

# Insights into the salt tolerance mechanism in barley (*Hordeum vulgare*) from comparisons of cultivars that differ in salt sensitivity

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Received: 29 June 2007 / Accepted: 21 September 2009 / Published online: 10 November 2009  
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**Abstract** Although barley (*Hordeum vulgare* L.) is a salt-tolerant crop, the underlying physiological and molecular mechanisms of salt tolerance remain to be elucidated. Therefore, we investigated the response of salt-tolerant (K305) and salt-sensitive (I743) cultivars to salt stress at both physiological and molecular levels. Salt treatment increased xylem sap osmolarity, which was attributed primarily to a rise in  $\text{Na}^+$  and  $\text{Cl}^-$  concentration; enhanced accumulation of the ions in shoots; and reduced plant growth more severely in I743 than K305. The concentration of  $\text{K}^+$  in roots and shoots decreased during 8 h of salt treatment in both cultivars but with no marked difference between cultivars. Hence, the severe growth reduction in I743 is attributed to the elevated levels of (mainly)  $\text{Na}^+$  in shoots. Analysis of gene expression using quantitative RT-PCR showed that transcripts of  $\text{K}^+$ -transporters (*HvHAK1* and *HvAKT1*), vacuolar  $\text{H}^+$ -ATPase and inorganic pyrophosphatase (*HvHVA/68* and *HvHVP1*) were more abundant in shoots of K305 than in shoots of I743. Expression of *HvHAK1* and  $\text{Na}^+/\text{H}^+$  antiporters (*HvNHX1*, *HvNHX3* and *HvNHX4*) was higher in roots of K305 than in I743 with prolonged exposure to salt. Taken together,

these results suggest that the better performance of K305 compared to I743 during salt stress may be related to its greater ability to sequester  $\text{Na}^+$  into sub-cellular compartments and/or maintain  $\text{K}^+$  homeostasis.

**Keywords** *Hordeum vulgare* · Ion accumulation ·  $\text{K}^+$ -transporter ·  $\text{Na}^+/\text{H}^+$  antiporter · Vacuolar  $\text{H}^+$ -ATPase

## Introduction

Soil salinity is one of the major environmental constraints limiting crop production in many parts of the world. It is prevalent under irrigated agricultural and marginal lands associated with poor drainage and high water tables. Nevertheless, crop production must be boosted in order to sustain the ever-growing world population. Since most farmers, especially in developing countries, cannot afford the cost of reclaiming saline soils, increasing salt tolerance of crops is crucial (Munns et al. 2006). For this purpose, understanding plant responses to salinity is essential, and the molecular biology of these responses needs to be characterized.

Crop species, and even cultivars of the same species, vary in their response to NaCl salinity in terms of growth and mineral element content of the shoots (Marschner 1995; Garthwaite et al. 2005). *Hordeum vulgare* and *Gossypium* spp., are the most salt-tolerant crops, followed by *Triticum aestivum*, while most legumes are sensitive (Munns 2001). Growth inhibition by  $\text{Na}^+$  and  $\text{Cl}^-$  is among the most common effect of soil salinity (Tester and Davenport 2003). For some plants, especially woody perennials such as *Citrus* and *Vitis vinifera*,  $\text{Na}^+$  is retained in the woody roots and stems and it is  $\text{Cl}^-$  that accumulates in the shoot and is most damaging to the plant (often by

**Electronic supplementary material** The online version of this article (doi:10.1007/s10265-009-0272-2) contains supplementary material, which is available to authorized users.

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inhibiting photosynthesis; Flowers 1988). However, for other plants (such as poaceae crops),  $\text{Na}^+$  is the primary cause of ion-specific damage.

The inhibition of plant growth by salt stress involves both osmotic and ionic components (Munns et al. 2006). In salt-sensitive plants, shoot- and, to a lesser extent, root-growth is reduced permanently within hours of salt stress, and this effect does not appear to depend on  $\text{Na}^+$  concentration in the growing tissues, but rather is a response to the osmolarity of the external solution (Munns et al. 2000; Munns 2002).  $\text{Na}^+$ -specific damage is also associated with the accumulation of  $\text{Na}^+$  in leaf tissues. High concentration of  $\text{Na}^+$  in the shoot can cause a range of osmotic and metabolic problems in plants (Bhandal and Malik 1988; Rubio et al. 1996; Maathuis and Amtmann 1999; Tester and Davenport 2003; Munns et al. 2006).

The capacity of plants to maintain a high cytosolic  $\text{K}^+/\text{Na}^+$  ratio is likely to be one of the key determinants of plant salt tolerance (Maathuis and Amtmann 1999; Garbarino and Dupont 1989). Several genes and transporters that contribute to cytosolic  $\text{K}^+/\text{Na}^+$  homeostasis have been identified and characterized. Members of the HAK/KUP/KT family are involved in both low and high-affinity  $\text{K}^+$  transport and, although no firm data exist, are presumably localized at different membranes (Banuelos et al. 2002). Such genes have been identified in many plant species including *H. vulgare* (Santa-Maria et al. 1997) and *Arabidopsis thaliana* (Quintero and Blatt 1997; Fu and Luan 1998; Kim et al. 1998). HKT1 functions as a potential  $\text{Na}^+$  uptake pathway in *Arabidopsis* (Rus et al. 2001), *T. aestivum* (Laurie et al. 2002) and *Oryza sativa* (Golldack et al. 2002; Horie et al. 2007), and antisense expression of this transporter in *T. aestivum* led to a decrease in unidirectional  $\text{Na}^+$  influx, providing direct evidence that it functions as a root  $\text{Na}^+$  uptake mechanism. In *Arabidopsis*, loss of function in HKT1 suppressed the sodium oversensitive (SOS3) phenotype and largely restored growth of the *sos3* mutant at low external  $\text{K}^+$ . Heterologous expression of HKT1 in *Xenopus laevis* oocytes (Rubio et al. 1995) showed that HKT1-mediated  $\text{K}^+$  transport depends on the presence of  $\text{Na}^+$ , with two  $\text{K}^+$  transported per one  $\text{Na}^+$ . Maathuis and Amtmann (1999) summarized that HKT2;1 represents a putative pathway for high-affinity  $\text{K}^+$  uptake and low-affinity  $\text{Na}^+$  uptake. Recently, Horie et al. (2007) showed that OsHKT2;1 is responsible for a major portion of nutritional  $\text{Na}^+$  uptake and distribution in  $\text{K}^+$ -starved rice plants, and that OsHKT2;1 functions in  $\text{Na}^+$ -selective influx in planta. After analyzing *athkt1-1 sos3* and *athkt1-2 sos3* double mutants, Horie et al. (2006) reported that the external  $\text{Ca}^{2+}$  concentration strongly impacts the  $\text{Na}^+$  stress response of *athkt1 sos3* double mutants. Furthermore, they showed that SOS3 and AtHKT1 are physiologically distinct major determinants of salinity resistance, such that

*sos3* more strongly causes  $\text{Na}^+$  overaccumulation in roots, whereas *athkt1* causes an increase in  $\text{Na}^+$  levels in the xylem sap and shoots and a concomitant  $\text{Na}^+$  reduction in roots. Recently, Davenport et al. (2007) showed that *AtHKT1;1* contributes to the control of both root accumulation of  $\text{Na}^+$  and retrieval of  $\text{Na}^+$  from the xylem, but is not involved in root influx or recirculation in the phloem.

Multiple mechanisms, including morphological and biochemical adaptations, are probably involved in maintaining a low cytoplasmic  $\text{Na}^+/\text{K}^+$  ratio. In barley (*H. vulgare*), two such mechanisms include the extrusion of  $\text{Na}^+$  into the vacuole across the tonoplast membrane, and extrusion of  $\text{Na}^+$  into the external medium across the plasma membrane (Pitman et al. 1981; Garbarino and Dupont 1989). Intracellular compartmentation of  $\text{Na}^+$  involves the pumping of  $\text{Na}^+$  into the vacuole before concentration increases in the cytoplasm (Tester and Davenport 2003). This process is accelerated by a pH gradient generated by a vacuolar proton-translocating  $\text{H}^+$ -ATPase and inorganic pyrophosphatase ( $\text{H}^+$ -PPase; Fukuda et al. 1998; Golldack and Dietz 2001; Kluge et al. 2003; Vera-Estrella et al. 2005). Increased  $\text{Na}^+/\text{H}^+$  antiporter activity upon  $\text{Na}^+$  addition has been reported in roots of *H. vulgare* (Garbarino and Dupont 1989), *Beta vulgaris* (Blumwald and Poole 1985, 1987), *Lycopersicon esculentum* (Wilson and Shannon 1995) and *Helianthus annuus* (Ballesteros et al. 1997). This increase was more pronounced in salt-tolerant species or cultivars (Staal et al. 1991; Fukuda et al. 1999). Consistent with the increase in  $\text{Na}^+/\text{H}^+$  antiporter activity are the increased expression of *AtNHX1*, *AtNHX2* and *AtNHX5* genes by NaCl treatment (Yokoi et al. 2002). Similarly, Kader et al. (2006) reported that *OsVHA* expression increased more rapidly and was higher in a salt-tolerant cultivar of *O. sativa* than a salt-sensitive cultivar. Other cytoplasmic adjustments that influence salt tolerance include pH, osmolytes and energy phosphates (Fan et al. 1989).

Several previous studies have reported that variation in salt tolerance exists among *H. vulgare* cultivars (Royo and Aragüés 1999; Leonova et al. 2005). Evaluating the response of two barley cultivars (K305 and I743) under salt stress conditions, Mano (1996) and Katsuhara et al. (2001) reported that K305 was damaged less by salt stress than I743 based on visual observation and root elongation. The former has been used as a standard salt-tolerant, and the latter as a standard salt-sensitive cultivar. However, detailed investigation to elucidate the physiological and molecular basis of salt tolerance in these barley cultivars has not been conducted. Hence, the current study was aimed at understanding the physiological and molecular basis of salt tolerance/sensitivity in barley cultivars. We determined the relative salt tolerance of the two cultivars based on plant growth, accumulation of ions in xylem sap and plant tissues, and expression of various genes involved in ion homeostasis under salinity stress conditions.

