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Developmental acquisition of salt tolerance in the halophyte *Atriplex halimus* L. is related to differential regulation of salt inducible genes

Reham M. Nada · Gaber M. Abogadallah

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Abstract The present study investigated the developmental tolerance of *Atriplex halimus* to osmotic and/or ionic stress. *A. halimus* was exposed to NaCl (0, 100, 250 and 400 mM), KCl (0, 100, 250 and 400 mM) and sorbitol (0, 200, 500 and 800 mM) at the level of germination, seedling emergence and vegetative stages. The response of *A. halimus* to different salts was stage dependent especially to NaCl that had a remarkable effect on *A. halimus* growth at each stage. At the germination stage, the growth reduction could be attributed to osmotic effect and *HRD* may have a role in that osmotic sensitivity. At this stage, the accumulation of Na^+ into vacuole could be a strategy for alleviating the osmotic effect. At the seedling emergence stage, the inhibition of growth could be mainly attributed to the ionic effect that may have resulted from excessive accumulation of Na^+ along with inconsistent regulations of Na^+ manipulating genes. *A. halimus* at the vegetative stage was an obligate halophyte with regulated mechanisms of tolerance to both ionic and osmotic components of salt stress. *A. halimus* exhibits glycophytic features at the early growth stages but it is an obligate halophyte at the vegetative stage.

Keywords *Atriplex halimus* · Germination · Seedling emergence · Vegetative · Osmotic · Ionic

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R. M. Nada (✉) · G. M. Abogadallah
Department of Botany, Faculty of Science, Damietta University, New Damietta 34517, Egypt
e-mail: rnada@du.edu.eg

Introduction

Halophytes are salt tolerant species whose growth is improved at moderate levels of salt (NaCl) concentrations that are sufficient to dramatically reduce the growth of glycophytes. Many dicotyledonous halophytes show an optimal growth in concentrations of 50–200 mM NaCl (Flowers et al. 1986), while monocotyledonous halophytes need just low concentrations (about 50 mM or less) to initiate the growth (Glenn et al. 1997, 1999). Halophytes grown in non-saline environments resort to accumulation of K^+ instead of Na^+ to assure stem succulence (e.g. *Suaeda maritima*, Yeo and Flowers 1986; *Halosarcia pergranulata*, Short and Colmer 1999). However, K^+ (KCl) cannot substitute Na^+ in many halophytes. The vegetative growth of some members of Chenopodiaceae like *S. maritima* (Yeo and Flowers 1986), *Atriplex nummelaria* and *Atriplex inflata* (Ashby and Beadle 1957) was reduced when grown in media containing 340 and 200 mM KCl, respectively. Yeo (1981) attributed that to some halophytes can retain Na^+ but not K^+ into vacuoles for osmotic adjustment.

Halophytes, in contrast to glycophytes, cope with salt stress by initiating different mechanisms (Flowers et al. 1977; Munns 2002; Parida et al. 2004; Turkan and Demiral 2009) depending mainly on controlled uptake, compartmentalization, extrusion and exclusion of salts (Flowers and Colmer 2008). The vacuolar Na^+/H^+ antiporter (NHX1) has been reported to reduce the toxicity of Na^+ by sequestering Na^+ into vacuole under the electrochemical gradient of proton generated by H^+-ATPase and H^+-PPase to regulate the intracellular pH and Na^+ level in cytosol. A plasma membrane salt overly sensitive (SOS1) is responsible for Na^+ exclusion from cytosol in exchange of H^+ generated by plasma membrane H^+-ATPase (Blumwald et al. 2000).

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Another mechanism of salt and drought tolerance is the accumulation of compatible solutes like glycine betaine (GB) that act as osmoprotectants and/or osmolytes (Rhodes and Hanson 1993). In some higher plants, a rate limiting enzyme choline monooxygenase (CMO) converts choline to betaine aldehyde and then to GB by the action of betaine aldehyde dehydrogenase (Hayashi and Murata 1998).

Transcription factors also play a central role in regulating downstream genes contributing to salt tolerance (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2006). Dehydration responsive element binding transcription factor (DREB) plays a key role in regulating downstream stress-induced genes that contain DRE cis-acting element in their promoters (Liu et al. 1998; Knight and Knight 2001). DREB has been reported to be regulated by low temperature, high salt or drought stress (Nakashima and Yamaguchi-Shinozak 2006; Jain and Chattopadhyay 2013). Moreover, DREB has been induced by osmotic not by ionic factor of salt stress (Khedr et al. 2011). Hardy (HRD), another transcription factor that belongs to AP2/ERF, classified as IIIb group (Nakano et al. 2006). HRD has been identified in Arabidopsis by gain of function mutant (Karaba et al. 2007) and its expression was detected in the inflorescence parts of Arabidopsis (Zimmermann et al. 2004). Therefore, its role in protecting these reproductive tissues from desiccation was suggested (Karaba et al. 2007).

Germination is a limiting stage in any plant's life especially in saline environment and it determines whether that plant can grow and survive successfully in such habitat or not (Song et al. 2005). Halophytes as well as non-halophytes respond similarly to salinity at germination stage; the initial germination is delayed under salinity (Keiffer and Ungar 1997; Khan and Ungar 1997). To date, many studies have attributed the inhibition of germination by salt stress to many reasons that may differ from one plant to another. In some plants, the inhibitory effect of salinity on germination could be due to osmotic effect rather than ionic one (Egan et al. 1997; Bajji et al. 2002; Almansouri et al. 2001). However, other studies (Petruzzelli et al. 1992; Poljakoff-Mayber et al. 1994) have attributed the inhibition of germination under salt stress to osmotic and/or specific ion effect. Also K^+ deficiency could inhibit the germination, where K^+ is involved in protein synthesis and in many other metabolic processes (Song et al. 2005).

Salt tolerance is a developmental stage specific phenomenon (Flowers and Colmer 2008). Evaluating salt tolerance during different developmental stages especially in halophytes could lead to understand the different mechanisms involved in salt response and consequently develop a tolerance program for crop species that have a limited range of genetic diversity (Glenn et al. 1999; Orsini et al. 2011; Shabala 2013). Many studies have reviewed the effect of salinity during germination and vegetative growth however, little is known about the different roles of stress inducible genes in stress response during these stages. (Sekmen et al. 2012) have suggested different antioxidant metabolism in *Gypsophila oblanceolata* during germination and vegetative growth in response to salt stress. Compartmentalization of toxic ions (Na^+) and essential ones (K^+) during seed germination in saline environment warranted an investigation on the role of ion transporters in response of seed to salt stress during germination as suggested by (Hariadi et al. 2011).

