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Effects of salinity on the DNA methylation pattern in barley shoots

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Abstract

Salinity is a crucial factor which inhibits crop production worldwide. Recent pieces of evidence reveal that epigenetic mechanisms modulate the gene expression in plants undergoing environmental stresses. Obviously, when the epigenetic regulation of plant growth and response to these stresses are truly understood, a novel heritable variation could be developed for crop improvement. The present study attempted to evaluate the DNA methylation alteration made by salt stress in two barley (*Hordeum vulgare* L.) cultivars differing in salt tolerance, namely salt-tolerant $Sahara_{3771}$ and salt-sensitive Clipper. Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA) was used to detect changes in the methylation pattern of the sequence CCGG in the nuclear genome of the plants growing under salinity stress (100 mM NaCl) and normal conditions. Leaf samples for DNA extraction were harvested 24 hours, 3 weeks, and 5 weeks after salt treatment. The results revealed that the average number of sites showing an increase in the methylation level at the three growth stages with the salt-stress imposition was higher in $Sahara_{3771}$ (26.21%) than in Clipper (16.32%). Moreover, the number of sites with an increase in methylation under salt stress in $Sahara_{3771}$ and Clipper, 24 hours and 5 weeks after imposing stress, respectively, was higher than the number of sites at the other stages. These results indicated a significant alteration of DNA methylation in plants as a response to salt stress and the effect was dose-dependent. These changes could provide a mechanism for the adaptation of plants under salt stress.

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Introduction

Salinity, affecting more than 950 million hectares of land globally, is considered one of the worst environmental factors which restrict crop yield. Owing to the climate change and immoderate irrigation, it is estimated that more than 50% of all arable land may have been salinized by 2050 (Ladeiro, 2012). Salt stress disrupts ionic equilibrium, inhibits enzymatic activity, creates osmotic imbalance, causes membrane disorganization, inhibits cell division and expansion, reduces photosynthesis, and produces reactive oxygen species (ROS) (Wang *et al*., 2003, Mahajan and Tuteja, 2005). ROS cause oxidative damage to nucleic acids, particularly modified bases, single or double-strand breaks in DNA, and make alteration to cytosine methylation (Weitzaman *et al*., 1994; Imlay, 2003).

Epigenetic mechanisms such as DNA methylation have a key role in regulating gene expression in plants' response to environmental stresses (Habu *et al*., 2001). DNA methylation is a form of enzymatic modification involving the addition of a methyl group to the carbon 5 (C5) position of cytosine (Zemach *et al*., 2010). The DNA methylation pattern changes in a variety of tissues at different growth and development stages and under biotic and abiotic stresses. This process plays an important role in regulating gene expression under stress conditions and making ecological adaptations (Suzuki and Bird, 2008). Plants have high levels of 5-methylcytosine (5mC). Depending on the species, it ranges from 6% to 25% of total cytosine (Grativol *et al*., 2012). Therefore, DNA methylation could be a regulatory mechanism.

In order to investigate the DNA methylation pattern in plants under different conditions, diverse methods are utilized; however, CRED-RA (Coupled Restriction Enzyme Digestion-Random Amplification) is a simple, easy, and economical technique for the initial study of DNA methylation. In this method, first, DNAs are separated into two groups. Each group is digested with one of the *Hpa*II/*Msp* Iisoschizomer enzymes. Next, digested products are amplified using random, 10-mer primers. Polymorphism between these products shows the methylation pattern of the band.

The two enzymes recognize the sequence 5ˊCCGGˊ3; however, *Hpa*II is not able to digest either of the methylated cytosines. Nevertheless, *Msp*I is capable of digesting DNA when the methylation of the inner cytosine happens. Both of the enzymes are able to digest DNA when no cytosine is methylated (McClelland *et al*., 1994).

Plant epigenetics has recently gained serious attention for both applied and basic research since understanding the epigenetic regulation of plant growth and development creates new genetic variation for the enhancement of crop productivity and adaptation to stress environment (Mirouze and Paszkowski, 2011). Bystudying the methylation pattern of the resistant and sensitive lines of rice under drought stress, Wang *et al*., (2011) demonstrated that there was a significant difference between the two lines in terms of methylation and demethylation sites. In addition, methylation changes induced by drought stress involved 12.1% of the total methylation changesin the rice genome. Approximately 70% of these changes reversed to their original status after recovering from stress and close to 29% remained unchanged. Erturk *et al*., (2013) applied the CRED-RA technique to the study of DNA methylation changes in corn (*Zea mays* L.) exposed to 5 Boron concentrations (0, 5, 10, 20, and 40 mM). The DNA methylation pattern revealed that Boron treatments, compared with the control, had caused an increase inthe amount of DNA methylation. Bednarek *et al*. (2007) studied epigenetic changes in the DNA methylation pattern in barley tissue culture using the isoschizomeric combinations *Acc*65I/*Mse*I and *Kpn*I/*Mse*I and reported that some somaclonal variations in calluses derived from changes in the DNA methylation pattern. In their study, the somaclonal variation related to DNA methylationdetected by pairs of enzymes was 6% and 1.7%, respectively. It is implied that epigenetic mechanisms get involved in the regulation of environmental stresses.