## Materials and methods

### Plant growth

Salt-tolerant (K305) and salt-sensitive (I743) cultivars of *H. vulgare* were used in the current study. The seeds were surface sterilized with 10% H<sub>2</sub>O<sub>2</sub> for 10 min. After rinsing five times with deionized water, the seeds were soaked overnight with continuous aeration. The germinating seeds were further rinsed and spread on a mesh placed on top of a 3.5 L pot filled with 0.25 mM CaSO<sub>4</sub>·2H<sub>2</sub>O solution. The seeds were covered with moist paper towel that was immersed in the Ca-solution to protect the germinating seeds from drying out. The pots were then covered with aluminum foil and the seedlings were grown in a dark and ventilated room at 25°C. After 2 days, the seedlings were transferred to an aerated nutrient solution containing 4 mM KNO<sub>3</sub>, 1 mM NaNO<sub>3</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 1 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, and micronutrients (1 ppm Fe, 0.5 ppm B, 0.5 ppm Mn, 0.05 ppm Zn, 0.02 ppm Cu, and 0.01 ppm Mo). The pH of the nutrient solution was adjusted to 5.5 with NaOH.

To determine the effect of NaCl on plant growth, after 2 days in 0.25 mM CaSO<sub>4</sub>·2H<sub>2</sub>O solution, seedlings were transferred to a complete nutrient solution and cultured in a growth chamber (Model MLR-351H, Sanyo, Osaka, Japan) on a cycle of 14 h day and 10 h night continuously at 25°C, and a light intensity of 150 μmol m<sup>-2</sup> s<sup>-1</sup> using Sanyo FL40SS.W/37 florescent lamps. After 2 days, the seedlings were transferred to fresh nutrient solution with or without NaCl (100 or 200 mM). Ten plants were grown per pot for another 5 days and fresh weight was determined.

### Determination of xylem sap osmolarity

The xylem sap osmolarity was determined from 5-day old plants previously treated with 100 mM NaCl for 2 h. The shoots were excised at 80 mm above the root/shoot junction and transferred to humid plastic boxes containing moist paper towel. Xylem sap was collected with thin pipette tips for 1 h or until sufficient sap was obtained. The amount of xylem sap exuded from NaCl-treated K305 and I743 was compared. The osmolarity was determined from 10 μL sap using a vapor pressure osmometer (model 5520, WESCOR, Logan, UT).

### Ion concentration in the xylem sap and plant tissue

To determine the concentrations of Na<sup>+</sup>, Cl<sup>-</sup> and K<sup>+</sup> in the tissue, plants were treated with 100 mM NaCl for 0, 2, 4, 6 and 8 h. The plants were then transferred to aerated 10 mM CaCl<sub>2</sub> solution to release free ions in the apoplast. The shoots and roots were rinsed with deionized water, and then dried at 70°C for about 48 h. The ion content in the dry

matter (0.1 g) was extracted with 5 ml 0.1 N acetic acid at 90°C for 2 h. The samples were then centrifuged at 1,000 g for 10 min. Supernatants of the extracts were diluted to determine ion concentration. The concentration of Na<sup>+</sup> and K<sup>+</sup> was determined by the atomic absorption method using a flame emission spectrophotometer (AA-8200, Nippon Jarrell-Ash, Kyoto, Japan) with standards of known concentration as a reference, while the Cl<sup>-</sup> concentration was determined with an ion chromatographic analyzer (IC 500 S-0, Yokogawa Hokushin Electric, Tokyo, Japan) and quantified by comparing peak areas with those of known standards. Ion concentration in the xylem sap was also determined directly from diluted sap samples.

### RNA isolation

For gene expression analysis, 0.1 g apical root and shoot (about 2 cm below the tip) tissues were sampled after 100 mM NaCl treatment and immediately frozen in liquid nitrogen. The frozen plant material was disrupted in a mortar and pestle. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) following the manufacturer's recommended procedure. For gene cloning, 1 g apical root tissue was excised and frozen in liquid nitrogen. The plant material was then lysed with Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA). RNA was isolated by a modified acid guanidinium thiocyanate-phenol-chloroform method (Chomezynski and Sacchi 1987). RNA samples were stored at -80°C for subsequent use.

### Gene cloning

cDNAs for K<sup>+</sup> transporters (*HvHAK1* and *HvAKT1*), a K<sup>+</sup>/Na<sup>+</sup> symporter (*HvHKT2;1*), Na<sup>+</sup>/H<sup>+</sup> antiporters (*HvNHX1*, *HvNHX2*, *HvNHX3* and *HvNHX4*) and vacuolar H<sup>+</sup>-ATPase (*HvHVA/68*) and vacuolar H<sup>+</sup> inorganic pyrophosphatase (*HvHVPI*) were cloned using gene specific primers (Table S1). For this purpose, mRNA was prepared from total RNA using the QuickPrep micro mRNA purification kit (Amersham Bioscience, Little Chalfont, UK). One microgram mRNA was reverse transcribed by the Superscript first strand synthesis system for RT-PCR (Invitrogen/Life Technologies). The cDNA fragments were amplified using a TaKaRa Ex Taq Kit (TAKARA BIO INC, Shiga, Japan). PCR products with expected size were purified from gels using a Qiaquick Gel Extraction Kit (Qiagen, Tokyo, Japan) and cloned into the cloning vector TOPO TA (Invitrogen/Life Technologies).

### Analysis of gene expression

Gene expression was studied by the quantitative real-time RT-PCR technique using gene specific primers (Table S1).

First strand cDNA synthesis was performed in a 20  $\mu$ L reaction containing 2  $\mu$ g total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster city, CA). PCR reactions were initiated by adding 1  $\mu$ L of the first strand cDNA to 50  $\mu$ L mixture containing 1 $\times$  Power SYBG PCR master mixture (Applied Biosystems), 200 nM sense- and antisense-primers (Table S1). Quantitative PCR was performed on the 7300 real-time PCR system (Applied Biosystems) with PCR conditions of 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 56°C for 30 s, and 60°C for 1 min. For absolute quantification of transcript copy numbers, the partial sequence of the genes was cloned into the TOPO TA cloning vector (Invitrogen/Life Technologies). The vector containing the genes was linearized by *NotI*—a unique restriction site. Complementary RNA was generated using the MEGA Script<sup>®</sup> T7 Kit (Ambion, Austin, TX). A serial dilution of the cRNA of known copy number ( $10^5$ – $10^{10}$ , calculated from the size and molecular weight of cRNA) was reverse transcribed along with the samples. The first strand cDNA from the cRNA was used as reference to determine the absolute transcript copy number of the genes in a given microgram of total RNA of each sample.

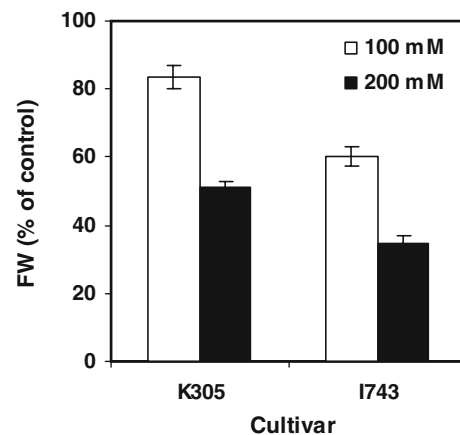
### Data analysis

Experiments were repeated at least twice. Transcript copy number was quantified from mRNA of five biological replicates pooled from several plants, and two technical replicates were used per each RNA sample. Statistical tests of significance were performed using the PROC GLM procedure in SAS. Following significant *F* tests, the Tukey multiple comparison procedure was used to separate the means (Westfall et al. 1996).

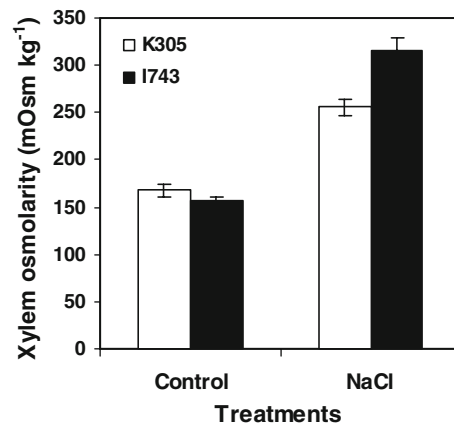
### Results

#### Effect of NaCl treatment on plant growth

Five-day-old seedlings of K305 and I743 were transferred to a nutrient solution containing 0, 100 or 200 mM NaCl. The seedlings were grown for another 5 days and fresh weight per plant was determined. Plant growth in both cultivars was inhibited by NaCl treatment in a concentration-dependent manner (Fig. 1), but more severely in I743 than K305. In the absence of salt, I743 grows faster than K305 (visual observation), but no difference was observed in plants exposed to salt. In Fig. 1, considering growth in control plants as 100%, treatment with 100 mM NaCl resulted in a 40% growth reduction in the sensitive cultivar, while growth of the tolerant cultivar was inhibited only by



**Fig. 1** The effect of NaCl on fresh weight. Plants were cultured as described in “Materials and methods”. Fresh weight in control plants (not exposed to NaCl) was considered as 100%. Bars mean  $\pm$  SE of 20–40 plants; two independent experiments were conducted



**Fig. 2** Effect of NaCl (100 mM) on xylem sap osmolarity. Seedlings were treated for 2 h, xylem sap was collected and osmolarity of the sap was determined as described in “Materials and methods”. Bars mean  $\pm$  SE of four replicates; four independent experiments were conducted

15%. Treatment with 200 mM NaCl further inhibited plant growth by 50 and 65% in K305 and I743, respectively, indicating a clear cultivar difference in salt tolerance.