During the vegetative stage, *A. halimus* shows a high tolerance to salt stress (Bajji et al. 1998; Martinez et al. 2003, 2005; Ben Hassine et al. 2008) but its seeds are inhibited from normal germination by salt stress (Bajji et al. 2002).

Although salt inducible genes have previously been studied in many halophytes and their roles in salt tolerance were evaluated, the regulations of these genes during different developmental stages and also their roles in the stress tolerance during early growth periods were not well documented. Therefore, the objectives of this study were to test the developmental response of *A. halimus* to different kinds of stresses and to investigate the roles of salt manipulating genes in stress tolerance at each developmental stage.

In the present study, three developmental stages of *A. halimus* were exposed to either salt stress (NaCl or KCl) or osmotic stress (sorbitol) after 28 days of germination (vegetative stage, 28-day old seedlings), 7 days of germination (seedling emergence stage, 7-day old seedlings) or during the course of germination (germination stage, 0-day old seedlings). The tolerance range at each stage in response to ionic or osmotic stress was evaluated.

Materials and methods

Plant material

Seeds of *A. halimus* used in this study were collected from Baltim area, Egypt. For all the following treatments, seeds were germinated in growth room at 28/20 °C day/night temperature, 16 h photoperiod, 60 % relative humidity and 300 µmol m^{-2} s^{-1} light intensity.

Three experiments were designed to examine the response of *A. halimus* to osmotic and/or ionic stress during its developmental stages. NaCl and KCl concentrations were adjusted to produce the equivalent osmotic potentials (ψ_s).

Exp. 1: Effect of NaCl, KCl or sorbitol treatment on A. halimus germination

About 300 mg of seeds was germinated in 10 × 25 cm trays containing vermiculite. Seeds were divided into three groups treated with NaCl, KCl or sorbitol in different concentrations. Each group included four sub-groups that were irrigated with Long Ashton nutrient solution (Hewitt 1966) supplemented with 0, 100, 250 and 400 mM NaCl or KCl or with 0, 200, 500 and 800 mM sorbitol. The nutrient solutions were made up to volume everyday to compensate for the evaporation and replaced every 2 days. Three replicates for each treatment were used. Germination percentage was determined as a percentage of the control for each treatment.

Exp. 2: Testing the tolerance range of germination, seedling emergence and vegetative stages to ionic and/or osmotic stress

This experiment was designed to evaluate the response of *A. halimus* at three growth stages to various kinds of stresses at the same time. Three groups of seeds were germinated at three time intervals. The first group will be used for the vegetative stage; seeds of *A. halimus* were germinated in 7 cm pots (10 mg per each pot) containing vermiculite and watered with Long Ashton nutrient solution. After 20 days of sowing, one seedling per pot was kept and the others were removed. After 21 days of germination onset for the first group, another group was germinated as previously mentioned and this group was for the seedling emergence stage. The third group was germinated on the same day of starting the stress and this group was for the germination stage. After 28 days (the vegetative stage), 7 days (the seedlings emergence stage) and 0 days (the germination stage) of seeds sowing, salt or osmotic treatment was started up by irrigated plants with Long Ashton nutrient solutions supplemented with the required amount of NaCl, KCl or sorbitol as previously mentioned in the germination experiment (see above). Plant materials were harvested after 20 days of stress onset. For each treatment, five plants were used to assay growth parameters. Leaves were collected in liquid nitrogen and then frozen in −80 °C until used in further analyses.

Exp. 3: Follow up the growth rate of *A. halimus* under 0 or 100 mM NaCl

Seeds of *A. halimus* were grown in 10 × 25 trays containing vermiculite and irrigated with Long Ashton nutrient solution for 5 days. Two groups of 5-day old seedlings were irrigated with Long Ashton nutrient solution supplemented with 0 or 100 mM NaCl. Seedlings were harvested after 5, 10, 15, 20, 25, 30 days of stress onset to follow up the growth rate and to determine Na^+-dependent age.

Na^+ and K^+ measurements

Na^+ and K^+ were extracted as described by Hansen and Munns (1988). About 100 mg of frozen leaf tissue was homogenized in liquid nitrogen, and then 2 ml of UHP H_2O was added. The samples were heated in water bath at 100 °C for 1 h. after cooling the samples, the residues were removed by centrifugation at 14,000 rpm for 20 min. Then the diluted supernatants (1:10) were used in measuring Na^+ and K^+ by flame photometer (PFP7, Jenway, Essex, UK). For each treatment at each stage, five independent measurements were made from five different extracts.

Glycine betaine measurement

Glycine betaine was extracted and measured according to methods described by Grieve and Grattan (1983). About 0.5 g of frozen leaves was ground in liquid nitrogen and then extracted by a mechanical shaking in 20 ml deionized H_2O for 48 h at 25 °C. The samples were filtrated and then diluted with 2N H_2SO_4 (1:1). About 0.5 ml of aliquots was gently mixed with 0.2 ml of cold potassium iodide–iodine and then vortexed. Consequently, the samples were stored at 4 °C for 16 h and then centrifuged at 1,000 rpm for 15 min at 4 °C. The supernatant was aspirated and kept in cold tubes until the separation of periodite crystals. These crystals were then dissolved in 9 ml of 1,2 dichloro-ethan. The mixture was vigorously vortexed until complete dissolving of crystals. After 2.5 h, the absorbance was measured at 365 nm. GB concentrations were calculated from a standard curve using GB in the range of 50–200 μg ml^{-1}. For each treatment at each stage, five independent measurements were made from five different extracts.

Quantification of genes expressions by semi-quantitative RT-PCR

Total RNA was extracted from about 50 mg frozen leaves using TRI reagent (Sigma, UK) according manufacturer's protocol. To prevent DNA contamination, the extracted RNA was treated with DNA free kit (Ambion, UK) for 30 min at 37 °C. Poly A tail mRNA was then isolated by reacting 10 μg of RNA with 2 μl of Oligo $dT_{(18)}$ and 3 μl of nucleases free H_2O for 5 min at 70 °C and the reaction was terminated on ice for 2 min. The reverse transcription reaction was conducted by using MMLV reverse transcriptase kit according manufacturer's protocol (Promega, UK). Primers for each gene were designed to amplify the most conserved regions resulting from alignment of the characterized genes in other species related to *A. halimus*