Barley (*Hordeum vulgare* L.) is considered to be one of the most salt-tolerant cereal crops. It is the fourth most cultivated crop in the world and could be widely found in arid and semiarid regions (FAO, 2014). In addition to its agronomic and economic significance, barley is used as an old model in genetic and physiological studies due to the remarkable diversity in physiological and morphological traits and a small number of chromosomes (Zhang and Li, 2010). This study was aimed at examining the effect of salinity on the cytosine methylation pattern in two barley cultivars differing in response to salt stress using the technique of CRED-RA.

Materials and methods

Plant material and growth condition

Two barley genotypes, namely Clipper (salt-sensitive) and Sahara$_{3771}$ (salt-tolerant), were used in the experiment. Seeds were obtained from the University of Western Australia. Sahara$_{3771}$ is a North African landrace and Clipper is a commercial Australian cultivar.

The experiment was carried out in a hydroponic culture system under greenhouse conditions. The cultivars were examined under two NaCl concentrations (0 and 100 mM) and leaf samples were harvested at three sampling time points. The experiment was conducted using a randomized block design with three replicates in a factorial manner. Seeds were sterilized using sodium hypochlorite and were germinated in Petri dishes. The seven-day-old seedlings of uniform size were transferred into pots containing perlite under controlled greenhouse conditions (15-hour daylight, 600-800 µmol m^{-2} s^{-1} photosynthetic photon flux density [PPFD], thermo period 25±5°C day/night, and relative humidity 45/60% day/night). The pots were sub-irrigated four times daily using a modified Hoagland nutrient solution. The solution temperature, electrical conductivity, and pH were monitored every day. Seven days after transferring the seedlings, salt stress was imposed. NaCl concentrations rose to 100 mM in daily increments of 50 mM NaCl. $CaCl_2$ was added to NaCl to sustain a Na^+/Ca^{2+} concentration ratio of 10:1 on a molar basis.

Genomic DNA isolation and CRED-RA analysis

Genomic DNAs were extracted using the CTAB method (Saghai-Maroof *et al*., 1984). The quality of the DNAs was tested using0.8% agarose-formaldehyde gel electrophoresis. The DNA concentration and purity were determined using spectrophotometer (A260 for DNA, A230 for polysaccharides, and A280 for proteins).CRED-RA consisted of two reactions, namely amplification and digestion. In the first reaction, the genomic DNA was amplified using random primers of 10 nucleotides (Table 1) and then the amplified products were digested with the enzymes *Hpa*II and *Msp*I. In the second reaction, the genomic DNA was digested with *Hpa*II and *Msp*I and the restricted products were amplified with the same random primers.

The polymerase chain reaction (PCR)was performed in a volume of 10 µl with2 µl of the genomic DNA, 1 µl of the primer, 4 µl of 2X Master Mix (Ampliqon), and 0.2 µl of $MgCl_2$. Thermal cycles consisted of the initial denaturation at 94 °C for 5 minutes, 40 cycles of denaturation at 94 °C for 1 minute, the binding of primers at their specific binding temperature for 1 minute, extension at 72 °C for 1.5 minutes, and the final extension at 72 °C for 10 minutes. The amplified products were separately treated using 1 µl of the enzymes *Hpa*II and *Msp*I at 37 °C for 16 hours. In the other reaction, 5 µg of the genomic DNA was separately digested with 1 µg of *Hpa*II and *Msp*I for 16 hours and then2 µl of the digested products was used as a DNA template in the PCR using the same random primers.

The amplified and digested products were separated using 1.5% agarose gelstained with ethidium bromide. The molecular-weight size marker with the fragments ranging in size from 100 to 3000 bp (Fermentase, #SM0321) was used in both sides and middle of the gel to determine the size of amplified fragments. The amplified and restricted fragments were scored according to the methylation patterns presented in Table 2.