#### Effect of salt stress on xylem sap osmolarity

Seedlings were exposed to 100 mM NaCl for 2 h. Shoots were excised at the base and xylem sap was collected to determine osmolarity. In control conditions, the volume of the xylem sap collected in I743 was about fivefold higher than in K305. After a 2-h salt treatment, the volumes collected from both cultivars decreased significantly (data not shown). The decrease in sap volume due to NaCl treatment was more pronounced in K305. In both cultivars, salt treatment increased the osmolarity of the xylem sap (Fig. 2) compared to control plants. The osmolarity in

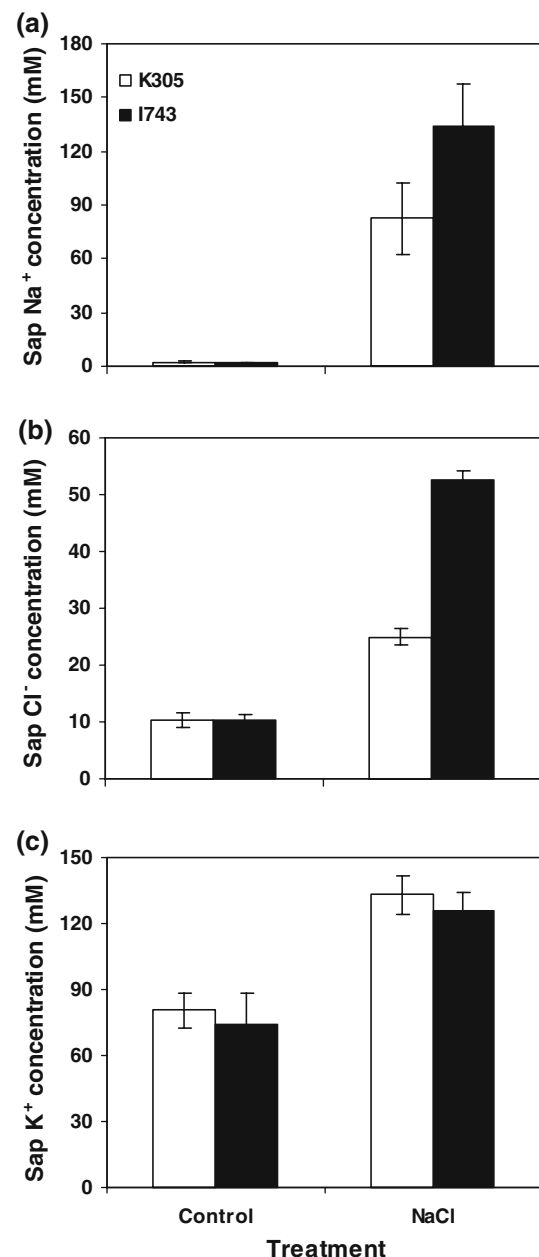
K305 was 1.5 times higher than control, whereas for I743 a twofold increase in osmolarity was observed after NaCl treatment. For both cultivars, the sap osmolarity in the control plants was the same, while sap osmolarity in I743 was 25% higher than K305 after salt treatment.

#### Salt stress enhances sap ion concentration

To monitor salt-induced changes in ion concentration, we determined the concentration of  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  in the xylem sap. As expected, the concentration of  $\text{Na}^+$  in the xylem sap increased in both cultivars after salt treatment. The concentration of  $\text{Na}^+$  increased by 35 and 75 times in K305 and I743, respectively, compared to the control. Interestingly, the  $\text{Na}^+$  concentration in the sap of I743 was higher than that in sap from K305 by 40% (Fig. 3a). Similarly, the concentration of  $\text{Cl}^-$  in the xylem sap of NaCl-treated K305 and I743 was 2.5 and 5 times higher than control plants, respectively (Fig. 3b). Xylem sap  $\text{Cl}^-$  concentration of I743 was twofold greater than the concentration in K305 sap after NaCl treatment. On the contrary, there was no difference in sap  $\text{K}^+$  concentration between cultivars. However, an increase in  $\text{K}^+$  concentration of 1.6 times was observed in both cultivars in response to salt stress (Fig. 3c).

The salt-sensitive cultivar accumulates more  $\text{Na}^+$  and  $\text{Cl}^-$  in shoots

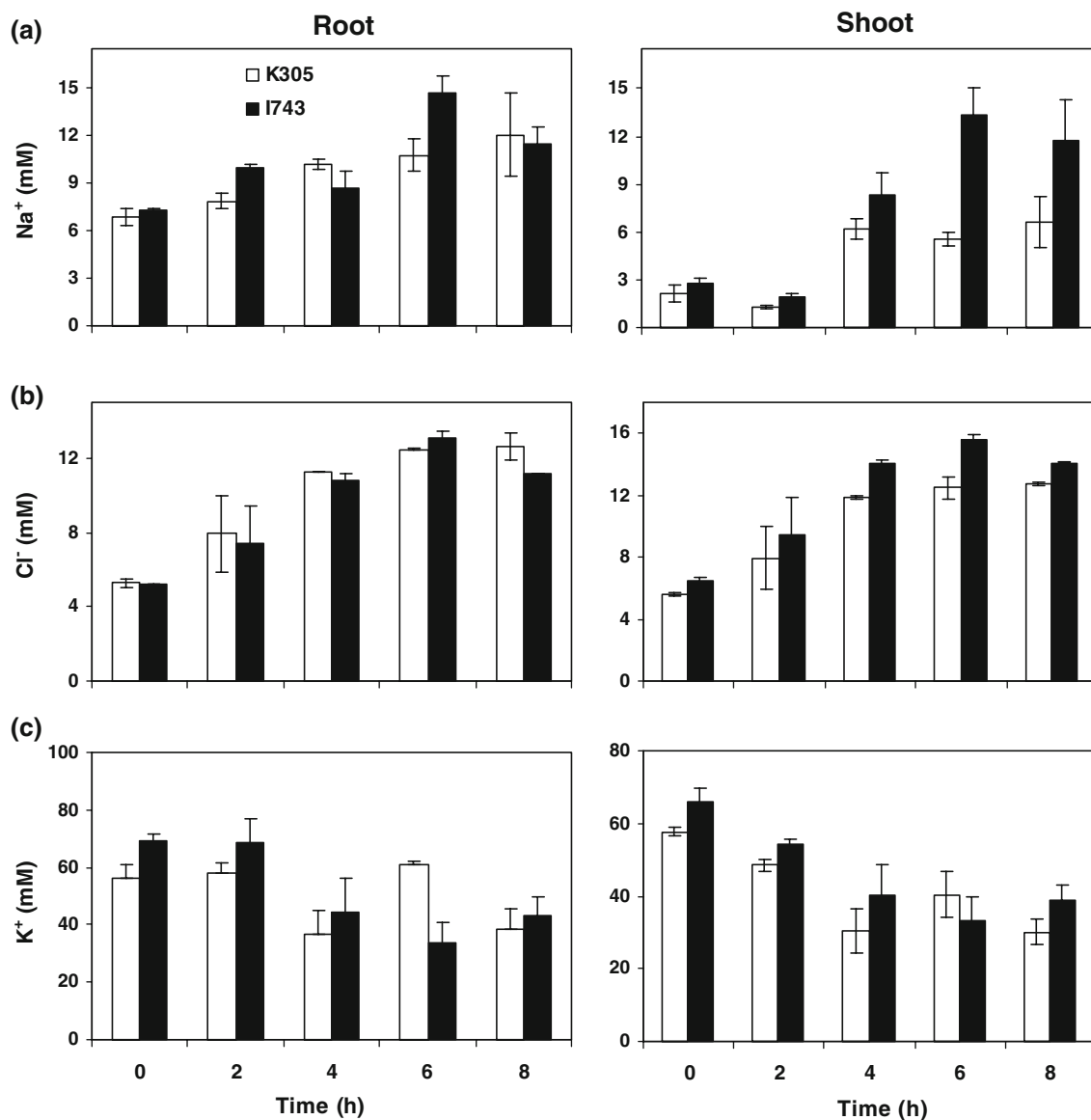
We also analyzed the concentration of  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  in roots and shoots of plants exposed to 100 mM NaCl for 8 h. The concentration of  $\text{Na}^+$  in roots and shoots of both cultivars increased with prolonged salt treatment (Fig. 4a). There was only a slight difference in  $\text{Na}^+$  concentration in roots. The  $\text{Na}^+$  concentration was higher in roots of I743 than of K305 at the 2- and 6-h time points. At the 4-h time point, the concentration was slightly higher in roots of K305 than of I743, whereas the concentration of  $\text{Na}^+$  in shoots of I743 was significantly higher than the concentration in K305 after 4 h. The concentration of  $\text{Cl}^-$  increased significantly with prolonged salt treatment in both roots and shoots (Fig. 4b). The difference in  $\text{Cl}^-$  concentration in roots between cultivars was not significant. The  $\text{Cl}^-$  concentration in shoots of I743 was slightly higher than the concentration in K305 after 4 h of treatment, indicating that the sensitive cultivar accumulates more  $\text{Na}^+$  and  $\text{Cl}^-$  in shoots. By comparison, the concentration of  $\text{K}^+$  in roots and shoots of both cultivars decreased throughout the salt treatment (Fig. 4c). The  $\text{K}^+$  concentration in roots and shoots was more or less similar in both cultivars except at the 6-h time point when the  $\text{K}^+$  concentration was significantly higher in K305 than in I743.



**Fig. 3** The effect of 100 mM NaCl on xylem sap ion concentrations. **a**  $\text{Na}^+$ , **b**  $\text{Cl}^-$ , **c**  $\text{K}^+$ . Seedlings were treated for 2 h and ion concentration was determined as described in “Materials and methods”. Bars mean  $\pm$  SE of four replicates; four independent experiments were conducted

#### Effect of NaCl on transcripts of $\text{K}^+$ -transporters

To determine the molecular mechanisms underlying salt tolerance in K305, plants were treated with 100 mM NaCl for 8 h, RNA was isolated from roots and shoots every 2 h, and expression of  $\text{K}^+$ -transporters (*HvHAK1* and *HvAKT1*) and a  $\text{K}^+/\text{Na}^+$  symporter (*HvHKT2;1*) was analyzed by quantitative RT-PCR. The transcripts of these three genes were more abundant in roots than shoots, suggesting that



**Fig. 4** Effect of 100 mM NaCl on ion concentrations in roots (*left panel*) and shoots (*right panel*). **a** Na<sup>+</sup>, **b** Cl<sup>-</sup>, **c** K<sup>+</sup>. Plants were treated for 8 h; root and shoot samples were taken every 2 h, and ion

concentration was determined as described in “[Materials and methods](#)”. Bars mean  $\pm$  SE of four replicates; at least two independent experiments were conducted

