Multiple Genes (*SOS, HKT, TVP*) Expression in Two Contrasting Bread Wheat (*Triticum aestivum* L.), Cultivars on In Vitro Saline Stress Conditions

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Abstract

Candidate genes expression for salinity tolerance was studied for both cationic transporters, namely: HKT1; 5 and HKT2; 1. The two genes were expressed in the root, and then, it showed that HKT1; 5 was better expressed in the root of HD cultivars. This suggests a more active function of HKT1; 5 genes in HD as a tolerant cultivar. Vacuolar antiporter Na^+/H^+, TNHX-1, expression was more elevated in roots, sheaths, and blades of HD than MD cultivar. Roots and sheaths of both two cultivars accumulate more transcripts of vacuolar TVP1 than the leaf blade. The two genes TNHX1 and TVP1 were expressed with high similarity in MD and HD cultivars, which suggesting equal efficiency storage in both genotypes. Na^+/H^+ antiporter localized in the plasma membrane (TaSOS1) was more accumulated in roots and sheaths of MD comparative to HD cultivar; suggesting that in addition to higher retention of Na^+ in sheaths, HD prevents the accumulation of Na^+ in the blade by activating its efflux via a high expression of the gene SOS1. Results showed that salinity tolerance in wheat (*Triticum aestivum* L.) is concordant to the ability to prevent Na^+ accumulation toxic levels, linked to a high osmoregulation capacity coupled with an acceptable K^+ level in the blade.

Keywords

Triticum aestivum L. • Saline stress • Ion transporters • Genetically expression

1 Introduction

Soil salinity is one of the major worldwide environmental constraints affecting agricultural production in arid and semiarid regions. Despite significant progress achieved toward understanding molecular mechanisms controlling a plant's response to salinity, there have been a few, if any, cereal cultivars with improved salinity tolerance. Glycophytes such as bread wheat (*Triticum aestivum*) cope with salinity stress by excluding Na^+ from shoots (Munns and James 2003; Colmer et al. 2005) and by tolerating high internal levels of Na^+, which is also referred to as tissue tolerance (Yeo and Flowers 1983; Colmer et al. 2005; Munns et al. 2006; Tammam et al. 2008). When grown in saline environments, bread wheat is generally more salt tolerant than durum wheat, which is likely to be largely due to its better Na^+ exclusion (Colmer et al. 2006).

Despite the complexity of salt tolerance, much of the recent work to improve the level of salt tolerance in wheat has focused on Na^+ exclusion in plant tissues as the most appropriate selection criteria. Genotypic differences in Na^+ exclusion (estimated by the Na^+ concentration in the leaf blade or whole shoot) can be demonstrated in wheat, but its relationship with salt tolerance is not consistent. Hexaploid bread wheat cultivars have slow rates of Na^+ transport to the shoot and maintain a high K^+/Na^+ ratio in leaves. This enhanced K^+/Na^+ discrimination trait contributes to salt tolerance (Dvořák et al. 1994; Tammam et al. 2008). A locus for this trait, *kna*1, was mapped to the distal region of chromosome 4DL (Dubcovsky et al. 1996) of the bread wheat cultivar, whereas the tetraploid durum wheat lacks this trait. A homolog of the *kna*1 locus has not yet been found on

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either the A or B genomes of tetraploid wheat species. A new source of Na^+ exclusion was found in durum wheat, Line 149, which had a low Na^+ concentration and high K^+/Na^+ ratios in the leaf blade similar to bread wheat (Munns et al. 2000). Genetic studies indicated two major loci, *Nax*1 and *Nax*2 (Na^+ exclusion loci), controlled leaf blade Na^+.

Sodium efflux from root cells prevents the accumulation of toxic levels of Na^+ in the cytosol and its transport to the shoot. Molecular genetic analysis of *Arabidopsis* SOS (salt overly sensitive) mutants has led to the identification of a plasma membrane Na^+/H^+ antiporter, SOS1, which plays a crucial role in sodium exclusion from root epidermal cells under salinity. Understanding the molecular basis of salt-stress signaling and tolerance mechanisms in wheat becomes today mandatory for engineering and/or screening for local wheat genotypes more tolerant to salt stress. This report performed physiological and molecular analysis on two Algerian bread wheat genotypes (*Triticum aestivum* L.), Mahon Demias and Hidhab, with contrasting salinity tolerance. Our data provide evidence for a functional correlation linking Na^+/fluxes and the expression patterns of SOS and HKT-type transporters to salt stress tolerance in bread wheat.

