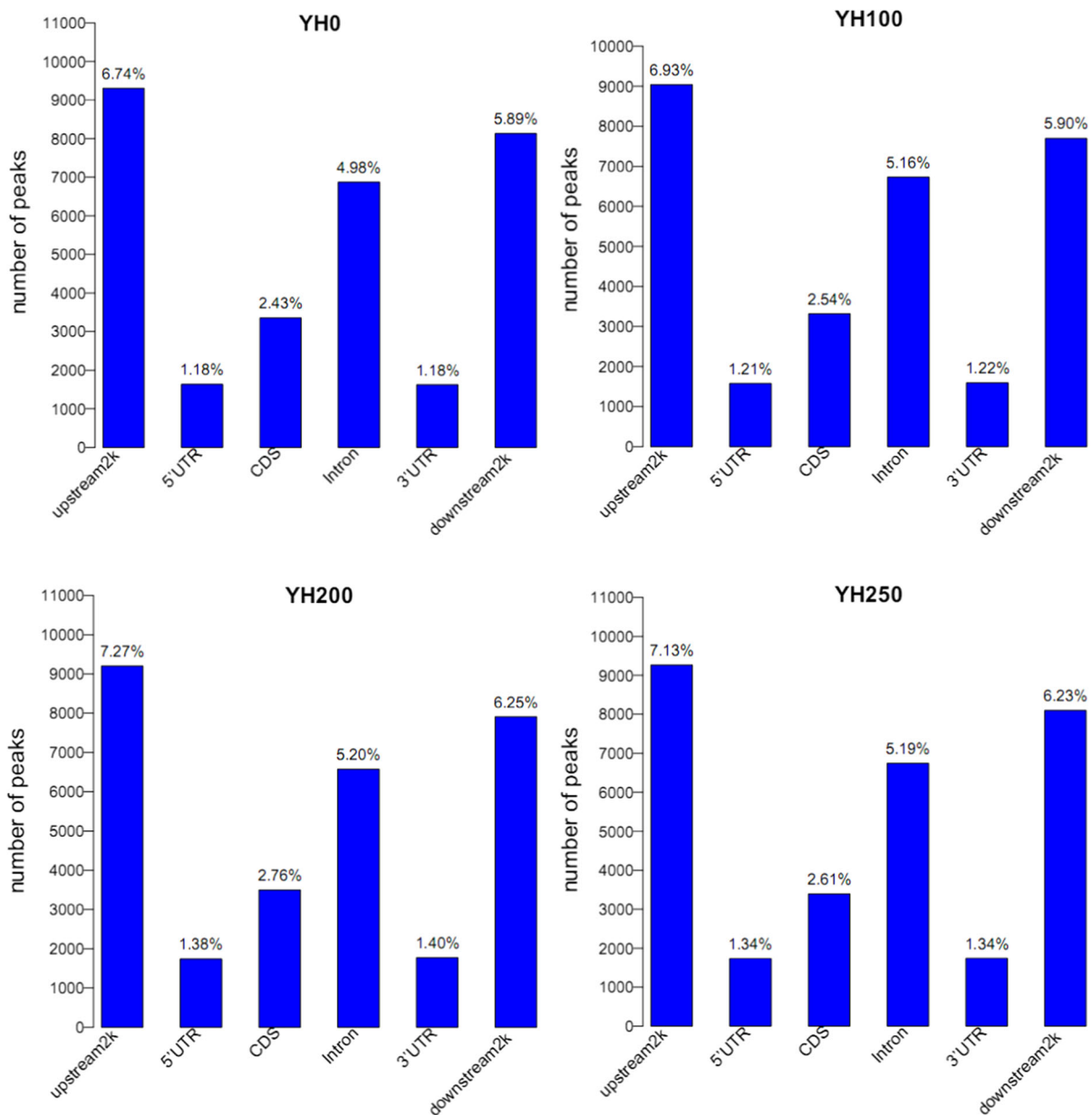


Fig. 3 HMRs distribution in different elements of genome in four samples. The x axis indicates different gene elements, and the y axis indicates the number of peaks in a specific gene element

occurred in different regions was list in the Table 4, and the detail information in the Additional file 14 through 16, which indicated that DNA methylated sites were mainly focused on the 2k upstream, intron, and 2k downstream regions. Integrated DMRs-associated genes of different regions and removed duplicate events, a total of 4402 DMR-associated genes were obtained: 566 hypermethylated and 828

hypomethylated genes in YH 100, 783 hypermethylated and 939 hypomethylated genes in YH 200, and 686 hypermethylated and 600 hypomethylated genes in YH 250 (Table 4), which suggested that YH 200 had numerous methylated genes than other treatments, and the number of hypomethylated genes was largest compared with hypermethylation genes in YH 100.