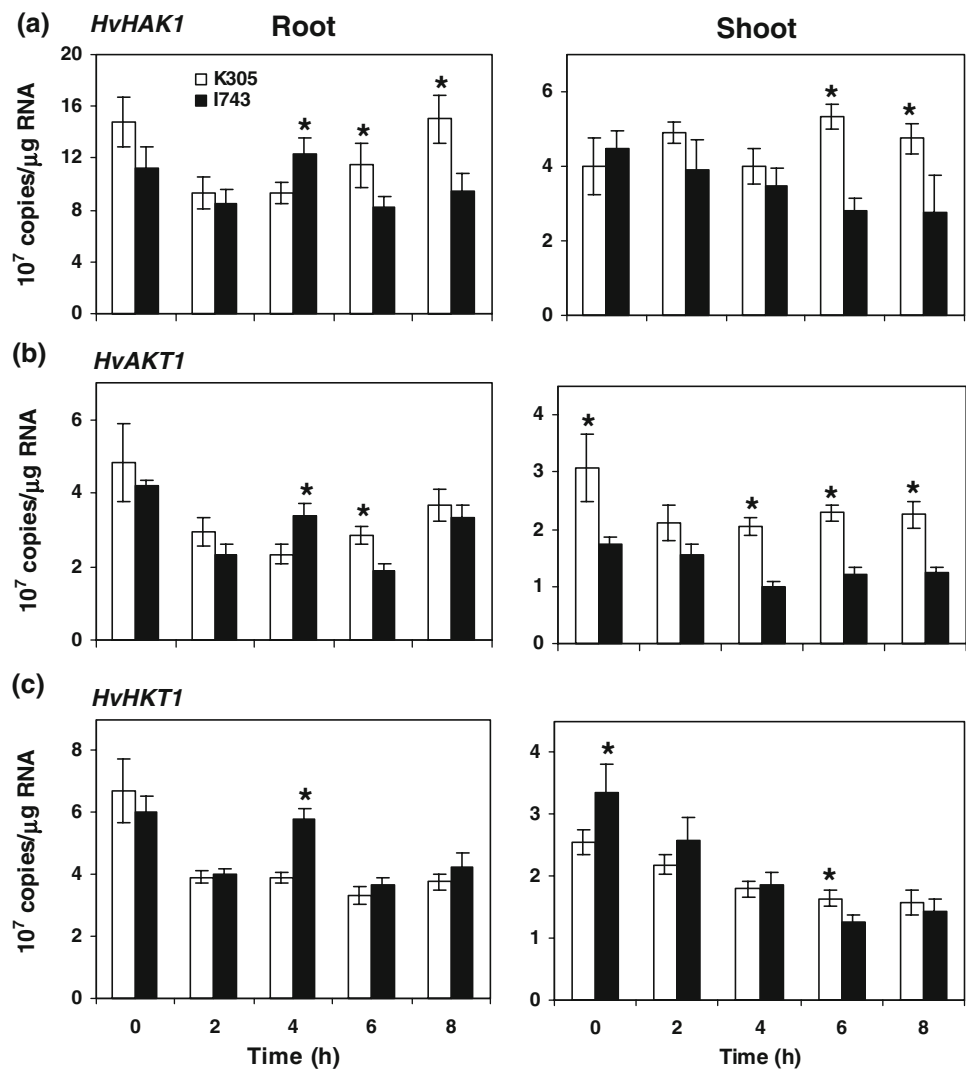
these genes are involved primarily in the uptake of ions in the root. In roots, expression of *HvHAK1* in K305 decreased during the first 2 h of salt treatment, but increased thereafter (Fig. 5a), while expression in I743 was not significantly affected. The *HvHAK1* transcript level decreased in shoots of I743, while in K305 expression increased slightly and remained significantly higher ( $P < 0.05$ ) than that in I743 (Fig. 5a). After 24 h, *HvHAK1* expression was twofold higher in roots of K305 than of I743 at 100 mM NaCl (Fig. 8a). Expression of *HvAKT1* was not significantly affected by salt treatment in roots and shoots of either cultivar during 8 h. There was no difference in the expression of *HvAKT1* in roots but *HvAKT1*

was more abundantly expressed in shoots of K305 than I743 (Fig. 5b). In roots, expression of *HvHKT2;1* decreased slightly in response to salt treatment (Fig. 5c). The decrease in the transcript of *HvHKT2;1* was more conspicuous in shoots, and the decrease was more rapid in I743 than in K305.

#### Effect of NaCl on transcripts of *HvHVA/68* and *HvHVP1*

The transport of ions across the vacuolar membrane is believed to be energized by vacuolar H<sup>+</sup>-ATPases and inorganic pyrophosphatase. Here we studied expression of

**Fig. 5** Effect of 100 mM NaCl treatment on the expression of **a** *HvHAK1* (accession number AF025292), **b** *HvAKT1* (accession number DQ465922), and **c** *HvHKT1* (accession number AM000056) in roots (*left panel*) and shoots (*right panel*). Seedlings were treated for 8 h and RNA was isolated every 2 h for gene expression analysis as described in “Materials and methods”. Bars mean  $\pm$  SE of five replicates; two independent experiments were conducted. Asterisks statistical difference between cultivars ( $P \leq 0.05$ )



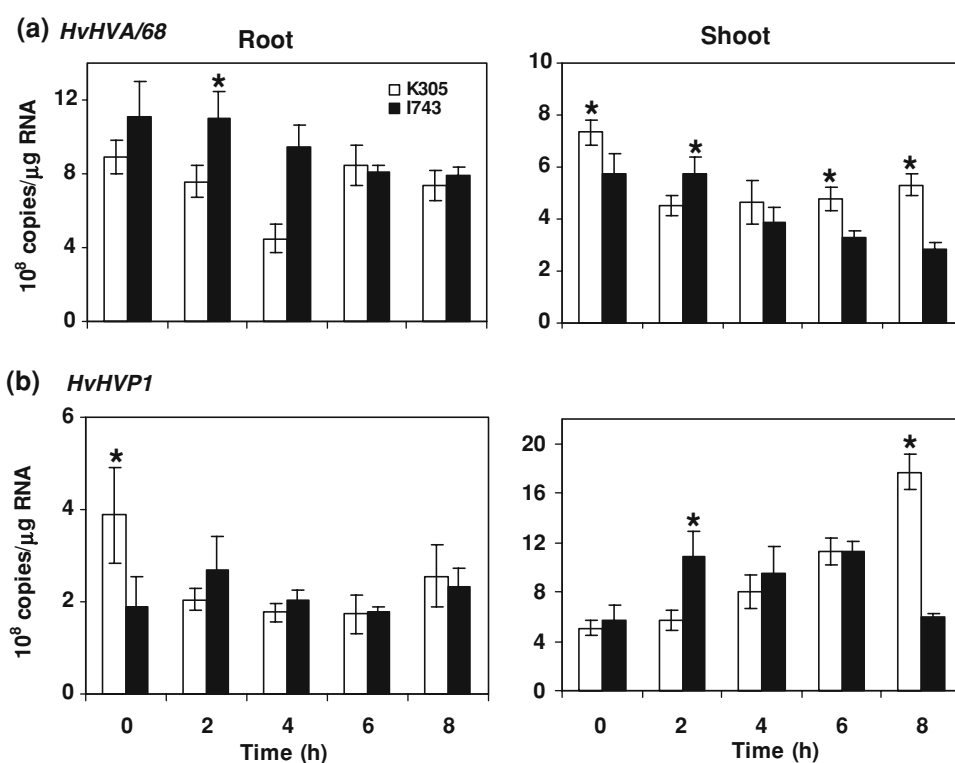
vacuolar H<sup>+</sup>-ATPase (*HvHVA/68*) and inorganic pyrophosphatase (*HvHVP1*), which may contribute to the sequestration of ions in the vacuole. The transcripts of *HvHVA/68* were slightly more abundant in roots while those of *HvHVP1* were more abundant in shoots of both cultivars. As shown in Fig. 6a, expression of *HvHVA/68* was not affected during 8 h of salt treatment in roots of K305, while expression in I743 decreased significantly ( $P < 0.05$ ). Moreover, expression of *HvHVA/68* was slightly higher in roots of I743 than in K305 during early hours of salt treatment. Expression of *HvHVA/68* decreased in shoots of I743, while transcript level was not affected in K305 after the first 2 h. After 8 h, expression of *HvHVA/68* in shoots was twofold higher in K305 than I743 (Fig. 6a). In roots, expression of *HvHVP1* was not significantly affected in either cultivar after salt stress (Fig. 6c), whereas expression in shoots of I743 increased during the first 2 h and decreased after 8 h. Transcripts of *HvHVP1* were more abundant in shoots of I743 than of K305 2 h after exposure

to salt. Intriguingly, expression of *HvHVP1* increased linearly during 8 h of salt treatment in K305 (Fig. 6d).

#### Effect of NaCl on Na<sup>+</sup>/H<sup>+</sup> exchangers

We also analyzed the expression of Na<sup>+</sup>/H<sup>+</sup> antiporters that are involved in Na<sup>+</sup>-homeostasis under salinity stress during 8 h (*HvNHX1*, *HvNHX2*, *HvNHX3* and *HvNHX4*) and 24 h after salt treatment (*HvNHX1*, *HvNHX3* and *HvNHX4*). Expression of most genes was not markedly affected by salt treatment in both cultivars during the initial hours of salt treatment (Fig. 7). In roots, expression of *HvNHX1* initially increased after NaCl treatment in I743 and decreased with prolonged salt treatment (Fig. 7a). Interestingly, 24 h after 100 mM NaCl treatment, transcripts of *HvNHX1* were twofold higher in roots of K305 than of I743 (Fig. 8b). During the first hours of salt treatment, expression of *HvNHX1* in shoots increased significantly in I743, and transcript levels remained higher in

**Fig. 6** Effect of 100 mM NaCl treatment on expression of **a** *HvHVA/68* (accession number AB032840), and **b** *HvHVP1* (accession number AB032839) in roots (*left panel*) and shoots (*right panel*). Seedlings were treated for 8 h and RNA was isolated every 2 h for gene expression analysis as described in “Materials and methods”. Bars mean  $\pm$  SE of five replicates; two independent experiments were conducted. Asterisks Statistical difference between cultivars ( $P \leq 0.05$ )



I743 than in K305 (Fig. 7a), whereas, with prolonged 100 mM salt treatment, expression in K305 was higher in both roots and shoots (Fig. 8b). The transcript level of *HvNHX2* was not significantly different in roots or shoots, except that expression in shoots of K305 was higher 8 h after salt treatment (Fig. 7b). In both roots and shoots, there was no significant difference in the level of *HvNHX3* transcripts during 8 h of salt treatment (Fig. 7c), whereas after 24 h *HvNHX3* transcripts were more abundant in roots of K305 as compared to I743 at 100 mM NaCl (Fig. 8c). In roots, expression of *HvNHX4* was not affected by NaCl treatment for 8 h in both cultivars (Fig. 7d) but decreased markedly in shoots of I743. After 24 h, transcripts of *HvNHX4* were more abundant in roots of K305 than I743 at 100 mM NaCl treatment (Fig. 8d), while no difference was observed in shoots.