2 Materials and Methods

2.1 Plant Material, Germination Assay, and Stress Conditions

The seeds of two bread wheat cultivars (*Triticum aestivum* L.) are Mahon Demias (MD, salt sensitive) and *Triticum aestivum* L. Hidhab (HD1220, salt tolerant). Seeds collected successively during the last crop years were supplied by the Agricultural Research Station of Sétif (ARSS-Algeria). Seeds of each line were sterilized in 0.5% NaOCl for 15 min, then washed three times with sterile water, and placed on Petri dishes with a single sheet of Whitman #1 filter paper for germination. The percentage of seed germination was determined as the number of seeds with radicals growing at least 2 mm long over the initial seed number soaked on wet Whitman paper. To test the response of the seeds to salt stress, 30 seeds of the two wheat varieties were germinated on various concentrations of NaCl (0, 50, 100, and 200 mM) and incubated at 25 °C in a growth chamber under a 16 h light/8 h dark photoperiod and 60 ± 10% relative humidity. Four-day-old seedlings were transferred to Eppendorf tubes floating on modified half-strength Hoagland's solution in containers (Epstein 1972). When plants reach the third leaf stage, NaCl concentrations (0, 50, 100, and 200 mM) were applied progressively (salt treatment). All seedlings were grown in a glasshouse at 25 ± 5 °C, under photosynthetically active radiation of 280 μmol m^2 s^{-1}, a 16 h photoperiods, and 60 ± 10% relative humidity. A first harvest was made at the beginning of salt treatment (initial harvest), and sequential harvests were made at different times (3, 7, 10, and 14 days) of exposure to salinity (final harvest). All the physiological tests were performed on leaves at the same developmental stage (leaf 1 or leaf 2).

2.2 RNA Extraction and RT-PCR Assay

Total RNA from roots, leaf sheaths, and leaf blades of 1-week-old plants treated with 100 mM NaCl for 3 days, were extracted using the RNeasy total RNA isolation kit (Qiagen). To remove contaminating DNA, RNAs (10 μg) were treated with RNase-free DNase (Promega). DNase-treated RNA samples (0.5 μg) were reverse-transcribed using M-MLV reverse transcriptase (*Invitrogen*). The reverse transcription (RT) reactions were performed at 37 °C for 1 h using 2 μM oligo-dT18. Two μl of the first strand cDNAs were used as templates for PCR amplification with specific primers of candidate genes (Table 1). A wheat *Actin* gene fragment was used as an internal control. Samples were denatured for 5 min at 94 °C and then ran for 35 cycles of 30 s at 94 °C, 45 s at 58 °C, and 2 min at 72 °C with a final extension of 5 min at 72 °C. The PCR products were separated by agarose gel (1%) electrophoresis.

3 Results

3.1 Germination and Seedling Growth

Under standard growth conditions, the overall seed germination rates of the cultivars Mahon Demias (MD) and Hidhab (HD) were >98%. However, in the presence of increasing concentrations of NaCl, a gradual decrease in the germination rates was observed (Fig. 1).

Under high salinity (200 mM NaCl), the germination of the sensitive genotype (MD) was severely affected and did not exceed 66%, whereas in the salt-tolerant HD genotype, it was maintained to ≈82%. Moreover, these salt stress treatments affect seedling growth and result in both leaf and root length reductions, greater in MD (Fig. 2). So consequently, the estimated total leaf area was more dramatically reduced in MD than in HD.

3.2 Ion Status

Following exposure to 100 mM of NaCl, Na^+ concentrations were measured in individual leaves of both wheat genotypes. At the leaf sheaths (leaf 1 and leaf 2), sodium accumulates at similar rates in both genotypes after the first 3 days of salt treatment but reaches later substantially higher levels in the

Table 1 List of the primers used for RT-PCR analysis of the wheat candidate genes

