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The influence of genes regulating transmembrane transport of Na^+ on the salt resistance of *Aeluropus lagopoides*

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Abstract. Plantlets of *Aeluropus lagopoides* (Linn.) Trin. Ex Thw. were grown at different NaCl concentrations (26, 167, 373 and 747 mM) for 3, 7 and 15 days; their growth, osmotic adjustment, gas exchange, ion compartmentalisation and expression of various genes related to Na^+ flux was studied. Plantlets showed optimal growth in non-saline (control; 26 mM NaCl) solutions, whereas CO_2/H_2O gas exchange, leaf water concentration and water use efficiency decreased under all salinity treatments, accompanied by increased leaf senescence, root ash, sodium content and leaf osmolality. A decrease in malondialdehyde (MDA) content with time was correlated with Na^+ accumulation in the leaf apoplast and a concomitant increase in Na^+ secretion rate. *A. lagopoides* accumulated a higher concentration of Na^+ in root than in leaf vacuoles, corresponding with higher expression of *V-NHX* and lower expression of *PM-NHX* in root than leaf tissue. It appears that *V-ATPase* plays a vital role during Na^+ transport by producing an electromotive force, driving ion transport. Leaf calcium increased with increasing salinity, with more rapid accumulation at high salinity than at low salinity, indicating a possible involvement of Ca^{2+} in maintaining $K^+ : Na^+$ ratio. Our results suggest that *A. lagopoides* successfully compartmentalised Na^+ at salinities up to 373 mM NaCl by upregulating the gene expression of membrane linked transport proteins (V-NHX and PM-NHX). At higher salinity (747 mM NaCl), a reduction in the expression of *V-NHX and PM-NHX* in leaves without any change in the rate of salt secretion, is a possible cause of the toxicity of NaCl.

Additional keywords: gene expression, growth, ion regulation, Na^+ sequestration, photosynthesis, salt stress.

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Introduction

Salinity is one of the most important environmental factors that limit plant growth and productivity worldwide (Koyro *et al*. 2011). Given this scenario, there is a need to improve salt tolerance of crops, a task requiring better understanding of this multi-genic trait. The detrimental effects of salinity on plants include osmotic stress and excessive Na^+ accumulation in the cytoplasm, which affects critical biochemical processes (Maathuis and Amtmann 1999), with disturbance of ion homeostasis, damage to plasma membrane integrity (Kawasaki *et al*. 2001) and impact on photosynthetic machinery (Abogadallah 2010). This complexity exists both in halophytes and glycophytes (Zhu 2001; Tester and Davenport 2003), although appropriate regulation of the transcriptome in halophytes controls an array of physiological processes that makes them salt resistant (Maathuis and Amtmann 1999; Zhu 2001).

The survival of plants on saline media depends on ion-specific mechanisms such as the compartmentalisation of K^+ in the cytoplasm and Na^+ in the vacuole, in addition to osmotic adjustment and defence against oxidative stress (Blumwald 2000; Chen *et al*. 2007; Cosentino *et al*. 2010). Plants may reduce the quantity of Na^+ and maintain higher $K^+ : Na^+$ ratios in cytoplasm, particularly in photosynthetic tissues, by limiting unidirectional Na^+ influx into roots (Wang *et al*. 2009), reducing entry of Na^+ into the xylem stream, retrieval of Na^+ from the xylem (Tester and Davenport 2003), re-translocation of Na^+ from

shoots to roots, compartmentalisation of Na^+ into vacuoles or cell walls and secretion from above ground tissues (Naz *et al.* 2009). The compartmentalisation of Na^+ in cellular organelles provides an efficient mechanism to prevent its deleterious effects in the cytosol and also to achieve a lower water potential compared with the growth media (Blumwald 2000; Tester and Davenport 2003; Flowers and Colmer 2008).

The active outward transport of Na^+ from the cell and compartmentalisation in vacuoles is mediated via salt overlay sensitive (SOS) pathway (*SOS1*: *PM-NHX*) and tonoplast $Na^+:H^+$ antiporter (*V-NHX*), respectively, using energy generated through H^+-translocating ATPases (*H^+-ATPase*) or pyrophosphatase (*H^+-PPase*). Tissue specific Na^+ partitioning is an important strategy which is species dependent (Flowers and Colmer 2008). Monocots do accumulate Na^+ in a lower ratio with K^+ in shoots compared with dicots (Flowers and Colmer 2008; Marcum 2008) and ~15% of monocotyledonous halophytes excrete Na^+ and Cl^- through bi-cellular epidermal salt glands (Adams *et al.* 1998). Overexpression of genes related to Na^+ flux has been reported to improve salt tolerance in some plants (Xue *et al.* 2004; He *et al.* 2005). Such studies indicate that Na^+ compartmentalisation into the vacuole and expression of *V-NHX*, *H^+-ATPase*, *H^+-PPase* and *SOS1* play a major role in salt tolerance (Oh *et al.* 2009). Moreover, Na^+ sequestration in the vacuole also implies prevention of its leaking back into cytosol (Shabala and Mackay 2011).

Aeluropus lagopoides (Linn.) Trin. Ex Thw. (Poaceae) is a stoloniferous, salt secreting, perennial grass that is distributed in semi-desert climates (Vaziri *et al.* 2011). This grass can survive in up to 1 M NaCl; however, salinity greater than 300 mM NaCl is considered toxic (Gulzar *et al.* 2003). *A. lagopoides* is being utilised as forage in some parts of India and the Iranian plateau because of low shoot sodium concentration (Torbatinejad *et al.* 2000). *A. lagopoides* can also be used to prevent soil erosion and aid sand dune stabilisation due to its highly developed network of roots and high salinity tolerance (Tewari 1970). *A. lagopoides* is also reported as a wild relative of wheat (Razavi *et al.* 2006). Therefore, it is a suitable model plant to understand the salt resistance mechanism in grasses and could help to develop salt resistance of cereals (Flowers and Colmer 2008). The possibility of improving salt resistance in wheat by asymmetric somatic hybridisation along with its wild relative has been reported (Yue *et al.* 2001).

Several other ecological and physiological studies on *A. lagopoides* have been conducted (Waghmode and Joshi 1982; Waghmode and Hegde 1984; Joshi and Bhoite 1988; Sher *et al.* 1994; Bhaskaran and Selvaraj 1997; Abarsaji 2000), which indicates that it accumulates substantial amounts of Na^+ and Cl^- in its roots. Sobhanian *et al.* (2010) reported that its salts secreting ability helps in regulating shoot Na^+ of *A. lagopoides* seedlings raised in 450 mM NaCl for 10 days. They also determined, using proteomics, that 2.1% of proteins were upregulated, whereas 2.4% were downregulated. Those upregulated were associated with energy formation, amino acid biosynthesis, C_4 photosynthesis and detoxification, which may be involved in salt resistance of *A. lagopoides*. Similarly, Razavi *et al.* (2006) using differential display amplified fragment length polymorphism (DD-AFLP) predicted a possible involvement of genes related to the signalling cascades, regulation of gene expression and osmotic adjustment in the survival of *A. lagopoides* under harsh conditions (such as drought). However, information is lacking about changes in gene expression as related to changes in growth and physiological and biochemical mechanisms. Therefore, we investigated early and late responses of plant growth, physiological mechanisms and expression of Na^+ manipulating genes, as well as the regulation of Na^+, in *A. lagopoides* in various salinities (26, 167, 373 and 747 mM NaCl). The objectives of the present work were to: (1) investigate the changes in the response of growth, gas exchange, water relations and Na^+ flux under NaCl; (2) investigate the coordination between root and leaf tissues for modulating Na^+ flux; (3) observe the relationship between kinetics of Na^+ accumulation and expression of Na^+ manipulating genes; and (4) understand the importance of temporal variation in salt secretion and the expression of Na^+ manipulating genes in NaCl resistance of *A. lagopoides*.

