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Assessment of Some Physiological Parameters and HAK1-type Transporter Transcriptional Changes in *Aeluropus littoralis* under NaCl Stress

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Maintenance of internal status of K^+ ion is one of the important mechanisms for regulation of potassium balance under salt stress. In the present study, we use *Aeluropus littoralis* as a halophytic monocot model to investigate transcriptional changes in HAK1 gene that code a H^+-K^+transporter for acquire potassium under condition of limited availability of K^+ and also measurement of some physiological parameters such as Dry weight, Ash and K^+ content, Photosynthetic pigments and Carbohydrate content under salt stress. The Comparison of HAK1 gene expression in pattern in shoot and root revealed that transcript levels of HAK1 in shoot induced by salt androot contained less levels of HAK1 mRNA than shoot at high concentrations of NaCl. Our results suggest that HAK1-type transporters can play an important role in potassium uptake and cell internal diffusion of K^+ ion to tissue that can be effective in salt tolerance in this grass.

Key words: Photosynthetic pigments, Soluble sugar and starch, Semi–quantative RT-PCR, K^+transporter, Salinity.

The ability of plants to neutralize impact of salinity stress depends on internal status of K^+ ions and the maintenance of a high cytosolic K^+/Na^+ ratio which are considered as essential factors for salt tolerance in plants (Aharon *et al*., 2003). The plants to maintain an adequate potassium status have developed multiple mechanisms for potassium acquisition and transportation. Plants employ high- and low-affinity K^+ uptake systems to act at different external K^+ concentrations (Epstein *et al*., 1963). High-affinity K^+ uptake frequently have been mediated by potassium transporters (Maathuis and Sanders, 1997). HAK1-type transporter is a major K^+ transporter that mediates high-affinity K^+ uptake and belongs to group I of the KT/HAK/KUP family (Rubio *et al*., 2000, Grabov, 2007). HAK1-type transporters probably function as an H^+-K^+symporter and play a key role in potassium acquisition particularly in conditions that K^+ availability is limited by starvation or low contents of this ion (Haro *et al*., 1999; Rodriguez-Navarro, 2000; Banuelos *et al*., 2002; Rodriguez-Navarro and Rubio, 2006). Several genes encoded K^+ transporters of the KT/HAK/KUP family have been identified in some of salt-sensitive and salt-tolerant plants including crops

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(Banuelos *et al*., 2002; Santa-Maria *et al*., 1997;Martinez-Cordero *et al*., 2004; Nieves-Cordones*et al*., 2007), Arabidopsis thaliana (Rubio *et al*., 2000), *Aeluropus littoralis* (Su *et al*., 2007), *Mesembryanthemum crystallinum* (Su *et al*., 2002), *Phragmite saustralis* (Takahashi *et al*., 2007), *Thellungiella halophila* (Aleman *et al*., 2009). *Aeluropus littoralis* as a genetic source for drought and salinity resistance is one of the promising species for genetic improvement and performance of crop plants. The monocot halophyte is a close relative of the most important agronomy plants and seems to show an effective way to improve salt tolerance of this kind of crops. Since halophytes are plants that can tolerate high salinity of soil and complete its life cycle or surviving in saline conditions, this possible which they be best materials to understand molecular mechanism of membrane transporters. This study presents the effect of salinity stress induced by NaCl on the some physiological characteristics and expression pattern of HAK1 gene encoding a K+ membrane transporter to understand that K+ homeostasis in response to salt can be effective on growth and development in *A.littoralis* as a halophyte grass.

MATERIALS AND METHODS

Plant material preparation

Seeds of *A. littoralis* were surface sterilized and then planted in plastic pots containing acid washed and nutrient free sand and grown in a growth chamber (temperature of 25/ 16°C and photoperiod of 14/10 h for day/night and 45-65% relative humidity). The pots were irrigated daily with Hoagland basal-medium. After 45 days, they were treated with complete Hoagland medium containing different concentrations (0, 100, 200, 300, 400 mM) of NaCl. Salinity treatments were gradually increased by daily increments of 100 mM until reach the maximum level of each treatment for avoiding of salt shock. Plants were kept under saline conditions. On the 14th day, plants were harvested in two parts shoot and root and washed with distilled water. Measurement of physiological parameters was carried out on the oven-dried plant materials (for 72 h at 60) and for RNA extraction the samples were immediately frozen in liquid nitrogen and stored in -80 .

