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Research Article

Comparison of Salinity-Induced Changes in Two Cultivars of Barley

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Abstract

In vitro effects of salinity were compared in two cultivars of barley (*Hordeum vulgare* cv. Bornova-92 and Hilal). Mature embryos were cultured on Murashige and Skoog media supplemented with 0, 50 and 100 mM NaCl for 20 days. NaCl-treatment decreased maximum shoot length, total soluble protein and DNA contents in both cultivars but decreased maximum root length in Hilal. Changes in fresh and dry weight and water content were not statistically-significant. Inhibitory effects were more dramatic in Hilal. Salinity did not cause genotoxic effects in both cultivars yet slightly affected protein patterns in Bornova-92. However, salinity altered cytosine methylation patterns from CCG to CG in Bornova-92, from CG to CCG in Hilal. Bornova-92 and Hilal may be regarded as salt-tolerant and salt-susceptible, respectively and the relative salt-tolerance of Bornova-92 may be due to cytosine methylation patterns and/or regulation of protein synthesis.

Keywords: Cytosine methylation, *Hordeum vulgare* L., RAPD, Salt stress, SDS-PAGE.
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İki Arpa Varyetesinde Tuzluluğun Etkisiyle Oluşan Değişimlerin Karşılaştırılması

Özet

Tuzluluğun *in vitro* ortamdaki etkileri 2 arpa varyetesinde (*Hordeum vulgare* cv. Bornova-92 and Hilal) karşılaştırıldı. Olgun embriyolar 0, 50 ve 100 mM NaCl içeren Murashige ve Skoog besiortamında 20 gün boyunca kültüre alındı. NaCl uygulaması maksimum sürgün boyunu, total çözünebilir protein ve DNA içeriğini her 2 varyetede de azaltırken; maksimum kök uzunluğunu Hilal varyetesinde azalttı. Taze ve kuru ağırlıktaki ve su içeriğindeki değişimler istatistik olarak anlamlı bulunmadı. İnhibe edici etkiler Hilal varyetesinde daha belirgindi. Tuzluluk her 2 varyetede de genotoksik etki oluşturmamasına karşın Bornova-92 varyetesinde protein profilini biraz etkiledi. Tuzluluk, Bornova-92 varyetesinde sitozin metilasyonunu CCG'den CG olacak şekilde etkilerken; Hilal varyetesinde CG'den CCG olacak şekilde etkiledi. Bornova-92 ve Hilal, sırayla tuza dayanıklı ve duyarlı olarak tanımlanabilir ve Bornova-92'nin görece tuzluluk dayanıklılığı sitozin metilasyon profilinden ve/veya protein sentezinin düzenlenmesinden kaynaklı olabilir.

Anahtar kelimeler: Sitozin metilasyonu, *Hordeum vulgare* L., RAPD, Tuz stresi, SDS-PAGE.

Introduction

Salinity causes nutrient deficiencies due to the competition of sodium (Na^+) and chloride (Cl^-) with potassium (K^+), calcium (Ca^{2+}) and nitrogen. These minerals have many vital roles such as protein and nucleic acids synthesis, photosynthesis, membrane function and cell division (Hu and Schmidhalter 2005). Higher concentrations of sodium ions inhibit the activity of many enzymes; cause reduction in photosynthesis and the production of reactive oxygen species (ROS) and disrupt the integrity of cellular membranes (Zhu 2001; Tuteja 2007). ROS are also harmful for proteins, lipids and nucleic acids (Hernandez et al. 1993). DNA damage occurs as modified bases and single and double strand breaks (Imlay 2003). Salinity causes nucleotide variations (Lu et al. 2007); alters cytosine methylation (Tan 2010) and affect protein profiles in plants (Rasoulnia et al. 2011). Barley (*Hordeum vulgare* L.) is regarded as a salt-tolerant crop whereas barley cultivars and *Hordeum* species show different levels of tolerance to salinity (Fatehi et al. 2012). In this study, the aim was to compare salinity responses of two Turkish barley cultivars. The effects of NaCl on in vitro germination, growth, DNA integrity, cytosine methylation and polypeptide composition were investigated. For this purpose, maximum root and shoot lengths, fresh weight (FW), dry weight (DW), total soluble protein and DNA contents were measured; water contents (WC) were calculated. Genetic and epigenetic effects were studied by Random Amplified Polymorphic DNA (RAPD) and Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA), respectively. Protein profiles were analyzed by Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Responses of two cultivars were compared and discussed in the light of literature.

Materials and methods

Plant material

Barley mature seeds were provided by the Aegean Agricultural Research Institute (AARI). Bornova-92 and Hilal, both two-rowed cultivars, were developed by AARI and registered in 1992 and 2010, respectively.