Materials and methods

Plant material

Aeluropus lagopoides (Linn.) Trin. Ex Thw. was collected from coastal habitats of Hawks Bay, Karachi, Pakistan (24°52′21.87″N, 66°51′24.58″E) and a perpetual culture in a green net-enclosed system that reduces light by 60% and facilitates air circulation (semi-ambient environmental condition) was established. Plantlets originating from tillers of *A. lagopoides* growing in beach sand were sub-irrigated with half-strength Hoagland nutrient solution (Hoagland and Arnon 1950).

Growth conditions

Plantlets of *A. lagopoides* were transferred to pots (one plant per pot; pot size: 6 × 10 cm) containing washed soil collected from natural populations of *A. lagopoides* and subirrigated with nutrient solution. Plantlets were grown under the semi-ambient environmental conditions described above. Plantlets of similar size (1 month old; six leaf stage) were selected for the experiment and divided into four groups (26, 167, 373 and 747 mM NaCl; 48 plantlets per group) which were further distributed into four subgroups (0, 3, 7 and 15 days; 12 plantlets per sub-group). The control group was irrigated with nutrient solution (which contained 26 mM NaCl) and saline treatments were given using NaCl in Hoagland solution solutions (Table 1). The salinity was increased stepwise (150 mM NaCl daily) until the desired concentration was reached. The volume of nutrient solutions

Table 1. Concentration of Na^+ and K^+, electrical conductivity, pH and osmotic potential in used NaCl treatments

NaCl treatment (mM)	Na^+ (mM)	K^+ (mM)	EC (mS cm^{-1})	pH	OP (mosmol kg^{-1} H_2O)
0	26	4.5	1.7	7.5	61
150	167	5.3	18.8	7.3	329
300	373	3.4	29.6	7.4	625
600	747	5.0	55.3	7.3	1186

was adjusted with water every alternate day to compensate for evaporation and was replaced after 5 days.

Harvest and growth parameters

Plants were harvested on 0, 3, 7 and 15 days after the desired salt concentrations were reached. Each individual was carefully removed from the soil and the leaves washed by dipping twice in distilled water for a few seconds and then wiped with tissue paper and roots were washed with respective NaCl solution followed by ice-cold 10 mM $CaCl_2$ solution (to remove sodium from surface). Roots and shoots were separated and FW determined for each plant. A portion of the fresh samples were immediately frozen in liquid nitrogen, stored at −80°C and subsequently freeze-dried. The remainder of the samples was oven-dried at 75°C for 48 h (when no weight change was observed). Tissue water concentration (WC) for shoot and root was determined separately using the equation: $WC = ((FW - DW)/FW) \times 100$. The growth rate of *A. lagopoides* was determined using four individual, from each salinity treatment. Non-destructive parameters (shoot length, leaf length and number of leaves including total and senesced leaves were recorded after 0, 3, 7 and 15 days from the start of the experiment.

Measurement of Na^+, K^+ and Ca^{2+} in tissue sap

To determine the concentration of Na^+, K^+ and Ca^{2+} in leaf and root tissues 'press-sap' was used. Root and fully-expanded young leaves (third node from the tip) were rinsed (as indicated above) immediately before collection from individuals growing in different NaCl treatments after 0, 3, 7 and 15 days. Sampled were wrapped in aluminium foil and immediately frozen at −18°C. Close to the time of measurements, frozen samples were thawed and hand squeezed to extract all the sap as described by Cuin *et al*. (2009). Extracted samples were mixed thoroughly, diluted and used for the determination of Na^+, K^+ and Ca^{2+} by atomic absorption spectrometer (AA-700; Perkin Elmer, Santa Clara, CA, USA).

We estimated the selective absorption ratio of K^+ vs Na^+ from the medium (SA) and the selective transport ratio of K^+ vs Na^+ from root-to-shoot (ST) was calculated using the following formulae (Debez *et al*. 2010):

$$SA = \frac{[K/(K+Na)]_{root}}{[K/(K+Na)]_{medium}}, \quad (1)$$

$$ST = \frac{[K/(K+Na)]_{leaf}}{[K/(K+Na)]_{root}}. \quad (2)$$

Determination of Na^+ concentration in the leaf apoplast and symplast

Five plants were harvested from 26 and 747 mM NaCl treatments after 15 days of treatment. Aboveground parts of plants were divided into three equal zones (lower (first 3 nodes from ground), middle (4–6 nodes) and upper (7–9 nodes)). Leaves were collected from each zone and sap from the apoplast and symplast collected separately by centrifugation (Yu *et al*. 1999). The samples were spun at 2000*g* for 15 min at 4°C to obtain the apoplastic sap. After centrifugation, samples were frozen at −80°C for 4 h and then thawed at room temperature before extraction of the symplasmic sap by centrifugation at 2000*g* for 15 min at 4°C. Na^+ concentration in the sap was determined by atomic absorption spectrometer (Perkin Elmer).

Secretion of Na^+

To determine the rate of Na^+ secretion, leaves were rinsed with 2 mL deionised water in Eppendorf tubes (Eppendorf, San Diego, CA, USA). Fully expanded young leaves (third node from the top) of three plantlets were tagged from each subgroup (0, 3, 7 and 15 days) of NaCl treatments (26, 167, 373 and 747 mM). Tagged leaves of each subgroup were prewashed 3 days before the collection of data (0, 3, 7 and 15 days of experiment). The amount of Na^+ secreted was determined by atomic absorption spectrometer (Perkin Elmer) and the area of rinsed leaves was determined by ImageJ software ver. 1.45 (http://rsb.info.nih.gov/ij/, accessed 10 September 2010). The rate of Na^+ secretion was expressed in μmol Na^+ cm^{-2} day^{-1}.

Leaf osmolality

Sap was extracted (as indicated above) from the frozen leaves and used directly to determine the osmolality by vapour pressure osmometer (VAPRO-5520; Wescor Inc., Logan, UT, USA) (Gucci *et al*. 1991). The contribution of Na^+ and K^+ to leaf ψ_s was calculated by van't Hoff equation (Kramer and Boyer 1995).

