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Effect of short- and long-time salt treatment on root traits and expression pattern of *Atls1* gene in barley (*Hordeum vulgare* L.)

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Abstract
Barley (*Hordeum vulgare* L.) as a salt-tolerant crop species has considerable economic importance in salinity-affected arid and semiarid regions of the world. In the present study, three barley genotypes (Sahara$_{3771}$ and an Iranian advanced line as salt tolerant and Clipper as salt susceptible) were exposed to 100 and 200 mM NaCl at the seedling stage and non-NaCl treatment was used as check. The root samples were harvested 24 hours, three days and three weeks after salt treatment in three replications. The root length and root fresh and dry weight were measured and expression pattern of *Atls1* gene was analyzed on root samples by quantitative Real-time-PCR. The effects of genotype and sampling time were significant for root characters and with the advancement of salt treatment duration, root length and root fresh and dry weight were significantly reduced. The expression of *Atls1* gene was significantly affected by NaCl level, genotype, sampling time and their interactions. In all three genotypes, with the increase of NaCl concentration, expression of *Atls1* gene was reduced. Under 100 mM NaCl, mRNA level of *Atls1* was significantly decreased in Clipper as compared with the salt tolerant genotypes, Sahara$_{3771}$ and Advanced line. In addition, long-term salt treatment (three weeks) significantly reduced the expression of *Atls1* in all three genotypes. Down-regulation of *Atls1* gene under long term salt treatment indicates that this gene may be involved in response to salinity stress at the beginning of salt stress.

Keywords: *Atls1* gene; Barley; Real-Time PCR; Root growth; Salinity stress

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Introduction

Barley is the fifth most important crop plant following corn, wheat, rice, and soybean. Due to its adaptation to unfavorable environmental conditions, stability under different environments and moderate tolerance to salinity, barley is a good candidate for studying salinity tolerance mechanisms (Munns and Tester 2008).

Salinity affects more than 950 million hectares of land in the world and is regarded as one of the most important environmental factors which affects crop growth and yield adversely. To feed the world's growing population, it is necessary to utilize more than 50% of world arable lands by 2050 (Ladeiro 2012).

Salinity stress decreases water absorption capacity of root systems and increases water loss from the leaves due to osmotic stress imposed by high salt accumulation in soil and plants (Munns 2005). Furthermore, high concentrations of salts impose some toxic effects on the important physiological and biochemical processes of cytosol (Tester and Devenopert 2003), depending on severity, growth stage and duration of the stress (Munns 2005; Rahnama *et al*. 2010; James *et al*. 2011) and significantly contribute to the reduced growth and productivity of plants (Tester and Devenopert

2003). The germination and seedling stages are the most sensitive stages of barley growing under salinity, determining the plant tolerance and hence ultimate performance under salinity condition (Greenway 1965).

Roots are the first organs exposed to water deficiency in the soil and are the place of salinity sensing. Since the root system contributes to efficient water uptake and ion exclusion (Ristova and Busch 2014), changes in root system architecture (RSA) are likely to affect the plant performance under salt stress condition (Julkowska and Testerink 2015). RSA is made up of structural features and the degree of root plasticity depends on variation in root number, extension and growth direction (Giehl *et al*. 2014). Root length and length of lateral roots exhibit great plasticity in response to environmental changes. It is critical to develop plants with more efficient roots (Khan *et al*. 2016). Thus, the changes in RSA affect the above-ground biomass and, therefore, stability of growth under salinity is one of the major determinants of salt tolerance (Paez-Garcia *et al*. 2015).

Molecular studies indicate the alteration of expression of numerous genes in the response to salinity. Studies using microarray (Ozturk *et al*. 2002) and differential detection methods (Ueda *et al*. 2002) indicate the involvement of a large number of genes, transcripts and proteins in the response paths to salinity stress, while their exact role in stress tolerance/sensitivity is yet to be explored. Analysis of gene expression at different growth stages and plant organs indicated that gene expression varies with plant organ and growth stage (Sakurai *et al*. 2005). Transcriptomics analysis extensively has been used in salinity stress studies (Urano *et al*. 2010).