Gene	N° access	Primer	Sequence
HKT1; 5	DQ64633	HKT8_F3 HKT8_R3	5′-CTGTCGCTCTTCTGCGCCAT-3′ 5′-TTATACTATCCTCCATGCCT-3′
HKT2; 2	DQ015706	HKT2_F3 HKT8_R3	5′-GATCCACTCAACTTCTCCAC-3′ 5′-TCATACTTTCCAGGATTTAC-3′
TNHX1	AY296910	B3_F B4_R	5′-TCGGAAAATTCCTCTACCTA-3′ 5′-AGAACAACAATGATTGTGCT-3′
TVP1	AY296911	TVP_F TVP_R	5′-GTCAGCAGAGCTGGTGTGAAG-3′ 5′-TCAGCTTGATGAGGATGTTGA-3′
TaSOS1	AY326952	KM1_F KM2_R	5′-GCATCTTATTGGAAGGATTT-3′ 5′-CCTCTCAGGTGAGACTGCTA-3′
Actin	AB181991	Act_F Act_R	5′-GTGCCCATTTACGAAGGATA-3′ 5′-GAAGACTCCATGCCGATCAT-3′

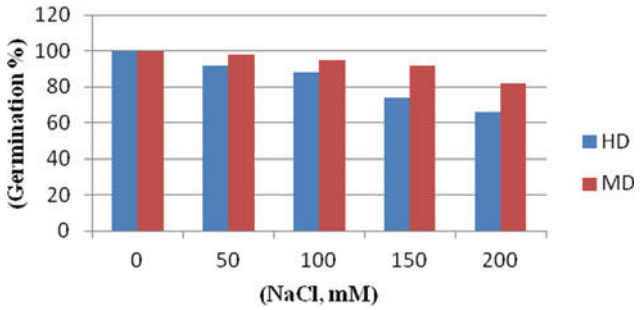

Fig. 1 Germination percentage of both two varieties, Hidhab (HD 1220) and Mahon Demias (MD)

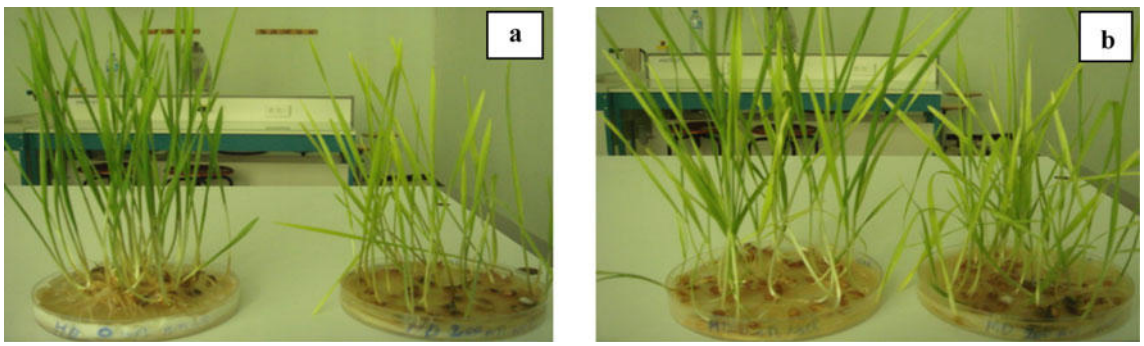

Fig. 2 Salt stress affecting using (200 mM, NaCl) in both HD (**a**) and MD (**b**) varieties seedlings

HD variety, especially at day 7 (Fig. 3a). However, the leaf blades of MD accumulate Na^+ up to 250 mM, a concentration 5 times higher than the values registered in HD variety (Fig. 3b). By contrast, in the root of both genotypes, similar Na^+ concentrations were registered (Fig. 3c). Storage of Na^+ in the two wheat genotypes was investigated further by measuring the Na^+ content in the leaf sheaths and the leaf blades (leaves 1 and 2) after 7 days of exposure to increasing NaCl concentrations. Both wheat varieties accumulated Na^+ at different levels in the leaf sheath, and HD accumulated a substantially higher Na^+ concentration than MD with no evidence of storage saturation (Fig. 3d). The two genotypes seem to have a contrasting capacity to store Na^+ in the leaf sheath, and their leaf sheath cells may differ in their ability to extract Na^+ from the xylem stream. This possibility was supported by genotypic differences in the proportion of total leaf Na^+ content stored in the leaf sheath.