Gas-exchange measurements

The rate of net photosynthesis (P_n), respiration (R), stomatal conductance (g_s) and transpiration (E) were measured on randomly chosen plantlets ($n = 4$) from each salinity treatment after 0, 3, 7 and 15 days from the day of NaCl application. Measurements were taken on fully-expanded leaves (from third node) using 2 × 3 cm chamber (6400–08 clear chamber bottom) of a portable photosynthesis system (6400XT, Li-Cor Inc., Lincoln, NE, USA) at ambient RH 60–80%, reference CO_2 of 380 μmol m^{-2} s^{-1}, flow rate of 500 μmol s^{-1} and photosynthetically active radiation of 600 μmol m^{-2} s^{-1}. Each leaf was first equilibrated in the chamber until constant readings were obtained.

Lipid peroxidation/malondialdehyde content

The level of lipid peroxidation in leaf samples was determined in terms of malondialdehyde (MDA) content (Heath and Packer 1968). For the MDA assay, 0.1 g fresh tissue (root and shoot) was homogenised with 1 mL trichloroacetic acid (TCA) 1% and centrifuged at 10 000*g* for 5 min at 4°C (Sorval Evolution RC, Kendro, Newtown, CT, USA). For measurement of MDA concentration, 4 mL of 20% trichloroacetic acid containing 0.5% 2-thiobarbituric acid was added to a 1 mL aliquot of the supernatant. The mixture was heated at 95°C for 30 min, cooled quickly in an ice bath and then centrifuged at 10 000*g* for 10 min at 4°C. The absorbance of the supernatant was read at 532 and 600 nm. The concentration of MDA was calculated using the MDA extinction coefficient of 155 mM^{-1} cm^{-1}. The result of MDA was expressed as μg mg^{-1} FW.

Quantification of gene expression by qRT–PCR

RNA was extracted from *A. lagopoides* leaf and root tissues ($n = 4$), harvested at 0, 3, 7 and 15 days from each treatment

(26, 167, 373 and 747 mM NaCl). Samples were frozen in liquid nitrogen, ground fine and RNA extracted with an RNAqueous Kit (Ambion, Austin, TX, USA). Quality and quantity of RNA was checked using a Spectrophotometer (DU Series 700, Beckman Coulter) and integrity by electrophoresis on 1% agarose gel. DNA was removed from RNA samples using DNase (DNA free kit, Ambion) following the manufacturer's instructions. First-strand of cDNA was synthesised from 1 µg RNA (DNA free) by using the protocol of cDNA Takara RNA PCR Kit (AMV; ver 3.0). The cDNA was cooled to 4°C and stored at −20°C for subsequent use for expression analysis of different genes.

For quantitative real time PCR (qRT–PCR), primers (see Table 2) were designed using selected gene sequences of *A. lagopoides* present in the NCBI database. *Actin* gene of *A. lagopoides* was used as an internal reference for qRT–PCR. Quantitative RT–PCR of different genes was performed by using the protocol for 20 µL reaction mixture in light cycler-carousel-based system (Roche Diagnostics, Alameda, CA, USA). Dilutions of the extracted plasmid (from 10^{-4} to 10^{-8} pg) were used in qRT–PCR to plot standard curves of selected genes. Dilutions for standard curve, cDNA of samples (from each NaCl treatment and time period) and negative controls were run in duplicate. PCR efficiency was calculated with all standard curves having an R^2 of 0.99 or higher. The data was quantified using LightCycler software ver. 4.0 (Roche).

Statistical analyses

Statistical analysis was conducted using SPSS ver. 11.0 for windows (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to identify significant effects of NaCl concentration and duration of salinity stress on growth, gas exchange, water relation, MDA content, cations and expression of genes, and significance level was $P < 0.05$. A Bonferroni test was performed to compare individual means if the effects were significant. Data in the form of means and standard errors were used to construct graphs by Sigma Plot for Windows ver. 10.0 (Systat Software, San Jose, CA, USA). Correlation analyses were conducted using the correlation functions in Excel. Pearson coefficients were calculated to assess correlations between different variables.

Results

Growth and water content

Leaf elongation rate, number of leaves and height of plant decreased with the increases in salinity, whereas leaf elongation stopped at 747 mM NaCl. Increase in leaf senescence was observed at the highest salinity (Fig. 1). Leaf

Table 2. Primer sequences and NCBI-records used for selected genes

Gene	Primer name	Sequence (5′→ 3′)	NCBI-ID
PM-NHX	PMN-F PMN-R	TATCGAATGGTGCTCGGAAGA, AGCCCAGCCACAGTACCGATA	*ISHU-Ala-4* GW796824
V-NHX	V-NHX-F V-NHX-R	GCAGGTCCTCAATCAGGATG, ACTCCAAGGAAGGTGCTTGA	*AlaNHX* GU199336
H^+-ATPase	ATP-F ATP-R	GAGGACTGCAAGAGCGGATTAC, TACCAAGCTCAGAAATCTGTCG	*ISHU-Ala-3* GW796823
ACTIN	ACT-F ACT-R	TACGAAGGGTTTACGCTTCCT, TCTCCAACTCCTCCTCGTAAT	*ISHU-Ala-2* GW796822

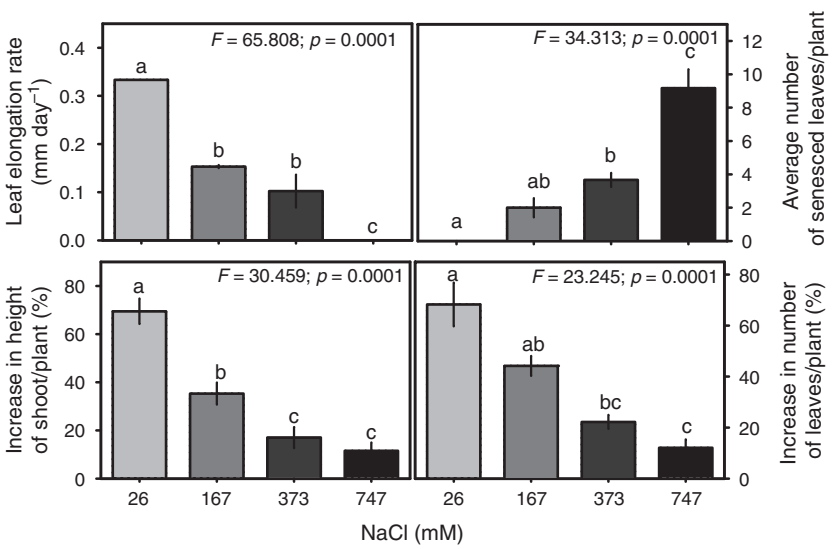

Fig. 1. Growth parameters of *Aeluropus lagopoides* in four NaCl concentrations for 15 d. *F*- and *P*-values were obtained from one-way ANOVA. In the lower two graphs, the % increase is compared with initial time value. Values with similar Bonferroni letter were not significantly different at $P < 0.05$.