Atls1 is a light-inducible gene which is down regulated by salinity in barley (Qian *et al*. 2007; Temel and Gozukirmizi 2015) and wheat (Temel and Gozukirmizi 2015). The root is the first organ, perceives salinity, and according to reports, a number of inducible genes exhibit further increase in expression in the root rather than other plant organs (Yan *et al*. 2005). However, there is no report of *Atls1* gene expression alternation under salinity in barley root. The aim of this study was to assess the effect of NaCl concentration and duration on root length and root wet and dry weight as well as expression of *Atls1* gene in the roots of salt tolerant and susceptible barley genotypes.

Materials and Methods

A hydroponic experiment was conducted to examine the effect of salinity (Na^+ and Cl^-) on root characters and expression pattern of *Atls1* gene in barley, cv. $Sahara_{3771}$ and cv. Clipper, as well as an Iranian advanced line (A-Line). $Sahara_{3771}$ is a six-row salt tolerant landrace from Algeria. Clipper is an improved Australian two-row variety and susceptible to salinity. Seeds of Clipper and $Sahara_{3771}$ were obtained from the University of Western Australia. Iranian advanced line is a highly salt tolerant line and is released by the Plant and Seed Improvement Institute from a cross of Kavir and Sahara varieties.

A split-plot experiment was conducted using randomized complete block design with three replicates. Salinity concentrations of 100 and 200 mM NaCl and a check (with no salt) were arranged in the main plots and genotypes in the sub-plots.

Seeds were surface sterilized in 10% sodium hypochlorite for 15 min and then washed thoroughly with distilled water for 15 minutes. The seeds were germinated on filter papers for 48-72 hours. Then, the seedlings at the similar germination stage were transferred to gravel under hydroponic system. Plants were irrigated with half-strength Hoagland's nutritious solution for three days and thereafter with full-strength solution. Hoagland's nutrient solution composed of 6 mM KNO_3, 5 mM Ca $(NO_3)_2$, 2 mM $MgSO_4$, 100 mM $ZnSO_4$, 8 mM H_3BO_3, 2 mM $MnCl_2$, 2 mM $CuSO_4$, 2 mM $H2MoO_4$, 4 mg/l Fe-EDTA, 6 mM KH_2PO_4. Salt stress was imposed when the seedlings were in the three-leaf stage. The initial salinity level was 50 mM and then elevated up to 100 and 200 mM in daily intervals. Plants were grown in greenhouse under controlled conditions at 28/18◦C day/night temperature, with a photoperiod regime of 16/8 h day/night, and70% relative humidity.

To measure root characters and RNA extraction, the roots samples were harvested 24 hours, 3 days and 3 weeks after the salt treatment. Then root length and root fresh and dry weights were measured. Total RNA was extracted using the RNX-Plus Kit (Cinna Gen, Iran) following the manufacturer protocols. The cDNA was synthesized in a 20 µl volume reaction containing 2 µl total RNA using the Revert Aid first strand cDNA synthesis kit (Thermo Scientific, USA). The *Atls1* gene specific primer pair was used in quantitative real-time PCR and *a-tubulin2* was used as housekeeping gene (Table 1). Real-time qPCR was performed in the total volume of 10 µl by adding 1 µl of the cDNA, 0.7 µl forward and reverse primers (5 ng/µl), 3.5 µl ddH2O and 4 µl SYBG premix Ex *Taq*TMII PCR master mixture (TAKARA, Japan). qPCR was performed on the C1000™ thermal cycler system (Bio-Rad, USA) with PCR conditions of 94 °C for 5 minutes, 40 cycles of 94 °C for 45 seconds, primer specific annealing temperature for 45 seconds, and 72 °C for 45 seconds and final extension at 72 °C for 5 minutes.