Table 4 Numbers of differentially methylated genes in different gene regions

| Contrast | YH0 versus YH100-Hypo | YH0 versus YH100-Hyper | YH0 versus YH200-Hypo | YH0 versus YH200-Hyper | YH0 versus YH250-Hypo | YH0 versus YH250-Hyper |
|---------------|-----------------------|------------------------|-----------------------|------------------------|-----------------------|------------------------|
| Upstream 2k | 265 | 192 | 289 | 281 | 193 | 244 |
| 5'-UTR | 39 | 20 | 53 | 41 | 34 | 39 |
| CDS | 88 | 81 | 113 | 122 | 89 | 103 |
| Intron | 251 | 207 | 353 | 239 | 215 | 214 |
| 3'-UTR | 59 | 34 | 46 | 78 | 29 | 45 |
| Downstream 2k | 307 | 171 | 290 | 262 | 182 | 232 |
| Total DMRs | 828 | 566 | 939 | 783 | 600 | 686 |

Hyper means hypermethylation; Hypo means hypomethylation

We also compared the DMRs-associated genes of YH 100, YH 200, and YH 250 compared with YH0 using venny 2.1 (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>). The results indicated that the majority of DMRs-associated genes were sample specific, and only 70 and 38 genes were commonly methylated between YH 100, YH 200, and YH 250. The two samples showing the greatest number of conserved methylated genes were YH100 and YH 200, sharing 210 common hypomethylated genes and 133 common hypermethylated genes (Fig. 4a, b). These results suggest that DNA methylation was dramatically affected by different salt concentrations stress and the higher number methylated genes, especially possible the hypomethylation genes, might play a vital role in the adaptation to environmental stress of Hei maize line.

Function annotation of DMR-associated genes

In order to examine the biological functions of DMR-associated genes, Gene Ontology (GO) Annotation (<http://www.geneontology.org>) was performed to categorize the methylated genes (Additional file 17). Figure 5 shows the classification of biological functions of DMR-associated genes in YH200; the corresponding figures for other treatments are presented in Additional file 18. These figures indicated that the significant enrichment of hyper-/hypomethylated genes was related to cellular and metabolic process, signal transduction, catalytic activity, etc. In addition, the number of hypomethylated genes was higher than the number of hypermethylated genes. We have also

listed some known genes in Additional files 19 through 24. These DMR-associated genes were related to the F-box protein, protein kinase, transposon protein, protein phosphatase, MYB DNA-binding transcription factor, MADS-box transcription factor, etc., and their further analysis might elucidate the functions of methylated genes under salt stress in maize.

Transcriptional analysis of selected genes associated with differential methylation

To further analyze the relationship between gene expression levels and DNA methylation levels under salt stress, real-time qRT-PCR was performed to assess seven DMR-associated genes (listed in the Table 5), which might play a significant role in salt resistance of maize. The results showed that the expression levels of *leucine-rich repeat protein kinase protein* (GRMZM2G097313), which is homologous to *Setaria italica leucine-rich repeat protein* gene, and *heat shock protein 70-4 isoform* (GRMZM2G340251) were dramatically lower under 100 mmol L⁻¹ NaCl (YH100) stress than in the control (YH0) (Fig. 6), suggesting that the expression of these two genes might be associated with the hypermethylation levels in the 2k upstream and 2k downstream/3'-UTR regions, respectively (Table 5). In addition, the expression levels of *WRKY DNA-binding domain protein* (GRMZM2G029292), and *SBP domain transcription factor protein* (GRMZM2G109354) showed a clear correlation with the methylation levels detected in the MeDIP-seq, as a further indication of methylation affecting gene expression. Moreover, the