Measurement of physiological parameters

Two weeks after applying the treatments, twenty plants randomly selected within each pot was used to assess shoot salt secretion. Detached shoots from each treatment rinsed with cold doubled distilled water to remove all secreted salts from shoot surfaces. Recorded electrical conductivity of each solution was considered as secreted salt. Potassium concentration of the latter solution and doubled distilled water-washed tissues (shoot and root) were determined. Dried shoot and root samples were ashed using incubation at 540 temperatures for 18h and the resulted ash weighted and digested by adding hydrogen chloride. K^+ content of the digested samples and the solutions from dissolving secreted salt were determined using a flame Photometer (Corning-EEL Model 430).

Photosynthetic pigments (Chlorophyll a and b) were determined according to Hiscox and Israestam (1979) method. In brief, 20 mg oven-dried leaf samples were ground and placed in a tube containing 2 ml of dimethylsulphoxide (DMSO). Tubes were incubated at 70°C for 60 minutes. Absorbance of the liquid extract was measured at 646 nm and 663 nm by spectrophotometer (Biowave II, Biochrom Ltd, England). Related calculation on chl data were carried out using the equations proposed by Lichtenthaler (1987). Soluble sugar and starch content in the shoot and root tissues were determined by the phenol-sulfuric acid method (Kochert, 1978) at wave length 485 nm.

Semi-quantitative RT-PCR analysis

RNA isolation and cDNA preparation

Shoot and Root samples were frozen in liquid nitrogen and kept at -80. The frozen samples (80-100 mg FW) were homogenized with mortar and pestle. Total RNA was extracted from samples with the TRIZOL reagent (Invitrogen. Inc., CA,USA) according to the manufacturer instruction manual. Total RNA (4µg) was treated with RNase-free DNaseI (Fermentas. Cat. No. EN0521) for removal of genomic DNA contamination. Single-strand cDNA was synthesized from 2µg of total RNA using the RevertAid™ Reverse Transcriptase (Fermentas. Cat. No. EP0441). semi qRT-PCR (Semi quantitative reverse transcription-polymerase chain reaction) of HAK1 gene were carried out using specific primers that were designed using Oligo 7 software according to the highly conserved

amino acid sequence of genes obtained from GenBank to amplify a specific fragment around 600 bp. The forward primer for the HAK1 was 5'-CATCATCTACGGTGACATCG-3', and the reverse primer 5'-ATTGCCACAGAGATCCAGAC-3'. The specific primers for actin as internal refrence gene designed according to the cDNA sequence of actin from *A.littoralis* and amplified a 330bp fragment. The forward primer was 5'-GTGCCCATTTA CGAAGGATA-3' and the reverse primer 5'-GAAGACTCCATGCCGATCAT-3'. PCR condition for actin amplification were: 95°C for 3 min; 25 cycles of 94°C for 45s, 55°C for 45s followed by 72°C for 1 min; and a final extension at 72 for 2 min. Optimized RT-PCR conditions was accomplished for HAK1 gene. The PCR products were separated on 1.2% (w/v) agarose gels and stained with ethidium bromide. Gel images were obtained and quantified using Total Lab TL120 software with accordance to GeneRuler 1Kb DNA ladder (Fermentas, SM0333) bands.

Statistical analysis

Data were statistically analyzed with SPSS 16, using Duncun test to determine statistical significance.