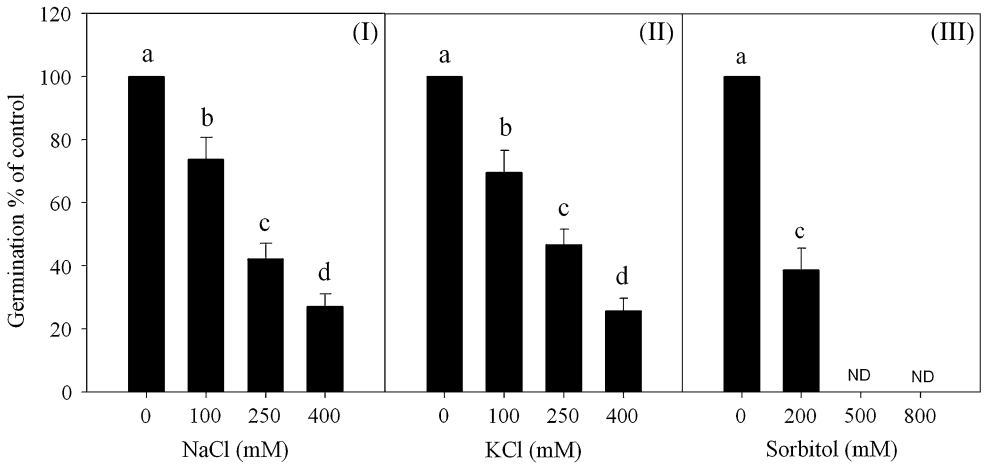

Fig. 1 Effect of NaCl (I), KCl (II) and sorbitol (III) treatments on seed germination. Data is mean plus or minus SE. *Bars* labelled with *different letters* are significantly different at $p \leq 0.05$

from NCBI database. The primers used for amplifying *NHX1*, *H*$^+$*-PPase*, *SOS1*, *CMO*, *DREB*, *HRD* and *18S rRNA* are listed in supplementary data, table S1. The PCR conditions were adjusted as follows: initial denaturation at 94 °C for 3 min followed by 22–30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 50 s. For each gene, the numbers of PCR cycles were optimized to show the maximum differences among samples (quantitative cycle) within the linear phase of amplification as shown in supplementary data, table S1. The conditions and cycle numbers were determined to avoid the DNA saturation. PCR products were resolved on 1 % Agarose gel stained with EtBr in 1× TAE buffer (Tris–Acetic acid-EDTA) using BioRad equipment and visualized by gel documentation system UVi-Tec. The band volumes were measured by using LabImage 2.7.2 software. The measurements were normalized for equal 18S *rRNA*. For each gene, three replicates from different RNA extractions were made.

Statistical analyses

All measurements were replicated as mentioned under each section. To compare among samples, One-Way ANOVA (LSD) was performed using SPSS v 12.0.1 at significant level of $p \leq 0.05$.

Results

Effect of ionic and/or osmotic stress on *A. halimus* germination

Both NaCl and KCl treatments had the same effect on *A. halimus* seed germination, where 100, 250 and 400 mM NaCl or KCl significantly reduced the germination to about 70, 43 and 26 % of the control, respectively (Fig. 1I, II). Osmotic stress with 200 mM sorbitol significantly lowered the germination to about 38 % of the control (Fig. 1III). The germination was completely inhibited at 500 and 800 mM sorbitol.

The growth rate of *A. halimus* in response to ionic and/or osmotic stress at different stages

At the germination stage, iso-osmotic solutions of NaCl and KCl had the same effect on the seedling fresh weights (FWt), where the FWts were significantly decreased at 100, 250 and 400 mM to about 75, 68 and 34 % of the control, respectively (Fig. 2I, II). Osmotic stress with 200 mM sorbitol reduced the FWt to about 31 % of the control. No germinated seedlings were detected at 500 and 800 mM sorbitol (Fig. 2III).

At the seedling emergence stage, 100 mM NaCl or KCl did not change the FWts compared to the control. FWts of 250 and 400 mM NaCl-treated seedlings were reduced to 79 and 22 % of the control, respectively (Fig. 2IV). In contrast to NaCl, 250 mM KCl did not change the FWt of treated-seedlings compared to the control, but 400 mM KCl significantly decreased the FWt to 56 % of the control (Fig. 2V). Osmotic stress with 200 and 500 mM sorbitol significantly lowered the FWts to about 60 and 36 % of the control, respectively. No survived seedlings were detected at 800 mM sorbitol (Fig. 2VI).

At the vegetative stage, the optimum growth was recorded at 100 and 250 mM NaCl (Fig. 2VII). Moreover, 400 mM NaCl did not change the FWt of treated seedlings compared to the control. All levels of KCl did not change the FWt compared to the control (Fig. 2VIII). The FWts of seedlings treated with 200, 500 or 800 mM sorbitol were significantly lowered to 57, 37 and 19 % of the control, respectively (Fig. 2IX).

From this data, the impact of NaCl treatments on *A. halimus* growth was different from one stage to another (either inhibitory or inductive effect). Therefore, the subsequent measurements will be focused on plants treated with NaCl during developmental stages.