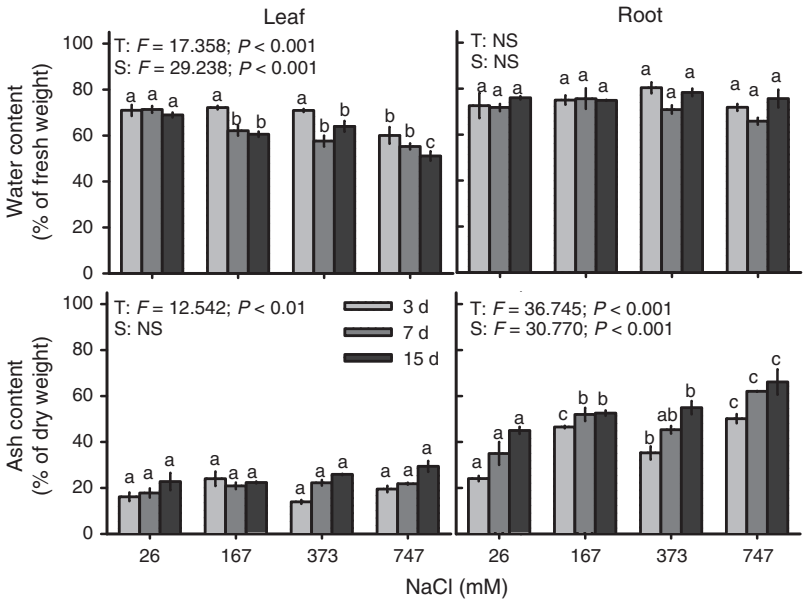

Fig. 2. Effect of NaCl concentrations and time of exposure to salinity on water and ash contents in leaf and root tissues of *Aeluropus lagopoides*. *F*- and *P*-values were obtained from one-way ANOVA by using T (time period of salinity exposure) and S (NaCl treatment). For each time period, values at different salinity levels with similar Bonferroni letter were not significantly different at $P < 0.05$ (NS, non-significant).

water concentration (water as % of FW) was significantly reduced at 747 mM NaCl, whereas no change was recorded in root water concentration. Ash content increased significantly ($P < 0.001$) in roots with salinity, but no such changes were recorded in leaves (Fig. 2).

Flux in Na^+, K^+ and Ca^{2+}

Na^+ in both root and leaf tissue increased at least 10-fold when plantlets of *A. lagopoides* were exposed to 747 mM NaCl concentrations for an extended period (15 days, Fig. 3). Moreover, the amount of leaf Na^+ was at least three times lower in 747 mM NaCl compared with the root tissues after 3 and 7 days of experiment (Figs 3, 4*e*).

Leaf K^+ remained unchanged in up to 373 mM NaCl. However, there was a significant reduction with time at 747 mM NaCl (Fig. 3). Root K^+ was generally lower compared with leaf K^+. At low salinity, root K^+ concentration increased with time, although there was no change at higher salinity concentration. Leaf Ca^{2+} was higher than root and showed a rapid increase in 167 to 373 mM NaCl although there was no significant change in root Ca^{2+} (Fig. 3).

The ratio between leaf Na^+ and K^+ increased with increase in salinity and time of exposure, with approximately a 10–12 fold higher $Na^+ : K^+$ observed at 747 mM NaCl, relative to control after 15 days (Fig. 4*a*, *b*). Roots had more Na^+ content than leaves in 373 and 747 mM NaCl (Fig. 4*e*). The values for selective transport (ST) of K^+ were lower than those for selective absorption (SA) (Fig. 4*c*, *d*). Slightly higher values of ST were recorded in 373 and 747 mM NaCl treatments than in the control (Fig. 4*c*). However, the values of SA were higher than the control values in all NaCl treatments (Fig. 4*c*). SA was lower (~35%) in plants treated with 747 mM than 373 mM NaCl at the beginning of experiment but there was no difference after 15 days (Fig. 4*c*).

Secretion of Na^+

Secretion of Na^+ from leaf tissue increased with an increase in salinity and duration of NaCl treatments (Fig. 4*f*). The rate of Na^+ secretion in 167 mM NaCl was similar to the control (which contained 26 mM NaCl; Table 1). However, a 2-fold increase was recorded in Na^+ secretion with a further increase in salinity. An earlier increase in Na^+ secretion rate was observed in 747 mM when compared with 373 mM NaCl (Fig. 4*f*).

Concentration of Na^+ in apoplast and symplast

Leaf Na^+ concentrations were similar in both apoplast and symplast under non-saline control (Fig. 5). An increase in concentration of Na^+ in the apoplast of leaves (selected from lower three nodes of the plant) was recorded when plants were exposed to 747 mM NaCl (Fig. 5).

Osmolality of leaf sap and contribution of Na^+ and K^+ to osmolality

There was no difference in leaf osmolality of plants grown for 3 days at 26, 167 and 373 mM NaCl, but ~3-fold increase observed in 747 mM NaCl (Fig. 6*a*). Maximum osmolality (~2500 mosmol kg^{-1} H_2O) was found in leaves from plantlets treated with 373 and 747 mM NaCl for 7 and 15 days. At the time of harvest, the osmotic contribution of Na^+ and K^+ was 40% up to 167 mM NaCl and it increased to 60% in 373 mM and 75% in 747 mM NaCl (Fig. 6*b*). The contribution of Na^+ to osmolality