Table 1. Gene specific primers for the amplification of *a-tubulin, Atls1* gene and its annealing temperature

Gene	Sequence	Annealing temperature
a-Tubulin 2	5'-AGTGTCCTGTCCACCCACTC-3'	65 ˚C
	5'-AGCATGAAGTGGATCCTTGG-3'	
Atls1	5'-TACGGTTTTCTGCCTCTGTCACA-3'	68 ˚C
	5'-ACAA CATCTGGTCATACTGCCG-3'	

Data analysis

The data for root characters were analyzed using linear model of split-plot design based on randomized block design. Before analysis of variance, the normality of residuals was tested using Kolmogorov-Smirnov test. The means were compared by Duncan's multiple range test with critical probability level of 0.05. For gene expression data analysis, ΔCt for each genotype was calculated under various treatments and replications by subtracting *Alts1* gene Ct from that of *a-Tubulin*2. The ΔΔCt was then calculated by subtracting the ΔCt of each genotype under salt treatments from that of normal condition. In

addition, ΔΔCt for salt tolerant genotypes (Sahara3771 and Advanced line) was calculated by subtracting their ΔCt from that of susceptible genotype (Clipper). Finally, $2^{-\Delta\Delta Ct}$ was used for the statistical analysis. Analysis of variance was carried out based on the split-plot design and means were compared by Duncan′s multiple range test with critical probability level of 0.05. MSTAT-C and SPSS v22 statistical software were used for the data analyses.

Results and Discussion

Effect of NaCl treatment on root characters

Significant differences were observed among genotypes and sampling times for root length and root fresh and dry weights. The salt × sampling stage and genotype × sampling stage were also significant for all root characters, but three-way interaction was only significant for root fresh and dry weights (Table 2). Maximum root length was observed three weeks after 100 mM NaCl treatment, however, there was no significant difference in root length between 100 and 200 mM NaCl treatments after three weeks (Figure 1A). Among the genotypes, A-Line showed the highest root length, three weeks after NaCl treatment which was significantly higher than those of $Sahara_{3771}$ and Clipper. After 24 hours and three days of salt treatment, no significant differences were observed among the genotypes for root length (Figure 1B). Maximum root fresh weight was recorded three weeks after 100 and 200 mM NaCl treatment for A-Line and three weeks after 100 mM NaCl treatment for $Shahra_{3771}$. However, 24 hours and three days after salt treatment, no significant differences were observed among the genotypes for root fresh weight (Figure 2). After three weeks of 200 mM NaCl treatment, A-Line root dry weight was significantly higher than those of $Sahara_{3771}$ and Clipper and three days after 100 mM NaCl treatment, A-Line and $Shahara_{3771}$ produced significantly higher root dry weight than the salt susceptible cultivar Clipper. In total, minimum and maximum root dry weight were recorded 24 hours and three weeks after 100 mM NaCl treatment in A-Line, respectively (Figure 3).

Table 2. Mean square of root properties of studied genotypes under salinity stress

SV	df	Mean squares			
		Root length	Root fresh weight	Root dry weight	*Atls1*
Replication	2	42.92^{ns}	0.14^{ns}	0.14^{ns}	0.01
Salinity	2	17.47^{ns}	0.15^{ns}	0.14^{ns}	6.77^{**}
Error 1	4	22.87	0.39	0.20	0.05
Genotype	2	23.92^{*}	0.65^{**}	0.37^{**}	5.33^{**}
Salinity × Genotype	4	3.43^{ns}	0.06^{ns}	0.04^{ns}	4.71^{**}
Sampling stage	2	72.85^{**}	7.76^{**}	1.86^{**}	1.09^{**}
Salinity × Sampling stage	4	18.69^{*}	0.15^{**}	0.21^{**}	8.35^{**}
Genotype × Sampling stage	4	22.15^{**}	0.26^{**}	0.21^{**}	2.77^{**}
Salinity × Genotype × Sampling stage	8	5.85^{ns}	0.18^{*}	0.09^{*}	1.30^{**}
Error 2	48	5.64	0.06	0.03	0.09

ns, *, **: not significant and significant at 5% and 1% probability levels, respectively.

