



Genetic and epigenetic effects of salinity on *in vitro* growth of barley

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Abstract

Morphological, physiological and molecular changes were investigated in *in vitro* salt-stressed barley (*Hordeum vulgare* L. cv. Tokak). Mature embryos were cultured in Murashige and Skoog medium containing 0 (control), 50 and 100 mM NaCl for 20 days. Both concentrations inhibited shoot growth, decreased fresh weight and protein content, and increased SOD (EC 1.15.1.1) activity in a dose-dependent manner. The lower concentration increased root growth. Salinity caused nucleotide variations in roots, but did not affect shoot DNAs. The higher concentration caused methylation changes, mainly hypermethylation in shoots. This is the first study on genetic and epigenetic effects of salinity in barley.

Keywords: *Hordeum vulgare*, Salt stress, Tissue culture, RAPD, CRED-RA.

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Introduction

The term salinity refers to the total dissolved concentration of major inorganic ions (Na, Ca, Mg, K, HCO₃, SO₄, and Cl) in irrigation, drainage and groundwaters. Certain ions, e.g. sodium (Na), are toxic to plants when present in excessive concentrations (FAO, 1992), so germination and growth of most plant species are inhibited by salinity (Anuradha and Rao, 2001; Agarwal and Pandey, 2004; Kirmizi and Bell, 2012). Salt stress causes disruption of ionic equilibrium, inhibition of enzymatic activity, osmotic imbalance, membrane disorganization, inhibition of cell division and expansion, reduction in photosynthesis and production of reactive oxygen species (ROS) (Mahajan and Tuteja, 2005). ROS cause oxidative damage to nucleic acids, including modified bases, single or double strand breaks in DNA, and alter cytosine methylation (Weitzman *et al.*, 1994; Imlay, 2003). Stress conditions alter cytosine methylation levels, independently of genetic variation (Lira-Medeiros *et al.*, 2010). Rapid adaptation to unfavorable conditions is achieved by regulating gene expression through cytosine methylation (Lewis and Bird, 1991; Causevic *et al.*, 2005; Lukens and Zhan, 2007). Stress-induced genetic (Liu *et al.*, 2005; Guangyuan *et al.*, 2007) and epigenetic (Guangyuan *et al.*, 2007; Labra *et al.*, 2002; Tan, 2010) variations were observed using molecular markers such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Methylation Sensitive Amplified Polymorphism

(MSAP) and Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA). Barley is a salt-tolerant crop (Ozturk *et al.*, 2002) and has been thoroughly investigated for many aspects of salinity (Mian *et al.*, 2011). Increase of lipid peroxidation, proline content, peroxidase, electrolyte leakage, activity of superoxide dismutase (SOD), ascorbate peroxidase, catalase and glutathione reductase, and decrease in relative water content and pigment content in barley were reported (El-Tayeb, 2005; Kim *et al.*, 2005; Mian *et al.*, 2011). Yet, to our best knowledge, there is no previous paper in the literature dealing with salinity, DNA damage and DNA methylation in barley. In this study, genetic and epigenetic variations were assessed in *in vitro* salt-stressed barley plants. For this purpose, we measured growth and SOD activity, the primary scavenger of ROS, and used RAPD and CRED-RA to investigate genetic and epigenetic variations, respectively, Random Amplified Polymorphic DNA (RAPD) to investigate genetic variations and DNA damage, and Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA) to investigate cytosine methylation changes. RAPD is a simple and fast technique for studying genetic diversity (Samal *et al.*, 2012). CRED-RA (Methylation-Sensitive-RAPD) was developed to detect DNA methylation (Uthup *et al.*, 2011). In this method, DNAs are divided into two groups and each group is digested with one of the *HpaII/MspI* isoschizomer pair restriction enzymes, after which the digestion products are amplified with random, 10-mer primers. Polymorphism between digestion products indicates the methylation pattern of the band. Both enzymes recognize the sequence 5'CCGG3', but *HpaII* cannot digest either of the methylated cytosines. *MspI* can digest DNA when the inner cyto-

sine is methylated. Both enzymes can digest DNA when none of the cytosines are methylated. Salinity (50 and 100 mM) affected fresh weight, shoot and root length, and SOD activity. Banding patterns of root DNAs of NaCl-treated plants were altered compared to control, while shoot DNAs of control and NaCl-treated plants revealed similar banding patterns. However, shoot DNAs exhibited a few changes in methylation status when treated with NaCl.

Materials and Methods

Plant material and culture conditions

Barley (*Hordeum vulgare* cv. Tokak) seeds were provided by the Aegean Agricultural Research Institute and cultured as described previously (Temel and Gozukirmizi, 2012). Embryos were cultured in MS (Murashige and Skoog) medium supplemented with 0 (Control), 50 and 100 mM NaCl, in a growth chamber (Sanyo). On the 20th day, 10 seedlings were used for estimating the percentage of germination, maximum shoot and root length, and fresh weight.

