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TRANSCRIPTIONAL CHANGES OF BFRUCT3, NHX1, OMT AND PEAMT GENES IN ROOT OF BARLEY (*HORDEUM VULGARE* L.) UNDER SALINITY STRESS

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Abstract

The expression changes of *BFRUCT3*, *OMT*, *NHX1*, *PEAMT* genes in root of barley genotypes; Clipper (sensitive), Sahara$_{3771}$ (tolerant) and an Iranian advanced line (tolerant) were evaluated under 0, 100 and 200 mM NaCl at 24 hours, 3 days and 3 weeks after salt treatment. Expression of the genes was analyzed using Real-Time PCR Based on $2^{-\Delta\Delta CT}$ data obtaining from the comparison of different salinity treatments. Analysis of transcript level of *BFRUCT3* gene revealed up-regulation of this gene in the salt tolerant genotypes with prolonging of salt treatment duration and increase of salinity level from 100 to 200 mM NaCl. Whereas, *OMT* gene showed up-regulation in all the three genotypes with increase of salinity level at the earlier stage of salt treatment. With increase of salt level to 200 mM NaCl, *PEAMT* gene showed up-regulation in the advanced line at the early time points (24h and 3 days) and in the Sahara$_{3771}$ at the late time point (3 weeks) but in Clipper expression of this gene was down-regulated.

Key words: Barley, Gene expression, Salinity, Salt responsive genes.

Introduction

Salt stress is one of the most serious limiting factors for crop growth and production in the arid regions. Salinity reduces plant yield by disrupting the vital functions of the cell through limiting of water absorption and absorption of excess salt (Leopold & Willing, 1984). Under these conditions, plants maintain osmotic pressure and ionic balance by the absorption, transportation and distribution of water and solutions (Johansone *et al.*, 2001; Verdoucq *et al.*, 2008). Salt stress causes metabolic disorders in plant cells, which lead to excessive production of reactive oxygen species (ROS) causing progressive oxidative damage such as lipid oxidation, protein structure changes, enzyme inactivation, loss of pigments such as chlorophyll and ultimately cell death (Mittler, 2002). Plants vary in terms of tolerance or sensitivity to salinity. Increasing plant tolerance to salinity and selection of suitable genotypes for planting in saline areas has a great agricultural and economic significance (Kingsbury *et al*., 1984).

Barley *(Hordeum vulgare* L.) is one of the most tolerant cereals to drought and salinity (Ceccarelli, 1987). The mechanism of salt tolerance in barley, like most of the plants, includes absorption of less salt, tissue tolerance, accumulation of salt in vacuoles, distinction in absorption of ions such as K^+, Na^+ and So_4^{--} by the roots and transportation of them to the aerial part and various biochemical processes such as the production of enzymes, hormones and antioxidants (Spychalla & Desborough, 1990; Gorham, 1995; Begum & Karmoker, 1999). The response of barley to salinity stress depends on the stage of plant growth. The most sensitive stage to salinity is germination and seedling stage and with the increase of age, tolerance to salinity is increased (Storey & Jones, 1978).

Studies have shown that several genes are involved in response to salinity (Ozturk *et al.*, 2002; Ueda *et al.*, 2004). Gene expression in response to salt stress is different in various species and different growth stages of plant (Ligaba *et al.*, 2011). In the response to stress, gene expression, transcripts and numerous proteins are changed and significant correlation exists between them. But in most cases, the exact function of these attributes is unknown in tolerant or sensitive genotypes (Bray, 1997).