Culture conditions

Seeds were surface-sterilized with 20% commercial bleach and rinsed with distilled water. Mature embryos were aseptically removed, soaked in ethanol for 30 sec, rinsed with distilled water and dried on filter paper. Embryos were cultured on Murashige and Skoog medium supplemented with 0 (Control), 50 mM (0.29%) NaCl, 100 mM (0.58%) NaCl in a growth chamber (Sanyo). At the 20^{th} day, the percentage of germination, maximum shoot and root length, and fresh weight were recorded. Twenty-day-old seedlings were dried at 37°C for 7 days for dry weight measurement or frozen in liquid nitrogen and used for further experiments.

Estimation of total soluble protein and DNA content

Homogenisation of seedlings and estimation of total soluble protein and DNA content were described previously (Temel and Gozukirmizi 2012). Total soluble protein and DNA levels were expressed as mg protein or mg DNA per g fresh tissue.

RAPD

Genomic DNAs (gDNA) were isolated from 20-day-old seedlings according to Temel and Gozukirmizi (2012). Five, 10-mer random primers (Fernandez et al. 2002) were synthesized by AlphaDNA and provided by SACEM (Turkey). PCR mixture consisted of 1× buffer, 2.5 mM $MgCl_2$, 0.25 mM each dNTPs, 2 µM (20 pmol) primer, 0.5 U Taq polymerase (Solis BioDyne) and 10 ng gDNA template in a 10 µL reaction mix. No template control without DNA template was run in all amplifications. PCR was carried out in a thermocycler (Techne). Amplification conditions were as follows; 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. Amplification products were resolved on 1.8% agarose gel in 1× TAE buffer, stained with ethidium bromide and visualized under a UV transilluminator. Band sizes were determined by comparison with a 100 bp DNA marker (SM0321, Fermentas).

CRED-RA

One µg gDNA samples were digested with *Hpa*II (FD0514, Fermentas) and *Msp*I (FD0544, Fermentas) according to manufacturer's instructions, purified with phenol:chloroform:isoamyl alcohol (25:24:1) mixture (P2069, Sigma) and dissolved with 10 µL distilled water and used as template in CRED-RA with S1 primer. Amplification conditions were the same as RAPD. Amplification products were resolved on 2% agarose gel in 1× TAE buffer, stained with ethidium bromide and visualized under a UV transilluminator.

SDS-PAGE

Total proteins were extracted by homogenising plant samples with extraction buffer (56 mM Na_2CO_3, 56 mM DTT, 2% SDS, 12% sucrose, 2 mM EDTA) and incubation at 70°C for 15 min. After centrifugation at 15000 ×g, 4°C for 10 min, supernatants were transferred into new tubes. Protein concentration was determined by the Bradford method. An aliquot of supernatant was mixed with an equal volume of 2× SDS loading buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% gliserol, 10% 2-Mercaptoethanol, 0.2% bromophenol blue) and denatured at 95°C for 5 min. Appr. 30 µg of total proteins were separated by SDS-PAGE. Appr. 5 µg BSA (66 kDa) was also loaded on gel as control. Bovine serum albumin (A9614, Sigma) was dissolved in distilled water, mixed with an equal volume of 2× SDS loading buffer, denatured at 95°C for 5 min. Band sizes were determined with a protein marker (A8889, Applichem). SDS-PAGE was carried out as described by Laemmli (1970) using 15% (37.5:1) polyacrylamide gel (Mini Protean cell, 1.0 mm, 165-8001, Biorad). After electrophoresis, gel was fixed (5:1:4 methanol:acetic acid:distilled water) for 3 h; stained with 1% Coomasie R-250 in 5:1:4 methanol:acetic acid:distilled water overnight and destained (5:7:88 methanol:acetic acid:distilled water) for 4 hours.

Data analysis

Physiological experiments were repeated three times independently and each data point is the arithmetic mean of biological triplicates (n=3). Seeds were regarded as germinated when the radicle reached 0.5 cm length. Ten seedlings were used for the measurement of FW, DW, maximum root and shoot length. Three seedlings were used for the estimation of protein and DNA content. Data were analyzed by One-Way Analysis of Variance (ANOVA). After ANOVA, statistical significance between two groups were examined by Least Significant Data (LSD) test.

WC was calculated according to formula [(FW-DW)/FW] where FW and DW are fresh and dry weight, respectively (Zhang and Blumwald 2001).