Results

The DNA methylation pattern of the shoot of the cultivars Sahara$_{3771}$ and Clipper at three growth stages under salt treatments of 0 and 100 mM NaCl revealed an increase in both of the methylation and demethylation levels (Tables 3 and 4).

The average number of unchanged sites after imposing salt stress was 57.93% in Sahara$_{3771}$ and 72.41% in Clipper. Figures 1 and 2 show the number of unchanged, hypermethylated, and demethylated sites under salinity stress in Sahara$_{3771}$ and Clipper, respectively at different sampling time points.

Table 1. Primers used in CRED-RA analysis.

Number	Name	Sequence
1	465	5'-GGTCAGGGCT-3'
2	630	5'-CACTTAACCG-3'
3	MT25	5'-CCGTCTCTTT-3'
4	663	5'-CGTATAGCCG-3'
5	541	5'-GCCCCTTTAC-3'
6	454	5'-GCTTACGGCA-3'
7	428	5'-GGCTGCGGTA-3'
8	698	5'-CTAGACGTTG-3'
9	MT5	5'-CTCACCGTCC-3'
10	MT21	5'-CCCGCCGTTG-3'

*Hpa*II and *Msp*I digestion products of the control and 100 mM NaCl-treated plants of cultivars Sahara and Clipper were amplified with 10 random primers with length of 10 nt and different types of methylation alteration were detected (Fig. 3). Using primer #465, a total of 14 fragments were amplified at the three sampling stages (Tables 3 and 4). Salt stress increased the level of methylation in Clipper at all the stages. In Sahara$_{3771}$ at the 24-hour sampling time point, hypermethylation happened; however, at the 3-week and 5-week sampling time points, demethylation occurred.

Table 2. Methylcytosine sensitivity and restriction patterns of the enzymes (HpaII and MspI).

Type	Methylation pattern		HpaII	MspI
Type I	CCGG	CCGG	Active	Active
	GGCC	GGCC		
Type II	CCGG		Active	Inactive
	GGCC			
Type III	CCGG		Inactive	Active
	GGCC			
Type IV	CCGG		Inactive	Inactive
	GGCC			

Based on primer #630, 15 fragments were amplified at the three sampling stages under stress and control conditions in Sahara$_{3771}$ and Clipper (Tables 3 and 4). Compared with Clipper, Sahara$_{3771}$ showed a larger number of methylated fragments through the salt-stress imposition at all three stages.

In the cultivar Clipper, the lowest methylation changes pertained to the 3-week stage (2 demethylated sites) and the highest methylation levels related to the 5-weekstage (1 methylated and 3 demethylated sites).

Table 3. The methylation pattern of Sahara$_{3771}$ shoots under salt stress.

Primer	24 Hours			3 Weeks			5 Weeks		
	Without changes	Hypermethylation	Demethylation	Without changes	Hypermethylation	Demethylation	Without changes	Hypermethylation	Demethylation
465	11	2	1	4	4	6	8	2	4
630	8	6	1	8	6	1	8	4	3
MT25	6	1	7	4	6	4	7	4	3
663	2	15	0	13	1	3	9	6	2
541	9	1	3	10	3	0	11	1	1
454	11	6	1	12	5	1	14	4	0
428	11	2	1	5	3	6	9	1	4
698	6	0	6	8	3	1	5	4	3
MT5	1	12	1	11	2	1	8	6	0
MT21	10	2	2	12	0	2	11	2	1

The amplification of *Hpa*II and *Msp*I digestion products with primer MT25 revealed different types of methylation alteration. A total of 14 fragments were amplified at the three sampling stages (Tables 3 and 4).

In Sahara$_{3771}$, 24 hours after salt treatment, most of the changes were of the demethylation type. Methylated sites, 3 and 5 weeks after the salt-stress imposition, were more than demethylated ones.

Table 4. The methylation pattern of Clipper shoots under salt stress.

Primer	24 Hours			3 Weeks			5 Weeks		
	Without changes	Hypermethylation	Demethylation	Without changes	Hypermethylation	Demethylation	Without changes	Hypermethylation	Demethylation
465	9	5	0	11	2	1	9	3	2
630	12	2	1	13	0	2	11	1	3
MT25	13	1	0	7	2	5	7	4	3
663	11	2	4	15	1	1	9	6	2
541	12	0	1	12	0	1	6	7	0
454	16	2	0	15	3	0	14	2	2
428	9	4	1	13	0	1	10	3	1
698	10	0	2	10	1	1	5	3	4
MT5	11	1	2	11	2	1	9	5	0
MT21	6	5	3	11	2	1	8	2	4

In Clipper, 24 hours after imposing salinity, compared with the control treatment, only one fragment showed an increase in the methylation level; however, at the 3-week stage, most of the changes were of the demethylation type.

