

Salt Stress Induces Genotype-specific DNA Hypomethylation in *ZmEXPB2* and *ZmXET1* Genes in Maize

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(Received 5 September 2018; Accepted 22 November 2018;
Communicated by M. Molnár-Láng)

Maize, a moderately salt sensitive crop, first experiences osmotic stress that cause reduction in plant growth under salt stress. Fluctuation in cell wall elongation is one of the reasons of this reduction. Along with others, two important proteins expansins and xyloglucan endotransglucosylase are involved in regulation of cell wall elasticity, but the role of epigenetic mechanisms in regulating the cell wall related genes is still elusive. The present study was conducted with the aim of understanding the role of DNA methylation in regulating *ZmEXPB2* and *ZmXET1* genes. One salt sensitive and one salt tolerant maize cultivar was grown under hydroponic conditions at different levels of salt stress: T1 = 1 mM (control), T2 = 100 mM and T3 = 200 mM in three replicates. DNA and RNA were extracted from roots. After bisulfite treatment, Methyl Sensitive PCR was used for the DNA methylation analysis. It was revealed that fragment in promoter of *ZmEXPB2* gene showed high level of DNA methylation under T1 in both varieties. Comparison of different stress treatments revealed decrease in DNA methylation with the increase in salt stress, significantly lower methylation appearing in T3. Similarly, the fragment in promoter of *ZmXET1* gene also showed high levels of DNA methylation in T1. When different treatments were analysed, this gene significantly hypomethylated at T2 which continued to decrease in T3 in sensitive variety but remain stable in tolerant variety. Although, further in-depth analysis is required, our results demonstrate region-specific and genotype-specific methylation shift in the promoter of the *ZmEXPB2* and *ZmXET1* genes when subjected to the salt stress confirming the epigenetic regulation of these genes under stress conditions.

Keywords: salinity, DNA methylation, expansins, xyloglucan endotransglucosylase, *Zea mays* L.

Introduction

Maize (*Zea mays* L.), is the third most important cereal crop after rice and wheat. It is classified as moderately sensitive to salt stress (Chinnusamy et al. 2005). This salt stress causes the reduction in plant growth rate as well as per hectare yield. In maize, the high concentration of NaCl inhibits shoot growth, stomatal closure causing decreased photosynthesis, hinders the potassium uptake and production of reactive oxidative species

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(ROS) resulting in oxidative damages (SüMER et al. 2004; de Azevedo Neto et al. 2006; Shahzad et al. 2012).

Salt stress strongly affects the cell enlargement though influencing cell wall elongation. The elasticity of the cell wall depends upon various proteins like expansins (EXP), xyloglucan endotransglucosylase (XET), the plasma membrane proton pump (PM-H⁺-ATPase, MHA) and endo-1,4- β -D-endoglucanase (EGase) (Geilfus et al. 2011).

Expansins is a gene family encoding for plant cell wall loosening proteins and experiments with transgenes have confirmed their vital role in cell enlargement (Cosgrove 2000; Cosgrove et al. 2002). Though, the elongation growth of roots, internodes and leaves is highly related to the expression of expansin genes, each gene performs their particular role in plant development (Cho and Cosgrove 2000; Choi et al. 2003; Lee et al. 2003). Salt stress upregulates various genes of expansin gene family, i.e. *ZmEXPA1*, *ZmEXPA3*, *ZmEXPA5*, *ZmEXPB1*, *ZmEXPB2* whereas downregulates genes like *ZmEXB4* and *ZmEXPB6* (Li et al. 2014; Geilfus et al. 2015). These shifts in the gene expression are perceived as the adaptive mechanisms to mitigate the harmful effects of salt stress. The distinct role of each of these genes, in the plant's attempt to adapt and survive through regulation of root growth and development, under the stressful conditions is evident from the differential pattern of expression of different genes.