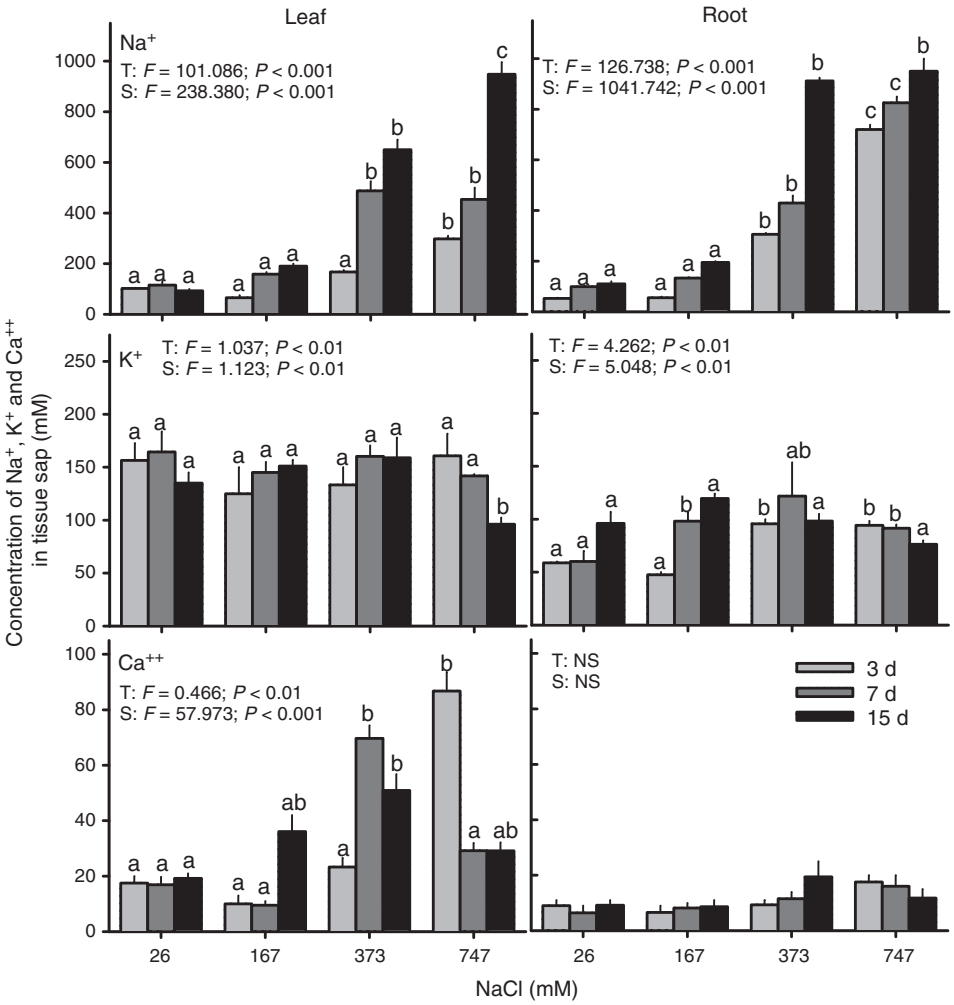

Fig. 3. Concentration of Na^+, K^+ and Ca^{++} in leaf and root tissues of *Aeluropus lagopoides* treated with different NaCl concentrations for 3, 7 and 15 d ($n = 3$). *F*- and *P*-values were obtained from one-way ANOVA by using T (time period of salinity exposure) and S (NaCl treatment). For each time period, values at different salinity levels with similar Bonferroni letter were not significantly different at $P < 0.05$ (NS, non-significant).

was substantially higher than K^+ in plants treated with NaCl (Fig. 6*b*).

Gas-exchange kinetics

The gas-exchange parameters remained unchanged in control plants with the passage of time (Table 3). After 3 days there was no change in net photosynthesis (P_n) in up to 373 mM NaCl and a gradual decrease was observed with increase in salinity and exposure time (Table 3). Respiration rate (R) increased transiently up to 373 mM NaCl on day 3 (Table 3). There was no initial change in stomatal conductance (g_s) and transpiration rate (E), although a 90% reduction was recorded in 747 mM NaCl. We noted that g_s and E gradually decreased during the latter part of the experiment with an increase in NaCl (Table 3). There was no major change in water use efficiency (WUE) among all treatments, with the exception at 747 mM salinity after 15 days, where a decrease of 66% in WUE was observed compared with other treatments (Table 3).

Peroxidation of lipid membrane

A progressive increase in MDA content was observed in leaves treated with NaCl after 3 days exposure, which gradually decreased with the passage of time. Around 40% increase in MDA was observed at 747 mM after 15 days in comparison to the control (Table 3).

Gene expression

A constitutive and unchanged expression of *AlaNHX*, *PM-NHX* and *H^+-ATPase* was observed in the control treatment in both leaf and root (Fig. 7). The expression of *AlaNHX* (*V-NHX*) in leaves progressively increased under all salinities relative to control, but this expression gradually decreased with time at 373 and 747 mM NaCl (Fig. 7*a*, *b*). The expression of *AlaNHX* in roots was higher in all NaCl treatments particularly at 747 mM NaCl where it was at least 12 times greater than respective non-saline control.

During initial periods, expression of *H^+-ATPase* was higher (2- to 4-fold) in leaves treated with NaCl than in the leaves from

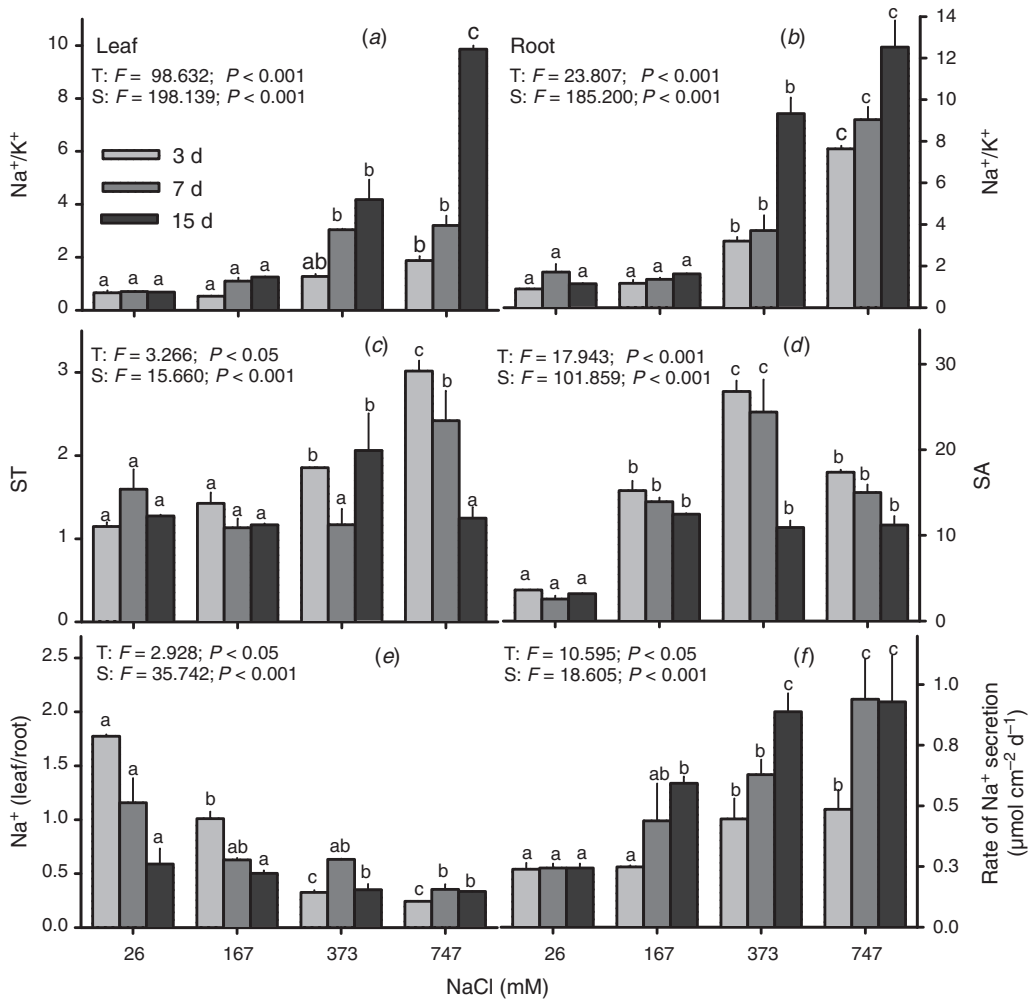

Fig. 4. $Na^+ : K^+$ ratio in leaf (*a*) and root (*b*), ST, selective transport ratio of K^+ vs. Na^+ from root to shoot (*c*) SA, selective absorption ratio of K^+ vs. Na^+ from the medium (*d*), quantity of Na^+ in leaf vs. root (*e*) and rate of Na^+ secretion from leaves (*f*) of *Aeluropus lagopoides* in different NaCl concentrations and their time of exposure to salinity. *F*- and *P*-values were obtained from one-way ANOVA by using T (time period of salinity exposure) and S (NaCl treatment). For each time period, values at different salinity levels with similar Bonferroni letter were not significantly different at $P < 0.05$.

control plantlets, whereas expression declined later at 373 and 747 mM NaCl (Fig. 7*c*). Expression of *H^+-ATPase* was 2-fold higher in leaves than in roots (Fig. 7*c*, *d*). Expression of *H^+-ATPase* gene in root increased with an increase in salinity and exposure time (Fig. 7*d*).