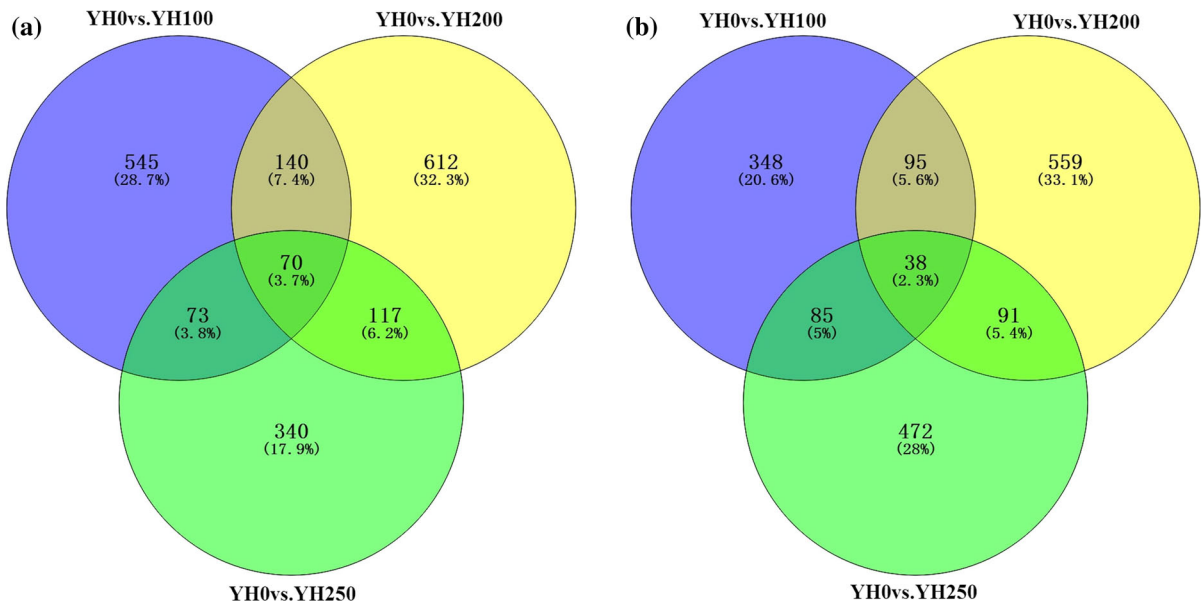


Fig. 4 Comparative analysis of the numbers of DMR-associated genes between the salt stress treatments (YH100, YH200, YH250) and control (YH0). **a** and **b**, Venn diagram of hypo- and hyper-methylated DMRs, respectively

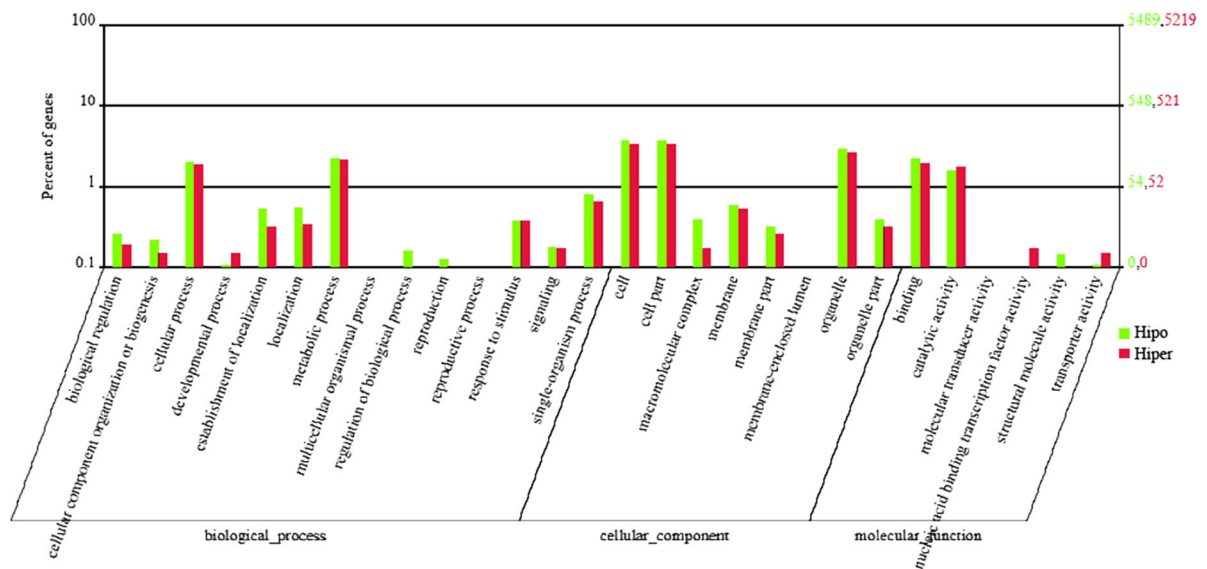


Fig. 5 GO classification of different genes in YH200. The x axis indicates the GO items, and the y axis indicates the proportion of genes involved, and the right vertical axis

indicates the exact number of genes. Hipo means hypomethylated of genes; Hiper means hypermethylated of genes

expression levels of *MYB DNA-binding domain protein* (GRMZM2G073826) also displayed in accordance with the MeDIP-seq result, but it was not significant, which might be due to the methylation not enough. However, the expression level of *RING zinc finger domain protein* (GRMZM2G085948) in YH100