On the other hand, XET is the most common hemicellulose in primary cell wall. The precise role of XET is still elusive, although its involvement in the plant response to shift in water potential under salt stress have been observed (Vincent et al. 2007; Kosová et al. 2013). At transcriptional level, salt stress causes a differential shift in XET gene expression in different species (Takeda and Fry 2004; Wu et al. 2005; Li et al. 2014) providing evidence of an important role of this gene in influencing cell wall elongation under abiotic stresses. Single transcript of XET has been classified in the maize plant *ZmXET1* whose positive commitment toward the increase in plant growth against the salt stress of 200 mM has been observed. This gene in maize shows an encouraging overexpression under salt stress in the primary root boosting the vigour and other growth-related parameters by supporting the cell wall extensibility (Li et al. 2014).

Gene activation and silencing during different developmental stages as well as in response to varying environmental conditions (abiotic stresses) is achieved by regulation of chromatin structure through epigenetic mechanisms like DNA methylation and histone modifications (Pecinka et al. 2010; González et al. 2013; Khan et al. 2013; Li et al. 2014). Plant epigenetic based adaptability mechanism toward the salt stress environment have been reported, but the level of their involvement is still not completely understood. Study on maize under salt stress validates the role of histone acetylation in the positive regulation of *ZmEXPB2* and *ZmXET1* genes both in their respective promoter region (Li et al. 2014). The association between the DNA methylation and histone acetylation for the gene regulation level has been reported. Therefore, it can be anticipated that DNA methylation in coordination with histone acetylation can be involved in the regulation of these genes.

The aim of this study was to investigate the role of DNA methylation shift in regulating *ZmEXPB2* and *ZmXET1* genes under salt stress in *Zea mays* L. Methyl Sensitive PCR primers were used for studying DNA methylation profile in the promoter regions of these

two genes. Both the genes showed decrease in the DNA methylation due to salt stress along with the increase in the gene expression indicating that salt stress induces region and gene specific hypomethylation at promoter of *ZmEXPB2* and *ZmXET1* genes.

Materials and Methods

Plant material and treatments

The seeds of two maize cultivars, EV-22 (salt sensitive; V1) and Syngenta 8441 (salt tolerant; V2) were provided by the Cereal Research Centre, Ayub Agriculture Research Institute, Faisalabad on demand. Seeds were germinated through sandwich method. In this method, seeds of both cultivars were soaked overnight in 1 mM CaSO₄ solution. On the next day, these seeds were placed between the two layers of tissue papers which were positioned between two sheets of the foam making a sandwich. This sandwich was then rolled and tied up. All the rolls were put in the container quarter filled with 1 mM CaSO₄ solution and kept at 28 °C in the dark conditions. Five days old seedlings were transplanted into hydroponic culture. Nutrient medium was composed as described in Zörb et al. (2015). The salt stress was induced on emergence of fourth leaf. Three treatments were given: Treatment 1 or control (T1): 1 mM NaCl, Treatment 2 (T2): 100 mM NaCl and Treatment 3 (T3): 200 mM NaCl. The whole experiment was replicated thrice. The plants were subjected to these treatments for seven days.

DNA and RNA extraction

DNA and RNA were separately extracted from 200 mg of roots from each plant. DNA extraction was done through modified protocol of CTAB method and was quantified by spectrophotometry. For RNA extraction, the samples were treated with the DNase to get good results. RNA integrity was also analyzed on 1% agarose gel and quantification was carried out on spectrophotometer.

Bisulphite treatment and analysis of converted PCR product

Bisulphite treatment on 350 ng of each DNA sample was carried out through EZ DNA Methylation Gold kit (ZYMO Research, Germany) by following the manufacturer's protocol. From 10 µl eluted solutions, 1 µl was used for each PCR reaction. DNA sequences for both *ZmEXPB2* and *ZmXET1* genes were obtained from the maize genome data base MaizeGDB (<http://www.maizegdb.org/>). The CpG islands and cis-regulatory elements were found out from the cis-regulatory element data base (<http://www.dna.affrc.go.jp/PLACE/>). Methyl Primer Express v.1.0 software (Applied Biosystems) was used for designing primers for Methylation Specific PCR. The sequence and fragment lengths of the primer pairs is provided in Table S1*. The PCR amplicons obtained through these MSPs were visualized on 1% agarose gel stained with ethidium bromide. The quantification of bands on gel was done using ImageJ software.