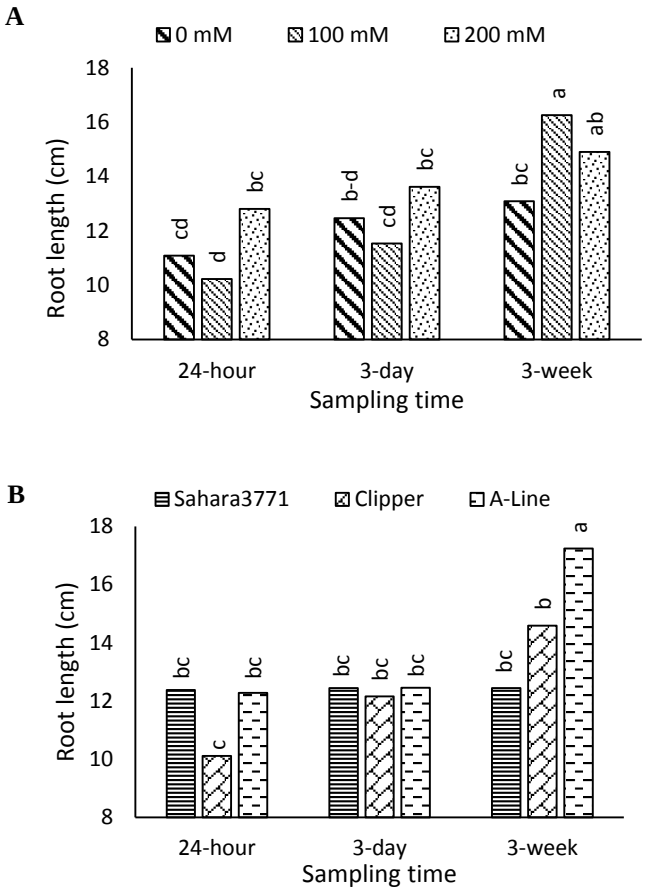

Figure 1. Mean comparison of root length for A) salinity × sampling stage and B) genotype × sampling stage combinations. Means with different letters are significantly different at 5% probability level based on Duncan's multiple range test.

Various studies have documented the differential responses of crop species and their genotypes to salinity. Identification of cultivars or genotypes with differential response to salinity is important in their classification into tolerant and susceptible genotypes that are useful in breeding for stress tolerance. In addition to genotypes, plants' responses to salinity varies by different plant growth stages (Greenway 1965). Therefore, we assessed the root growth and gene expression at three plant growth stages. It was reported that high concentration of salt results in the inhibition of enzymatic activity, membrane disorganization, inhibition of cell division and expansion and reduction of photosynthesis (Mahajan and Tuteja 2005) which finally reduces growth, yield and quality. Barley is regarded as a salt tolerant crop, but seedling stage is the most sensitive growth stage of this crop plant to salinity (Greenway 1965).

When grown hydroponically, the salt tolerant genotypes, A-Line and Sahara$_{3771}$, and the salt susceptible cultivar, Clipper, showed differential response to salt and A-Line and Sahara$_{3771}$ were less affected by salt treatments as compared with Clipper. However, the responses of salt tolerant genotypes to salinity stress was also different. Widodo *et al*. (2009) compared Sahara$_{3771}$ and Clipper growth response to salinity under hydroponic condition and reported that Sahara$_{3771}$