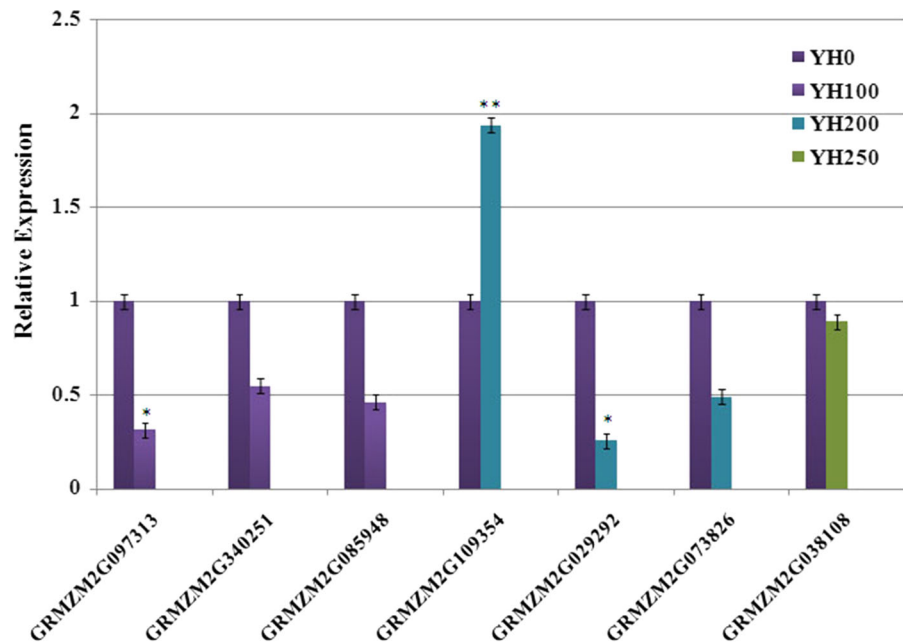
was in contrast to the result of the MeDIP-seq analysis, presumably due to the presence of complex methylated sites (2k downstream/5'-UTR/CDS/intron/3'-UTR). Similarly, the expression level of *activator of heat shock protein ATPase* (GRMZM2G038108) as assessed by real-time qRT-PCR in YH250, with

Table 5 The detected DMR-associated genes by real-time qRT-PCR

| Locus ID | DMR location | Annotation | Samples | Hyper-/Hypo- | P value |
|---------------|--------------------------------------|---|---------|--------------|-----------|
| GRMZM2G097313 | Upstream 2k | Leucine-rich repeat protein kinase protein [<i>Setaria italica</i>] | YH100 | Hyper- | 0 |
| GRMZM2G340251 | Downstream 2k/3'UTR | Heat shock protein70-4 isoform | YH100 | Hyper- | 0 |
| GRMZM2G073826 | Upstream 2k | MYB DNA-binding domain protein | YH200 | Hyper- | 2.43E-156 |
| GRMZM2G029292 | Downstream 2k/Intron | WRKY DNA-binding domain protein | YH200 | Hyper- | 2.85E-67 |
| GRMZM2G085948 | Downstream 2k/5'UTR/CDS/Intron/3'UTR | RING zinc finger domain protein | YH100 | Hypo- | 0 |
| GRMZM2G109354 | Downstream 2k/Intron | SBP domain transcription factor protein | YH200 | Hypo- | 0 |
| GRMZM2G038108 | Upstream 2k | Activator of heat shock protein ATPase | YH250 | Hypo- | 0 |

Hyper- hypermethylation, *Hypo-* hypomethylation

Fig. 6 Real-time qRT-PCR analyze of methylated genes. *Values significantly lower in salt stress sample than control at $p \leq 0.05$; **Values significantly higher in salt stress sample than control at $p \leq 0.01$



respect to the control, contrasted with the results from MeDIP-seq analysis (Fig. 6), presumably due to the serious injury and partial RNA degradation in plants under higher salt stress (250 mmol L⁻¹ NaCl). In addition, none of the two genes has significant difference for the qRT-PCR as shown in Fig. 6 and warrants further research. Finally, we may conclude that the DNA methylation of certain gene regions might affect transcriptional activity of genes, and some others may be not affected only by DNA methylation under salt stress in maize.

Discussion

Salt stress is one of the major environmental factors that severely affect plant growth and development, ultimately influencing the economic yield. Studies have demonstrated that DNA methylation plays a significant role in the regulation of plant stress tolerance. In rice, it was shown that salt stress triggered reduction of global methylation in most genotypes and mutations of epigenetic regulators resulted in better resistance to salt stress (Ferreira et al. 2015). Another study in rice showed that changes

in DNA methylation was highly correlated with rice salt. The study identified a series of stress related genes that were differentially methylated (Wang et al. 2015). In tomato, it was shown that down-regulating transgenic plant containing of a core epigenetic regulator exhibited enhanced tolerance to salt and drought stress compared to wild-type (Huang et al. 2016). Therefore, studying the relationship between DNA methylation and salt stress tolerance may help us better understand the epigenetic regulation of salt stress.