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

PCR reactions were carried out in a final volume of 20 μ l containing 1 μ l bisulfite-treated DNA, 1X buffer with 2 mM MgCl₂, 0.25 mM dNTPs, 0.4 μ M of forward and reverse primers, and 1 Unit of hotstart Taq DNA polymerase (BioBasic®). The PCR program included initial denaturation at 94 °C for 5 min, then 30 cycles of denaturation at 94 °C for 30 seconds, annealing at primer pair specific annealing temperatures (given in Table S1) for 45 seconds and elongation at 72 °C for 45 seconds followed by final elongation at 72 °C for 4 minutes.

Gene expression analysis

For gene expression analysis 2 μ g RNA of each sample was converted to cDNA using TOPscript™ cDNA Synthesis Kit (Enzynomics®) according to the given protocol. The semi-quantitative PCR was carried out using optimized primers (Table S1) for *ZmEXPB2*, *ZmXET1* and *ACT2* (housekeeping) genes and the PCR product was visualized on 1% agarose gel. The band intensity was quantified by ImageJ software for each gene and then normalized by the *ACT2* gene. The PCR program included initial denaturation at 95 °C for 10 min, then 30 cycles of denaturation at 94 °C for 45 seconds, annealing at primer pair specific annealing temperatures (given in Table S1) for 45 seconds and elongation at 72 °C for 1 minute followed by final elongation at 72 °C for 10 minutes.

Statistical analyses of methylation variation

To test the significance of methylation variation, analysis of variance was performed by using the model $Y_{ij} = \mu + T_j + \epsilon_{ij}$ where T represents treatment effect (control and salt stress) and ϵ_{ij} the residual. As the Y_{ij} are percentages in the ANOVA.

Results

Salt stress affects the plant growth

After seven days of different levels of salt stress treatments, both the varieties showed signs of wilting and reduction in leaf growth. A genotype-specific effect of these treatments on the phenotype of plants was observed. Comparison of the two genotypes revealed that V1 was more affected by salt stress compared to V2. V1 genotype showed pronounced reduction in the shoot growth in treatment T3 with the wilting of leaves and the appearance of purplish color on the leaf tips. Interestingly, an increase in primary root length and stunted secondary roots with the elevated salt treatment was observed. In V2 genotype, the shoot also showed reduced growth with signs of leaf wilting and yellowing of leaf tips at higher concentration of salt stress. The increase in the primary root length and stunted secondary roots was observed but not as pronounced as in V1 (Figure S1).

DNA methylation analysis of *ZmEXPB2*

DNA methylation profile at the promoter of *EXPB2* gene from control plants of both V1 and V2 under normal conditions at 1.1kb upstream region from the Transcription Start Site (will be represented as -1.1k region in rest of manuscript) in the promoter was evaluated by using MSP primer pairs (E2B(MSP) -1.1k-M-F/R, E2B(MSP) -1.1k-NM, Table: S1). Slightly different DNA methylation profile was observed for the two maize varieties. V1 showed DNA hemi-methylation as strong band with methylated primers and a faint band with the unmethylated primers appeared on the gel (Figure 1a). Quantification of these bands with ImageJ software showed that the methylation percentage was 82.16% (Figure 1b). In variety V2, complete DNA methylation (100%) was observed as only bright band in methylated primer pair and no band in non-methylated primer pair. These results indicate towards genotype specific pattern of DNA methylation in promoter of *ZmEXPB2* gene.