At the 5-week sampling time point, the methylation changes of Sahara$_{3771}$ and Clipper were the same but the differences at the 24-hour and 3-week sampling time points must have been the source of their different response to salt stress.

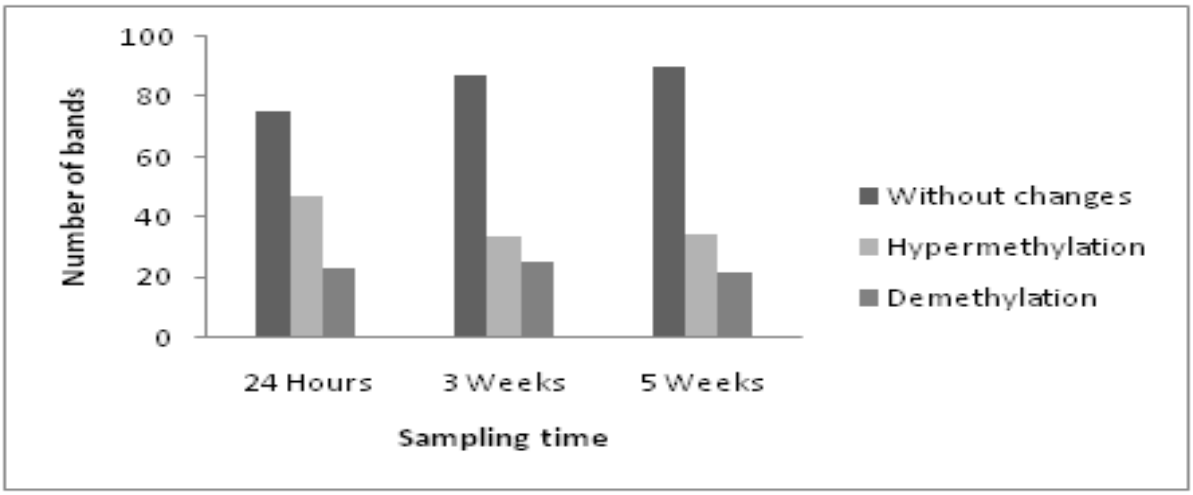

Fig. 1. The number of unchanged, hypermethylated and demethylated sites after imposing salt in a total of examined markers in the genotype Sahara$_{3771}$ at 3 sampling time points.

Seventeen fragments were amplified at the three sampling stages under stress and control conditions in Sahara$_{3771}$ and Clipper using primer #663 (Tables 3 and 4). After imposing salinity, the highest methylation changes at the enzyme recognition sites in the amplification fragments in Sahara$_{3771}$wereobserved at the 24-hour sampling stage (15 methylated sites). At the 5-week sampling time point, the methylation changes of Sahara$_{3771}$ and Clipper were the same but, at the 24-hour and 3-week sampling time points, Sahara$_{3771}$ revealed more changes in methylation than Clipper.