RAPD analysis was repeated twice with different DNA templates. Three seedlings were used in each gDNA extraction. Only clear and repeatable bands were scored in RAPD analysis. Disappearance of a normal band and appearance of a new band in comparison to the control group indicated polymorphism (Liu et al. 2005). Genomic template stability (GTS) values were calculated according to RAPD data using the formula GTS=(1-a/n)×100, where a indicates the RAPD polymorphic profiles in each sample and n is the number of total bands in the control (Aydin et al. 2012). CRED-RA analysis was also repeated twice with different DNA templates. An analysis of banding patterns was described previously (Temel et al. 2008).

Results

Salinity did not affect germination and germination percentages were 100% for control and NaCl-treatment groups and in both cultivars. Roots and leaves were shorter and leaves were strikingly yellowish in treatment groups especially at 100 mM concentration in both cultivars. Salinity decreased ($p<0.05$) shoot length, protein and DNA content in both cultivars; decreased ($p<0.05$) FW in Bornova-92 (Table 1), decreased ($p<0.05$) root length in Hilal (Table 2). WC and DW were not affected by salinity ($p>0.05$).

A total of 40 bands ranging from 125-1800 bp were amplified in Bornova-92 with 5 primers (Table 3); only 1 (2.56%) band was polymorphic. A total of 45 bands, all monomorphic, ranging from 125-1800 bp were amplified in cv. Hilal (Table 4). S10 primer

Table 1. FW (mg/seedling) WC (%) and DW (mg/seedling) of cv. Bornova-92 and cv. Hilal treated with different concentrations of NaCl. Data were presented as mean (*n*=3) ± SE. * and ** indicate data significant from control *P* at 0.05 and 0.01 levels.

		FW	WC	DW
Bornova-92	Control	114.73 ± 13.16	87.09 ± 2.44	14.33 ± 2.09
	50 mM	79.69 ± 13.53	86.25 ± 3.09	10.2 ± 0.75
	100 mM	46.5 ± 16.06	79.89 ± 5.12	7.71 ± 1.69
Hilal	Control	89.66 ± 7.53	92.11 ± 1.17	6.91 ± 1.08
	50 mM	49.83 ± 7.67*	88.29 ± 5.26	5.1 ± 1.46
	100 mM	12.5 ± 2.56**	59.23 ± 20.93	4.02 ± 1.27

Table 2. Maximum root and shoot length (cm), protein content (mg/g) and DNA content (mg/g) of cv. Bornova-92 and cv. Hilal treated with different concentrations of NaCl. Data were presented as mean (*n*=3) ± SE. * and ** indicate data significant from control *P* at 0.05 and 0.01 levels.

		ROOT	SHOOT	PROTEIN	DNA
Bornova-92	Control	10 ± 3.51	9.83 ± 1.58	33.43 ± 6.29	17.08 ± 0.53
	50 mM	8.1 ± 3.15	8.66 ± 0.33	19.89 ± 0.78	15.26 ± 0.78
	100 mM	2.13 ± 0.81	4.26 ± 1.1	10.20 ± 1.14*	7.01 ± 0.05**
Hilal	Control	9.46 ± 2.05	8.63 ± 1.7	31.14 ± 7.05	25.51 ± 5.77
	50 mM	6.23 ± 0.66	4.23 ± 0.4	17.25 ± 0.78	10.51 ± 0.14
	100 mM	2.33 ± 0.88	2.66 ± 0.33*	7.85 ± 1.31*	4.29 ± 1.88*

Table 3. Molecular sizes (bp) of bands of cv. Bornova-92 treated with different concentrations of NaCl.

cv.	Primer	Control	50 mM	100 mM
Bornova-92	S1	125, 300, 400, 500, 580, 800, 900, 1100, 1800	125, 300, 490, 510, 580, 800, 900, 1100, 1800	125, 300, 490, 510, 580, 800, 900, 1100, 1800
	S7	280, 320, 390, 430, 500, 650	280, 320, 390, 430, 500, 650	280, 320, 390, 430, 500, 650
	S10	310, 350, 430, 490, 550, 700, 800, 900	310, 350, 430, 490, 550, 700, 800, 900	310, 350, 430, 490, 550, 700, 800, 900
	S13	190, 220, 380, 390, 410, 590, 690	190, 220, 390, 410, 590, 690	190, 220, 390, 410, 590, 690
	S19	180, 320, 450, 490, 550, 700, 800, 950, 1150, 1300	180, 320, 450, 490, 550, 700, 800, 950, 1150, 1300	180, 320, 450, 490, 550, 700, 800, 950, 1150, 1300

Table 4. Molecular sizes (bp) of bands of cv. Hilal treated with different concentrations of NaCl.