RESULTS AND DISCUSSION

Present results from measurement of growth parameters showed that *A. littoralis* maintained optimal growth at 200mM, and growth declined with increasing saline concentration. At high saline concentrations, growth diminution might be caused by reasons such as reduction of ability to adjust osmotic balance due to saturation of solute uptake system (Munns *et al*., 1983) or excessive energy requirement of such systems (Gale and Zeroni, 1985) and nutrient deficiencies (Marschner 1995). Shoot dry weight was increased to 200 mMNaCl level and declined with a further increase in salinity (Table 1). Increment of dry mass production at 200mM NaCl may be due to ion uptake and enhancement of inorganic ions content (Khan *et al*., 2001; Flowers *et al*., 1977), because growth and survival of halophytes is dependent on the high level of ion accumulation for the maintenance of turgor and osmotic adjustment (Flowers *et al*., 1977). Similar results were shown in some of salt-tolerant plants such as *Suaeda maritima* (Clipson *et al*., 1985), *Atriplex halimus* (Bajji*et al*., 1998), *Suaeda fruticosa* (Khan *et al*., 2000), *Salicornia rubra* (Khan *et al*., 2001) and *Aeluropus lagopoides* (Gulzar*et al*., 2003). Ash content was increased at 200 mM and decreased with 400 mM salt concentration (Table 1). Enhancement of ash content from 0 to 200 mMNaCl is due to ions increment and mineral elements uptake and also reduction of ash content at high salinities might be cause of increase salt secretion or decrease essential nutrient uptake. The secreted potassium content was increased 7 folds in comparison with control (Table 1), While potassium concentration of the shoot, in contrast to root, decreased with increasing in salinity levels (Figure 1). The increment of potassium content of the root, in contrast to aerial part, under salinity is probably mediated by two different hypothesis: (1) potassium uptake is increased (2)Potassium for maintenance of K^+/Na^+ ratio is not translocated to shoot and this ion aggregated in roots. In present study, a decrease in all measured photosynthetic parameters was observed under salt stress. This result, in accordance with data obtained previously on *Suaeda salsa* (Congming *et al*., 2002), *Salvadora persica* (Dagar *et al*., 2004), *Aegiceras corniculatum* (Parida *et al*., 2004) and sorghum (Azooz *et al*., 2004). Contents of photosynthetic pigments (chlorophyll a and b) and total chlorophyll were significantly decreased in response to salinity (Table 2). Chlorophyll a and b showed significant reduction by 1.3 and 1.6 folds at 400 mMNaCl in comparison with control, respectively (Table 2).

The ratio of chlorophyll a/b also increased under high saline condition especially at 400 mMNaCl (Table 2). Chlorophyll content decline under saline stress is a commonly reported phenomenon and possibly has been attributed to different reasons such as membrane deterioration, destruction of chlorophyll pigments, the instability of the pigment-protein complex due to changes in the lipid protein ratio of pigment-protein complexes, interference of salt ions with the de novo synthesis of proteins, inhibition of synthesis of chlorophyll or accelerating in degradation of chlorophyll and chlorophyllase activity increment (Levit, 1980; Reddy and Vora, 1986; Sudhakar *et al*., 1991; Iyengar and Reddy, 1996; Jaleel *et al*., 2007). Chlorophyll a content predominated over chlorophyll b. Imbalance in chlorophyll a and

chlorophyll b sensitivity in response to salinity caused to chlorophyll a/b ratio increment. Root carbohydrate content (soluble sugars and starch) showed an increase in response to salinity while the highest amount of soluble sugars and starch content in the shoot were obtained in 200 mM and 300 mMNaCl, respectively (Table 3). The accumulation of carbohydrates (soluble sugar and starch) in many of the plants as an osmoregulation agent has been widely reported in response to salinity or drought and major roles have been suggested for these carbohydrates such as osmo protection, osmotic adjustment, carbon storage and radical scavenging despite of a significant decrease in CO_2 assimilation rate (Dubey, 1999; Stivesev *et al*., 1973; Strogonove *et al*., 1970). The decrease in sugar content of shoot in high salinities could be either due to high respiration or a decrease in photosynthetic activity accompanied by reduction in growth rate (Sivasankaramoorthy *et*