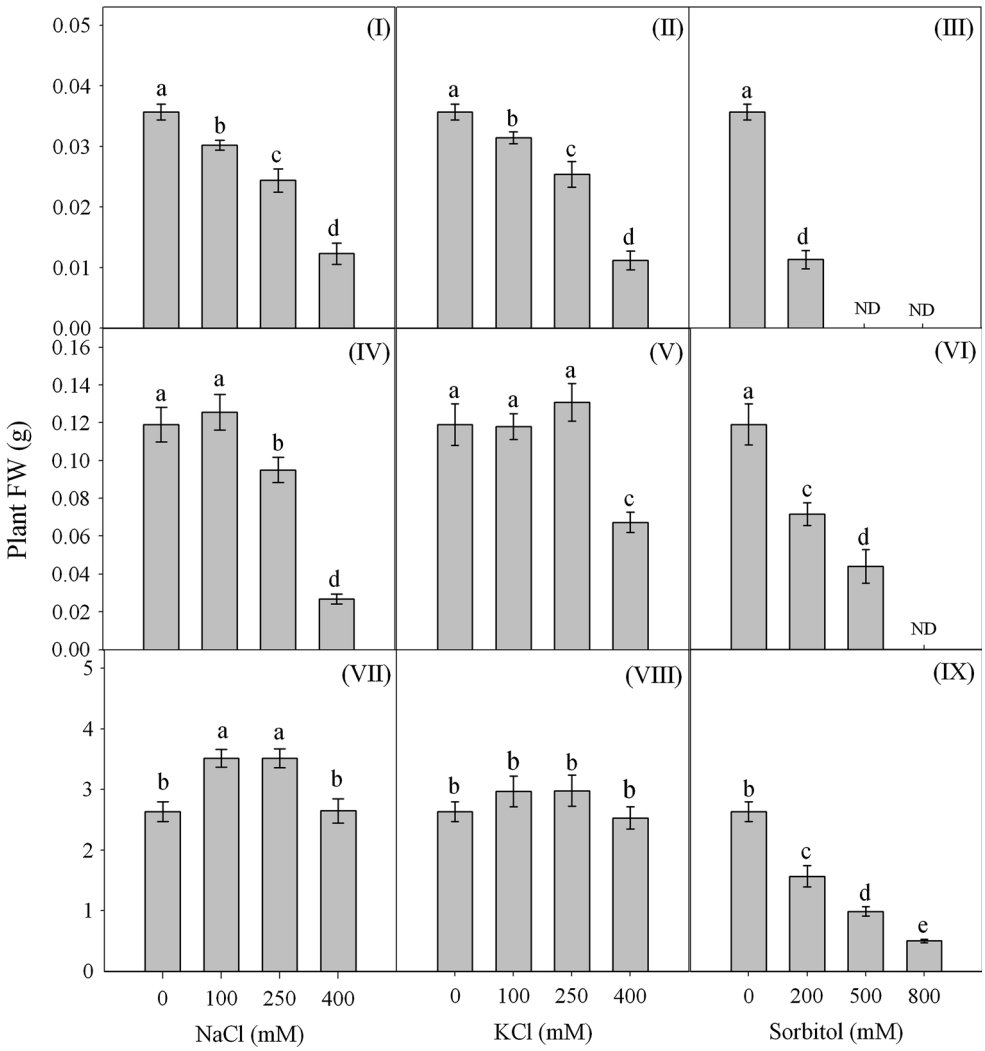

Fig. 2 Effect of NaCl (I, IV and VII), KCl (II, V and VIII) and sorbitol (III, VI and IX) treatments on shoot fresh weights at the germination (I, II and III), the seedling emergence (IV, V and VI) and the vegetative (VII, VIII and IX) stages. Data is mean plus or minus SE. *Bars* labelled with *different letters* are significantly different at $p \leq 0.05$

Na^+ and K^+ accumulations

Leaf Na^+ was progressively accumulated with increasing outer concentrations of NaCl at each stage (Table 1). The highest increase was observed in leaves treated with 400 mM NaCl (3.3-fold increases of the control) at the seedling emergence stage.

In contrast, K^+ content (Table 1) in treated leaves was significantly decreased with increasing external NaCl concentrations. The lowest decreases were detected at the germination and the seedling emergence stages in leaves treated with 400 mM NaCl (about 1.35 and 1.38-fold decreases of the control, respectively).

Glycine betaine accumulation

Table 1 shows that GB content was significantly increased to about 2.2, 2.3 and 1.7 folds of the control inside leaves stressed with 400 mM NaCl at the germination, the seedling emergence and the vegetative stages, respectively.

Expression of *NHX1*

Salt stress with 100, 250 and 400 mM NaCl increased the expression level of *NHX1* to 1.8, 2 and 2.3 folds of the control, respectively at the germination stage. At the seedling emergence stage, the transcript level of *NHX1* was significantly similar to that of the control within leaves treated with 100 mM NaCl, while 250 and 400 mM NaCl significantly induced the transcript levels to be 2 and 2.3 folds higher than that of the control, respectively. At the vegetative stage, the only significant increase was detected in 400 mM NaCl treated-leaves (Fig. 3a, b).

Expression of H^+-*PPase*

The H^+-*PPase* expression level was two folds higher than the control at the germination stage with all NaCl levels. At the seedling emergence stage, the H^+-*PPase* transcript level in stressed leaves was statistically similar to that in non-stressed ones. At the vegetative stage, the transcript

Table 1 Na^+, K^+ and glycine betaine (GB) contents in leaves treated with different concentrations of NaCl

Developmental stages	Parameters									
	Na^+ (mmol/gFWt)				K^+ (mmol/gFWt)				GB (µmol/gFWt)	
	NaCl concentrations				NaCl concentrations				NaCl concentrations	
	0 mM	100 mM	250 mM	400 mM	0 mM	100 mM	250 mM	400 mM	0 mM	400 mM
Germination	293.7 ± 20e	314.9 ± 5de	583.5 ± 30cd	785.1 ± 30b	168.2 ± 18d	60.9 ± 19f	62.9 ± 14f	59.2 ± 10f	21.5 ± 5A	48 ± 10*
Seedling emergence	279.4 ± 1.5e	506.7 ± 20de	698.3 ± 38bc	942.2 ± 10a	175.4 ± 11d	90.8 ± 19e	97.3 ± 7e	68.7 ± 15f	22.4 ± 3A	50 ± 15*
Vegetative	335.6 ± 2e	458.7 ± 11de	617.9 ± 14c	860.7 ± 25b	180. ± 4d	124.8 ± 12de	109.9 ± 22e	110.4 ± 7e	41 ± 4 A	69 ± 13*

Values are mean plus or minus SE. Values labelled with different letters are significantly different at $p \leq 0.05$

* Significantly different at $p \leq 0.05$ from A at each stage

levels were increased by about 1.4 and 1.6 folds of the control at 250 and 400 mM NaCl, respectively (Fig. 4a, b).

Expression of *SOS1*

At the germination stage, the expression of *SOS1* showed slight increases with all NaCl levels (about 1.5 fold of the control). At the seedling emergence stage, the transcript level of *SOS1* was up-regulated to about 2 and 2.7 folds of the control at 250 and 400 mM NaCl, respectively. At the vegetative stage, the highest increase was observed at 400 mM NaCl (about twofold increase of the control) (Fig. 5a, b).

Expression of *CMO*

At all stages, salt stress with 250 and 400 mM NaCl significantly increased the transcript level of *CMO* (Fig. 6a, b). The highest increase was recorded within 400 mM NaCl treated-leaves (2.5 folds of the control) at the vegetative stage.

Expression of *DREB*

DREB expression levels recorded highly significant increases within 250 and 400 mM NaCl-treated leaves at the germination stage (about 2.3 and 3.4 folds of the control, respectively). No further changes in the expression level were observed at the seedling emergence and the vegetative stages (Fig. 7a, b).

Expression of *HRD*

The transcript level of *HRD* was significantly decreased by 2 and 2.3 folds of the control in leaves treated with 250 and 400 mM NaCl, respectively at the germination stage. Thereafter, the transcript level remained statically similar to the control at the seedling emergence and the vegetative stages (Fig. 8a, b).