We measured K^+ in the leaf blade and leaf sheath of both genotypes during 14 days of growth in the presence of 100 mM NaCl. K^+ was accumulated to similar levels in the

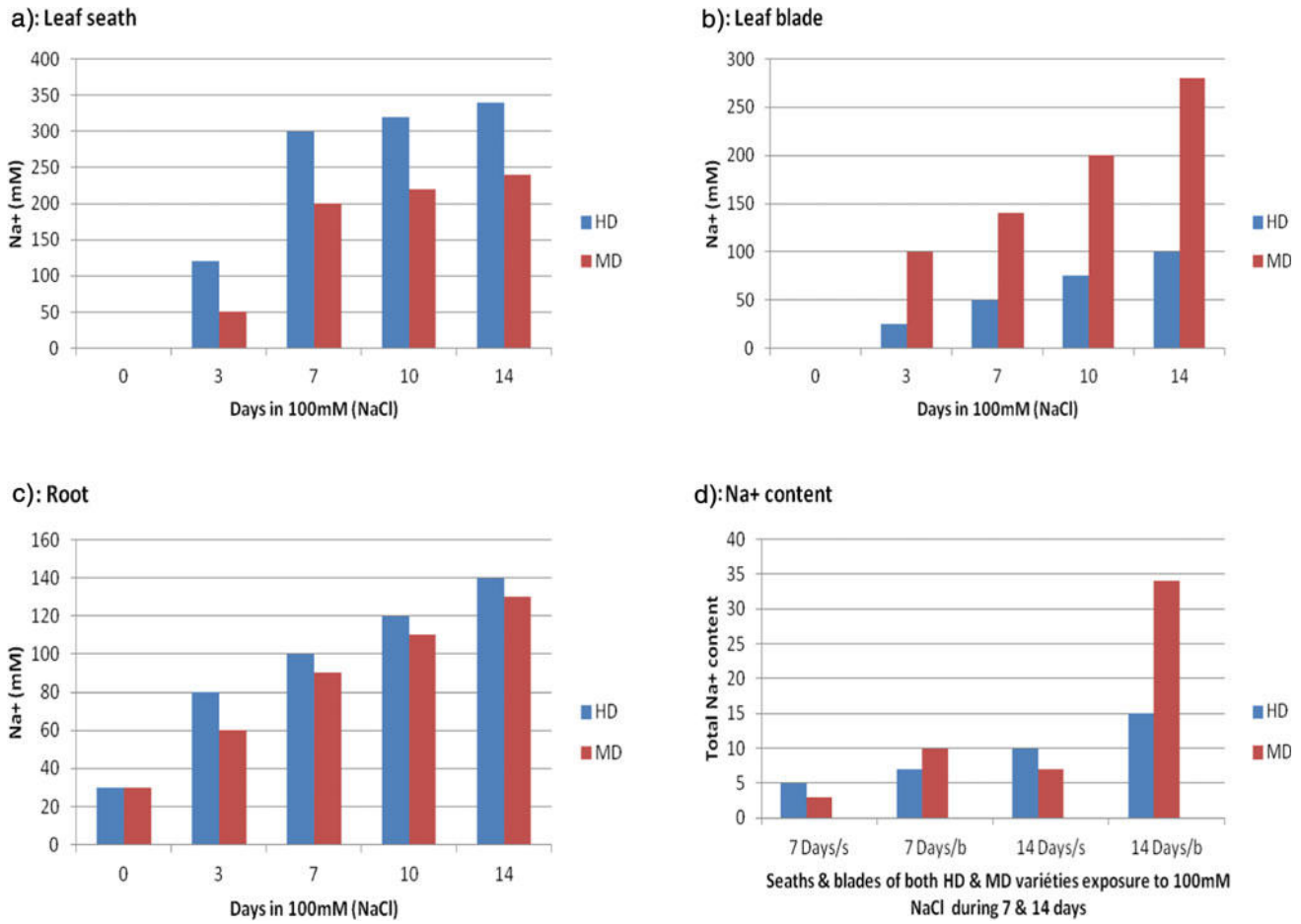

Fig. 3 Increase in Na^+ concentrations in the leaf sheaths (**a**) and leaf blade (**b**), and roots (**c**), of salt-tolerant HD and salt-sensitive MD and total Na^+ content in blade and sheath in both HD and MD varieties (**d**) during 14 days exposure to 100 mM NaCl

leaf sheath of each genotype, whereas in leaf blades, more K^+ accumulated in HD than MD (Fig. 4a, b), giving a higher K^+/Na^+ ratio in the salt-tolerant genotype (Fig. 4c). In roots, K^+ content was higher in HD genotype compared to MD genotype (Fig. 4d). The root's ability to retain K^+ correlates with higher salt tolerance in HD, compared to the MD genotype.