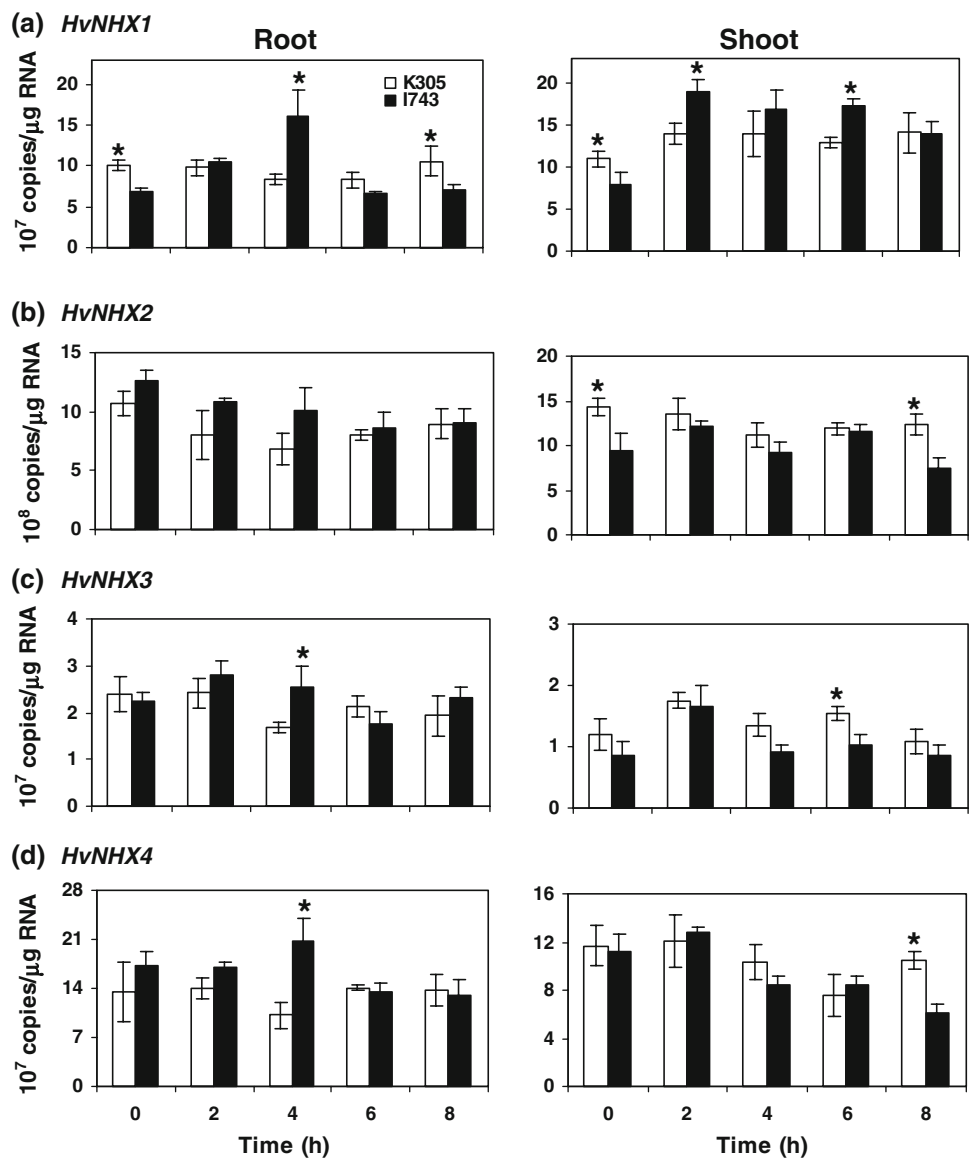
## Discussion

While barley is known as one of the more salt-tolerant crops, variation does exist among different cultivars. The current study compared the growth responses of two cultivars (K305 and I743) after 5 days of treatment with 100 or 200 mM NaCl. The results showed that 5 days of salt stress (100 mM NaCl) resulted in a 40% growth reduction in the sensitive cultivar (I743), while growth of the tolerant cultivar (K305) was inhibited only by 15% (Fig. 1).

Similarly, plant growth was severely inhibited by 50% in K305 and 65% in I743 in response to 200 mM NaCl. Previously, Mano (1996) reported that leaves of I743 were more seriously affected by a 3-week exposure to 500 mM NaCl than leaves of K305. And Katsuhara et al. (2001) showed that 100 mM NaCl treatment for 24 h did not affect root elongation in K305 while root elongation was inhibited by 40% in I743, indicating that K305 is more salt tolerant than I743. However, no further investigation has been conducted to determine the underlying physiological and molecular mechanisms potentially regulating salt tolerance in these barley cultivars. Evaluating various barely cultivars based on yield and ion accumulation in response to salinity, Royo and Aragüés (1999), Royo et al. (2000) and Leonova et al. (2005) have also reported the existence of differential responses in salt stress tolerance among barley cultivars.

To understand the physiological processes involved in regulating ion transport to the shoot under salt stress conditions, xylem sap was compared in the two cultivars. Salt treatment increased the xylem sap osmolarity in both cultivars (Fig. 2). This increase was consistent with the increase in the concentration of  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$ . The xylem sap osmolarity in I743 was 25% higher than that of K305. This is attributed mainly to the difference in the concentration of  $\text{Na}^+$  and  $\text{Cl}^-$  since the concentration of  $\text{K}^+$  did not vary between the two cultivars (Fig. 3). The increase in sap  $\text{Na}^+$  and  $\text{Cl}^-$  is higher in I743 after salt

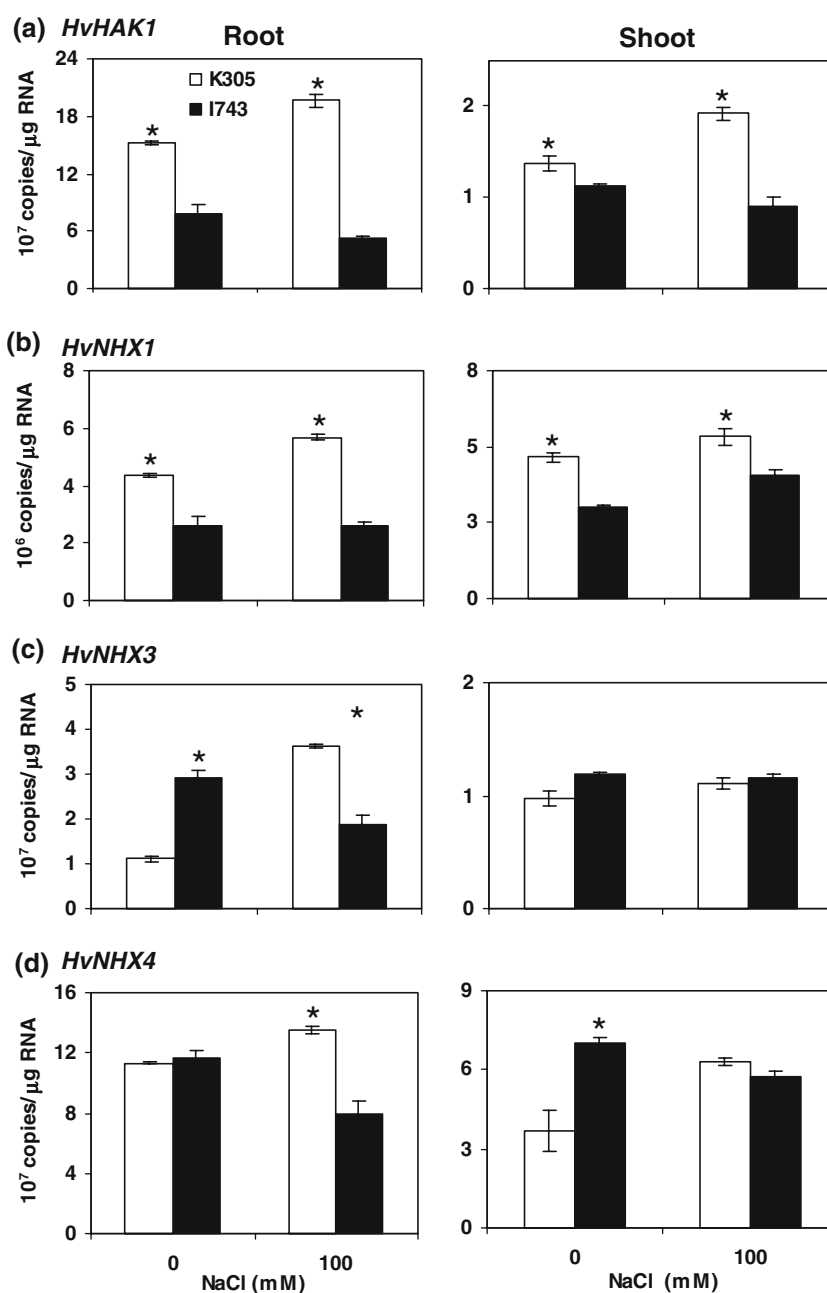
**Fig. 7** Effect of 100 mM NaCl treatment on expression of **a** *HvNHX1* (accession number AB089197), **b** *HvNHX2* (accession number AY247791), **c** *HvNHX3* (accession number DQ372061), and **d** *HvNHX4* (accession number DQ314285) in roots (*left panel*) and shoots (*right panel*). Seedlings were treated for 8 h and RNA was isolated every 2 h for gene expression analysis as described in “Materials and methods”. Bars mean  $\pm$  SE of five replicates; two independent experiments were conducted. Note that expression of *HvNHX2* is in the range of  $10^8$  copies. Asterisks statistical difference between cultivars ( $P \leq 0.05$ )



treatment. Contrary to the rapid accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  in the xylem sap as a salt stress response observed here, previous reports have pointed out that apoplastic movement of water and solutes from the cortex to the stele is restricted by the presence of the Casparian band, which contains lignin and suberin (Robards et al. 1973; Enstone et al. 2003; Zhu et al. 2006; Dinneny et al. 2008). In 3- to 8-week-old barley roots, the development of a Casparian band on the radial walls of the endodermal cells has been shown to prevent the apoplastic movement of solutes between the root cortex and the central stele (Robards et al. 1973). Since we treated 5-day old seedlings with 100 mM NaCl for 2 h, rapid accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  ions might have occurred in root apices without a fully developed endodermis, as also suggested by Peterson (1988). Although barley is a relatively salt-tolerant species, we

think that such a short term (2 h) treatment with 100 mM NaCl might not have induced a marked change in root anatomy or morphology. In rice, a relatively salt-sensitive species, Krishnamurthy et al. (2009) observed no significant morphological or anatomical changes in seedlings treated with 200 mM NaCl for 2 days. However, treatment with a moderate stress of 100 mM NaCl for 1 week resulted in dramatic changes in both root morphology and development of hydrophobic barriers. Furthermore, more suberin deposition was observed in soil-grown roots than in hydroponically grown roots. The rapid accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  ions in the xylem sap was consistent with the accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  in shoots (Fig. 4). Therefore, the severe reduction in growth in I743 may be attributed primarily to the accumulation of  $\text{Na}^+$  in the shoots, which might have caused metabolic toxicity to plants.