Follow up the growth rate of *A. halimus* under 0 or 100 mM NaCl

No significant differences were observed between the growth of the control (0 mM) and 100 mM NaCl-treated plants after 5, 10, 15 and 20 days of stress (Fig. 9). However after 25 and 30 days of treatment, the FWt of 100 mM-treated seedlings was significantly higher (by about 1.5 and 2 folds, respectively) than that of the control.

Discussion

Halophytes like *Atriplex* species are unique plants that able to grow and complete their life cycle under salt stress

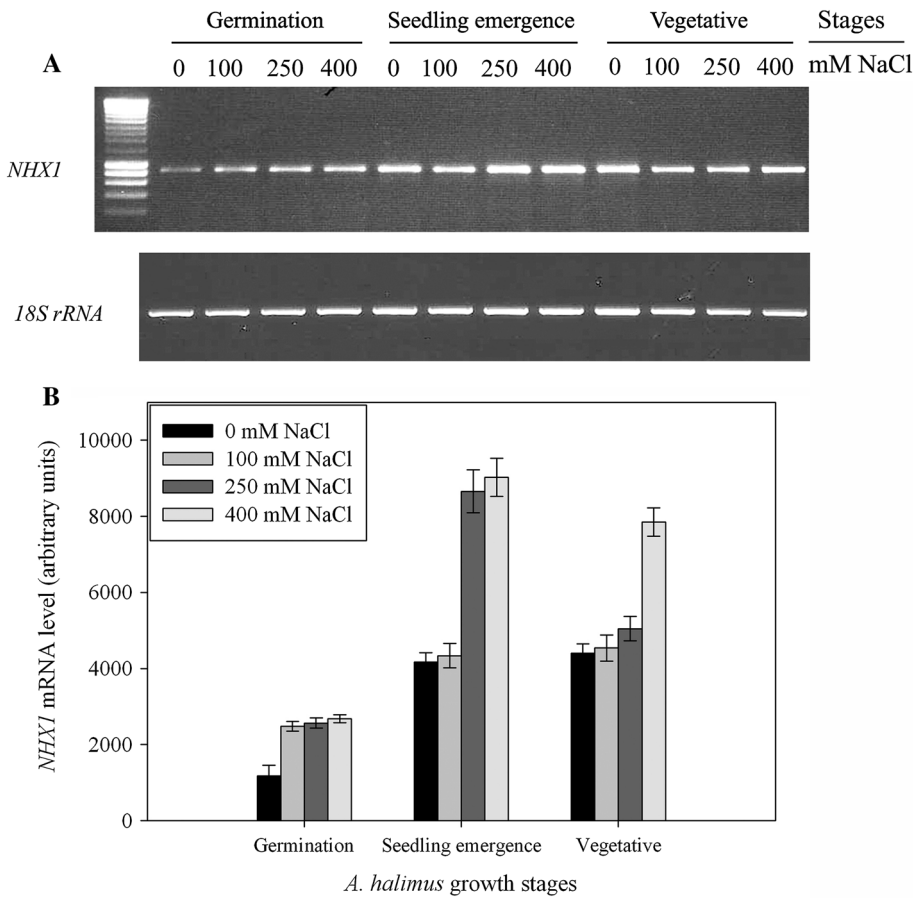

Fig. 3 Developmental response of *NHX1* to NaCl. **A** Semi-quantitative RT-PCR.**B** quantification of mRNA levels normalized with *18S rRNA* as internal control. Data is mean plus or minus SE

(Winicov and Bastola 1997; Volkmar et al. 1998). Moreover, their growth is improved at moderate levels of NaCl (Glenn et al. 1999; Flowers and Colmer 2008). However, seeds of halophytes show an optimal growth in fresh water rather than in saline environment (Boorman 1968; Macke and Ungar 1971; Waisel and Ovadia 1972; Ungar 1982; Khan and Ungar 1984) and showed a reduction in germination by increasing salt concentration in soil (Waisel and Ovadia 1972; Albregts and Howard 1973; Ungar 1982). Therefore, the germination stages of halophytes exhibit less tolerance to salt stress than their vegetative ones (Meyer and Poljakoff-Mayber 1963; Ungar 1995, 1996).

In the present study, the response of *A. halimus* to salt stress (NaCl or KCl) or to osmotic one was evaluated at the level of germination, seedling emergence and vegetative stages. Germination percentages were decreased with increasing salt concentrations of NaCl or KCl (Fig. 1I, II), while sorbitol (500 and 800 mM) totally inhibited the germination (Fig. 1III). These results may indicate that germination of *A. halimus* was more negatively affected by osmotic stress than by salt one. Ions could alleviate the effect of osmotic stress (Song et al. 2005) that is why high concentrations of NaCl or KCl did not completely inhibit the germination but reduced it. Consequently, seedlings germinated in different concentrations of NaCl or KCl showed significant growth reductions (Fig. 2I, II) but they could survive and retain growth in contrast to seedlings germinated in different concentrations of sorbitol (Fig. 2III). Therefore, the osmotic component of salt stress could be the main cause for inhibiting the germination rather than the ionic one. Contrarily, Katembe et al. (1998 and ref. therein) have found that water uptake in germinating seeds has been inhibited by application of NaCl rather than by iso-osmotic solution like PEG.

Exposing *A. halimus* to either salt or osmotic stress at the seedling emergence stage significantly reduced the growth of treated plants compared to the control (Fig. 2IV). Moreover, salt stress with NaCl had a deleterious effect on the growth compared to that with KCl. In contrast to NaCl, 250 mM KCl did not change the growth compared to the control (Fig. 2V) that could be attributed to the importance of K^+ for growth and development of the seedling at this stage. It has been reported that growing tissues of halophytes need K^+ like that of glycophytes (Gorham and Wyn Jones 1983; Flowers et al. 1986). K^+, in optimum concentrations, plays different roles in plant growth; it contributes in