This study reported a comprehensive genome-wide epigenetic survey of different salt stress treatments, based on directly sequenced plant DNA methylomes. The data showed that a greater number of uniquely mapped reads for DNA methylation were located in CpG islands compared with other gene regions (Table 2), which is consistent with the results from other studies (Li et al. 2012a; Hu et al. 2015). Blackledge and Klose demonstrated that besides the promoters of genes as transcription start sites, CpG island elements utilize chromatin-based processes to create environments that contribute to the transcriptional potential of the associated genes (Blackledge and Klose 2011). Thus, highly methylated CpG islands might participate in regulation of gene transcription in our study. Moreover, there was a significantly high HMR distribution in the 2k upstream, intronic, and 2k downstream regions in this study, which is in agreement with the results from DMRs (Fig. 3). Furthermore, the number of hypomethylated DMR-associated genes was much higher than that of hypermethylated genes in YH100 and YH200 plants. Hypomethylation in plants is regulated by the DNA glycosidase subfamily, including DEMETER (DME) and Repressor of Silencing 1 (ROS1) (Martínez-Macías et al. 2012; Zhu 2009). Generally, the hypomethylated regions can lead to active genes expression. Previous studies have also reported that hypomethylation is a common feature associated with adaptive response to various stresses (Wang et al. 2014; Uthup et al. 2011; Angers et al. 2010). For example, hypomethylated sites were more frequent in drought-tolerant rice genotypes under drought stress (Joel 2013). Therefore, the high levels of hypomethylation in YH100 and YH200 might help activate genes relevant to salt stress tolerance. In addition, these DMR-associated genes are involved in many biological functions, and can be classified into many metabolic pathways based on the

GO annotation, which indicated that hypomethylation may be responsible for regulating many stress-related genes under salt stress.

Furthermore, seven DMR-associated genes were selected based on MeDIP-seq to detect the expression levels by real-time qRT-PCR. GRMZM2G097313 is hypermethylated in its upstream 2k region under salt stress treatment and belongs to the leucine-rich repeat protein kinase family. Its *Arabidopsis* homolog is responsible for mediating salt stress tolerance (Van der Does et al. 2017). GRMZM2G340251 encodes a heat shock protein and is hypermethylated in its downstream 2k region as well as in 3' UTR. Its *Agrostis stolonifera* homolog was strongly induced by salt, and may function as a protein chaperone to negatively regulate plant responses to adverse environmental stresses (Sun et al. 2016). GRMZM2G073826 is a MYB transcription factor and is hypermethylated in its upstream 2k region. Its rice homolog is involved in regulating plant growth as well as salt stress (Zhu et al. 2015). GRMZM2G029292 is a WRKY transcription factor and is hypermethylated in its downstream 2k as well as the intron region. GRMZM2G109354 is a *SQUAMOSA* promoter binding protein-like (SPL) transcription factor and is hypermethylated in its downstream 2k and intron region, previous study showed that SPL family transcription factors are responsive to abiotic stress treatment (Mao et al. 2016). GRMZM2G085948 encodes a zinc finger domain protein and is hypomethylated in its downstream 2k as well as gene body and UTR regions. Its *Arabidopsis* homolog is involved in regulating reactive oxygen species (ROS) and sodium/potassium homeostasis (Zang et al. 2016). GRMZM2G038108 is an activator of heat shock protein ATPase and is hypomethylated in its upstream 2k region. All seven genes are functionally related to abiotic stress and contain DMRs in their proximity and were therefore chosen for qRT-PCR validation.

The results showed that some genes were negatively correlated with methylation, while others displayed positive correlation to methylation (Fig. 6). Generally, the DNA methylation levels are dramatically higher in the 5' and 3' flanking regions of genes than in the gene body, and DNA methylation at promoter sites and around the transcriptional termination region (TTR) represses gene expression (Li et al. 2012b; Jones 2012; Tolley and Woodfield 2012; Baek et al. 2011). However, a previous study

demonstrated that the negative correlation between methylated promoters and the expression of downstream genes is still unclear to some extent, as the effects of DNA methylation are associated with imprinting and repression of mobile elements (Walsh and Bestor 1999). Even if it is possible, this effect could be overcome by an enhancer (Boyes and Bird 1992). Moreover, Rishi showed that in some cases, methylation is required for activation of transcription (Rishi et al. 2010). In addition, Medvedeva demonstrated that direct and selective methylation of certain transcription factor binding sites that prevents transcription factor binding is restricted to special cases and cannot be considered as a general regulatory mechanism of transcription (Medvedeva et al. 2014). Therefore, our results indicated that the expressions of some genes might be negatively/positively correlated with methylation via interference with promoter and transcriptional termination sites, under salt stress. In addition, gene body methylation may affect alternative splicing in plants (Wang et al. 2016a, b). Meng also reported that targeting of introns by plant microRNA might be a possible novel mechanism of gene regulation (Meng et al. 2013). Therefore, gene body methylations as well as microRNA may play a role in gene expression in this study.

Conclusion

We systematically analyzed the methylomes of a salt-tolerant inbred maize line under different concentrations of NaCl stress, using MeDIP-seq. The results showed that there were more hypomethylated regions than hypermethylated ones. Differences were observed in the total number of HMRs and DMRs, which were tightly associated with salt tolerance. In addition, our results revealed that some of the DNA-methylated regions might influence the transcription of the corresponding genes, whereas others do not.