**Fig. 8** The effect of NaCl treatment on the expression of **a** *HvHAK1*, **b** *HvNHX1*, **c** *HvNHX3*, and **d** *HvNHX4* in roots (*left panel*) and shoots (*right panel*). Seedlings were treated for 24 h and RNA was isolated for gene expression analysis as described in “Materials and methods”. Bars mean  $\pm$  SE of four replicates; two experiments were conducted. Asterisks statistical difference between cultivars



Metabolic toxicity of  $\text{Na}^+$  occurs through inhibition of various enzymes that require  $\text{K}^+$  as a cofactor (Tester and Davenport 2003; Munns et al. 2006). Such enzymes are sensitive to high levels of  $\text{Na}^+$  or a high  $\text{Na}^+/\text{K}^+$  ratio. Therefore, the ability to maintain a high  $\text{K}^+/\text{Na}^+$  ratio (either retention of  $\text{K}^+$  or preventing  $\text{Na}^+$  from accumulating in leaves as observed in K305) is an important mechanism of salt tolerance. Chloride toxicity is another constraint associated with salinity. For example, in *Citrus* (Maas 1993) and legumes (Chavan and Karadge 1980; Marschner 1995),  $\text{Cl}^-$  toxicity can cause severe leaf chlorosis and depression of photosynthesis. In the current

study, however, NaCl-induced growth reduction in I743 is probably not caused by  $\text{Cl}^-$  toxicity but rather by  $\text{Na}^+$  toxicity due to the high  $\text{Na}^+/\text{K}^+$  ratio in shoots. After 5 days of salt treatment, no clear symptom of  $\text{Cl}^-$  toxicity was observed in either cultivar (data not shown). Moreover, during the first hours of salt stress, the difference in shoot  $\text{Na}^+$  concentration between the cultivars was more pronounced than the difference in  $\text{Cl}^-$  concentration. Since, there must be a proportional increase in anion concentration for electroneutrality, the concentration of other inorganic anions such as  $\text{NO}_3^-$ ,  $\text{PO}_4^{2-}$  and  $\text{SO}_4^{2-}$ , and organic anions such as lactate, oxalate, malate, pyruvate etc., may

increase with increasing  $\text{Na}^+$  concentration. Malate is an important osmoticum and acts as a major anion compensating for increasing concentrations of intracellular  $\text{K}^+$  and  $\text{Na}^+$  ions. In Arabidopsis, comparing wild type and vacuolar malate transporter (*AtDT*) knockout lines treated with NaCl showed a significant increase in leaf malate content in wild-type plants, suggesting that this increase in malate ions may compensate for the charge difference between  $\text{Na}^+$  and  $\text{Cl}^-$  taken up by plants (Emmerlich et al. 2003). Accumulation of compatible solutes such as proline, glycinebetaine, and polyols is one mechanism by which plants survive under salt stress conditions (Di Martino et al. 2003; Meloni et al. 2001), and genotypic differences in proline and glycinebetaine accumulation has been reported in cereals (Colmer et al. 1995; Yang et al. 2003). Therefore, we cannot rule out the possibility that the two cultivars differ in their accumulation of compatible solutes. On the contrary, Chen et al. (2007) compared barley genotypes contrasting in their salt tolerance and reported a twofold higher leaf and root proline and leaf glycinebetaine accumulation in salt-sensitive cultivars. Based on this observation, they suggested that hyperaccumulation of major compatible solutes in barley does not appear to play a major role in salt tolerance, implying that involvement of compatible solutes in salt tolerance may depend on plant species.

Since the current study showed a clear ion imbalance during short-term treatment with NaCl, we analyzed expression of several genes involved in  $\text{K}^+$  and  $\text{Na}^+$  homeostasis. Our results showed that transcripts of *HvHAK1* (in roots and shoots) and *HvAKT1* (in shoots) were more abundant in K305 than in I743, especially after short-term salt treatment (Fig. 5a, b). Likewise, with prolonged NaCl treatment, expression of *HvHAK1* was enhanced threefold in roots and twofold in shoots of K305 compared to I743 (Fig. 8a). In Arabidopsis, the gene encoding the outward rectifying  $\text{K}^+$  channel (*SKOR*) and *AKT2,3* were upregulated in roots and shoots, respectively, under salt stress (Maathuis 2006). Similarly, Walia et al. (2006) reported that an inward rectifying potassium channel (*KAT1*) was induced after 3 h in response to salinity. *KAT1* is structurally similar to the animal *Shaker*  $\text{K}^+$  type channel and does not depend on external  $\text{K}^+$  concentration for inward rectification (Latorre et al. 2003). This may facilitate maintenance of a low  $\text{Na}^+/\text{K}^+$  ratio by enhancing the uptake and distribution of  $\text{K}^+$  in the plant through the vascular tissue. Similarly, higher expression of *HvHAK1* and *HvAKT1* may be a salt stress tolerance mechanism in K305, alleviating the problem of  $\text{K}^+$ -deficiency induced by high  $\text{Na}^+$  concentrations in shoots. Santa-Maria et al. (1997) previously reported that the *HvHAK1* transcript was detected exclusively in roots and that its expression was enhanced more than fivefold in  $\text{K}^+$ -starved plants. They

also showed that, not only could *HvHAK1* mediate high-affinity  $\text{K}^+$  uptake in *Saccharomyces cerevisiae* but that it can mediate low-affinity  $\text{Na}^+$  uptake as well.

On the other hand, expression of a  $\text{K}^+/\text{Na}^+$  symporter (*HvHKT2;1*) slightly decreased in roots of both cultivars after salt treatment (Fig. 5c). In shoots, transcript levels decreased with salt treatment in both cultivars but more rapidly in I743 than K305. *HKT2;1* functions as a potential  $\text{Na}^+$  uptake pathway in both *T. aestivum* (Rubio et al. 1995; Laurie et al. 2002) and Arabidopsis (Rus et al. 2001). Schachtman and Schroeder (1994) showed that *HKT2;1* isolated from *T. aestivum* encodes a high-affinity  $\text{K}^+$  transporter, and that it is expressed in roots and leaves. However, *AtHKT2;1* could not complement growth of *S. cerevisiae* cells deficient in  $\text{K}^+$  uptake when cultured in  $\text{K}^+$ -limiting medium (Uozumi et al. 2000). On the contrary, expression of *AtHKT2;1* did rescue *Escherichia coli* mutants carrying deletions in  $\text{K}^+$  transporters. And, in *O. sativa*, Horie et al. (2001) isolated two isoforms of HKT1 that transport  $\text{Na}^+$  (*OsHKT2;1*) or facilitate  $\text{Na}^+/\text{K}^+$  symport (*OsHKT2;2*), indicating that the HKT family of proteins may facilitate the uptake of  $\text{Na}^+$ ,  $\text{K}^+$  or both ions. Kader et al. (2006) reported that *OsHKT2;2* transcripts were induced immediately after salt stress in a salt-tolerant rice cultivar as compared to a salt-sensitive cultivar, suggesting a role for *OsHKT2;2* in salt stress tolerance. Since there was no difference in *HvHKT2;1* expression between the cultivars used in the current study, the difference in salt tolerance between I743 and K305 might not be explained by the function of *HvHKT2;1* at least at the transcript level. However, we cannot rule out the possibility that other homologues of *HKT2;1* may facilitate the uptake of  $\text{Na}^+$ ,  $\text{K}^+$  or both ions in barley.

One mechanism by which plants survive under salt stress conditions is via the removal of toxic  $\text{Na}^+$  from the cytoplasm by the action of  $\text{Na}^+/\text{H}^+$ -antiporter proteins that are localized in plant plasma and vacuolar membranes. The process of  $\text{Na}^+$  compartmentation into the vacuole is a tolerance mechanism to salinity stress and is accelerated by a pH gradient generated by a proton-translocating  $\text{H}^+$ -ATPase and inorganic pyrophosphatase ( $\text{H}^+$ -PPase; Garbarino and DuPont 1989; Fukuda et al. 1998; Gollmack and Dietz 2001; Kluge et al. 2003; Vera-Estrella et al. 2005). Our results showed that transcripts of *HvHVA/68* and *HvHVP1* were more abundant in shoots of K305 than shoots of I743 after short-term salt stress (Fig. 6a, b). Moreover, transcripts of *HvHVP1* in shoots of K305 increased significantly upon prolonged salt treatment. This may suggest that *HvHVP1* and *HvHVA/68* are involved in salt stress tolerance. In line with this, Fukuda et al. (1998) showed that salt stress increased the transcript levels of *HvHVP1*, *HvHVP10*, *HvVHA-A*, and *HvNHX1* in roots. Similarly, in rice, *OsVHA* transcripts were induced

immediately after salt stress in a salt-tolerant cultivar whereas induction was very low and delayed in a salt-sensitive cultivar, supporting the conclusion that proton-translocating  $H^+$ -ATPase and inorganic pyrophosphatase are involved in salt stress tolerance.