cv.	Primer	Control	50 mM	100 mM
Hilal	S1	125, 225, 300, 400, 490, 500, 580, 750, 800, 900, 1100, 1190, 1210, 1800	125, 225, 300, 400, 490, 500, 580, 750, 800, 900, 1100, 1190, 1210, 1800	125, 225, 300, 400, 490, 500, 580, 750, 800, 900, 1100, 1190, 1210, 1800
	S7	280, 320, 500, 650	280, 320, 500, 650	280, 320, 500, 650
	S10	150, 275, 350, 430, 490, 540, 550, 650, 800	150, 275, 350, 430, 490, 540, 550, 650, 800	150, 275, 350, 430, 490, 540, 550, 650, 800
	S13	190, 220, 380, 410, 590, 690, 750, 1000	190, 220, 380, 410, 590, 690, 750, 1000	190, 220, 380, 410, 590, 690, 750, 1000
	S19	180, 225, 320, 490, 550, 700, 800, 950, 1150, 1300	180, 225, 320, 490, 550, 700, 800, 950, 1150, 1300	180, 225, 320, 490, 550, 700, 800, 950, 1150, 1300

detected a decrease in intensity of a band (350 bp); S13 primer detected disappearance of a band (380 bp) in Bornova-92 under salinity stress. Moreover, intensity of 2 bands (690 and 700 bp) amplified with S13 and S19 respectively decreased with salinity (Fig. 1). GTS values of 50 and 100 mM NaCl-treated groups were 97.5%. No polymorphism was detected in Hilal. Therefore, GTS values were 100% in Hilal. Briefly, salinity did not cause genotoxicity in both cultivars.

*Hpa*II and *Msp*I digestion products of control and 100 mM NaCl-treated plants of both cultivars were amplified with S1 primer and detected different types of methylation alterations. Six bands (150, 210, 300, 350, 500, 600) were amplified in Bornova-92; 2 (500 and 600 bp) of them represent a change in methylation pattern from CCG to CG methylation (Fig. 2). Six bands (125, 210, 300, 350, 550, 600) were amplified in Hilal. Three bands (125, 550 and 600 bp) represent, two hyper- and one hypomethylation of CCG residues, respectively. Three bands (210, 300 and 350 bp) represent a change from CG to CCG methylation. Thirty-three % of bands were polymorphic in Bornova-92 whereas all bands were polymorphic in Hilal. Salinity caused changes in methylation pattern (CG vs CCG) in both cultivars but also level (both hypo- and hypermethylation) in Hilal. However, salinity favored CG and CCG methylation in Bornova-92 and Hilal, respectively.

Total proteins of two cultivars exposed to different concentrations of NaCl were analyzed by SDS-PAGE (Fig. 3). The level of a protein (50 kDa) decreased in Bornova-92 yet slightly-decreased in Hilal. The intensity of two proteins very close to each other (between 35-48 kDa) decreased by salinity in Bornova-92. The intensity of a protein (35 kDa) increased by salinity in Bornova-92. The protein profile of Hilal remained unaffected.

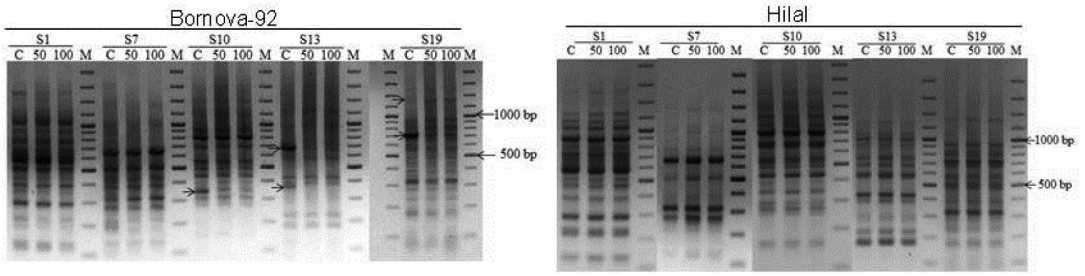

Figure 1. RAPD products of two cultivars treated with different concentrations of NaCl. Differences in band presence or intensity were indicated by arrow. M: Marker.

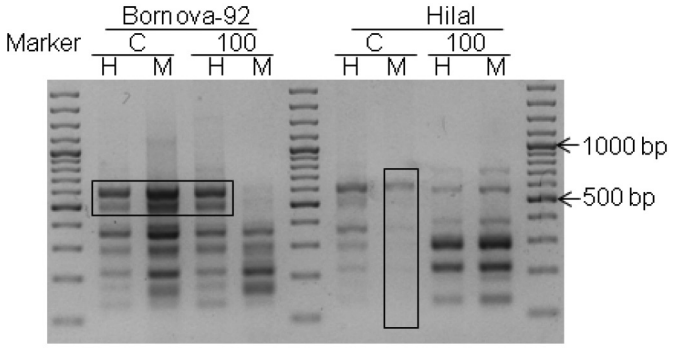

Figure 2. CRED-RA products of two cultivars treated with different concentrations (0 or 100 mM) of NaCl. Polymorphic bands were indicated in a rectangular. H: *Hpa*II, M: *Msp*I.