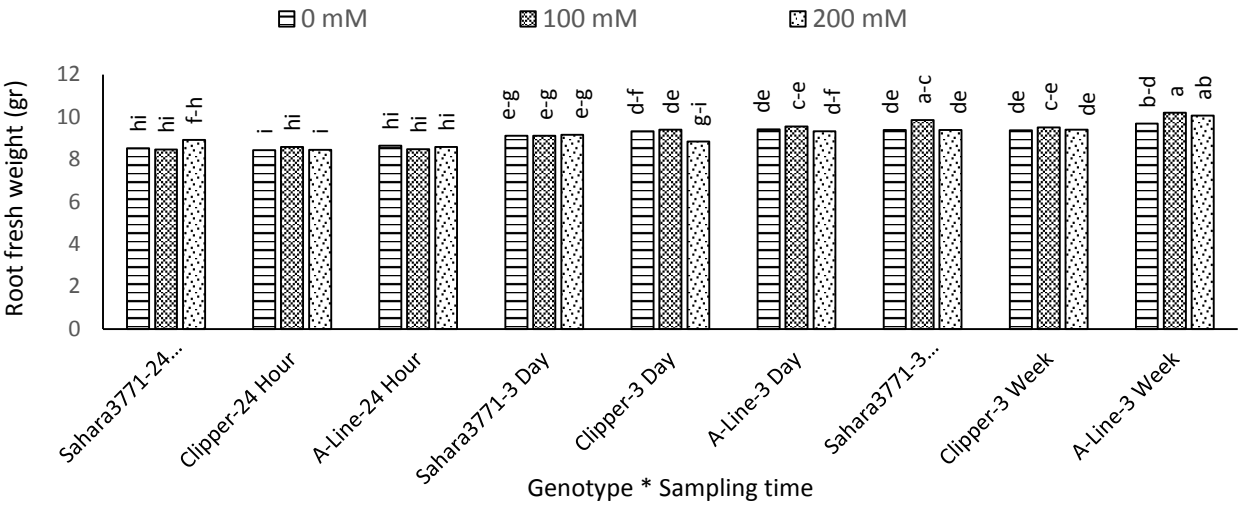

Figure 2. Mean comparison of root fresh weight for salinity × genotype × sampling time interaction. Means with different letters are significantly different at 5% probability level based on Duncan's multiple range test.

was less affected by 100 mM NaCl treatment, however, there was an obvious reduction in biomass of Clipper. In our study, based on root characters, A-Line showed higher tolerance to salt stress as compared with Sahara$_{3771}$. A-Line had 1.6-fold larger root length to shoot length ratio as compared with Sahara$_{3771}$ and Clipper.

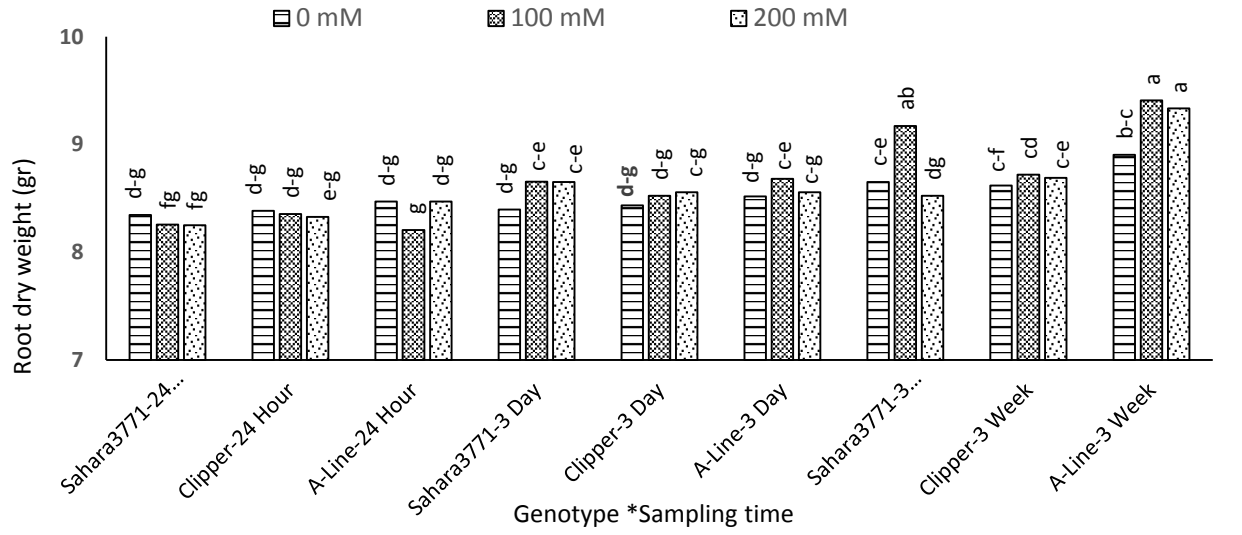

Figure 3. Mean comparison of root dry weight for salinity × genotype × sampling time combinations. Means with different letters are significantly different at 5% probability level based on Duncan's multiple range test.