Table 1. Effect of different NaCl concentrations on Dry weight, Ash content and secreted of K^+ contents of shoots of *Aeluropus littoralis* plants

NaCl (mM)	Dry weight (mg plant^{-1})	Ash content (mg DW^{-1})	K^+ (µmol g DW^{-1})
0	42.295 b	4.208 ab	30.442 b
100	39.462 b	4.346 ab	196.631 a
200	57.925 a	5.014 a	180.376 a
300	39.680 b	4.057 ab	229.149 a
400	43.067 b	3.321 b	198.009 a

Plants were kept under salinity treatments (0,100,200,300,400mM NaCl) during two weeks. Values followed by the same letter are not significantly different (P = 0.05) as described by Duncan's test. Data are means of three replications

Table 2. Effect of different NaCl concentrations on the chlorophyll (mg g^{-1}DW) content of *Aeluropus littoralis* plants

NaCl concen. (mM)	Chlorophyll a (mg g^{-1}DW)	Chlorophyll b (mg g^{-1}DW)	Total Chlorophyll (mg g^{-1}DW)	chlorophyll a/b (mg g^{-1}DW)
0	2.38 a	0.92 a	3.30 a	2.64 b
100	2.41 ab	0.88 a	3.29 a	2.78 b
200	2.09 b	0.69 b	2.78 b	3.03 ab
300	1.95 b	0.66 b	2.61 b	2.97 ab
400	1.85 b	0.56 b	2.41 b	3.31 a

Values followed by the same letter are not significantly different (P = 0.05) as described by Duncan's test. The data are means of three Replications

Table 3. Effect of different NaCl concentrations on the Soluble sugar and starch contents in Shoots and Roots (mg g^{-1} DW) of *Aeluropus littoralis* plants

NaCl	Soluble sugars (mg g^{-1}DW)		Starch (mg g^{-1}DW)	
concen. (mM)	Shoot	Root	Shoot	Root
0	25.75 c	5.77 b	90.70 b	49.09 c
100	37.55 b	7.09 b	88.91 b	35.05 d
200	74.52 a	17.27 a	110.66 b	77.48 b
300	42.14 b	4.86 b	158.89 a	66.38 b
400	42.14 b	17.83 a	73.48 b	96.61 a

Values followed by the same letter are not significantly different (P = 0.05) as described by Duncan's test. The data are means of three Replications

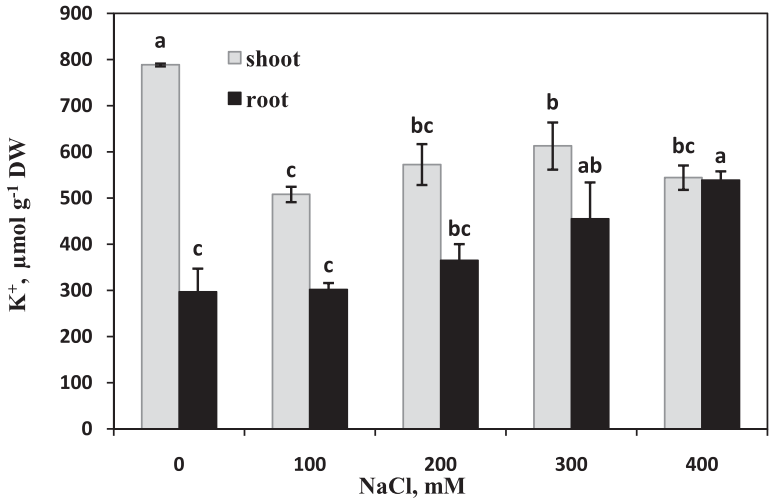

Fig. 1. Effect of NaCl concentrations on K^+ contents in shoots and roots of *Aeluropus littoralis*. Plants were kept under saline conditions supplied with different NaCl concentrations for 2 weeks (see "Materials and methods" for details of sand culture). Values are Means±SE of three Replications and bars indicate SE.Columns with different letters indicate significant differences at P = 0.05 by Duncan's test