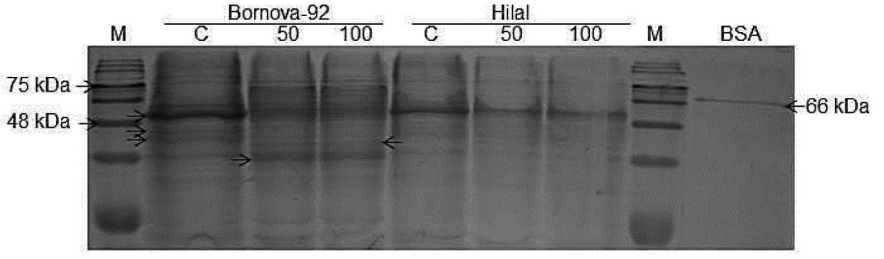

Figure 3. SDS-PAGE profiles of two cultivars treated with different concentrations of NaCl. Polymorphic bands and differences in intensity of bands were indicated by arrow. M: Marker.

Discussion

In this study, salinity did not inhibit WC and germination yet decreased protein and DNA content and shoot length. It may be hypothesized that 50 and 100 mM NaCl treatments may not be very harmful in both cultivars. In the study of Demirkiran et al. (2013) barley (*H. vulgare* cv. Tokak) mature embryos were germinated in the presence of 50 and 100 mM NaCl for 20 days. Both concentrations decreased FW and protein content and inhibited shoot growth (Demirkiran et al. 2013). Salinity-induced reduction in water uptake is reflected as decreased WC and causes growth inhibition (Sairam and Srivastava 2002). Water uptake in barley was not significantly affected by salt stress (Pesserakli et al. 1991). However, 150 mM NaCl may not inhibit germination in wheat (Yumurtaci et al. 2007). Maintenance of high WC may provide salt tolerance. Salinity decrease WC less in tolerant varieties (Sairam and Srivastava 2002).

In the present study, salinity did not cause genotoxicity. However, DNA degradation and nuclear deformation occur under saline

conditions (Liu et al. 2000). It was reported that salt stress caused random mutations and the effects were dose-dependent (Lu et al. 2007). Appearance of novel bands, loss of bands and changes in intensity of bands represent mutational events *e.g.* mutations, deletions, insertions (Atienzar et al. 2000). Demirkiran et al. (2013) reported that 100 mM NaCl-treatment caused nucleotide variations in roots of barley seedlings but not in shoots. RAPD has been used for detection of DNA damage and mutations.

In this study, salt treatment affected cytosine methylation in both cultivars in different ways. Demirkiran et al. (2013) reported hypermethylation in shoots of seedlings germinated under saline (100 mM) conditions. Both *de novo* methylation and demethylation events occur in salt-stressed plants (Lu et al. 2007; Tan 2010). Differences in salt tolerance among cultivars are also related to methylation levels (Peng and Zhang 2009). In the case of Sp1, a transcription factor, methylation of a CpG in Sp1 binding site does not interfere with protein binding (Ben-Hattar et al. 1989) whereas methylation of the outer cytosine (or both) is inhibitory (Clark et al. 1997). CRED-RA has been used to detect changes in DNA methylation (Cai et al. 1996).

Salinity caused alterations in protein profiles in Bornova-92. The appearance of new polypeptides, and an increase or decrease of levels of some polypeptides occur in NaCl-treated plants (Rasoulnia et al. 2011). Proteome response of salt-sensitive and tolerant cultivars might be different (Fatehi et al. 2012).

In the present study, altered salt responses of two barley cultivars (Bornova-92 and Hilal) were compared at either physiological or molecular level. Salinity caused similar physiological changes in both cultivars. Inhibitory effects of salinity were more severe in Hilal than Bornova-92. Interestingly, epigenetic changes were the most discriminating feature. However, salinity did not cause genotoxic effects and had little effect on protein patterns, especially in Hilal. Epigenetic changes may not be associated with genetic changes and may not reflect on protein synthesis. It may also be supposed that Bornova-92 is more salt-tolerant than Hilal. Relative salt-tolerance of Bornova-92 may be explained with altered cytosine methylation and/or protein profiles.

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