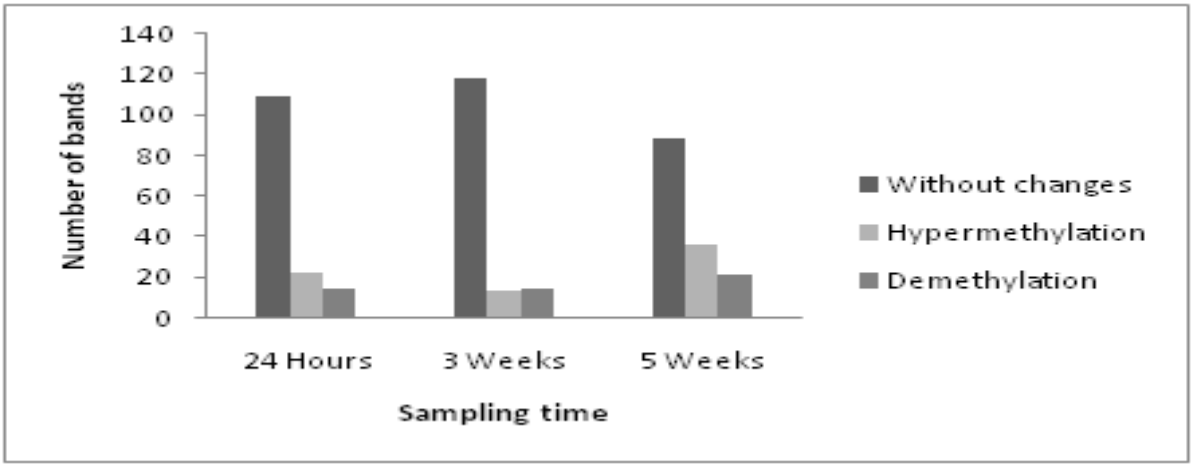

Fig. 2. The number of unchanged, hypermethylated and demethylated sites after imposing salt in a total of examined markers in the genotype Clipper at 3 sampling time points.

*Hpa*II and *Msp*I digestion products of control and 100 mM NaCl-treated plants of cultivars Sahara and Clipper were amplified with primer 541. A total of 13 fragments were amplified at the three sampling stages (Tables 3 and 4). Most changes in the methylation pattern were observed in the genotype Sahara$_{3771}$at the 24-hour stage (1 methylated and 3 demethylated sites) and in the genotype Clipper at the 5-week stage (7 methylated sites).

In total, 18 fragments were amplified at the three sampling stages under stress and control conditions in Sahara$_{3771}$ and Clipper based on primer #454 (Tables 3 and 4). In Sahara$_{3771}$, most of the changes at the three sampling stages were related to hypermethylation. The methylation changes in Sahara$_{3771}$ at the 24-hour and 3-week sampling time points were more than those in Clipper.

At the 5-weekstage, all the changes in Sahara$_{3771}$ were related to hypermethylation but, in Clipper, both hypermethylation and demethylation were observed.

Usingprimer #428, in total, 14 fragments were amplified at the three sampling stages under stress and control conditions in Sahara$_{3771}$ and Clipper (Tables 3 and 4). Most changes were observed in Sahara$_{3771}$ after 3 weeks (3 methylated and 6 demethylated sites) and in Clipper after 24 hours (4 methylated and 1 demethylated sites) of salinity treatment.

*Hpa*II and *Msp*I digested DNA of control and 100 mM NaCl-treated plants of the cultivars Sahara and Clipper was amplified with primer #698. A total of 12 fragments were amplified at the three sampling stages (Tables 3 and 4). In Sahara$_{3771}$ and Clipper, changes in the methylation level 5 weeks after salt treatment were more than those at the other two stages. However, Sahara$_{3771}$ showed more changes in the methylation than Clipper did at the 24-hour and 3-week sampling time points.

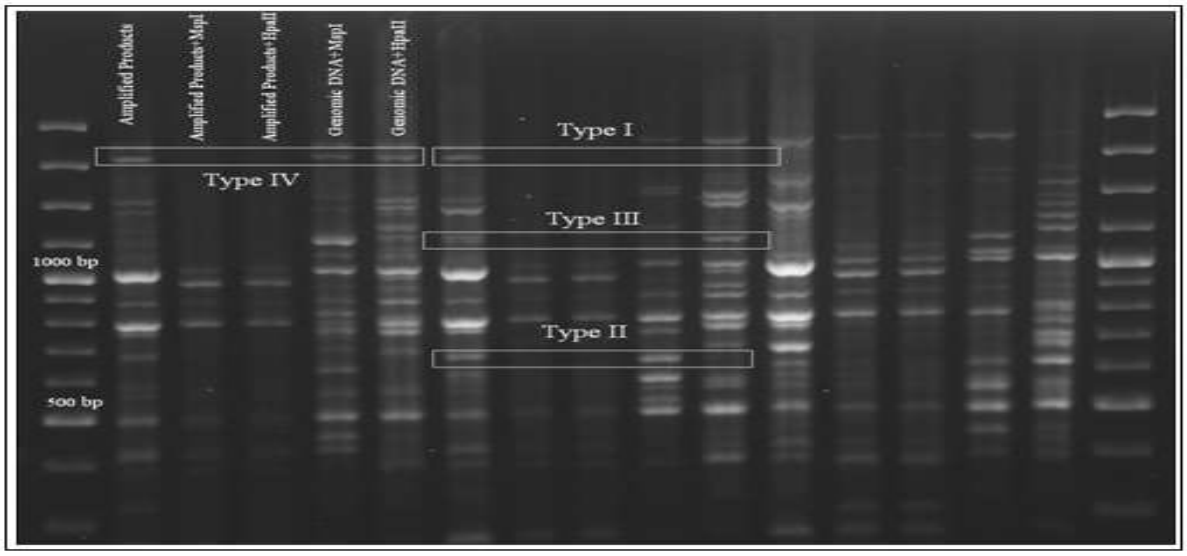

Fig. 3. CRED-RA profiles of control and 100 mM Nacl-treated plants of Sahara and Clipper cultivars.