Acknowledgments This study was supported by the Heilongjiang Provincial Youth Science Foundation of China (QC2015029), “Young innovative talents” project of Heilongjiang Bayi Agricultural University in China (CXRC2017002), Public welfare industry (agriculture) special funds for scientific research projects of China (201303007), and Doctoral instructor fund for higher education of Chinese educational ministry (20122305110001). In addition, we also thank Zhang H M of Life Science College of Heilongjiang Bayi

Agricultural University for preparing DNA isolation reagents in this study.

Authors contribution Conceived and designed the experiments: GSR and YKJ. Performed the experiments: DJ, WYF, and ZYF. Analyzed the data: SLF and WX. Contributed reagents/materials/analysis tools: MXF. Wrote the paper: SLF. Revise the paper: CJ. All authors read and approved the final version of the manuscript.

References

- Angers B, Castonguay E, Massicotte R (2010) Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after. *Mol Ecol* 19:1283–1295
- Baek D, Jiang JF, Chung JS, Wang BS, Chen JP, Xin ZG, Shi HZ (2011) Regulated *AtHKT1* gene expression by a distal enhancer element and DNA methylation in the promoter plays an important role in salt tolerance. *Plant Cell Physiol* 52(1):149–161
- Bell CG, Xia Y, Yuan W, Gao F, Ward K, Roos L, Mangino M, Hysi PG, Bell J, Wang J, Spector TD (2016) Novel regional age-associated DNA methylation changes within human common disease-associated loci. *Genome Biol* 17(193):1–14
- Blackledge NP, Klose R (2011) CpG island chromatin: a platform for gene regulation. *Epigenetics* 6(2):147–152
- Boyes J, Bird A (1992) Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. *EMBO J* 11:327–333
- Downen RH, Pelizzola M, Schmitz RJ, Lister R, Downen JM, Nery JR, Dixon JE, Ecker JR (2012) Widespread dynamic DNA methylation in response to biotic stress. *PNAS* 109(32):2183–2191
- Farooq M, Hussain M, Wakeel A, Siddique KHM (2015) Salt stress in maize: effects, resistance mechanisms, and management. A review. *Agron Sustain Dev* 35(2):461–481
- Ferreira LJ, Azevedo V, Maroco J, Oliveira MM, Santos AP (2015) Salt tolerant and sensitive rice varieties display differential methylome flexibility under salt stress. *PLoS ONE* 10(5):1–19
- Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 473:398–402
- Fu Y, Gao SR, Yang KJ, Yin KD (2011) Effects of salt stress on several physiological and biochemical indicators in seedling of salt tolerant line and salt sensitive line of maize (*Zea mays* L.). *Plant Physiol J China* 47(5):459–462
- Gao M, Huang Q, Chu Y, Ding CJ, Zhang BY, Su XH (2014) Analysis of the leaf methylomes of parents and their hybrids provides new insight into hybrid vigor in *Populus deltoides*. *BMC Genet* 15(1):1–17
- Garg R, Chevala VN, Shankar R, Jain M (2015) Divergent DNA methylation patterns associated with gene expression in rice cultivars with contrasting drought and salinity stress