Comparison of DNA methylation profile of both genotypes (V1 and V2) under normal and salt stress conditions revealed reduction in the DNA methylation level of both the genotypes with the increase in the salt stress conditions in -1.1k region in promoter of the *ZmEXPB2* gene. This state of DNA hypomethylation was more prominent in plants treated with 200 mM NaCl (T3) (Figure 1c). Quantification of observed bands on the agarose gel (through ImageJ software) revealed that both the genotypes were significantly different from each other in terms of DNA methylation shift though the pattern of DNA methylation variation was found to be similar in both genotypes. The genotype V2 showed higher level of DNA methylation compared to V1 (data not shown).

The quantification values for control (T1) were kept constant as shown in Figure 1b (V1T1 as 81.08% and V2T1 as 100%) to avoid any confusion. It was found that DNA

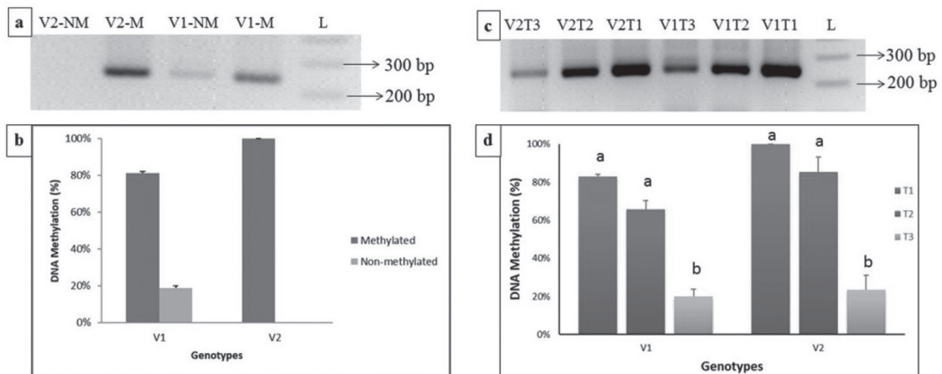


Figure 1. DNA methylation profile of EV-22 (V1) and Syngenta 8441 (V2) at the region of -1.1k of the promoter of *ZmEXPB2* gene under normal and salt stress conditions. a) PCR amplification of -1.1k region with methylated and non-methylated primers. M represents methylated primers and NM represents non-methylated primers. b) Comparison of DNA methylation profile of two genotypes through gel quantification data. c) PCR amplification of -1.1k region with methylated primers of both varieties at different salt treatments. d) Comparison of shift in DNA methylation profile (with methylated primer pair) of two genotypes at different salt treatments through gel quantification data

methylation decreased from 81.08% in T1 (control) to 65.63% in T2 (100 mM NaCl) and 20.03% at the level of T3 (200 mM NaCl) in V1. In V2 genotype, from 100% in T1 to 85% in T2 to 23% in T3. Statistical analysis revealed that the DNA methylation observed at T3 (200 mM) was significantly lower than that of other two treatments in both genotypes, whereas the difference in DNA methylation between T1 and T2, though visible, was nonsignificant (Figure 1d). V1 showed more methylation decline as compare to the V2 indicating that the V1 response to salt stress is more influenced by DNA methylation levels as compare to the V2.

DNA methylation analysis of *ZmXET1*

DNA methylation pattern in the promoter region of *ZmXET1* gene was investigated under normal conditions by studying 0.4 kb upstream region from the Transcription Start Site (will be represented as -0.4k region in rest of manuscript) through MSP primer pairs (X1(MSP) -0.4k-M-F/R, X1(MSP) -0.4k-NM, Table 1). Similar to *ZmEXPB2* gene, both V1 and V2 genotypes showed high level of DNA methylation in normal conditions though the pattern was slightly different. Software based quantification of bands showed that genotype V1 showed 78% DNA methylation whereas V2 revealed 100% DNA methylation under control conditions (Figure 2a). These results confirm that this gene is DNA hypermethylated in control conditions.

Plants, treated with different concentrations of NaCl, were compared to evaluate the influence of salt stress on the DNA methylation profile in the promoter region of *ZmXET1* gene. The results of amplification of -0.4k region of promoter revealed a marked decrease in the DNA methylation when the plants were treated with 100 mM NaCl and this decreased DNA methylation state continues even in higher salt concentrations (Figure 2a).