Estimation of total soluble protein levels and SOD activity

Seedlings were crushed in 5 volumes of ice-cold phosphate buffer (0.1 mol/L, pH 7.0) using a pre-chilled porcelain mortar and pestle. The homogenates were transferred into cold centrifuge tubes and centrifuged at 15,000 g, at 4°C for 20 min. The supernatants were used to estimate total soluble protein and SOD levels. The total soluble protein contents (mg/g) were estimated by a Bradford assay, using immunoglobulin (Sigma) as protein standard. SOD activity was measured spectrophotometrically, based on the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). One mL reaction mixture contained 50 mmol/L phosphate buffer (pH 7.0), 50 mmol/L sodium carbonate, 0.1 mmol/L EDTA, 13 mmol l-methionine, 75 µmol/L NBT (Sigma), 2 µmol/L riboflavin and 2 µL of 5 x diluted plant extract. The samples were illuminated with three 15-W white fluorescent lamps for 30 min at room temperature. An enzyme-free sample was used as positive control. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction at 560 nm. Enzyme activity was expressed as enzyme unit per µg protein per min (U/µg protein/min).

Genomic DNA isolation

Twenty-day-old seedlings were ground in liquid nitrogen and genomic DNAs (gDNA) were extracted (Tchorbadjieva and Pantchev, 2004). The gDNAs were resolved on 1% agarose gel in 1x TAE buffer, stained with ethidium bromide (0.5 µg/mL) and visualized under a UV transilluminator.

RAPD analysis

Sequences of five, 10-mer random primers (S1: CTAAGTGCCT, S7: TCCGATGCTG, S10: ACCGTTCCAG, S13: GTCGTTCCCTG, S19: GAGTCAGCAG) described elsewhere (Fernandez *et al.*, 2002) were used; the primers were provided by SACEM (Turkey). The PCR mixture consisted of 1x buffer [80 mM Tris-HCl, 20 mM (NH₄)₂SO₄, 0.02% Tween-20], 2.5 mM MgCl₂, 0.25 mM of each dNTP, 2 µM (20 pmol) primer, 0.5 U *Taq* polymerase (Solis BioDyne) and 10 ng gDNA template in a 10 µL reaction mixture. The amplification conditions were: an initial denaturation step of 5 min at 95°C, 40 cycles of 60 s at 94°C, 60 s at 50°C and 90 s at 72°C, and a final extension step of 10 min at 72°C. The amplification products were resolved on 1.8% agarose gel in 1x TAE buffer at 70 V/cm, stained with ethidium bromide (0.5 µg/mL) and visualized under a UV transilluminator. Band sizes were determined by comparison with a 100 bp DNA ladder (Fermentas).

CRED-RA analysis

One µg gDNA samples were digested with 1 µL (1 FDU) *Hpa*II (FD0514, Fermentas) and 1 µL (1 FDU) *Msp*I (FD0544, Fermentas) according to the manufacturer's instructions, purified with a phenol:chloroform:isoamyl alcohol (25:24:1) solution, dissolved in 10 µL of 10 mM Tris-HCl (pH 8.0) and used as template in CRED-RA. The primers used were S1 and S10. The CRED-RA mixture was the same as used for RAPD, except for the 3 µL digestion product as template. A 3 µL aliquot of the first PCR mixture was used as template in the second PCR assay. The amplification conditions were: an initial denaturation step of 5 min at 94°C, 40 cycles of 60 s at 94°C, 60 s at 50°C and 90 s at 72°C, and a final extension step of 5 min at 72°C. The amplification products were resolved on 2% agarose gel in 1x TAE buffer at 70 V/cm, stained with ethidium bromide (0.5 µg/mL) and visualized under a UV transilluminator.

Statistical analysis

The physiological experiments were repeated three times independently and each data point is the arithmetic mean of triplicates ($n = 3$). Data were analyzed by One-Way Analysis of Variance (ANOVA), and the statistical significance of the difference between two groups was determined by the Least Significant Data (LSD) test. Molecular assays (RAPD, CRED-RA) were repeated twice with different genomic DNAs isolated from biological replicates.

Results

Phenotypic and physiological analyses

Mature embryos were cultured in 0 (control), 50 and 100 mM NaCl-supplemented MS media for 20 days. All

embryos germinated in the control and 50 mM groups. However, the 100 mM NaCl concentration inhibited germination (85.5%, $p < 0.05$). Salinity also caused physiological changes (Table 1). Both NaCl concentrations decreased the protein content ($p > 0.05$), fresh weight ($p < 0.05$) and shoot length ($p < 0.05$), while root growth ($p < 0.05$) was induced by 50 mM NaCl. Both concentrations increased SOD activity ($p < 0.05$). Decrease in fresh weight [$r(2) = (-0.99)$, $p < 0.01$] and shoot length [$r(2) = (0.93)$, $p > 0.05$] and increase in SOD activity were dose-dependent: the higher concentration (100 mM) inhibited root growth, while the lower concentration (50 mM) stimulated it.