Expression of *PM-NHX* was 10-fold higher in leaf than root tissue (Fig. 7*e*,*f*). Moreover, expression in leaves did not change under low salinity, but increased under high salinity (Fig. 7*e*). Expression of *PM-NHX* in root tissue increased with an increase in salinity and duration of exposure to NaCl solutions (Fig. 7*f*).

Discussion

Salt resistance in plants is based on a chain of events, including physiological and molecular processes triggered through differential expression of genes (Abogadallah 2010; Yang *et al*. 2010). However, any change in these events could negatively affect salt resistance of a particular species, which depends both on the extent and length of exposure to salinity (Munns 2002; Abogadallah 2010). Some halophytes grow best in the presence of some salinity, whereas others (especially grasses) in non-saline condition even if they have high salt resistance (Gulzar *et al*. 2003; Barhoumi *et al*. 2007; Flowers and Colmer 2008). *A. lagopoides* may survive in more than 1000 mM NaCl, but its best growth was recorded in non-saline condition (Fig. 1; Gulzar *et al*. 2003). There was no change in WC of roots throughout the experiment, whereas inorganic content increased with the increase in salinity as also reported before in *A. lagopoides* (Sobhanian *et al*. 2010) and other salt tolerant grasses (Bell and O'Leary 2003; Barhoumi *et al*. 2007). The water concentration of leaf tissue decreased by 10% at 373 mM and ~30% in 747 mM NaCl as a delayed response of NaCl treatments, which had affected leaf osmolality. However, the values of WC remained in the normal range (65–85%) for grasses (Tiku and Snaydon 1971; Howard and Mendelssohn 1999). Halophytic grasses commonly reduce leaf transpiration rate to minimise

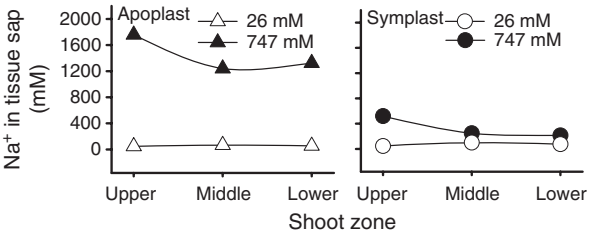

Fig. 5. Distribution of Na^+ between apoplast and symplast in leaves collected from three zones (lower, 0–3 nodes; middle, 4–6 nodes; upper, 7–9 nodes)) of *Aeluropus lagopoides* plants treated with 26 and 747 mM of NaCl for 15 days.

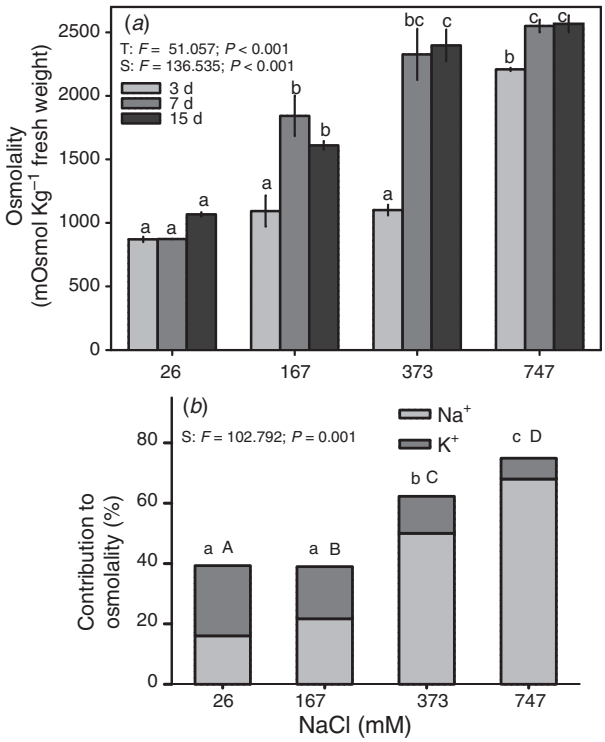

Fig. 6. Osmolality (*a*) and relative osmotic contribution of Na^+ and K^+ (*b*) in leaf tissue of *Aeluropus lagopoides* in different NaCl concentrations. *F*- and *P*-values were obtained from one-way ANOVA by using T (time period of salinity exposure) and S (NaCl treatment). For each time period, values at different salinity levels with similar Bonferroni letter (lower case and upper case letters were used for Na and K respectively) were not significantly different at $P < 0.05$.

Na^+ uptake, though at the cost of reduced growth and photosynthesis (Munns 2002; Flowers and Colmer 2008). An increase in leaf osmolality in plants under salinity, indicating osmotic adjustment, is frequently observed (Munns and Tester 2008). Osmotic adjustment is attributed to the accumulation of vacuolar ions or production of compatible organic solutes (Flowers and Colmer 2008). There was little change in leaf osmolality up to 373 mM NaCl during the first 3 days, but a 2-fold increase was observed in 747 mM NaCl, indicating decrease in water content under highly saline conditions. However, after 15 days, high leaf osmolality in 373 mM NaCl with almost 50% contribution of Na^+ indicates an efficient Na^+ compartmentalisation in plants. A contribution of Na^+ and K^+ of >40% of osmolality is a common feature of salt-tolerant grasses (Marcum 2008), which may results in decreased metabolic cost for osmolyte production (Hasegawa *et al*. 2000; Bell and O'Leary 2003). However, ion toxicity may occur if the concentration of Na^+ in tissue sap exceeds from 650 mM as indicated by accumulation of higher MDA content at 747 mM NaCl.