High concentrations of sodium ions due to its destructive effect on the enzymes activities, photosynthesis and metabolism, could adversely affect the growth of plants (Niu *et al.*, 1995). Plants apply different mechanisms such as limiting the entry of sodium into the cell, increasing transfer of sodium out of the cell and storage of sodium in the vacuole to reduce its toxic effects (Blumwald *et al.*, 2000; Aharon *et al.*, 2003). Releasing of sodium out of cells or its storage in vacuoles are mediated by the activity of Na^+/H^+ antiporter (NHX) in the plasma and vacuolar membrane (Barkla & Blumwald, 1991). A key enzyme in the synthesis of choline in plants is *PEAMT* which catalyzes N methylation of phosphoethanolamine, phospho monoethylethanolamine and phospho dimethylethanolamine for conversion of phosphoethanolamine to phosphocholine that phosphocholine is converted to choline during the stage. Choline is a vital precursor in the plant because it is required for the synthesis of phosphatidylcholine (PC), which constitutes 40-60% of the cell membrane (Mou *et al.*, 2002). Certain plants use choline to produce glycine-betaine that is an osmoprotectant and causes resistance to salinity, drought and other stresses (Gorham, 1990). O-methyltransferase (OMT) is a large family of enzymes that methylate the oxygen atom in the secondary metabolites such as phenylpropanoids, flavonoids and alkaloids. It plays an important role in the biosynthesis of lignin, abiotic stress tolerance and disease resistance in

plants (Lam *et al.*, 2007). It is reported that invertase protein, alone or in combination with plant hormones, is involved in regulation of plant development, classification of carbohydrates as well as biotic and abiotic interactions. *BFRUCT3* gene codes invertase protein (Acid beta-fructofuranosidase3, EC 3.2.1.26), which is a key metabolic enzyme to split sucrose into beta-D- fructose and alpha-D-glucose (Fotopoulos, 2005).

In the present study, we report the transcriptional response of barley cv. Sahara$_{3771}$ and Clipper and an advanced line to 100 mM and 200 mM NaCl at 24 hours, 3 days and 3 weeks after salt treatment using real-time PCR technique.

Materials and Methods

Plant materials: To investigate the effect of salinity stress on gene expression changes in barley root, three genotypes; Clipper (salt sensitive), Sahara3771 (salt tolerance) and an Iranian advanced line (salt tolerance) derived from a cross between Sahra and Kavir were used in the experiment. Seeds of the genotypes were obtained from the University of Western Australia and Seed and Plant Improvement Institute, Karaj, Iran. Sahara3771 is a North African landrace and Clipper is a commercial Australian Variety.

Salt Treatment and experimental condition: The experiment was conducted in hydroponic culture system at greenhouse. The genotypes were evaluated under 100 and 200 mM NaCl treatments and root sample for RNA extraction was harvested 24h, 3 days and 3 weeks after reaching a final NaCl concentration of each treatment. The experiment was performed using a split plot-factorial design with three replicates and salinity treatment was used as main factor and combinations of genotypes and sampling time as sub factor. Seeds were sterilized with sodium hypochlorite and germinated in petri dishes and seven day old seedlings of uniform size were transferred into large sand tanks under controlled greenhouse (15 h daily light, 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 tanks were sub irrigated and flushed four times daily with a modified Hoagland nutrient solution. Electrical conductivity, pH and solution temperature were monitored daily. Salt stress was imposed seven days after the seedlings were transferred. NaCl concentrations were brought up to 100 and 200 mM NaCl by increments of 50 mM NaCl per day. CaCl2 was added with NaCl to maintain a Na+/Ca2+ concentration ratio of 10:1 on a molar basis.

RNA isolation, cDNA synthesis and Real-time PCR: Total RNA was extracted from barley root samples using RNX-Plus kit according to the manufacturer's protocol and treated with RNase-free DNase I. Quality and quantity of the RNA samples were tested by 1% agarose–formaldehyde gel electrophoresis and PicoDrop spectrophotometer, respectively. To synthesize cDNA, Fermentas superscript III Reverse Transcriptase kit with an oligo(dT)20 primer was used, following the manufacturer's instructions. Real-time qPCR was done for each gene in total volume of 10 µl by adding 1 µl of the cDNA, 1.5 µl forward and reverse primers, 3.5 µl ddH2O and 4 µl SYBG premix Ex TaqTMII PCR master mixture (TAKARA, Japan). The analysis was carried out with two replicates for each sample. qPCR was performed with PCR conditions of 94°C for 5 minutes, 44 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. Primers were designed to determine the expression of a number of key genes involved in Na+ compartmentation and ROS detoxification and included members of the BFRUCT3, NHX1, OMT and PEAMT genes (Table 1). α-tubulin was used as reference gene and data was quantified using the comparative CT method (2-ΔΔCT method) based on CT values (Livak & Schmittgen, 2001). Analysis of variance was performed based on linear model of the split-plot factorial design and mean comparison was performed by Duncan′s multiple range test with critical value of $p \leq 0.05$.