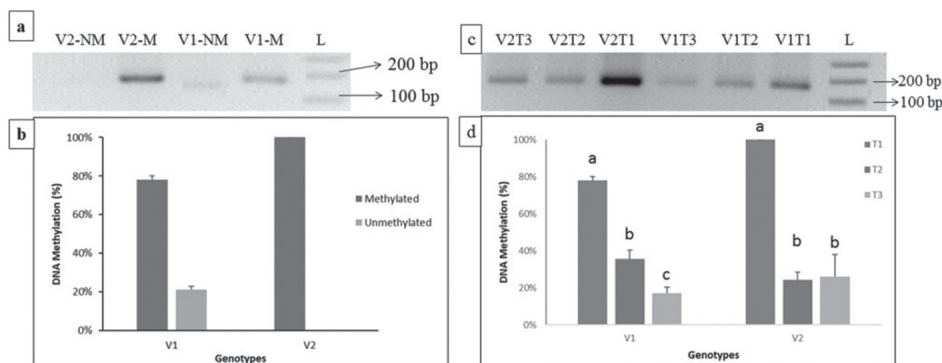


Figure 2. DNA methylation profile of EV-22 (V1) and Syngenta 8441 (V2) at the region of -0.4k of the promoter of *ZmXET1* gene under normal and stress conditions. a) PCR amplification of -0.4k region with methylated and non-methylated primers. M represents methylated primers and NM represents non-methylated primers. The V1-NM band is slightly lower because the expected band size is smaller than V1-M band b) Comparison of DNA methylation profile of two genotypes through gel quantification data. c) PCR amplification of -0.4k region with methylated primers of both varieties at different treatments. d) Comparison of shift in DNA methylation profile of two genotypes through gel quantification data

Quantification of band intensities through ImageJ software revealed that DNA methylation significantly decreased from 78% in control conditions to 35% in 100 mM NaCl (T2) to 17% in 200 mM NaCl (T3) in V1 genotype (Figure 2d). In V2 genotype the observed pattern is a bit different. The salt stress of 100 mM NaCl significantly decreased the DNA methylation, of -0.4k region in the promoter, to 24% from 100% in control. Interestingly, the level of DNA methylation did not significantly decrease further in the 200 mM NaCl concentration (Figure 2d). These results indicate towards genotype specific DNA methylation shift due to increased levels of NaCl concentrations.

Gene expression analysis of *ZmEXPB2* and *ZmXET1* genes

Semi-quantitative RT PCR was performed to analyze the shift in gene expression of *ZmEXPB2* and *ZmXET1* genes. *ACT2* gene was used as housekeeping gene for normalization purpose. In *ZmEXPB2* gene, the salt treatment significantly increased the expression of this gene at T2 (200 mM NaCl) in both V1 and V2 in comparison to control (Figure 3a). In contrast, the pattern of change in gene expression was different in *ZmXET1* gene. In V1, a gradual increase in the gene expression was observed whereas, in V2, the expression of *ZmXET1* gene significantly increased at treatment T2 (Figure 3).

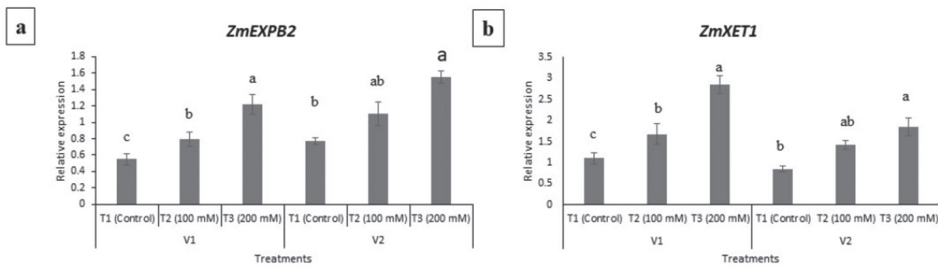


Figure 3. Relative gene expression levels of *ZmEXPB2* and *ZmXET1* genes in of EV-22 (V1) and Syngenta 8441 (V2). T1 = 1 mM (control), T2 = 100 mM and T3 = 200 mM of NaCl