3.3 Expression Analysis of Candidate Genes HKT1; 5, HKT2; 2, TNHX1, TVP1, and TaSOS1 in the Two Bread Wheat Varieties

Many genes were previously shown to play important roles in maintaining K^+ or Na^+ homeostasis in higher plants. We have studied the expression levels of five candidate genes involved in controlling uptake, transport, and sequestration of Na^+ ions. RT-PCR analysis of two HKT encoding genes: HKT1; 5 (previously named HKT8) and HKT2; 2 (previously named HKT2) in cultivars MD and HD exposed to 100 mM NaCl showed high expression levels of both genes in roots but neither in leaf sheaths nor in leaf blades (Fig. 5). However, HKT2; 2 shows similar expression patterns in the roots of the two wheat genotypes, and HKT1; 5 transcripts seem to accumulate to higher levels in HD (Fig. 5). Regarding the vacuolar Na^+/H^+ antiporter TNHX-1, the transcript levels in the roots, sheaths, and blades were greater in HD than in the MD genotype. More transcripts seem to accumulate in the roots and leaf sheaths than leaf blades in the two genotypes (Fig. 5). The expression level of the vacuolar H^+-Pyrophosphatase TVP1 was comparable to that observed with TNHX1. In fact, the roots and sheaths of the two genotypes accumulated more TVP1 transcript than the leaf.

4 Discussion

Salt tolerance reflects the plant's ability to exclude Na^+ as well as the mechanisms linked to the tolerance of the tissues to accumulate Na^+. These two salt tolerance components are likely to operate independently, making salt tolerance