Genetic and epigenetic analyses

All five primers produced a total of 44 clear and reproducible bands. New bands were observed in salt-treated roots (Figures 1 and 2). The intensity of the same bands also increased in treated roots. No polymorphism was detected in shoot samples, in other words, the shoot DNA samples from different NaCl concentrations were monomorphic. The root and shoot RAPD patterns of the control group were different, but those of the NaCl-treated groups were similar. gDNA samples of control and NaCl-treated shoots were restricted with *HpaII* and *MspI* enzymes and then amplified using primers S1 and S10 (Figure 3).

A total of 21 bands were amplified, and five (23%) of them represent methylation changes. Band type 1 (500 bp) was cut by *HpaII* only in the control DNA. *HpaII* was not able to cut this amplicon in treated plant DNA. Inner cytosine was methylated in the control group, but none of the cytosines were methylated in the treatment groups. Band

type 1 represents a decrease in methylation by salt stress. Type 2 bands (250 and 650 bp) were cut by *MspI* but not by *HpaII* in the control group. Both enzymes failed to cut these

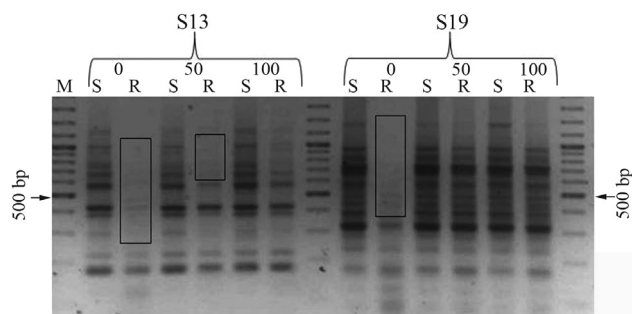


Figure 2 - RAPD results of primers S13 and S19. 0, 50 and 100 represent NaCl concentrations (mM). Polymorphic bands are indicated by rectangles.

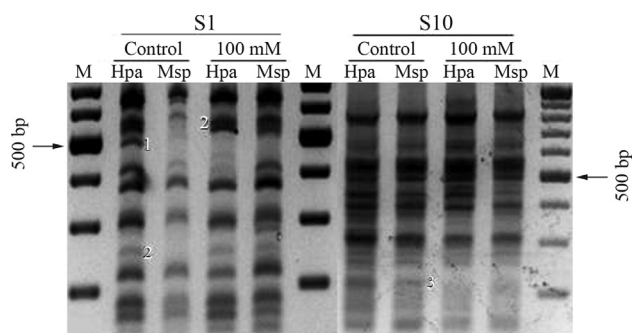


Figure 3 - CRED-RA results of primers S1 and S10 in 100 mM NaCl-treated plants and controls. Polymorphic bands are indicated with numbers. Detailed interpretation can be found in the text.

Table 1 - Effects of salt stress on total soluble protein content, SOD activity, fresh weight, max shoot and root length.

NaCl concentration (mM)	0 (control)	50	100
Protein content (mg/g)	51.28 ± 13.9	44.52 ± 15	39.12 ± 8.83
SOD activity (U/mg protein.min)	79.3 ± 0.02a	100.3 ± 0.034a	180.8 ± 0.059a
Fresh weight (mg/plant)	257 ± 0.022b	203 ± 0.007	157 ± 0.013b
Max shoot length (mm)	102.6 ± 1.09a	96 ± 0.115a	64.3 ± 0.87a
Max root length (mm)	30.1 ± 0.36a	44.5 ± 0.6a	22.7 ± 0.39a

a = $p < 0.01$; b = $p < 0.05$ (LSD).

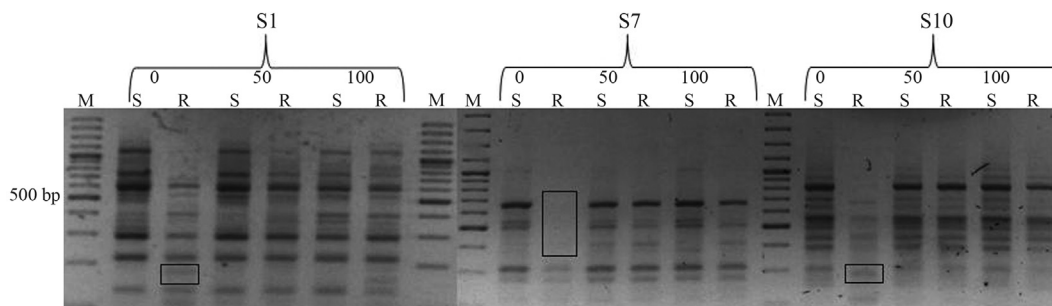


Figure 1 - RAPD results of primers S1, S7 and S10. 0, 50 and 100 represent NaCl concentrations (mM). Polymorphic bands are indicated by rectangles.