Results

Transcriptional changes in the gene expression profile: The expression of 4 genes potentially involved in plant adaptive responses to salinity was examined in the root of hydroponically-grown plants. Plants were exposed to 100 and 200 mM NaCl and the expression patterns of BFRUCT3, NHX1, OMT and PEAMT genes in root were tested in Clipper, Sahara3771 and advanced line genotypes at three time points of salt treatment: the early stage (24 hours of salt), the intermediate stage (3 days of salt), and the late stage (3 weeks of salt). Analysis of variance for gene expression revealed significant changes in the expression of NHX1 and OMT genes. Transcriptional changes in the gene expression profile were significant for all the genes among time point of salt treatments and among genotypes except NHX1 gene. Salinity x genotypes, salinity x time point and genotype x time point two interactions and salinity x genotype x time point three ways interaction were significantly affected the expression profiles of the genes except NHX1 gene which was not significantly affected by salinity x genotype and salinity x genotype x time point interactions (data not shown).

Table 1. Gene specific primers for the amplification of *PEAMT, OMT, BFRUCT3, NHX1* and *α-tubulin 2*.

Gene	reverse primer	forward primers
PEAMT	TTACAAAGCGAGTCTCGCTCG	CCAGGAGGATTACGACGACATC
OMT	AACCTTTCCCCCCATTTCG	TCCCTCGTCCCACTATCATACC
BFRUCT3	CAAACATTGCCGGTC	GGTTGATCACTCCATCGTGGA
NHX1	ACAACATCTGGTCATACTGCCG	TACGGTTTTCTGCCTCTGTCACA
α-tubulin 2	AGCATGAAGTGGATCCTTGG	AGTGTCCTGTCCACCCACTC

BFRUCT3 gene: At the early stage of salt treatment (24 h), *BFRUCT3* gene was highly up-regulated in the salt tolerant advanced line compared with the varieties under 100 mM NaCl as compared with control. With prolonging salt treatment duration, transcript level of this gene was decreased in Clipper and advanced line. In the roots of Sahara$_{3771}$, transcript level of *BFRUCT3* was increased from 24h to 3 days, but at late time point (3 weeks) it decreased again (Fig. 1a). Under 200 mM NaCl compared with the control, expression of *BFRUCT3* was increased at late stage of treatment in salt tolerant Sahara$_{3771}$ and advanced line and decreased in salt susceptible Clipper (Fig. 1b). Under 200 mM NaCl compared with 100 mM NaCl, *BFRUCT3* gene up-regulated in Sahara$_{3771}$ and advanced line and down-regulated in Clipper genotype with increased salt stress duration (Fig. 1c).

OMT gene: Analysis of transcript level of *OMT* gene revealed that with prolonging salt treatment duration, it down-regulated salt sensitive Clipper and up regulated in salt tolerant Sahara$_{3771}$ varieties under 100 mM NaCl compared with the control. In advanced line, expression of this gene increased with prolonging of salt duration from 24h to 3 days and then decreased in late time point (Fig. 2a). Under 200 mM NaCl compared with control, expression of this gene was decreased in Clipper and advanced line with prolonged salt treatment duration, whereas in Sahara$_{3771}$, its transcript level was decreased from 24h to 3 days and then increased in late time point (Fig. 2b). In the all three genotypes, expression of OMT gene was significantly reduced with increase of salinity level from 100 to 200 mM NaCl with prolonged salt treatment duration (Fig. 2c).