Aeluropus lagopoides appears to maintain K^+ homeostasis up to 373 mM NaCl, but $Na^+ : K^+$ ratio increased progressively with increasing NaCl concentration. K^+ homeostasis in both roots and leaves is likely to be due to selective absorption and transport (Bell and O'Leary 2003; Gulzar *et al*. 2003; Wang *et al*. 2009). Plants may achieve selective absorption and transport of K^+ over Na^+ across the membranes through stelar K^+ outward rectifiers (SKORs) and KUP-HAK protein channels (Santa-María *et al*. 1997; Gaymard *et al*. 1998). In addition, the prevention of K^+ loss from the cell also appears to be a crucial part of K^+ maintenance (Chen *et al*. 2007). When plantlets of

Table 3. Gas-exchange parameters (P_n, photosynthesis; R, respiration; g_s, stomatal conductance; E, transpiration; WUE, water use efficiency) and MDA content in *Aeluropus lagopoides* after 3 and 15 days of various NaCl concentrations

Values are means ± s.e. *P*-values were obtained from one-way ANOVA by using T (time period of salinity exposure) and S (NaCl treatment) (NS, non-significant). Values with different Bonferroni letters are significantly different at $P < 0.05$

Parameter	Day	NaCl (mM)				ANOVA
		26	167	373	747	
P_n (μmol CO_2 m^{-2} s^{-1})	3	14.47 ± 0.59a	14.29 ± 2.96a	9.59 ± 3.83a	0.9 ± 0.37c	T: $P < 0.05$ S: $P < 0.001$
	15	14.47 ± 0.59a	7.67 ± 2.01b	2.12 ± 0.92c	0.3 ± 0.07d	
R (μmol m^{-2} s^{-1})	3	–3.41 ± 0.59b	–3.57 ± 0.40b	–4.78 ± 0.24a	–1.03 ± 0.33c	T: $P < 0.05$ S: $P < 0.001$
	15	–3.72 ± 1.45a	–1.48 ± 0.63ab	–1.09 ± 0.40b	–0.79 ± 0.19c	
g_s (mol H_2O m^{-2} s^{-1})	3	0.10 ± 0.02a	0.10 ± 0.03a	0.11 ± 0.02a	0.01 ± 0.01b	T: $P < 0.05$ S: $P < 0.001$
	15	0.12 ± 0.01a	0.07 ± 0.01b	0.02 ± 0.00c	0.01 ± 0.00d	
E (mmol H_2O m^{-2} s^{-1})	3	4.00 ± 0.86a	3.85 ± 1.18a	3.77 ± 0.82a	0.34 ± 0.16b	T: $P < 0.001$ S: $P < 0.001$
	15	4.81 ± 0.39a	2.66 ± 0.42b	0.88 ± 0.07c	0.32 ± 0.04d	
WUE (μmol CO_2 $mmol^{-1}$ H_2O)	3	2.31 ± 0.22b	3.84 ± 0.41a	2.44 ± 0.49b	2.77 ± 0.16b	T: NS S: $P < 0.05$
	15	3.04 ± 0.37a	2.82 ± 0.38a	2.41 ± 1.03a	0.90 ± 0.12b	
MDA (μmol g^{-1} FW)	3	10.62 ± 0.08d	12.24 ± 0.24c	13.41 ± 0.41b	18.32 ± 0.32a	T: $P < 0.001$ S: $P < 0.001$
	15	9.60 ± 0.41b	10.53 ± 0.53b	10.07 ± 0.07b	13.47 ± 0.47a	

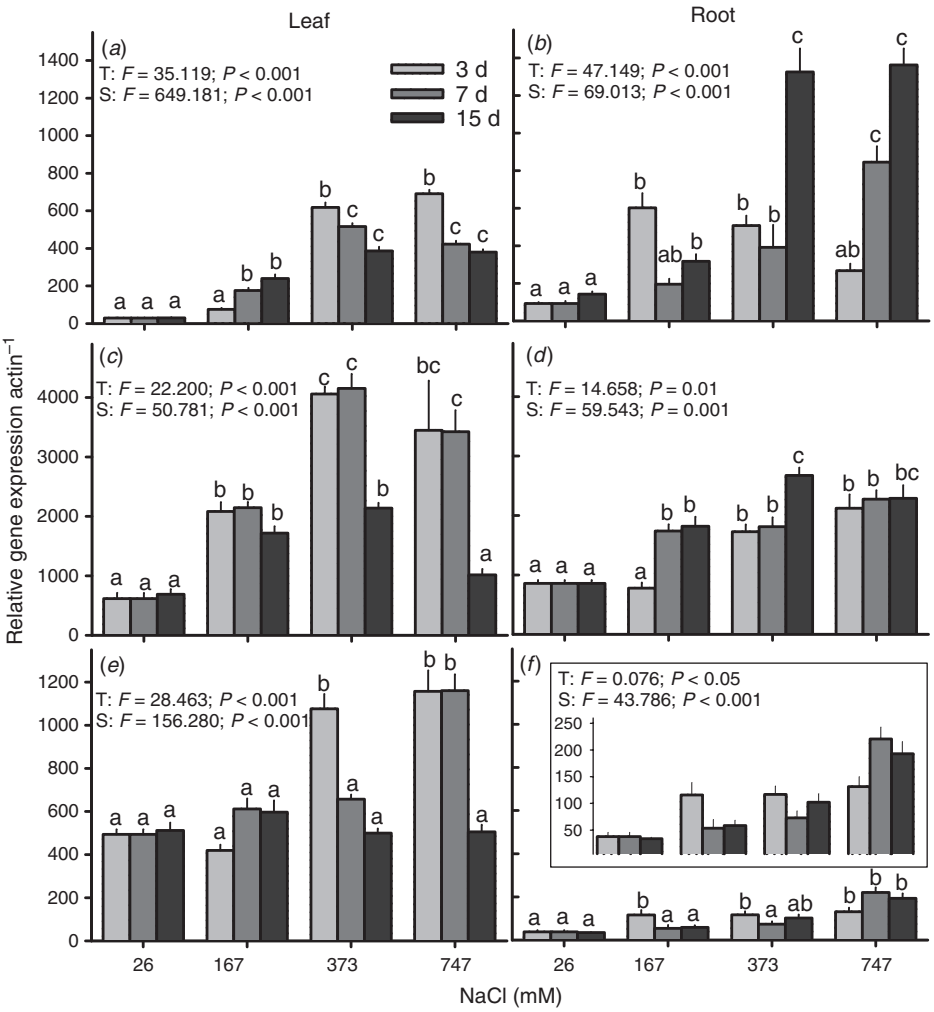

Fig. 7. Relative expression of selected genes against acting (housekeeping gene) in *Aeluropus lagopoides* treated with different NaCl concentrations for 3, 7 and 15 days. Data for gene expression of *AlaNHX* (*V-NHX*; *a*, *b*), H^+-*ATPase* (*c*, *d*) and *PM-NHX* (*e*, *f*) are shown in leaf (*a*, *c*, *e*) and root (*b*, *d*, *f*) respectively. Insert: insert shows the same graph (*f*) with different scale. *F*- and *P*-values were obtained from one-way ANOVA by using T (time period of salinity exposure) and S (NaCl treatment). For each time period, values at different salinity levels with similar Bonferroni letter were not significantly different at $P < 0.05$.

A. lagopoides were exposed to 747 mM NaCl for a longer period (15 days), K^+ deficiency in leaves was observed which could have led to restricted growth and loss of selective K^+ absorption from saline medium as well as selective K^+ transport through xylem (Hu and Schmidhalter 2005; Wang *et al*. 2009).