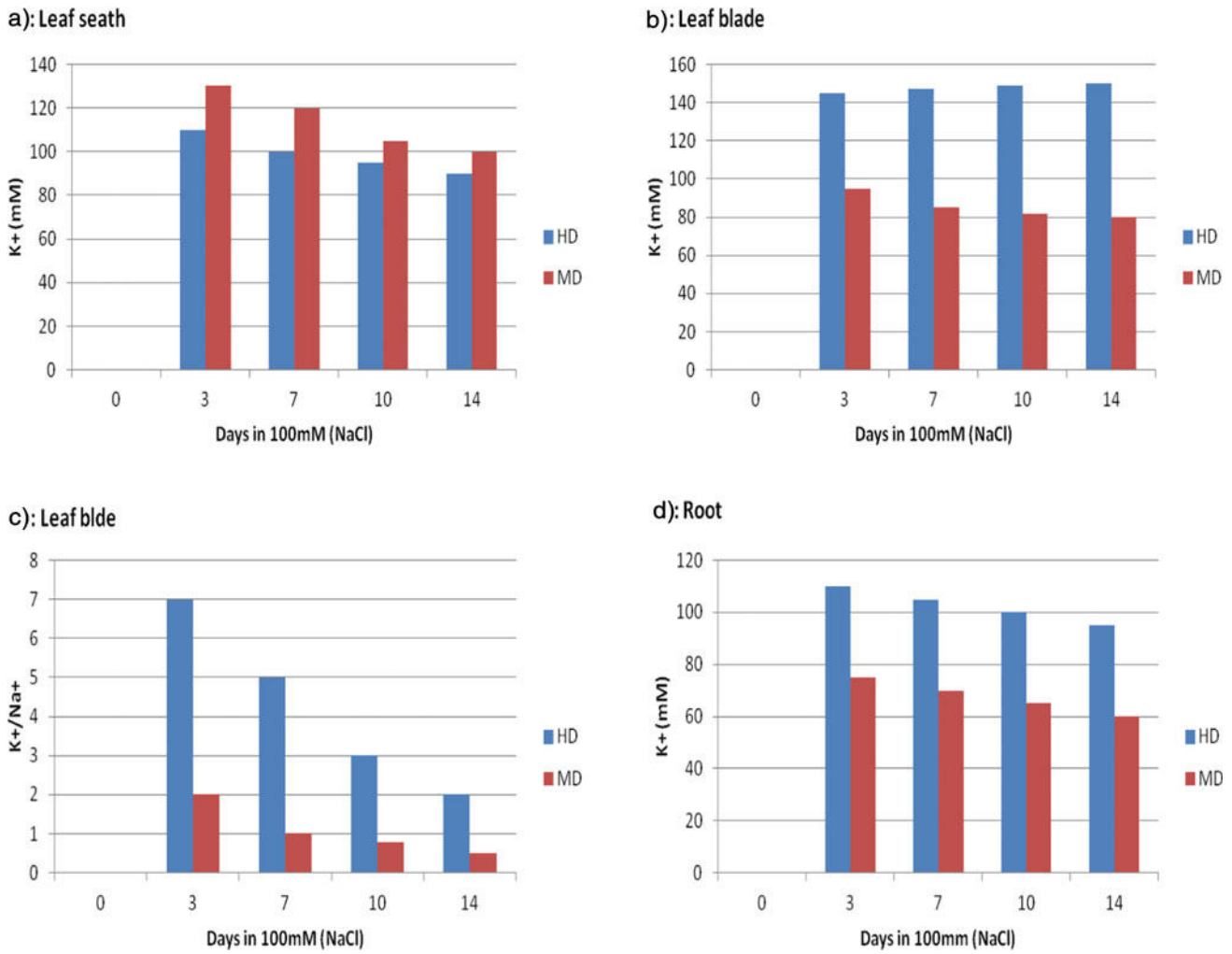

Fig. 4 Effect of salt stress on the accumulation of K^+ ions in leaf sheaths (**a**), in leaf blades (**b**) and roots (**c**); K^+/Na^+ ratio in leaf blade (**d**) in salt-tolerant HD and salt-sensitive MD values are means ± SD (n = 5)

dependent on their relative effects. Accordingly, salt tolerance of two Algerian bread wheat genotypes was evaluated in this study using a cluster of physiological and molecular parameters. Our data show that MD appeared to be more sensitive to salt than HD at the germination stage (Fig. 1). While the two wheat genotypes studied here appeared to have similar Na^+ storage capacity in the roots, they showed different Na^+ accumulation rates in leaf blades. Na^+ accumulated more in the sheaths of leaf 1 and 2 of HD compared to MD (Fig. 4). Sheath storage capacity may represent an advantage for HD by limiting the loading of Na^+ to the leaf blade, thus, preserving its photosynthetic capacity.

However, sheath retention of Na^+ itself would only delay the accumulation of Na^+ in leaf blades until a threshold was reached above which the Na^+ will reach leaf blades of both cultivars. It is possible that this trait of preferential sheath retention of Na^+ would interact with the low xylem loading. Similarly, differential sheath retention of Na^+ has been previously reported on two durum wheat varieties showing marked differences in salt and drought stress (Brini et al. 2009). Uptake of K^+ into leaf sheaths of salt-treated plants showed no differences between the genotypes. This suggests that sheath sequestration of Na^+ could be Na^+ specific. However, enhanced uptake of K^+ in leaves of HD compared to MD resulted in a higher K^+/Na^+ ratio in leaf blades, which may benefit cellular homeostasis. Salt tolerance is associated with low rates of transport of Na^+ to shoots with high selectivity for K^+ over Na^+, and therefore, K^+/Na^+ ratio in young leaves is suggested as an important factor for metabolism and growth (Dvořák and Gorham 1992; Husain et al. 2004; Poustini and Siosemardeh 2004). K^+/Na^+ ratio is controlled by a QTL linked to *Kna*1 locus located at the distal region of chromosome 4DL of bread wheat (Gorham et al. 1987; Dubcovsky et al. 1996). Increasing evidence shows that, during root uptake, enhanced discrimination of K^+ over Na^+ is an important trait contributing to salt