PEAMT gene: Under 100 mM NaCl compared with the control, 3 days after salt treatment, the highest level of *PEAMT* gene was observed in Clipper and transcript level of this gene was decreased in Sahara$_{3771}$ with prolonged salt duration time but in Clipper transcript level of this gene was increased at intermediate time point and then decreased at late time point. In advanced line, at the intermediate time point, the transcript level of *PEAMT* was decreased and then increased at the late time point (Fig. 3a). Compared with the control, under 200 mM NaCl, differential expression of the gene at different time points of salt treatment was recorded at all the three genotypes. In the advanced line and Clipper prolonged salt treatment duration from 24 hours to 3 days resulted in down-regulation of *PEAMT*, whereas up-regulation was observed from 3 days to 3 weeks, but this up-regulation was not significant. For Sahara$_{3771}$ the expression was increased from 24h to 3 days and then decreased at late time point (Fig. 3b). Under 200 mM NaCl compared with 100 mM NaCl, with prolonged salt treatment duration, the transcript level of this gene was increased in Sahara3771 but it was decreased in Clipper at intermediate time point and then increased at late time point. In advanced line, changes were not significant from 24h to 3 days but in 3 week time point expression was highly decreased (Fig. 3c).

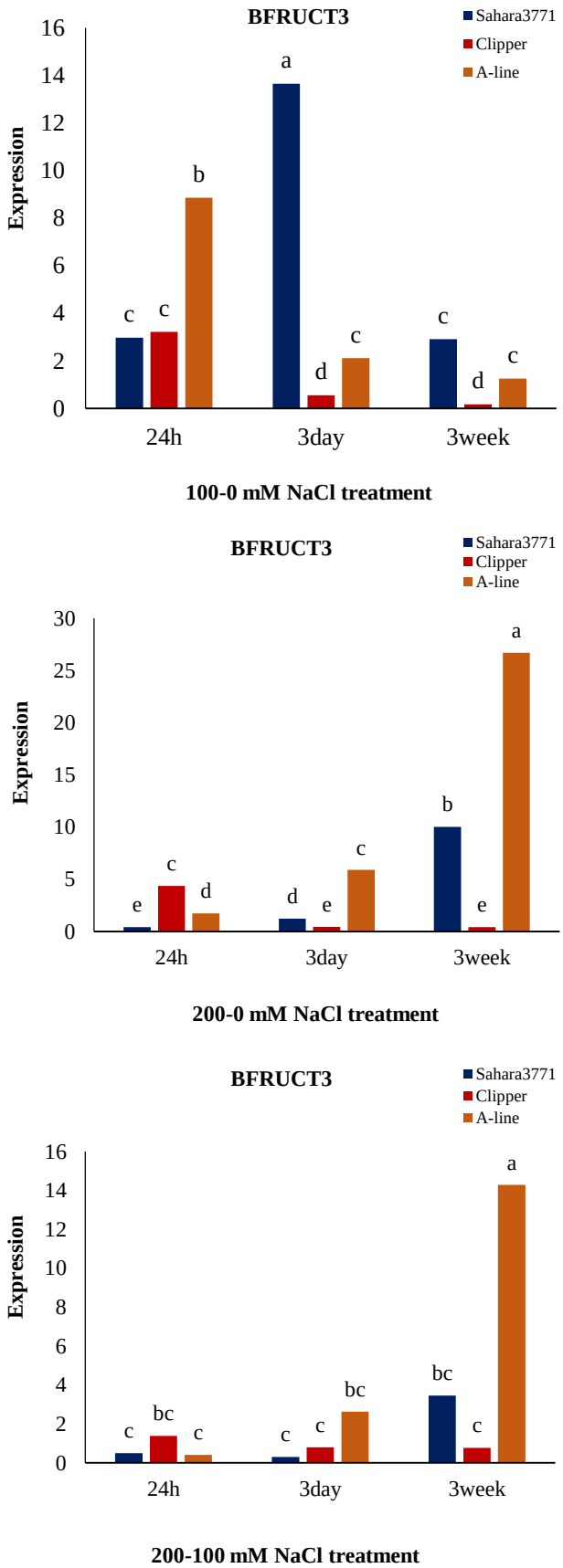
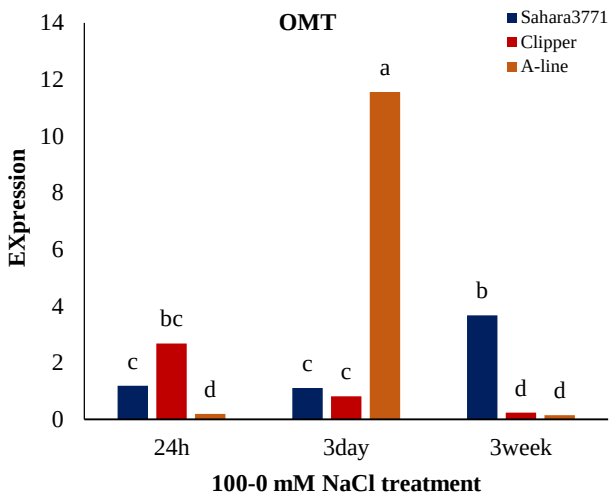
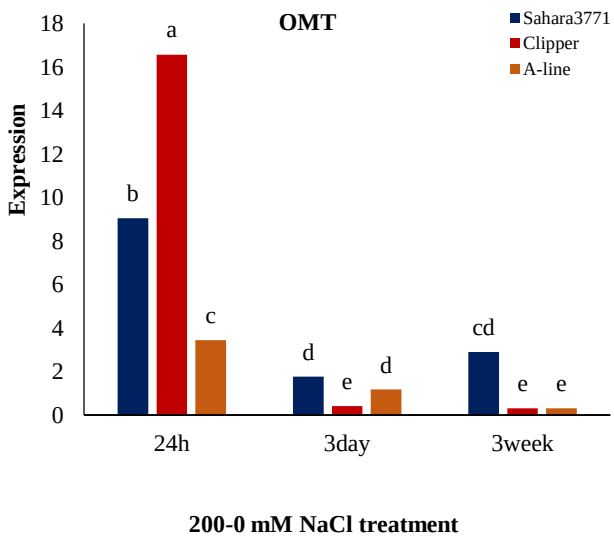