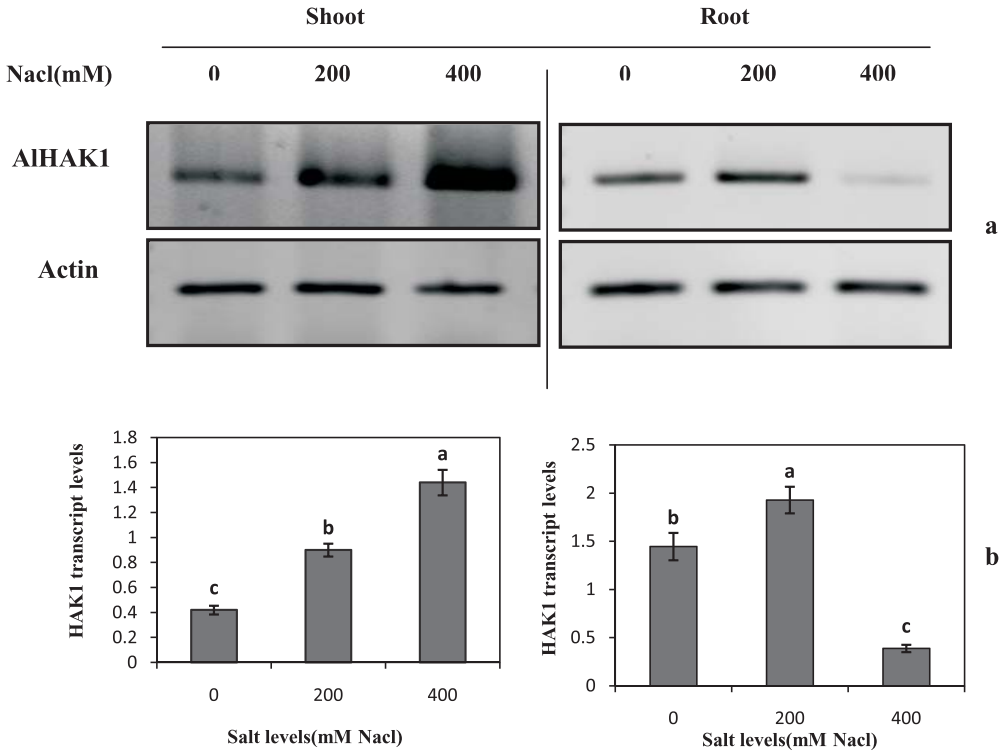

Fig. 2. Expression of AlHAK1 in shoot and root of *A.littoralis* under different concentrations of NaCl. (a)Semi-quantitative RT-PCR analysis of AlHAK1 mRNA in plants treated with 0,100,200, 300 and 400 mM NaCl for two weeks. ACTIN was used as an internal control. Experiments were repeated at least three times to obtain similar results.Only the optimal pictures are shown. (b)The relative expression level of AlHAK1 (related to ACTIN) under different concentrations of NaCl. Values are Means±SD and bars indicate SD.Columns with different letters indicate significant differences at P = 0.05(Duncan'stest)

al., 2011). The high accumulation of starch content in root and shoot could be as a source for soluble sugars synthesis under salinity conditions.

AlHAK1 gene expression was up regulated in shoot by 2.3 and 3.5 folds under 200 mM and 400mM of NaCl, respectively, while in root, transcript levels of this gene was declined about 4 folds in response to 400 mM (Figure 2).This result, in coincidence with data obtained by Hua *et al*. (2002) on Mesembryanthemumcrystallinum.In Thellungiellahalophila, the expression of ThHAK5 as high-affinity transporter observed in K+ starvation conditions (Aleman *et al*., 2009). Whereas, HAK1-type transporter is a major high-affinity K^+ transporter that play a key role **in**potassium acquisition, particularly in conditions that K^+ availability is low or limited by K^+ starvation thus reduction of potassium content of shoot causes induction in AlHAK1 expression in shoot. Also, in root with increment of potassium content, AlHAK1 expression decreased due to potassium uptake decline and maintenance of K^+/Na^+ ratio.

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