In order to determine whether *H. vulgare* cultivars vary in the level of  $Na^+$  compartmentation, we further analyzed expression of  $Na^+/H^+$ -antiporter genes (*HvNHX1*, *HvNHX2*, *HvNHX3*, *HvNHX4*). During 8 h of 100 mM NaCl treatment, expression of most genes was not significantly affected, and the difference between the two cultivars was not significant except for *HvNHX1* in roots and shoots, and *HvNHX2*, *HvNHX3* and *HvNHX4* in shoots (Fig. 7a–d). However, after 24 h of 100 mM NaCl treatment, expression of *HvNHX1*, *HvNHX3* and *HvNHX4* was greater in roots of K305 than I743 (Fig. 8b–d). Members of the NHX family of proteins have been reported to function as  $Na^+/H^+$ -antiporters (see Apse et al. 1999; Yokoi et al. 2002; Zhu 2002). Overall, enhanced expression of *HvHVP1*, *HvHVA/68*, *HvNHX1*, *HvNHX3* and *HvNHX4* in shoots of K305 with prolonged salt treatment may suggest a salt stress tolerance mechanism in this cultivar by which toxic  $Na^+$  is sequestered into the vacuole. Our result is consistent with previous reports showing an increase in mRNA of  $Na^+/H^+$  antiporters in response to salt stress in salt-tolerant cultivars compared to salt-sensitive cultivars. In cotton, the salt-induced mRNA level of *GhNHX1* was three- and seven-times higher in a salt-tolerant cultivar than in two salt-sensitive cultivars (Wu et al. 2004). Similarly, in soybean, mRNA of *GmNHX1* was higher in NaCl-treated roots of salt-tolerant cultivars than similarly treated roots of salt-sensitive cultivars (Yanxiang et al. 2006). Our results also showed a transient increase in the expression of *HvHVP1*, *HvNHX1* and *HvNHX3* in I743 following salt treatment (Figs. 6b, 7a, c). Furthermore, transcripts of *HvHVA/68* in roots and *HvNHX1* in shoot were slightly more abundant in I743 than in K305 shortly after salt treatment (Figs. 6a, 7a); nevertheless, this appears to have no significant role in salt stress tolerance since this higher expression was not maintained with prolonged exposure to salt. On the other hand, we cannot rule out the possibility that the function of such transporters may be regulated not only at a transcript level but may also involve posttranslational and/or transcriptional modification of the protein. Protein phosphorylation is a posttranslational modification that is implicated in regulating the activity of various proteins, including ion channels (Armstrong et al. 1995; Bethke and Jones 1997; Köhler and Blatt 2002). In barley root tonoplast,  $Na^+/H^+$  exchange activity was induced by  $Na^+$  within 15 min even in the presence of a protein synthesis inhibitor, indicating that  $Na^+/H^+$  exchange activity was due to the activity of an existing protein rather than to de novo protein synthesis (Garbarino and Dupont 1989).

Such a rapid response involves the modification of the existing protein by phosphorylation or dephosphorylation (Salimath and Marme 1983), or other chemical modification such as acylation or reduction of disulfide bonds. Furthermore, binding of proteins such as CaM (calmodulin-like protein) can modulate the function of ion transporters. A CaM isoform (AtCaM15) that is localized in the plant vacuolar compartment plays a role in binding and regulating the activity of a tonoplast transporter (AtNHX1) from within a vacuole (Yamaguchi et al. 2003, 2005). These latter authors also showed that the interaction between AtNHX1 and AtCaM15 is  $Ca^{2+}$ - and pH-dependent, and that the binding of  $Ca^{2+}$ -AtCaM15 to the C-terminus of AtNHX1 decreased at higher vacuolar pH, indicating a pH-dependent signaling component. Horie et al. (2007) reported that OshKT2;1-dependent  $Na^+$  influx is rapidly downregulated by posttranslational and transcriptional mechanisms that monitor and restrict the amount of  $Na^+$  influx via OshKT2;1.

In conclusion, severe growth reduction in I743 may be caused primarily by the accumulation of  $Na^+$  in shoots, hence by Na-toxicity. Higher expression of transporter genes involved in  $K^+$  homeostasis and  $Na^+/H^+$  exchange under salt stress conditions in K305 suggest that the greater salt tolerance of K305 could be attributed to the intracellular compartmentation of  $Na^+$  into the vacuole and/or a better capacity for regulating  $K^+$  homeostasis. Further investigation of transporter genes is essential in order to understand salt tolerance mechanisms in barley and to manipulate salt tolerance traits in plants.

**Acknowledgments** We are very grateful to Prof. Kazuyoshi Takeda for kind provision of barley seeds, Prof. Isao Aoyama for permitting the use of the atomic absorption and flame emission spectrophotometer and for ion chromatography, and Mr. Hisao Nishizaki for technical assistance. This research was supported mainly by a postdoctoral fellowship awarded by the Japan Society for the Promotion of Science (to A.L.) and partly by the Program for Promotion of Basic Research Activity for Innovative Bioscience (PROBRAIN, to M.K.).

## References

- Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar  $Na^+/H^+$  antiport in *Arabidopsis*. *Science* 285:1256–1258
- Armstrong F, Leung J, Grabov A, Brearley J, Giraudat J, Blatt MR (1995) Sensitivity to abscisic acid of guard cell  $K^+$  channels is suppressed by *abi1-1*, a mutant *Arabidopsis* gene encoding a putative protein phosphatase. *Proc Natl Acad Sci USA* 92:9520–9524
- Ballesteros E, Blumwald E, Donaire JP, Belder A (1997)  $Na^+/H^+$  antiport activity in tonoplast vesicles isolated from sunflower roots induced by NaCl stress. *Physiol Plant* 99:328–334
- Banuelos MA, Garcíadeblas B, Cubero B, Rodríguez-Navarro A (2002) Inventory and functional characterization of the HAK potassium transporters of rice. *Plant Physiol* 130:784–795

- Bethke PC, Jones RL (1997) Reversible protein phosphorylation regulates the activity of slow-vacuolar ion channel. *Plant J* 11:1227–1235
- Bhandal IS, Malik CP (1988) Potassium estimation, uptake, and its role in the physiology and metabolism of flowering plants. *Int Rev Cytol* 110:205–254
- Blumwald E, Poole RT (1985)  $\text{Na}^+/\text{H}^+$  antiporter in isolated tonoplast vesicles from storage tissue of *Beta vulgaris*. *Plant Physiol* 78:163–167
- Blumwald E, Poole RT (1987) Salt tolerance in suspension cultures of sugar beet. *Plant Physiol* 83:884–887
- Chavan P, Karadge B (1980) Influence of sodium chloride and sodium sulphate salinization on photosynthesis carbon assimilation in pea nuts. *Plant Soil* 56:201–207
- Chen Z, Cui TA, Zhou M, Twomey A, Naidu BP, Shabala S (2007) Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance. *J Exp Bot* 58:4245–4255
- Chomezynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162:156–159
- Colmer TD, Epstein E, Dvorak J (1995) Differential solute regulation in leaf blades of various ages in salt-sensitive wheat and a salt-tolerant wheat X *Lophopyrum elongatum* (Host) A. Love amphiploid. *Plant Physiol* 108:1715–1724
- Davenport RJ, Muñoz-Mayo A, Jha D, Essah PA, Rus A, Tester M (2007) The  $\text{Na}^+$  transporter AtHKT1;1 controls retrieval of  $\text{Na}^+$  from the xylem in *Arabidopsis*. *Plant Cell Environ* 30:497–507
- Di Martino C, Delfino S, Pizzuto R, Loreto F, Fuggi A (2003) Free amino acids and glycine betaine in leaf osmoregulation of spinach responding to increasing salt stress. *New Phytol* 158:455–463
- Dinnyen JR, Long TA, Wang JY, Jung JW, Mace D, Pointer S, Barron C, Brady SM, Schiefelbein J, Benfey PN (2008) Roots to abiotic stress cell identity mediates the response. *Science* 320:942–944
- Emmerlich V, Linka N, Reinhold T, Hurth MA, Traub M, Martinoia E, Neuhaus HE (2003) The plant homolog to the human sodium/dicarboxylic cotransporter is the vacuolar malate carrier. *Proc Natl Acad Sci USA* 100:11122–11126
- Enstone DE, Peterson CA, Ma F (2003) Root endodermis and exodermis: structure, function, and responses to the environment. *J Plant Growth Regul* 21:335–351
- Fan TWM, Higashi RM, Norlyn J, Epstein E (1989) In vivo  $^{23}\text{Na}$  and  $^{31}\text{P}$  NMR measurement of a tonoplast  $\text{Na}^+/\text{H}^+$  exchange process and its characteristics in two barley cultivars. *Proc Natl Acad Sci USA* 86:9856–9860
- Flowers TJ (1988) Chloride as a nutrient and as an osmoticum. In: Tinker B, Läuchli A (eds) *Advances in plant nutrition*, vol 3. Praeger, New York, pp 55–78
- Fu HH, Luan S (1998) AtKUP1: a dual affinity K-transporter from *Arabidopsis*. *Plant Cell* 10:63–73
- Fukuda A, Yazaki Y, Ishikawa T, Koike S, Tanaka Y (1998)  $\text{Na}^+/\text{H}^+$  antiporter in tonoplast vesicles from rice roots. *Plant Cell Physiol* 39:196–201
- Fukuda A, Nakamura A, Tanaka Y (1999) Molecular cloning and expression of the  $\text{Na}^+/\text{H}^+$  exchanger gene in *Oryza sativa*. *Biochim Biophys Acta* 1446:149–155
- Garbarino J, DuPont FM (1989) Rapid induction of  $\text{Na}^+/\text{H}^+$  exchange activity in barley root tonoplast. *Plant Physiol* 89:1–4
- Garthwaite AJ, von Bothmer R, Colmer TD (2005) Salt tolerance in wild *Hordeum* species is associated with restricted entry of  $\text{Na}^+$  and  $\text{Cl}^-$  into the shoots. *J Exp Bot* 56:2365–2378
- Golldack D, Dietz KJ (2001) Salt-induced expression of the vacuolar  $\text{H}^+$ -ATPase in the common ice plant is developmentally controlled and tissue specific. *Plant Physiol* 125:1643–1654
- Golldack D, Su H, Quigley F, Kamasani UR, Muñoz-Garay C, Balderas E, Popova OV, Bennett J, Bohnert HJ, Pantoja O (2002) Characterization of a HKT-type transporter in rice as a general alkali cation transporter. *Plant J* 31:529–542
- Horie T, Yoshida K, Nakayama H, Yamada K, Oiki S, Shinmyo A (2001) Two types of HKT transporters with different properties of  $\text{Na}^+$  and  $\text{K}^+$  transport in *Oryza sativa*. *Plant J* 27:129–138
- Horie T, Horie R, Chan WY, Leung HY, Schroeder JI (2006) Calcium regulation of sodium hypersensitivities of *sos3* and *athkt1* mutants. *Plant Cell Physiol* 47:622–633
- Horie T, Costa A, Kim TH, Han MJ, Horie R, Leung HY, Miyao A, Hirochika H, An G, Schroeder JI (2007) Rice OsHKT2;1 transporter mediates large  $\text{Na}^+$  influx component into  $\text{K}^+$ -starved roots for growth. *EMBO J* 26:3003–3014
- Kader MA, Seidel T, Golldack D, Lindberg S (2006) Expressions of OsHKT1, OsHKT2 and OsVHA are differentially regulated under NaCl stress in salt-sensitive and salt-tolerant rice (*Oryza sativa* L.) cultivars. *J Exp Bot* 57:4257–4268
- Katsuhara M, Yamada M, Kasamo K (2001) Isolation of barley *salt* gene: its relation to salt tolerance and to hormonal regulation by abscisic acid and jasmonic acid. *Soil Sci Plant Nutr* 47:187–193
- Kim EJ, Kwak JM, Uozumi N, Schroeder JI (1998) AtKUP1: an *Arabidopsis* gene encoding high-affinity potassium transport activity. *Plant Cell* 10:51–62
- Kluge C, Lamkemeyer P, Tavakoli N, Golldack D, Kandlbinder A, Dietz KJ (2003) cDNA cloning of 12 subunits of the V-type ATPase from *Mesembryanthemum crystallinum* and their expression under stress. *Mol Membr Biol* 20:171–183
- Köhler B, Blatt MR (2002) Protein phosphorylation activates the guard cell  $\text{Ca}^{2+}$  channel and is a prerequisite for gating by abscisic acid. *Plant J* 32:185–194
- Krishnamurthy P, Ranathunge K, Franke R, Prakash HS, Schreiber L, Mathew MK (2009) The role of root apoplastic transport barriers in salt tolerance of rice (*Oryza sativa* L.). *Planta* 230:119–134
- Latorre R, Olcese R, Basso C, Gonzalez C, Munoz F, Cosmelli D, Alvarez O (2003) Molecular coupling between voltage sensor and pore opening in the *Arabidopsis* inward rectifier  $\text{K}^+$  channel KAT1. *J Gen Physiol* 122:459–469
- Laurie S, Feeney KA, Maathuis FJM, Heard PJ, Brown SJ, Leigh RA (2002) A role for HKT1 in sodium uptake by wheat roots. *Plant J* 32:139–149
- Leonova TG, Goncharova EA, Khodorenko AV, Babakov AV (2005) Characteristics of salt-tolerant and salt-susceptible cultivars of barley. *Russ J Plant Physiol* 52:774–778
- Maas EV (1993) Salinity and citriculture. *Tree Physiol* 12:195–216
- Maathuis FJM (2006) The role of monovalent cation transporters in plant responses to salinity. *J Exp Bot* 57:1137–1147
- Maathuis FJM, Amtmann A (1999)  $\text{K}^+$  nutrition and  $\text{Na}^+$  toxicity: the basis of cellular  $\text{K}^+/\text{Na}^+$  ratios. *Ann Bot* 84:123–133
- Mano Y (1996) Studies on breeding and evaluation of germplasm for salt tolerance in barley (in Japanese with English summary). Special report of the Barley Germplasm Center Okayama University 2:1–89
- Marschner H (1995) *Mineral nutrition of higher plants*, 2nd edn. Academic, London
- Meloni DA, Oliva MA, Ruiz HA, Martinez CA (2001) Contribution of proline and inorganic solutes to osmotic adjustment in cotton under salt stress. *J Plant Nutr* 24:599–612
- Munns R (2001) Avenues for increasing salt tolerance of crops. In: Horst J et al (eds) *Plant nutrition: food security and sustainability of agro-ecosystems*. Kluwer, Dordrecht, pp 370–371
- Munns R (2002) Comparative physiology of salt and water stress. *Plant Cell Environ* 25:239–250
- Munns R, Guo J, Passioura JB, Cramer GR (2000) Leaf water status controls day-time but not daily rates of leaf expansion in