Fig. 1. Mean comparison of expression level for *BFRUCT3* gene under a) 100 mM NaCl treatment compared with the control, b) 200 mM NaCl treatment compared with the control and c) 200 compared with 100 mM NaCl treatment 24h, 3 days and 3 weeks after treatment.





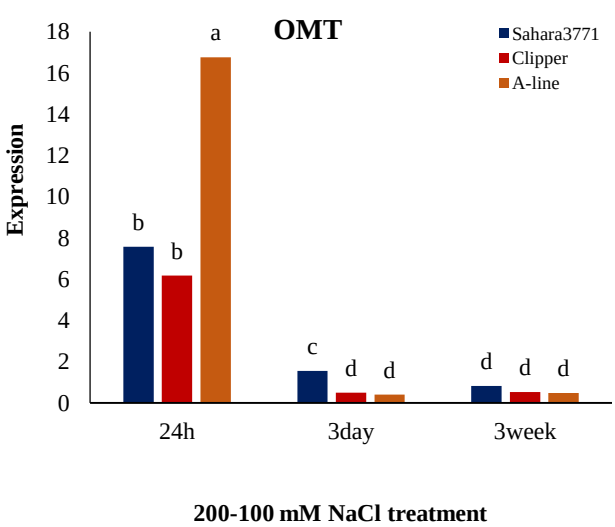
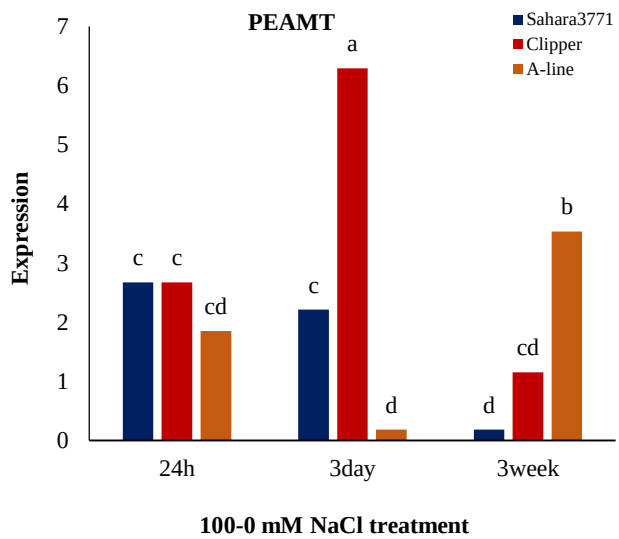
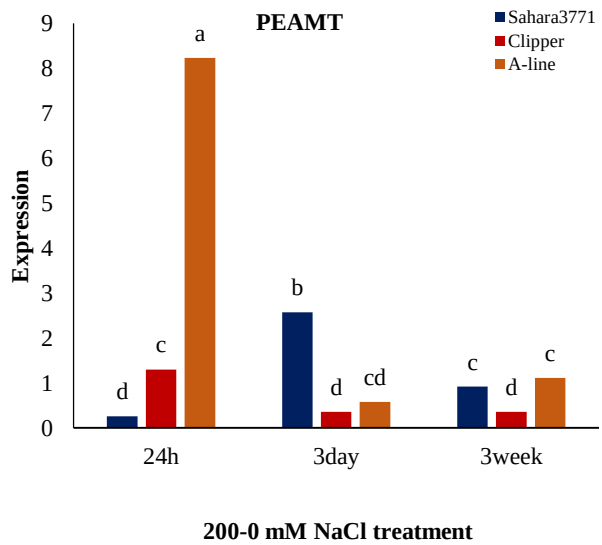


Fig. 2. Mean comparison of expression level for *OMT* gene under a) 100 mM NaCl treatment compared with the control, b) 200 mM NaCl treatment compared with the control and c) 200 compared with 100 mM NaCl treatment 24h, 3 days and 3 weeks after treatment.





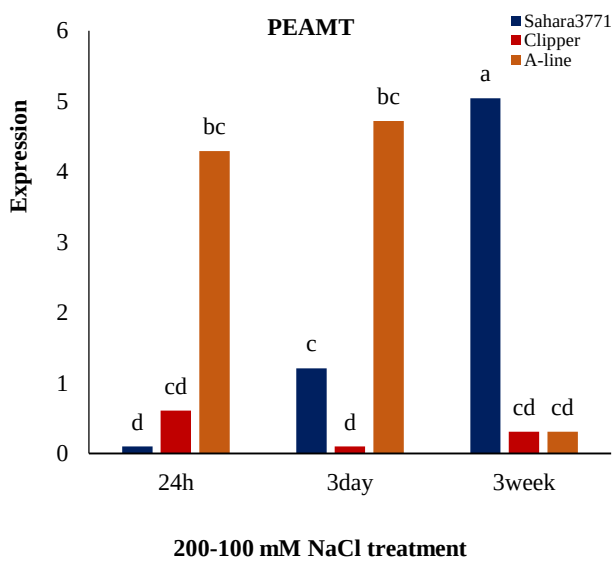


Fig. 3. Mean comparison of expression level for *PEAMT* gene under a) 100 mM NaCl treatment compared with the control, b) 200 mM NaCl treatment compared with the control and c) 200 compared with 100 mM NaCl treatment 24h, 3 days and 3 weeks after treatment.