Effect of NaCl treatment on *Atls1* gene expression pattern

Analysis of variance revealed significant effects of NaCl concentrations, genotypes and sampling times on expression of *Atls1* gene. All two-way and three-way interactions were also significant (Table 2). The expression of *Atls1* gene was down-regulated under 200-100 mM NaCl regime and significant reduction in mRAN level of the gene was observed under 200-100 mM NaCl as compared with 100-0 and 200-0 mM NaCl and check. There was no significant difference between 100-0 and 200-0 mM NaCl treatments (Figure 4a). Among the genotypes, higher expression of *Atls1* gene was recorded in A-Line and Clipper (salt susceptible) which was significantly higher than that of the salt-tolerant cultivar of $Shara_{3771}$. However, there no significant difference was observed between A-Line and Clipper (Figure 4b). Tamel and Gozulcirmizi (2015) by studying comparison the physiological and molecular changes in barley and wheat under salinity reported that expression of *Atls1* gene was down-regulated in salt tolerant varieties under 250 mM NaCl treatment. They found that barley had the capacity to regulate the expression of *Atls1* gene faster and maintain expression level as compared with wheat. With the increased duration of salt treatment from 24 hours to three days, the expression of *Atls1* gene was significantly down-regulated, however, there was no significant difference between 3-day and 3-week salt treatments regarding the mRNA level of *Atls1* gene (Figure 4c).

Compared with check, salinity decreased the expression of *Atls1* gene in barley roots under 200-100 mM NaCl treatments and the decrease in mRNA level of *Atls1* gene due to salinity was significantly higher in the salt susceptible cultivar of Clipper as compared with the salt tolerant genotypes (Figure 4). Under 100 mM NaCl at all sampling stages, the level of *Atls1* gene expression in the salt susceptible cultivar of Clipper was lower than that of $Sahara_{3771}$ and advanced line. However, under 200 mM NaCl, the transcript level of this gene in Clipper was higher than that of 100 Mm NaCl as compared to the control treatment. However, there was a slight increase in $Sahara_{3771}$ three days after treatment with 100 and 200 mM NaCl. In total, Advanced line was less affected by salt treatment as compared to $Sahara_{3771}$ and specially Clipper.

We compared gene expression patterns under long- and short- term salinity stresses. Observing *Atls1* transcript levels at 24-h, 3-days and 3-weeks salt treatments, revealed that the change in mRNA level was lower than the control under both NaCl treatments. On the average of all genotypes, the minimum level of *Atls1* gene expression was obtained 24 hours after NaCl treatment. The results clearly demonstrated that with increased concentration and duration of NaCl treatment, the transcript level of *Atls1* was less affected. The maximum level of this gene was observed in Sahara 3771 three days after 100 mM NaCl treatment.

Quantitative PCR is regarded as the most sensitive method for the quantification of gene expression levels. *Atls1* has been shown to down regulate by salinity in barley (Qian *et al*. 2007; Temel and Gozukirmizi 2015) and wheat (Temel and Gozukirmizi 2015). Nevertheless, there was no

report of *Atls1* gene expression alternation under saline conditions in the barley root.

In conclusion, our results showed that salinity decreased the expression level of *Atls1* gene in all genotypes and sampling stages and this was more sever in the salt susceptible cultivar of Clipper. Under 100 mM NaCl treatment, the reduction *of Atls1* gene expression was more highlighted than under 200 mM NaCl level. Differences between the control and salt treatments for mRNA level were decreased when time elapsed from 24 hours to three weeks after the salt treatment.