There was no significant change in the rate of photosynthesis up to 373 mM NaCl after 3 days, but a sharp decline was observed after long-term exposure to all concentrations of NaCl. A positive relationship among photosynthesis, stomatal conductance and transpiration ($r = 0.999$; $P < 0.001$) indicates that the reduction in photosynthesis is possibly linked to stomatal closure to prevent water loss (Carroll *et al*. 2001). Moreover, reduction in transpiration may also reduce the Na^+ loading into the xylem stream and subsequently its transport towards leaf tissues, so that the longevity of plant is increased by maintaining Na^+ at subtoxic level (Ma *et al*. 2004). A growth inhibition at 373 mM NaCl may indicate the ability of plant to cope with salinity by compromising CO_2 assimilation (Sabra *et al*. 2012). However, poor growth with substantially reduced photosynthesis at 747 mM NaCl could be a result of insufficient reactive oxygen species (ROS) quenching (Sobhanian *et al*. 2010), which is reflected by a higher MDA (40% of respective non-saline treatment) concentration. This oxidative damage could also be a consequence of the rapid increase in leaf Na^+ (three times higher than control) which might be injurious for light harvesting complex or enzymes related to CO_2 assimilation (Parida and Das 2005). Sobhanian *et al*. (2010) reported a decrease in photosynthesis with downregulation in proteins of both light reaction and Calvin cycle (LSU and SSU of RuBisCO), when *A. lagopoides* plantlets were exposed to 450 mM NaCl for 10 days. Respiration rate in plants treated with 373 mM NaCl was higher than other salinity levels used for 3 days, is consistent with a greater energy demand for Na^+ regulation and osmotic adjustment besides activating any defence system. A

Table 4. Representation of changes in different parameters of *Aeluropus lagopoides* after treatment with 373 and 747 mM NaCl for 15 days

The direction of arrow shows the change in comparison with the non-saline treatment and number of arrows shows a significant difference ($P < 0.05$) between 373 and 747 mM NaCl treatments

Parameters	NaCl (mM)	
	373	747
Leaves		
Growth rate	↓	↓↓
WC	↓	↓↓
Osmolality	↑	↑
P_n	↓	↓↓
R	↓	↓↓
WUE	–	↓
MDA	–	↑
Secretion rate of Na^+	↑	↑
K^+	–	↓
Na^+	↑	↑↑
VNHX	↑	↑
PMNHX	–	–
H-ATPase	↑	–
Roots		
K^+	–	–
Na^+	↑	↑
VNHX	↑	↑
PMNHX	↑	↑↑
H-ATPase	↑	↑

considerable decrease in respiration of *A. lagopoides* after 15 days at all NaCl concentrations indicates a possible shift of metabolism towards amino acid synthesis (Sobhanian *et al.* (2010). The low rate of respiration at 747 mM NaCl indicates ionic toxicity that is controlled by inappropriate expression of genes as well as inadequate secretion of salt (Table 3).

The removal of toxic ions such as Na^+ from the metabolically active cytoplasm is the key for salt resistance (Munns and Tester 2008; Oh *et al.* 2009) which is achieved either by Na^+ compartmentalisation in the vacuole (*V-NHX*) or continuous removal to and excretion from the apoplast (*PM-NHX*) (Cosentino *et al.* 2010) using energy in the form of electromotive force generated through protein encoded by *H^+-ATPase* and *H^+-PPase* genes (Hedrich *et al.* 1989). The extent of Na^+ compartmentalisation in vacuoles of root and shoot tissues varies with species (Abogadallah 2010; Yang *et al.* 2010). In general, halophytic grasses accumulate ions in roots (Marcum 2008), although shoot and leaf tissues play only a minor role in ion accumulation under NaCl stress (Barhoumi *et al.* 2007). Our data indicate that leaf versus root ratio of sodium content decreased with increase in NaCl concentrations. This also validates the finding that the expression of *V-NHX* gene was 2-fold higher in roots than leaves. In contrast, *PM-NHX* was higher in leaves than roots, which shows co-ordination with *V-NHX*. The expression of *V-NHX* and *PM-NHX* was dependent on the duration of NaCl treatment (Khedr *et al.* 2011). Higher expression of *V-NHX* and *PM-NHX* in leaves was a prompt response of NaCl treatment, which could have helped in decreasing Na^+ content in cytoplasm and to maintain water concentration (percentage of FW) (Khedr *et al.* 2011). However, the expression of both genes decreased with time of exposure to NaCl, which may have caused a shift to Na^+ secretion mechanism. In addition, a 4-fold increased concentration of Na^+ was noted in the apoplast than symplast, which could have enhanced the rate of Na^+ secretion through salt glands (Naz *et al.* 2009). Although, the expression of *PM-NHX* gene was downregulated with time but still Na^+ transfer to the apoplast is possible because of higher stability of PM-NHX protein under saline condition compared with non-saline control (Chung *et al.* 2008). The expression of both genes (*PM-NHX* and *V-NHX*) and salt secretion rate was similar in plantlets treated with 373 and 747 mM NaCl after 15 days, but the tissue sap concentration of Na^+ was 30% higher in 747 mM NaCl treatment, suggesting Na^+ toxicity in plants treated with 747 mM NaCl. An increase in Ca^{2+} concentration in plantlets exposed to 373 mM NaCl for 15 days possibly help in blocking Na^+ uptake via NSCC (non-selective cation channel; Demidchik and Tester 2002) and improving K^+ selectivity by KOR (potassium outward rectifying channel; Wang *et al.* 2009). The localisation of H^+-translocating adenosine tri-phosphatase enzyme is previously reported both in tonoplast and plasma membrane but in this study expression of *H^+-ATPase* gene was positively correlated with the expression of *V-NHX* gene in both leaves ($r = 0.821$; $P < 0.0001$) and roots ($r = 0.700$; $P < 0.0001$). Our results suggest that the expression of *H^+-ATPase* is of great importance in *A. lagopoides* to generate an electrochemical gradient so that an efficient sequestration of Na^+ in vacuoles may occur as reported for other species (Abogadallah 2010; Yang *et al.* 2010). The upregulation of ATP synthase in *A. lagopoides* under salt stress (Sobhanian *et al.* 2010) might be helpful in Na^+ partitioning through *H^+-ATPase*.

We conclude that NaCl induced changes in growth, physiological and molecular mechanisms of *A. lagopoides* could be related to ionic homeostasis in plants. Plantlets of *A. lagopoides* successfully compartmentalised Na^+ in 373 mM NaCl at the cost of growth reduction by achieving K^+ homeostasis and preventing damage. This partitioning is made possible by proper coordination of *AlaNHX*, *H^+-ATPase* and *PM-NHX* genes in leaf and root tissues (Table 4). Second, a reduction in gas exchange parameters helped plants reduce water loss to minimise Na^+ uptake. Increased secretion of Na^+ through leaves may have contributed in avoiding toxicity. However, 747 mM NaCl proved to be toxic as plants not only suffered from K^+ deficiency, but also showed apparent signs of damage. This damage could be a result of ineffective Na^+ secretion as well as inappropriate gene expression controlling Na^+ flux.

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