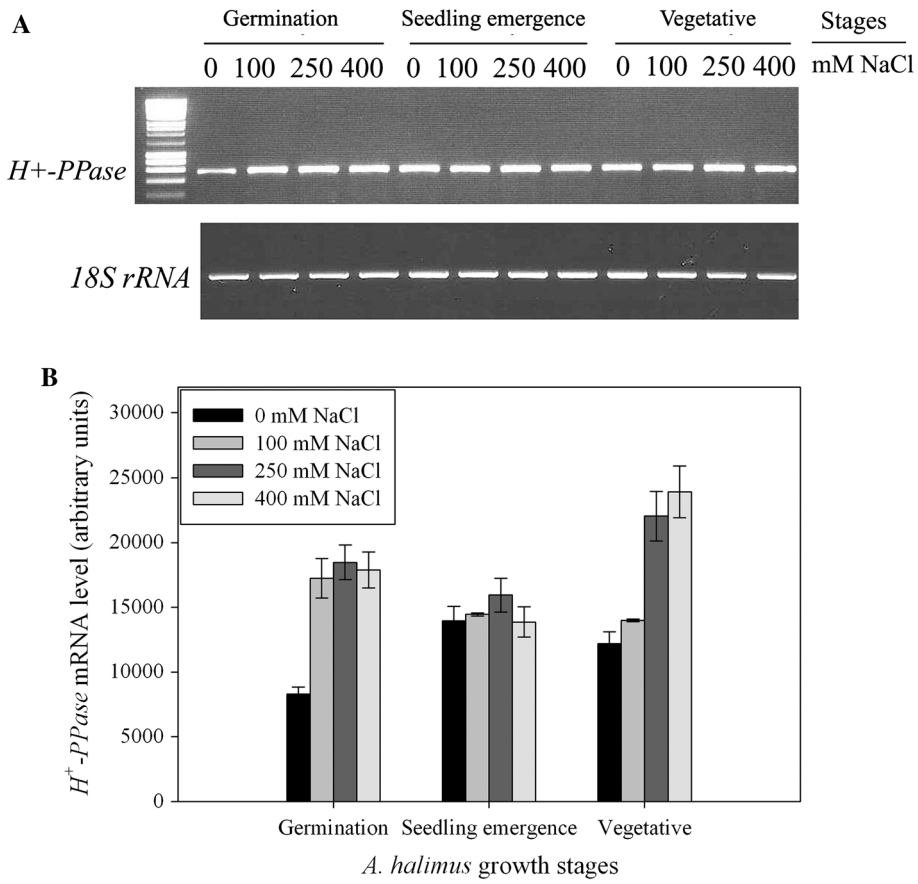

Fig. 4 Developmental response of H^+-PPase to NaCl. **A** Semi-quantitative RT-PCR. **B** quantification of mRNA levels normalized with *18S rRNA* as internal control. Data is mean plus or minus SE

photosynthesis, protein biosynthesis, maintaining turgor and reducing water loss and promoting cell elongation (Rao et al. 2006).

Although 400 mM NaCl and 400 mM KCl reduced the growth compared to the control, the reduction resulted from NaCl was more than that resulted from KCl (Fig. 2IV, V). That reduction might be due to a combination of ionic and osmotic effects but K^+ may alleviate the osmotic effect suggesting that K^+ was a preferable ion over Na^+ for *A. halimus* growth at this stage.

At the vegetative stage, *A. halimus* was an obligate halophyte, its growth was enhanced with moderate concentrations of NaCl (100 and 250 mM), meanwhile KCl at the same concentrations did not change the growth compared to the control (Fig. 2VII, VIII). In contrast to the pervious stage, K^+ cannot substitute Na^+ in many halophytes at the vegetative stage (see "Introduction"). KCl has been reported to cause a poorer water use efficiency compared to NaCl in halophytic species *Suaeda aegyptiaca* (Eshel 1985).

Na^+ and K^+ contents were measured at the three stages. The highest accumulation of sodium ions was detected in leaves treated with NaCl at the seedling emergence stage (Table 1). Therefore, the inhibition of growth at this stage could be mainly attributed to ion toxicity but also the osmotic effect would not be neglected. The lowest reductions in K^+ content were detected at the germination stage with all NaCl treatments (Table 1). The reduction of K^+ content along with the accumulation of Na^+ may inhibit the activities of some enzymes that play critical roles in seed germination and hence reduce the germination rate (Katembe et al. 1998). However, germinating *A. halimus* seeds in KCl did not enhance the growth of the seedlings compared to those germinated in NaCl (Fig. 2I, II). These results would confirm that the growth reduction at the germination stage could be attributed to osmotic effect rather than ionic one and both Na^+ and K^+ had the same effect on seed germination.

Ion sequestration (*NHX1*) and ion extrusion (*SOS1*) are two main mechanisms by which plant can survive and retain growth under salt conditions (Niu et al. 1995; Apse et al. 1999; Zhu 2001). In the present study, sodium manipulating genes showed different regulations at each developmental stage.

At the germination stage, the transcript levels of *NHX1, H^+-PPase* and *SOS1* were significantly increased in seedlings treated with NaCl (Figs. 3, 4, 5, respectively). This indicates that increases in vacuolar Na^+/H^+ antiporter and

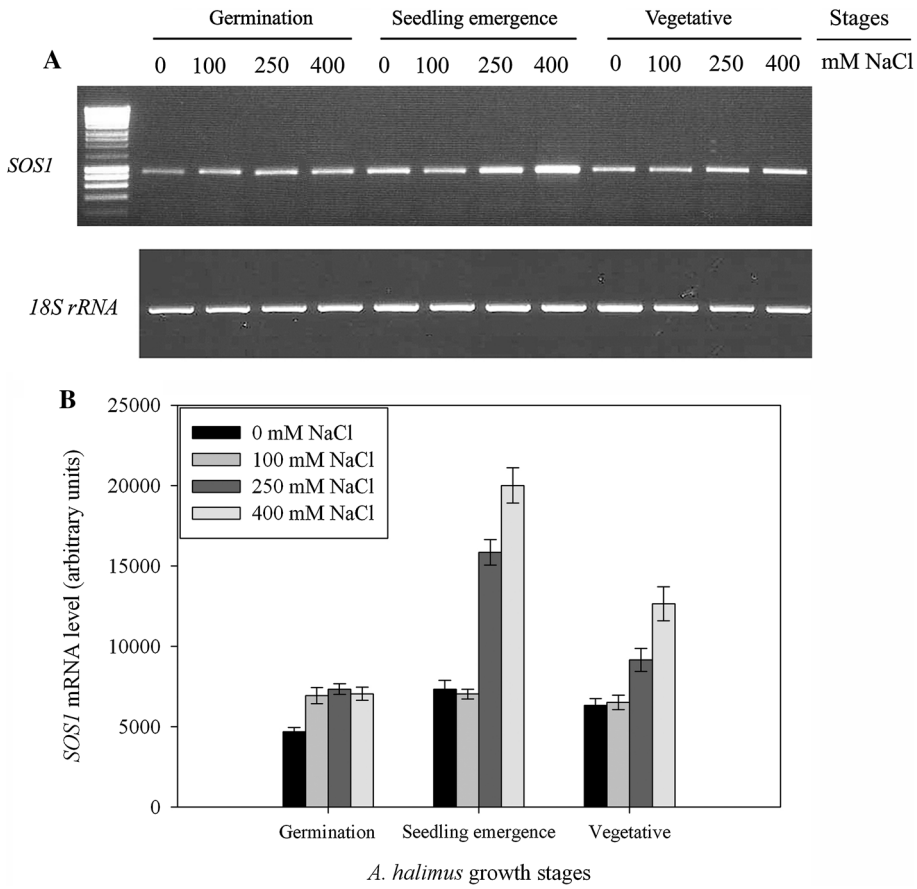

Fig. 5 Developmental response of *SOS1* to NaCl. **A** Semi-quantitative RT-PCR. **B** quantification of mRNA levels normalized with *18S rRNA* as internal control. Data is mean plus or minus SE