- increasing salt tolerance in monocotyledonous plants 1041 salt-treated barley. *Aust J Plant Physiol* 27:949–957
- Munns R, James AJ, Läuchli A (2006) Approaches to increasing the salt tolerance of wheat and other cereals. *J Exp Bot* 57:1025–1043
- Peterson CA (1988) Exodermal Casparian bands: their significance for ion uptake by roots. *Physiol Plant* 72:204–208
- Pitman MG, Lauchli A, Stelzer R (1981) Ion distribution in roots of barley seedlings measured by electron probe X-ray microanalysis. *Plant Physiol* 68:673–679
- Quintero J, Blatt MR (1997) A new family of K-transporters from *Arabidopsis* that are conserved across phyla. *FEBS Lett* 415:206–211
- Robards AW, Jackson SM, Clarkson DT, Sanderson J (1973) The structure of barley roots in relation to the transport of ions into the stele. *Protoplasma* 77:291–311
- Royo A, Aragüés R (1999) Salinity-yield response functions of barley genotypes assessed with a triple line source sprinkler system. *Plant Soil* 209:9–20
- Royo A, Aragüés R, Playán E, Ortiz R (2000) Salinity-grain yield response functions of barley cultivars assessed with a drip-injection irrigation system. *Soil Sci Soc Am J* 64:359–365
- Rubio F, Gassmann W, Schroeder JI (1995) Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. *Science* 270:1660–1663
- Rubio F, Gassmann W, Schroeder JI (1996) Technical comment. *Science* 273:978–979
- Rus A, Yokoi S, Sharkhuu A, Reddy M, Lee BH, Matsumoto TK, Koiwa H, Zhu JK, Bressan RA, Hasegawa PM (2001) AtHKT1 is a salt tolerance determinant that controls Na<sup>+</sup> entry into plant roots. *Proc Natl Acad Sci USA* 98:14150–14155
- Salimath BP, Marme D (1983) Protein phosphorylation and its regulation by calcium and calmodulin in membrane fractions from zucchini hypocotyls. *Planta* 158:560–568
- Santa-Maria GE, Rubio F, Dubcovsky J, Rodriguez-Navarro A (1997) The *HAK1* gene of barley is a member of a large gene family and encodes a high-affinity potassium transporter. *Plant Cell* 9:2281–2289
- Schachtman DP, Schroeder JI (1994) Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. *Nature* 370:655–658
- Staal M, Maathuis FJM, Elzenga TM, Overbeek HM, Prins HBA (1991) Na<sup>+</sup>/H<sup>+</sup> antiport activity in tonoplast vesicles from roots of the salt-tolerant *Plantago maritima* and the salt-sensitive *Plantago media*. *Physiol Plant* 82:179–184
- Tester M, Davenport R (2003) Na<sup>+</sup> tolerance and Na<sup>+</sup> transport in higher plants. *Ann Bot* 91:503–527
- Uozumi N, Kim EJ, Rubio F, Yamaguchi T, Muto S, Tsuboi A, Bakker EP, Nakamura T, Schroeder JI (2000) The *Arabidopsis* HKT1 gene homolog mediates inward Na<sup>+</sup> currents in *Xenopus laevis* oocytes and Na<sup>+</sup> uptake in *Saccharomyces cerevisiae*. *Plant Physiol* 122:1249–1259
- Vera-Estrella R, Barkla BJ, Garcia-Ramirez L, Pantoja O (2005) Salt stress in *Thellungiella halophila* activates Na<sup>+</sup> transport mechanisms required for salinity tolerance. *Plant Physiol* 139:1507–1517
- Walia H, Wilson C, Wahid A, Condamine P, Cui X, Close TJ (2006) Expression analysis of barley (*Hordeum vulgare* L.) during salinity stress. *Funct Integr Genomics* 6(2):143–156
- Westfall PH, Tobia RD, Rom D, Wolfinger RD, Hochberg Y (1996) Multiple comparisons of multiple tests using the SAS system. SAS Institute, Cary
- Wilson C, Shannon MC (1995) Salt induced Na<sup>+</sup>/H<sup>+</sup> antiport in root plasma membrane of a glycophytic and halophytic species of tomato. *Plant Sci* 107:147–157
- Wu CA, Yang GD, Meng QW, Zheng CC (2004) The cotton *GhNHX1* gene encoding a novel putative tonoplast Na<sup>+</sup>/H<sup>+</sup> antiporter plays an important role in salt stress. *Plant Cell Physiol* 45:600–607
- Yamaguchi T, Apse MP, Shi H, Blumwald E (2003) Topological analysis of a plant vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter reveals a luminal C terminus that regulates antiporter cation selectivity. *Proc Natl Acad Sci USA* 100:12510–12515
- Yamaguchi T, Aharon GS, Sottosanto JB, Blumwald E (2005) Vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter cation selectivity is regulated by calmodulin from within the vacuole in a Ca<sup>2+</sup>- and pH-dependent manner. *Proc Natl Acad Sci USA* 102:16107–16112
- Yang W-J, Rich PJ, Axtell JD, Wood KV, Bonham CC, Ejeta G, Mickelbart MV, Rhodes D (2003) Genotypic variation for glycine betaine in *Sorghum bicolor*. *Crop Sci* 43:162–169
- Yanxiang S, Dan W, Yanling B, Ningning W, Yong W (2006) Studies on the overexpression of the soybean GmNHX1 in *Lotus corniculatus*: the reduced Na<sup>+</sup> level is the basis of the increased salt tolerance. *Chin Sci Bull* 51:1306–1315
- Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM (2002) Differential expression and function of *Arabidopsis thaliana* NHX Na<sup>+</sup>/H<sup>+</sup> antiporters in the salt stress response. *Plant J* 30:529–539
- Zhu JK (2002) Salt and drought stress signal transduction in plants. *Annu Rev Plant Biol* 53:247–273
- Zhu H, Ding GH, Fang K, Zhao FG, Qin P (2006) New perspective on the mechanism of alleviating salt stress by spermidine in barley seedlings. *Plant Growth Regul* 149:147–156