Discussion

Salt stress can cause a decrease of 40–60% in vegetative growth in maize (Pitann et al. 2009/a). Salt concentrations of 0.25 M or higher have been reported to be lethal for maize plant due to lose of plant vigour and severe wilting ultimately leading to death in early stages of stress (Menezes-Benavente et al. 2004). In saline conditions, an important aspect that hampers the plant growth is fluctuations in cell wall extensibility through altering the function of cell wall regulating proteins (Fortmeier and Schubert 1995; SüMER et al. 2004). Salinity badly affects the apoplastic pH in maize which leads to the destruction of expansin protein family, one of the important proteins involved in cell expansion (Pitann et al. 2009/a).

In the current study, reduction in shoot growth has been observed with the increased salt concentration. Interestingly, the mild salt stress mainly showed the wilting effect and not affected much the plant height. The higher level of NaCl (200 mM NaCl) caused the reduction of shoot growth in addition to wilting of plants. These results are in accordance with the previous reports where reduction of shoot growth due to salt stress in maize have been reported (Pitann et al. 2009/a; Pitann et al. 2009/b; Szalai and Janda 2009; El Sayed 2011). Szalai and Janda (2009) reported the decrease in elongation rate as well as the number of elongating cells caused this reduction of shoot growth. The role of cell wall proteins like expansins has also been observed. In the apoplastic regions of leaf elongation zone, the expansins functions in an acid dependent manner to regulate the loosening of cell wall (Cosgrove 2000).

Salt stress affected root growth in rather unexpected fashion in this investigation. Though the stunted growth of secondary roots in both the genotypes was in line with the previous reports, the primary roots showed increase in their length in addition to root swelling with the raise in salt stress which is contrary to root behaviour reported in maize under saline conditions. This pattern was more prominent in V1 genotype. So, one hypothesis to explain this genotype specific behaviour of roots could be halotropism which is a type of tropism in which plants roots can circumvent to reduce their exposure to saline conditions. This pattern has been reported in different plants like Arabidopsis, tomato, *Glycin max*, tobacco and sorghum (Liu et al. 2000; Galvan-Ampudia et al. 2013), where plant roots show directional growth that allow them to move away from salt conditions. As this phenomenon was reported recently, complete understanding of this mechanism is still elusive. Particularly, in the perspective of current study, this hypothesis needs to be further evaluated in the context of gene regulation.

Various cell wall regulating genes have shown shift in their expression due to salt stress. This gene regulation can be mediated by epigenetic modifications. Among these genes, *ZmXET1* and *ZmEXPB2* were reported to show histone modifications under salt stress (Li et al. 2014). As the association between DNA methylation and histone acetylation have been observed (Cedar and Bergman 2009), therefore, DNA methylation profile of these genes have been investigated during this study.

Gene expression and DNA methylation in the promoter region has shown inverse relationship in many studies (Paszkowski and Whitham 2001; Choi and Sano 2007). This is due to the presence of *cis*-regulatory elements in the promoter that can interact with the transcription factors and influence gene regulation during stress conditions (Bjornson et al. 2016), and DNA methylation at these regions can cause the gene silencing. For this reason, 2000 bp upstream of the Transcription Start Site (TSS) for both the genes were evaluated through *cis*-regulatory element data base (<http://www.dna.affrc.go.jp/PLACE/>) for the presence of *cis*-regulatory elements.