H^+ electrochemical gradient generator activities would bring about an increase in the Na^+ sequestering activity into vacuole and an increase in plasma membrane Na^+/H^+ activity would prevent the toxicity of cytosol. These coordinate up-regulations of Na^+ manipulating genes along with controlled accumulation of Na^+ (Table 1) could suggest the ability of plants to tolerate the ionic components at this stage.

At the seedling emergence stage, the transcript levels of *NHX1*and *SOS1*were up-regulated (Figs. 3, 5, respectively), while the mRNA of *H^+-PPase* did not significantly change with all NaCl treatments (Fig. 4). This indicates increases in vacuolar and plasma membrane Na^+/H^+ antiporters activities but not in H^+ electrochemical gradient generating activity. However, the highest accumulation of Na^+ (about 940 mmol/g FWt) was detected at this stage assuming that sequestration and/or extrusion of Na^+ was not sufficient to manipulate ion build-up. Moreover, H^+ electrochemical gradient (H^+-PPase) may be limiting for Na^+ sequestration into vacuole. Overexpression of *AVP1* (*H^+-PPase* of Arabidopsis) has had a significant effect on transgenic Arabidopsis lines, where AVP1 enhanced phosphate proton pump activity, ion vacuole sequestration, K^+-uptake and root hair development (Undurraga et al. 2012). Therefore, the inhibition of growth at this stage could mainly result from the toxicity of Na^+.

At the vegetative stage, the coordinate inductions of *NHX1*, *SOS1* and *H^+-PPase* (Figs. 3, 5, respectively) in addition to the constitutive expression of these genes could explain the ability of *A. halimus* to survive and retain growth at this stage even under high salt concentrations (Khedr et al. 2011) in spite of Na^+ accumulation (Table 1). These coordinate regulations were also detected at the germination stage that would mainly suffer from osmotic components suggesting that the vegetative stage could tolerate the effects of both ionic and osmotic components of salt stress. It has been reported that halophytes have evolved complicated mechanisms to cope with osmotic and ionic components of salt stress (Parida and Das 2005).

H^+-ATPase did not show any significant increase at all developmental stages suggesting that the available amount of protein would be sufficient to cope with the applied stress (supplementary data, Fig. S1).

In addition to sodium sequestration and sodium extrusion, plants tolerate salt stress by setting-up other mechanisms such as producing compatible solutes that act as

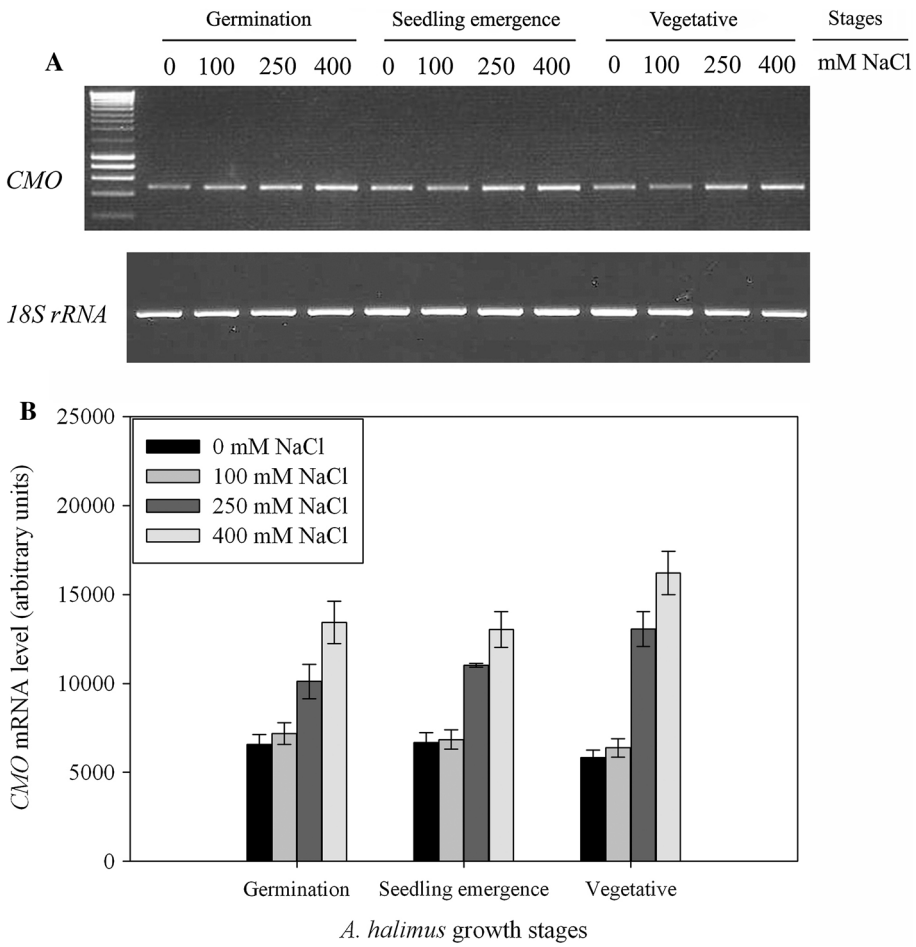

Fig. 6 Developmental response of *CMO* to NaCl. **A** Semi-quantitative RT-PCR. **B** quantification of mRNA levels normalized with *18S rRNA* as internal control. Data is mean plus or minus SE

omolytes and/or osmoprotectants. One of the most important compatible solutes, especially for chenopods, is GB (Rhodes and Hanson 1993). The key enzyme in its pathway is CMO (see "Introduction"). The transcript levels of *CMO* were significantly increased in NaCl-treated leaves at all stages (Fig. 6). The increase in CMO activity coincided with an increase in GB content at all developmental stages (Table 1). These consistent increases in GB content and CMO activity would suggest their vital roles in developmental tolerance of *A. halimus*.