MT5 primer -amplified14 fragments at the three sampling stages under stress and control conditions in Sahara$_{3771}$ and Clipper (Tables 3 and 4). In the genotype Sahara$_{3771}$, 24 hours after imposing salt stress, most changes in the methylation pattern were observed in response to salt (12 methylated and 1 demethylated sites). At the 3-week and 5-week sampling time points, the changes in the methylation pattern of Sahara$_{3771}$ and Clipper had no differences.

Based on primer MT21, 14 fragments were amplified at the three sampling stages under stress and control conditions in Sahara$_{3771}$ and Clipper (Tables 3 and 4). In Clipper, most change in the methylation level of the enzyme recognition site happened 24 hours after salt treatment (5 methylated and 3 demethylated sites) and fewest changes were observed 3 weeks after stress.

Unlike other primers, Clipper revealed more changes in the methylation pattern thanSahara$_{3771}$did at all of the three stages using this primer.

Discussion

In the present study, the average number of sites with an increase in the methylation level at three growth stages under salt stress was higher in Sahara$_{3771}$ than in Clipper. Zhong *et al.*, (2009) studied the methylation pattern of wheat salt-tolerant and sensitive cultivars under salinity of 100 mM NaCl and reported that the number of methylated sites in the tolerant cultivar was more than that in the sensitive one. Studying the methylated cytosine pattern in salt-tolerant and sensitive cultivars of rice 24 hours after salt treatment and control using 32 random primers, Karan *et al.*, (2012) reported that the level of methylation under salt stress increased in shoots of the salt-tolerant cultivar.

Moreover, a higher level of complete methylation, compared with incomplete methylation was observed in the root and shoot of the both cultivars under stress and control treatments. Demirkiran *et al*., (2013), using the technique of CRED-RA to study the epigenetic changes of barley under salt stress, reported that 23% of the fragments in the shoots howed a change in the methylation pattern.

The increase in the DNA methylation level could be a mechanism in salt-tolerant genotypes in response to salt stress. The number of sites with an increase in methylation under salt stress in Sahara$_{3771}$ and Clipper,24 hours and 5 weeks after salt treatment, respectively, was higher compared with the other stages. The increase in the methylation level of Sahara$_{3771}$ as a salt-tolerant genotype24 hours after salt treatment may shows that the high tolerance of this genotype to salinity was due to its initial quick response to stress, which developed tolerance to salinity at the other stages. The number of demethylated sites in the shoot of the salt-tolerant genotype Sahara$_{3771}$,24 hours and 3 weeks after salt treatment, was higher than that of the salt-sensitive cultivar Clipper; however, at the 5-weekstage,the pattern was the same in both cultivars. Karan *et al*., (2012) reported that, in the root of salt-tolerant genotype of rice, the number of methylated sites under salt treatment decreased.

Our study showed 38.3%, 31.7%, and 30% changes in the methylation pattern of the Sahara$_{3771}$ shoot 24hours, 3weeks, and 5 weeks after imposing salinity, respectively. In Clipper, 30% of the fragments 24hours, 22.5% of the fragments 3 weeks, and 47.5% of the fragments 5 weeks after salt treatment showed changes in the methylation pattern of the shoot. Having analyzed the methylation pattern of leaves in three maize inbred lines and their hybrids, 20, 32, 47, and 66, after planting, using the technique of CRED-RA, Tsaftaris and Polidoros (2000) reported that, at the four stages, 28.5%, 27.6%, 31.6%, and 31.4% of the sites were methylated, respectively.

Conclusion

The CRED-RA analysis indicates that salt stress brings about epigenetic variations in barley shoots. The number of sites with an increase in the methylation level in Sahara$_{3771}$ and Clipper was higher than the other stages 24 hours and 5 weeks after salt treatment, respectively. The methylation level of Sahara$_{3771}$ as a salt-tolerant cultivar increased 24 hours after salt treatment. The initial quick response to salt stress could be the reason for the high tolerance of this genotype to salinity stress. In this context, the late response of Clipper as a salt-sensitive genotype, compared with Sahara$_{3771}$, shows that this cultivar does not have enough time to compensate for salt damage. This study could broaden the understanding of the relationship between cytosine methylation and salt tolerance.

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