Acknowledgment

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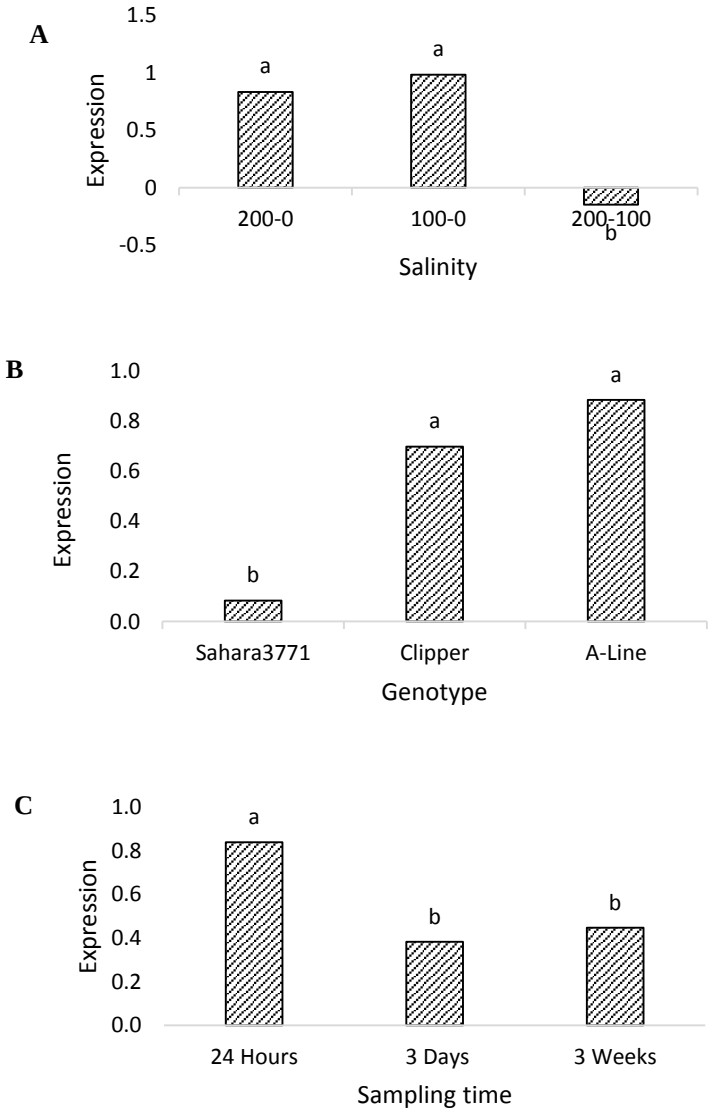

Figure 4. The effect of A) NaCl concentration, B) genotype and C) sampling time on expression of *Atls1* gene. Means with different letters within each factor are significantly different at 5% probability level based on Duncan's multiple range test.