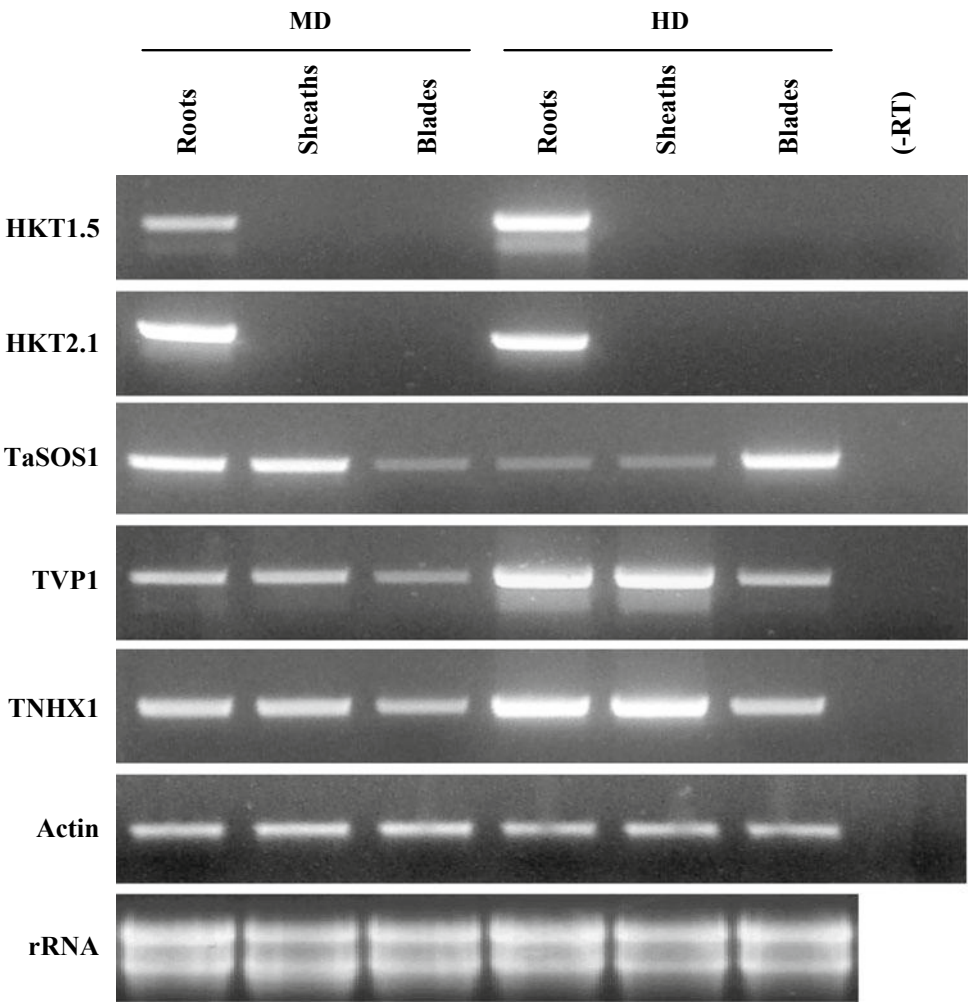

Fig. 5 RT-PCR analysis of the expression levels of HKT1; 5, HKT2; 2, TNHX1, TVP1, and TaSOS1, in roots, sheaths, and blades of salt sensitive MD and salt-tolerant HD genotypes using specific gene primers. The expected size of the cDNA HKT1; 5 is 600 pb, HKT2; 2 is 450 pb, TNHX1 is 640 pb, TVP1 is 550 pb, and TaSOS1 is 680 pb. (-RT): without reverse transcriptase; at 380 pb Actin, a fragment was amplified by RT-PCR as an internal control. The Ribosomal RNA samples stained by ethidium bromide are also indicated

tolerance, and therefore, K^+/Na^+ ratio in plant tissues is a widely used parameter in distinguishing genotypes for their tolerance to NaCl toxicity in wheat and other cereal species (Gorham et al. 1990; Santa-Maria and Epstein 2001; Munns and James 2003). Reducing salt-induced K^+ efflux would allow its contribution toward osmoregulation, negating the need for a high investment into the production of organic solutes and allowing the critical maintenance of optimal cytosolic K^+/Na^+ ratio (Cuin et al. 2008). Salt tolerance of plants depends on HKT transporters, which mediate Na^+ specific transport of Na^+, K^+ transport, and play a key role in regulating Na^+ homeostasis (Rodriguez-Navarro and Rubio 2006; Munns and Tester 2008).

Several genes belonging to the HKT family have been studied in wheat. TaHKT1 was the first HKT gene cloned from higher plants, showing cortical expression (Schachtman and Schroeder 1994). The down-regulation (by an antisense construct) of TaHKT2; 1 in wheat increased shoot fresh weight by 50–100% in 200 mM NaCl under conditions of K^+ deficiency (Laurie et al. 2002). Following the down-regulation of TaHKT2; 1, transgenic wheat had smaller Na^+ induced depolarization in roots cortical cells and low Na^+ influx, indicating that TaHKT2; 1 mediates Na^+ influx (Laurie et al. 2002). Further evidence using a root uptake system and a yeast transformation system also supported TaHKT2; 1 and HvHKT2; 1 functioned as a Na^+ uniport (Haro et al. 2005). In durum wheat, the gene homologous to TmHKT7-A2 (from *Triticum monococum*), which is the best candidate for *Nax*1, could control Na^+ unloading from the xylem in root and sheath of line 149 (salt tolerant) but not of Tamaroi (salt sensitive) (James et al. 2006). Upon salt stress, the expression of the two HKT genes used in this study was detected only in the two bread wheat genotypes' roots. While HKT2; 2 shows the same expression pattern in both varieties, a differential accumulation was observed for HKT1; 5 transcripts, which reach higher levels in HD roots. These findings suggest that both HKT genes might be involved in Na^+/K^+ transport through the root cortical cells' plasma membrane with a more active role of HKT1; 5 in the tolerant variety.