amplicons in the treatment groups. Only inner cytosine was methylated in the control group, while both cytosines were methylated in the treatment groups. Type 2 bands represent an increase in methylation (inhibition of *MspI* digestion) by salt stress. Two bands, very close to each other and approximately 575 bp in size, were also marked as number 2. Band type 3 (200 bp) may represent demethylation or mutation. Demethylation may have caused digestion and prevented amplification. Due to nucleotide variation, a recognition site may have occurred in the treated groups.

Discussion

Salt stress affects growth and germination (Anuradha and Rao, 2001). Root and shoot lengths and fresh weights of plants under salt stress are affected negatively, even at lower concentrations than the ones used in this study (Agarwal and Pandey, 2004). There are contradictory reports on protein content (Makela *et al.*, 2000; Zhu *et al.*, 2004) and SOD activity (Bor *et al.*, 2003; Dionisio-Sese and Tobita, 2004) of plants under salt stress. In this study, the 50 mM NaCl concentration did not cause a dramatic effect on growth and germination inhibition, while the 100 mM NaCl concentration did. In turn, root growth was induced at the 50 mM NaCl concentration. Protein content was decreased in both treatment groups. SOD activity was higher at both concentrations, especially at 100 mM NaCl that caused a dramatic increase. Tokak is also a drought-tolerant cultivar (Altinkut *et al.*, 2001) and has been used in several studies on salt or drought tolerance (Ozturk *et al.*, 2002). Salt stress also results in a water deficit condition in the plant, taking the form of a physiological drought (Mahajan and Tuteja, 2005). Therefore, we may postulate that Tokak is a salt-tolerant cultivar.

ROS are formed during salt stress and are harmful to macromolecules such as proteins, lipids and nucleic acids (Hernandez *et al.*, 1993). Oxidative DNA damage can affect the DNA methylation patterns (Franco *et al.*, 2008), and there are reports on salinity-induced DNA damage (Katsuhara and Kawasaki, 1996). However, this DNA damage was detected by agarose gel electrophoresis of DNA samples, which may fail to show less severe damage. Therefore, the use of molecular markers is crucial. Salt stress was reported to induce genetic modification (Guangyuan *et al.*, 2007), and the effect of NaCl-induced DNA damage was dose-dependent. It was also shown that salinity alters DNA methylation in plants (Dyachenko *et al.*, 2006). Salt stress-induced DNA methylation changes comprised both hypermethylation and hypomethylation events, but ended up leading to a net hypermethylation of the genome (Guangyuan *et al.*, 2007). In the present study, the RAPD patterns of shoot samples were monomorphic, as were the shoots and roots of NaCl-treated plants. However, some polymorphic bands were observed in roots of treated plants compared to controls. Fingerprinting patterns of shoots and roots of the control group were also altered. We

propose that NaCl caused nucleotide variations and appearance of new bands in roots which were exposed to NaCl before the shoots. The DNA samples of shoots were not affected by salt stress. Most of the polymorphic CRED-RA bands represent methylation of both cytosines in the 5'CCGG3' sequence of 100 mM NaCl-treated plants, allowing us to state that salt stress caused hypermethylation. However, the CRED-RA analysis was performed with shoot DNA samples only. There were two reasons for that: one was the low efficiency of DNA isolation from root samples, especially from treated ones, not yielding enough DNA for CRED-RA experiments. The second reason was the DNA polymorphism among root samples that does not allow to be sure whether the polymorphic CRED-RA bands are a result of DNA methylation or of DNA sequence variation.

In conclusion, although the biochemical and physiological effects of salinity were studied in detail, and mechanisms of salt stress and tolerance were reviewed exhaustively (Mahajan and Tuteja, 2005), there is still little information about the genetic and epigenetic effects of salinity in plants. We investigated here the physiological effects of salt stress at two NaCl concentrations (50 and 100 mM) in *H. vulgare* L. cv. Tokak, a drought-tolerant barley cultivar. The lower concentration of NaCl (50 mM) did not affect growth and germination dramatically, but interestingly favored root growth. DNA damage was observed only in roots, at both concentrations, and hypermethylation in shoots also occurred under salinity. Changes in methylation were observed when genetic variations were absent. This study may help understanding the relationship between cytosine methylation and salt tolerance.

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