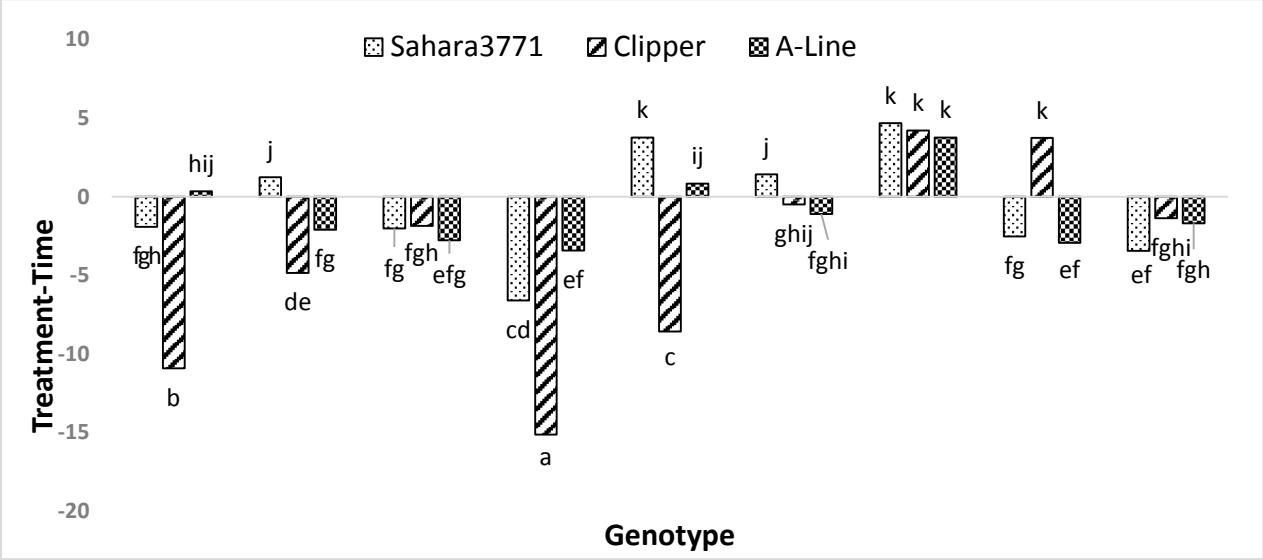

Figure 5. Mean comparison of *Atls1* gene mRNA level for salinity × genotype × sampling time treatment combinations. Means with different letters are significantly different at 5% probability level based on Duncan's multiple range test.

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تاثیر تنش شوری کوتاه و بلند مدت بر صفات ریشه و الگوی بیان ژن *Atls1* در جو (*Hordeum vulgare* L.)

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چکیده

جو (*Hordeum vulgare* L.) به عنوان یک گیاه متحمل به شوری دارای اهمیت اقتصادی قابل توجهی در نواحی خشک و نیمه خشک است. در این مطالعه، سه ژنوتیپ جو (Sahara3771 و لاین امید بخش به عنوان لاین‌های متحمل و Clipper به عنوان لاین حساس به شوری) در مرحله گیاهچه تحت تیمار شوری صفر، ۱۰۰ و ۲۰۰ میلی مولار NaCl قرار گرفتند. ۲۴ ساعت، سه روز و سه هفته پس از اعمال تیمار شوری نمونه برداری از ریشه در سه تکرار انجام شد. طول ریشه و وزن خشک و تر ریشه اندازه‌گیری شد و الگوی بیان ژن *Atls1* در نمونه‌های ریشه با تکنیک Real-time-PCR کمی مورد بررسی قرار گرفت. اختلاف بین ژنوتیپ‌ها و مراحل نمونه برداری برای صفات ریشه معنی‌دار بود. با پیشرفت مراحل نمونه برداری و طول مدت تیمار، طول ریشه و وزن خشک و تر آن افزایش معنی‌داری داشت. شوری، ژنوتیپ، مرحله نمونه برداری و اثر متقابل آن‌ها بیان ژن *Atls1* را به طور معنی‌داری تحت تاثیر قرار دادند. در هر سه ژنوتیپ، با افزایش غلظت NaCl، بیان ژن *Atls1* کاهش یافت. تحت شوری ۱۰۰ میلی مولار NaCl، سطح mRNA ژن *Atls1* در Clipper در مقایسه با ژنوتیپ‌های متحمل به شوری Sahara3771 و لاین امید بخش کاهش معنی‌داری داشت. علاوه بر این، در تمامی ژنوتیپ‌ها تحت تیمار شوری طولانی مدت (سه هفته) بیان ژن *Atls1* کاهش یافت. کاهش بیان این ژن در تیمار شوری بلند مدت نشان می‌دهد که احتمالا این ژن در مراحل ابتدایی پاسخ به تنش شوری دخالت دارد.

واژه‌های کلیدی: تنش شوری؛ جو؛ رشد ریشه؛ ژن *Atls1*؛ Real-Time PCR