In *ZmEXPB2* gene, *cis*-regulatory elements i.e. ABA-responsive elements (ABRE) and MYB core were found in 1.1 k region upstream of the TSS (in the promoter) (Figure S2a). In *ZmXET1* gene, MYB core and CAAT-box *cis*-regulatory elements were identified in the 0.4 kb upstream region of TSS (Figure S2b). Interestingly in both genes, Methyl Specific PCR showed high level of DNA methylation in both genotypes under

normal conditions in a genotype specific manner. Genotype V2 showed complete DNA methylation as compared to V1 where around 80% DNA methylation was recorded in both the genes. This genotype specific high level of DNA methylation is in accordance with the previous reports where DNA hypermethylation has been observed stress responsive genes under control conditions. In a recent study, high level of DNA methylation was revealed in ABRERATCAL and MYB-core *cis*-regulatory elements of the promoter region of *TaGAPC1* in wheat (Fei et al. 2017). Similar to our results, the relatively more tolerant wheat genotype showed higher level of DNA methylation under normal conditions. These results supported our hypothesis of involvement of DNA methylation in the regulation of this gene as many reports of epigenetically regulated genes have promoter methylation.

To further assess the effect of salt stress on the DNA methylation profile of *ZmEXPB2* and *ZmXET1* genes, plants treated with different levels of salt stress were evaluated. Since, both the genes showed relatively different pattern of DNA methylation, therefore, both the genes are discussed differently. In *ZmEXPB2* gene, both the genotypes showed significant DNA demethylation at 200 mM NaCl. Similar pattern of reduction in DNA methylation in the promoter has been reported in other salt responsive genes in various species like *AtHKT1*, *AtMYB74* in Arabidopsis (Baek et al. 2011; Xu et al. 2015), different transcription factors in soybean (Song et al. 2012) and *OsBZ8* in rice (Paul et al. 2017). Interestingly, the gene expression of *ZmEXPB2* have also shown significant increase at 200 mM NaCl treated plants indicating towards an association between DNA methylation and gene expression. These results provide preliminary confirmation of the involvement of DNA methylation, in addition to histone acetylation, in the regulation of *ZmEXPB2* gene.

In comparison to *ZmEXPB2* gene, the different pattern of reduction in DNA methylation in *ZmXET1* was revealed. This gene showed genotype-specific pattern in DNA methylation shift. A gradual DNA hypomethylation in genotype V1 (sensitive genotype) with increasing NaCl treatment was found. In comparison, genotype V2 (tolerant genotype) showed reduction with 100 mM NaCl treatment but then this DNA methylation remained unchanged with increasing NaCl concentration. In addition, the expression analysis also confirmed the genotype specific pattern of this gene. V1 gradually increased the gene expression with higher salt stress whereas genotype V2 showed no change at mild salt stress (T2) but showed a relative increase at 200 mM NaCl. Recent studies have shown that various stress responsive genes show genotype-specific pattern under stress conditions as shown in wheat (Kumar et al. 2017) and in rice (Paul et al. 2017). This could be due to the presence of different alleles in the two genotypes influencing the gene expression differently. As the gene from each of the used genotypes have not been sequenced this hypothesis needs to be tested.

Though further in-depth study to completely unveil the DNA methylation profile in these genes and its correlation with gene expression is recommended, we report the initial indication of presence of DNA methylation in promoter of two important genes in maize. A genotype-specific DNA hypomethylation in response to salt stress has been observed which upregulated the expression of these two genes. This shows that in addi-

tion to histone acetylation, DNA methylation in the promoter region plays its part to regulate these genes.

Acknowledgement

This research was supported by Higher Education Commission (HEC) of Pakistan under the project No. 21-472/R&D/HEC//2014. This paper is the part of MS thesis of Mr. Fawad Kaleem.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at <http://www.akademai.com/content/120427/>

Electronic Supplementary *Table S1*. Description of primer pairs with their sequence used for MSP and semi-quantitative Reverse transcriptase PCR

Electronic Supplementary *Figure S1*. Effect of different treatments of salt stress on plant growth of EV-22 (V1) and Syngenta 8441 (V2). T1 = 1 mM (control), T2 = 100 mM and T3 = 200 mM of NaCl

Electronic Supplementary *Figure S2*. Studied regions with their respective *cis*-regulatory sites in the promoters of *ZmEXPB2* and *ZmXET1* genes