- response. *Sci Rep* 5:144922. <https://doi.org/10.1038/srep14922>
- Hu J, Chen X, Zhang H, Ding Y (2015) Genome-wide analysis of DNA methylation in photoperiod-and thermo-sensitive male sterile rice Peiai 64S. *BMC Genom* 16(102):1–14
- Huang YZ, Sun JJ, Zhang LZ, Li C, Womack JE, Li ZJ, Lan XY, Lei CZ, Zhang CL, Zhao X, Chen H (2014) Genome-wide DNA methylation profiles and their relationships with mRNA and the microRNA transcriptome in bovine muscle tissue (*Bos taurine*). *Sci Rep* 4(6546):1–17
- Huang W, Xian Z, Hu G, Li Z (2016) SLAGO4A, a core factor of RNA-directed DNA methylation (RdDM) pathway, plays an important role under salt and drought stress in tomato. *Mol Breed* 36(3):1–13
- Joel AJ (2013) Epigenetic responses to drought stress in rice (*Oryza sativa* L.). *Physiol Mol Biol Plants* 19:379–387
- Jones PA (2012) Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 13(7):484–492
- Khan A, Yadav NS, Morgenstern Y, Zemach A, Grafi G (2016) Activation of Tag1 transposable elements in *Arabidopsis* dedifferentiating cells and their regulation by CHROMO-METHYLASE 3-mediated CHG methylation. *Biochim Biophys Acta (BBA)-Gene Regul Mech* 1859(10):1289–1298
- Kidwell KK, Osborn TC (1992) Simple plant DNA isolation procedures. *Plant Genomes*. https://doi.org/10.1007/978-94-011-2442-3_1
- Li R, Li Y, Kristiansen K, Wang J (2008) SOAP: short oligonucleotide alignment program. *Bioinformatics* 24(5):713–714
- Li N, Ye M, Li Y, Yan Z, Butcher LM, Sun J, Han X, Chen Q, Zhang X, Wang J (2010) Whole genome DNA methylation analysis based on high throughput sequencing technology. *Methods* 52(3):203–212
- Li MZ, Wu HL, Luo ZG, Xia YD (2012a) An atlas of DNA methylomes in porcine adipose and muscle tissues. *Nat Commun* 3:850. <https://doi.org/10.1038/ncomms1854>
- Li X, Zhu JD, Hu FY, Ge S, Ye MZ, Xiang H, Zhang G, Zheng X, Zhang H, Zhang S, Li Q, Luo R, Yu C, Yu J, Sun J, Zou X, Cao X, Xie X, Wang J, Wang W (2012b) Single-base resolution maps of cultivated and wild rice methylomes and regulatory roles of DNA methylation in plant gene expression. *BMC Genom* 13(300):1–15
- Liang D, Zhang Z, Wu H, Huang C, Shuai P, Ye C, Tang S, Wang Y, Yang L, Wang J, Yin W, Xia X (2014) Single-base-resolution methylomes of *populus trichocarpa* reveal the association between DNA methylation and drought stress. *BMC Genet* 15(1):1–11
- Manickavelu A, Kambara K, Mishina K, Koba T (2007) An efficient method for purifying high quality RNA from wheat pistils. *Colloids Surf B* 54:254–258
- Mao HD, Yu LJ, Li ZJ, Yan Y, Han R, Liu H, Ma M (2016) Genome-wide analysis of the SPL family transcription factors and their responses to abiotic stresses in maize. *Plant Gene* 6:1–12
- Martínez-Macías MI, Qian W, Miki D, Pontes O, Liu Y, Tang K, Liu R, Morales-Ruiz T, Ariza RR, Roldán-Arjona T, Zhu J (2012) A DNA 3′phosphatase functions in active DNA demethylation in *Arabidopsis*. *Mol Cell* 45(3):357–370
- Medvedeva YA, Khamis AM, Kulakovskiy IV, Ba-Alawi W, Bhuyan MSI, Kawaji H, Lassmann T, Harbers M, Forrest AR, Bajic VB (2014) Effects of cytosine methylation on transcription factor binding sites. *BMC Genom* 15(119):1–12
- Meng Y, Shao C, Ma X, Wang H (2013) Introns targeted by plant microRNAs: a possible novel mechanism of gene regulation. *Rice* 6(1):1–10
- Meng D, Dubin M, Zhang P, Osborne EJ, Stegle O, Clark RM, Nordborg M (2016) Limited contribution of DNA methylation variation to expression regulation in *Arabidopsis thaliana*. *PLoS Genet* 12(7):1–18
- Rishi V, Bhattacharya P, Chatterjee R, Rozenberg J, Zhao J, Glass K, Fitzgerald P, Vinson C (2010) CpG methylation of half-CRE sequences creates C/EBPalpha binding sites that activate some tissue-specific genes. *Proc Natl Acad Sci USA* 107:20311–20316
- Sati S, Tanwar VS, Kumar KA, Patowary A, Jain V, Ghosh S, Ahmad S, Singh M, Chandak GR, Raghunath M, Sridhar S, Chakraborty K, Scaria V, Sengupta S (2012) High resolution methylome map of rat indicates role of intragenic DNA methylation in identification of coding region. *PLoS ONE* 7(2):1–12
- Song Y, Ji D, Li S, Wang P, Li Q, Xiang F (2012) The dynamic changes of DNA methylation and histone modifications of salt responsive transcription factor genes in soybean. *PLoS ONE* 7(7):1–11
- Sun LF, Wu Y, Su SZ, Liu HK, Yang G, Li SP, Shan XH, Yuan YP (2012) Differential gene expression of maize (*Zea mays* L.) inbred line H99 during somatic embryogenesis. *Plant Cell Tissue Organ Cult* 109(2):271–286
- Sun LF, Liu TJ, Liu HK, Shan XH, Su SZ, Li SP, Yuan YP, Zhang J (2015) Analysis of DNA cytosine methylation patterns in maize hybrids and their Parents. *Biol Plant* 59(2):266–272
- Sun X, Sun C, Li Z, Hu Q, Han L, Luo H (2016) AsHSP17, a creeping bentgrass small heat shock protein modulates plant photosynthesis and ABA-dependent and independent signalling to attenuate plant response to abiotic stress. *Plant, Cell Environ* 39(6):1320–1337
- Tolley BJ, Woodfield H (2012) Light-regulated and cell-specific methylation of the maize PEPC promoter. *J Exp Bot* 63(3):1381–1390
- Uthup TK, Ravindran M, Bini K, Takurdas S (2011) Divergent DNA methylation patterns associated with abiotic stress in *Hevea brasiliensis*. *Mol Plant* 4:996–1013
- Van der Does D, Boutrot F, Engelsdorf T, Rhodes J, McKenna JF, Vernhettes S, Koevoets I, Tintor N, Veerabagu M, Miedes E, Segonzac C, Roux M, Breda AS, Hardtke CS, Molina A, Rep M, Testerink C, Mouille G, Höfte H, Hamann T, Zipfel C (2017) The *Arabidopsis* leucine-rich repeat receptor kinase MIK2/LRR-KISS connects cell wall integrity sensing, root growth and response to abiotic and biotic stresses. *PLoS Genet* 13(6):e1006832
- Walsh CP, Bestor TH (1999) Cytosine methylation and mammalian development. *Genes Dev* 13:26–34
- Wang H, Feng Q, Zhang M, Yang C, Sha W, Liu B (2010) Alteration of DNA methylation level and pattern in sorghum (*Sorghum bicolor* L.) pure-lines and inter-line F1 hybrids following low-dose laser irradiation. *J Photochem Photobiol B* 99(3):150–153
- Wang M, Qin L, Xie C, Li W, Yuan J, Kong L, Yu W, Xia G, Liu S (2014) Induced and constitutive DNA methylation in a