The expression level of the wheat Na^+/H^+ antiporter gene (TNHX1) following salt stress was also investigated. The

TNHX1 transcripts accumulate to higher amounts in roots and leaf sheaths of both MD and HD than leaf blades. The greater increase of TNHX1 expression in roots and sheaths treated with salt might respond to more Na^+ accumulating in the corresponding vacuoles. The expression level of TVP1 seems to be similar to TNHX1 in the different tissues of the plant of the two genotypes, MD and HD (Fig. 5). Thus, the Na^+/H^+ antiporter acts in concert with the vacuolar H^+-PPase and ATPase to sequester cations in the vacuole and pre-vacuolar compartments. The similar expression patterns of TNHX1 and TVP1 observed in MD and HD suggest that vacuolar compartmentation acts with comparable efficiency in both genotypes. High salinity induction of V-PPase gene expression in roots has been reported for AVP1, HVP1, HVP10, and TsVP (Fukuda et al. 2004; Gao et al. 2006). Vacuolar compartmentation of excess Na^+ would provide a cheap osmoticum for osmoregulation under saline conditions, an osmolarity that could be matched by cytosolic retention of K^+. Indeed, overexpression of the *Arabidopsis* tonoplast Na^+ $(K^+)/H^+$ antiporter AtNHX1 (that would increase Na^+ influx into vacuole) improved Na^+ tolerance without increasing Na^+ content of transgenic wheat plants (Xue et al. 2004). Transcript accumulation of the plasma membrane Na^+/H^+ antiporter, TaSOS1, was lower in roots and leaf sheaths of HD than in MD; whereas, in leaf blades, the expression of TaSOS1 in HD was slightly greater than in MD (Fig. 5).

These expression patterns suggest that besides efficient Na^+ retention in the sheath, the HD variety may avoid Na^+ accumulation in leaf blades by activating sodium efflux through a higher expression of SOS1 in this compartment. Similar results were previously confirmed by Brini et al. (2009). In fact, a correlation was obtained between the expression pattern of TaSOS1 in the roots and sheaths of both durum wheat varieties and the Na^+ fluxes from roots to leaves. However, other recent findings have reported no apparent correlation between leaf Na^+ content and wheat salt tolerance (Genc et al. 2007). Thus, it appears that excluding Na^+ is not itself always sufficient to increase plant salt tolerance, and other physiological traits should also be considered (Benderradji et al. 2011).

5 Conclusion

This study showed that more TaSOS-1 transcripts accumulate in the roots and sheath of MD compared to HD. This type of expression suggests that in addition to better retention efficiency of the Na^+ ion in the sheaths, the HD variety avoids accumulating the Na^+ ion in the leaf blade by activating its efflux via high expression. Of the SOS1 gene in this compartment, HKT1; 5 and HKT2; 2 are expressed in the roots, but not in the leaf sheath and leaf blade, with a better expression of the HKT1 gene; 5, which accumulates at higher levels in the roots of HD. This suggests that these two genes are involved in the transport of Na^+/K^+ ions, through the plasma membrane of the cortical cells of the roots, with, however, a more active role of the HKT1; 5 genes in the HD tolerant variety. The level of expression of TVP1 is comparable to that observed for transcripts of the TNHX1 gene. The roots and sheath of the two genotypes accumulate more TVP1 transcripts than the leaf blade. The similarity in the type of expression of the TNHX1 and TVP1 genes, noted in MD and HD, suggests that vacuolar compartmentalization acts with the same efficiency in the two genotypes. Tolerance to high saline concentrations in bread wheat seems to be related to an ability to avoid the accumulation of toxic levels of Na^+, an enhanced capacity for osmotic adjustment and maintaining adequate levels of K^+, especially in the leaf blade. This information will help select more adapted wheat varieties for future breeding programs.

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