Discussion

Although barley is known as one of the most salt-tolerant crop plant, variation does exist among different varieties. In this study, three genotypes of barley with differential response to salt stress were used and gene expression was analyzed at different time points after salt treatment. Salinity induces accumulation of soluble sugars and alters the activity of sugar metabolism enzymes in plants. The up-regulation of extracellular invertase (*BFRUCT3*) appears to be a common response to various biotic and abiotic stress-related stimuli such salt stress (Roitsch *et al.*, 2003). Our results showed that expression of *BFRUCT3* was increased with increase of salt stress duration and amount of salinity from 100 to 200 mM NaCl at salt tolerant Sahara$_{3771}$ and advanced line, but decreased in Clipper. (Walia *et al*., 2006) studied the expression of *BFRUCT3* gene using Morex variety, 3, 8 and 27 hours after salt treatment and reported that the expression of the gene decreased at the time period of 3 hours and increased at the 27 hours after the stress treatment. Dubey and Singh (1999) reported that in rice under salinity, acid invertase activity gene was decreased in shoots of the salt tolerant varieties and increased in salt sensitive varieties.

Many plants use sucrose as the major form of transported carbon (Nguyen-Quoc & Foyer, 2001) and the utilization of sucrose depends on its cleavage into glucose and fructose. This reaction is catalyzed by two enzymes; sucrose synthase, a cytosolic enzyme which is crucial to sucrose utilization in fruit development (Sun *et al.*, 1992; Wang *et al.*, 1993) and the invertase, a hydrolase cleaving sucrose into the two mono-saccharides, to maintain the cellular hexoses concentration (Yelle *et al.*, 1988; Scholes *et al.*, 1996). Increased level of invertase activity in the leaves of plants treated with high salt concentrations indicates a major request of hexoses (controlling osmotic potential and cell turgor), substrates necessary to respiratory processes (Muscolo *et al.*, 2003).

In plants, O-methylation is mediated by an enzyme family of O-methyltransferases (OMTs) that transfer the methyl groups from the methyl donor, S-adenosyl-L-methionine (AdoMet) to suitable phenolic acceptor molecules (Lam *et al.*, 2007). In our study, the *OMT* gene highly up-regulated at early stage of salt stress with increase salt level from 100 to 200 mM NaCl, but with prolonged salt duration the transcript level of this gene was decreased in all the three genotypes. This may indicate the short-term response of this gene to increased salt level in the all the three genotypes. (Sugimoto *et al*., 2003) reported that the *OMT* gene was expressed constitutively in the root of salt-tolerant barley variety and the expression level was increased 1.5 times by salt stress, but the salt-sensitive variety showed no changes in expression of the gene in roots and leaves. (Walia *et al*., 2006) reported that in barley transcript level of *OMT* gene increased 3 and 27 hours after salt treatment compared with the control.

Phosphoethanolamine N-methyltransferase (*PEAMT*) related to the synthesis and accumulation of glycinebetaine, a well known compatible solute for osmotic adjustment in plants under salt and drought stresses (Gorham, 1990). with increasing salt level from 100 to 200 mM NaCl up regulated the expression of *PEAMT* in sahara$_{3771}$ at late time points (3days and 3 weeks) and in advance-line at early time point (24h) but down-regulated its expression in Clipper at the all three time points. (Ueda *et al*., 2004) analyzed the expression of *PEAMT* gene in Haruna-Nijyo variety of barley under 100 and 200 mM NaCl and reported strong expression of this gene under salinity stress after 3 days and 3 weeks.

Conclusion

Real-time RT–PCR was used to analysis the expression pattern of a number of key genes involved in Na^+ compartmentation and ROS detoxification, namely *BFRUCT3*, *NHX1*, *OMT* and *PEAMT* in response to salt stress in the root of three barley genotypes. Salt stress increased the expression of *BFRUCT3* in tolerant genotypes with increased stress duration and increase of salinity level. This shows long-term response of this gene to salinity stress in the salt tolerant genotypes. Whereas, expression of *OMT* gene in all the three genotypes was significantly increased at the earlier stage of salt treatment with increasing amount of salinity. Therefore, this gene may be involved in the early response of salinity stress. It can be said that with increase of salt level from 100 to 200 mM NaCl, expression of *PEAMT* has significantly increased in advanced line at the early time point and in Sahara3771 at the late time point, but in Clipper expression of this gene was down-regulation.

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