- salinity-tolerant wheat introgression line. *Plant Cell Physiol* 55(7):1354–1365
- Wang W, Huang F, Qin Q, Zhao X, Li Z, Fu B (2015) Comparative analysis of DNA methylation changes in two rice genotypes under salt stress and subsequent recovery. *Biochem Biophys Res Commun* 465(4):790–796
- Wang B, Zhang M, Fu R, Qian X, Rong P, Zhang Y, Jiang P, Wang J, Lu X, Wang D, Ye W, Zhu X (2016a) Epigenetic mechanisms of salt tolerance and heterosis in Upland cotton (*Gossypium hirsutum* L.) revealed by methylation-sensitive amplified polymorphism analysis. *Euphytica* 208(3):477–491
- Wang X, Hu L, Wang X, Li N, Xu C, Gong L, Liu B (2016b) DNA methylation affects gene alternative splicing in plants: an example from rice. *Mol Plant* 9(2):305–307
- Xiang H, Zhu J, Chen Q, Dai FY, Li X, Li M, Zhang HY, Zhang GJ, Li D, Dong Y, Zhao L, Lin Y, Cheng DJ, Sun JF, Zhou XY, Ma K, He Y, Zhao YX, Guo SC, Ye MZ, Guo GW, Li Y, Li RQ, Zhang XQ (2010) Single base-resolution methylome of the silkworm reveals a sparse epigenomic map. *Nat Biotechnol* 28(5):516–520
- Xu R, Wang Y, Zheng H, Lu W, Wu C, Huang J, Yan K, Yang G, Zheng C (2015) Salt-induced transcription factor MYB74 is regulated by the RNA-directed DNA methylation pathway in *Arabidopsis*. *J Exp Bot* 66(19):5997–6008
- Yaari R, Noy-Malka C, Wiedemann G, Gershovitz NA, Reski R, Aviva Katz, Ohad N (2015) DNA METHYLTRANSFERASE 1 is involved in mCG and mCCG DNA methylation and is essential for sporophyte development in *Physcomitrella patens*. *Plant Mol Biol* 88(4–5):387–400
- Yan H, Kikuchi S, Neumann P, Zhang W, Wu Y, Chen F, Jiang J (2010) Genome-wide mapping of cytosine methylation revealed dynaSmallc DNA methylation patterns associated with genes and centromeres in rice. *Plant J* 63(3):353–365
- Yang H, Chang F, You C, Cui J, Zhu G, Wang L, Zheng Y, Qi J, Ma H (2015) Whole-genome DNA methylation patterns and complex associations with gene structure and expression during flower development in *Arabidopsis*. *Plant J* 81(2):268–281
- Zang DD, Li HY, Xu HY, Zhang WH, Zhang YM, Shi XX, Wang YC (2016) An *Arabidopsis* zinc finger protein increases abiotic stress tolerance by regulating sodium and potassium homeostasis, reactive oxygen species scavenging and osmotic potential. *Front Plant Sci* 7:1272
- Zhang Y, Liu T, Meyer CA, Eeckhoutte J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS (2008) Model-based analysis of ChIP-Seq (MACS). *Genome Biol* 9(9):R137
- Zhu JK (2009) Active DNA demethylation mediated by DNA glycosylases. *Annu Rev Genet* 43:143–166
- Zhu N, Cheng SF, Liu XY, Du H, Dai MQ, Zhou DX, Yang WJ, Zhao Y (2015) The R2R3-type MYB gene *OsMYB91* has a function in coordinating plant growth and salt stress tolerance in rice. *Plant Sci* 236:146–156