Both *DREB* (Yamaguchi-Shinozaki and Shinozaki 1994) and *HRD* (Karaba et al. 2007) belong to AP2/EREB super family. This family plays a key role in regulating downstream stress responsive genes that contain DRE cis-regulatory element in their promoters (Liu et al. 1998; Knight and Knight 2001). *HRD* has been reported to enhance the water use efficiency of transgenic *Oryza sativa* and *Trifolium alexandrinum* by reducing the transpiration and enhancing the photosynthesis (Karaba et al. 2007 and Abogadallah et al. 2011, respectively).

In the current study, both *DREB* and *HRD* transcript levels were measured to evaluate their roles in *A. halimus* response to NaCl stress at different stages. The change of their expression was observed only at the germination stage. *DREB* transcript level was significantly increased in leaves treated with 250 and 400 mM NaCl (Fig. 7). It has been reported that *DREB* was induced by osmotic component rather than ionic one in many plant species (Khedr et al. 2011 and references therein). This expression pattern supports that the germination stage would be affected by osmotic rather than ionic factor of salt stress. Contrarily, *HRD* transcript level showed significant decreases with increasing external NaCl concentrations (Fig. 8). *HRD* is expressed in inflorescence tissue including petals, inflorescence stem, pollen and mature seeds (Zimmermann et al. 2004). This gene could play a role in the maturation of inflorescence stage that requires protection of this tissue including seeds from desiccation (Karaba et al. 2007). The expression of *HRD* in *A. halimus* seeds may play the same role but the down regulation of its transcript level may be a

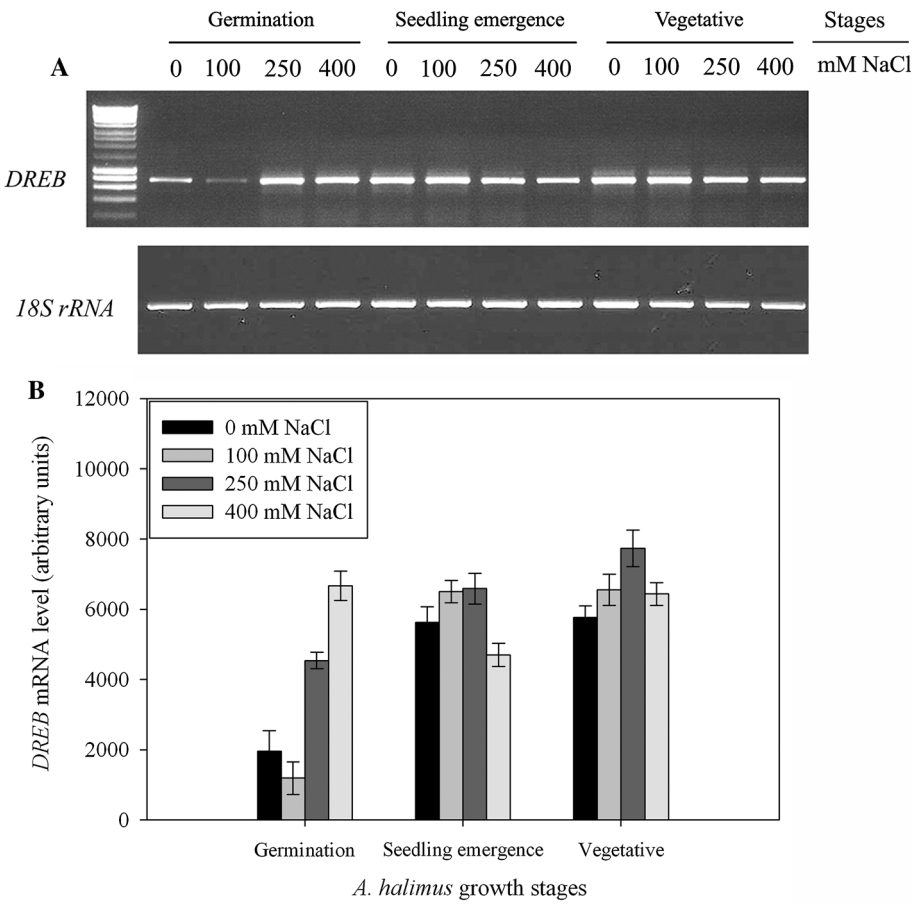

Fig. 7 Developmental response of *DREB* to NaCl. **A** Semi-quantitative RT-PCR. **B** quantification of mRNA levels normalized with *18S rRNA* as internal control. Data is mean plus or minus SE

reason for reducing the germination rate. The down regulation of *HRD* may result in suppressing downstream genes that are involved in protecting seeds from the osmotic stress. The lack of stable regulations between *DREB* and *HRD* may lead to inconsistent regulations of stress inducible genes and consequently lowering or inhibiting seed germination under saline conditions, which may explain the sensitivity of *A. halimus* to salt stress at this vital stage.

From the previous data, all the examined genes were constitutively expressed at all stages. The transcript levels of these genes within the controls (unstressed leaves, 0 mM NaCl treatment) showed different regulations that were gene dependent. On one side, the transcript level of *NHX1, H^+-PPase, SOS1* and *DREB* were significantly higher at the seedling emergence and the vegetative stages than that at the germination stage (Figs. 3, 4, 7). On the other side, *HRD* transcript level at the seedling emergence and the vegetative stages was significantly lower than that at the germination stage (Fig. 8). The transcript level of *CMO* did not show any difference among the three controls (Figs. 5, 6, respectively). Therefore, the regulation of genes could be a stage dependent and that may suggest different mechanisms control the stress tolerance at each stage.

Follow-up the growth rate of 5-day old seedlings exposed to either 0 or 100 mM NaCl showed that NaCl enhanced the growth of seedlings at the age of 30 days and below this age no significant change was detected (Fig. 9). Taken together, the previous data shows that *A. halimus*—at the vegetative stage—has an ability to control the mechanisms involved in salt tolerance to survive and retain growth even under high salt concentrations. Moreover, low to moderate concentrations of NaCl significantly enhance its growth. Alternatively, the most sensitive stage to NaCl stress seems to be the seedling emergence stage (see above) according to our data.

In conclusion, the response of *A. halimus* to osmotic and salt stresses was stage dependent. At the germination stage, the osmotic component could be the main reason for growth reduction, while the growth at the seedling emergence stage could be mainly affected by the ionic component. At the vegetative stage, Na^+ in moderate concentrations enhanced the growth of the plant compared to the control. The early growth stages of *A. halimus* were

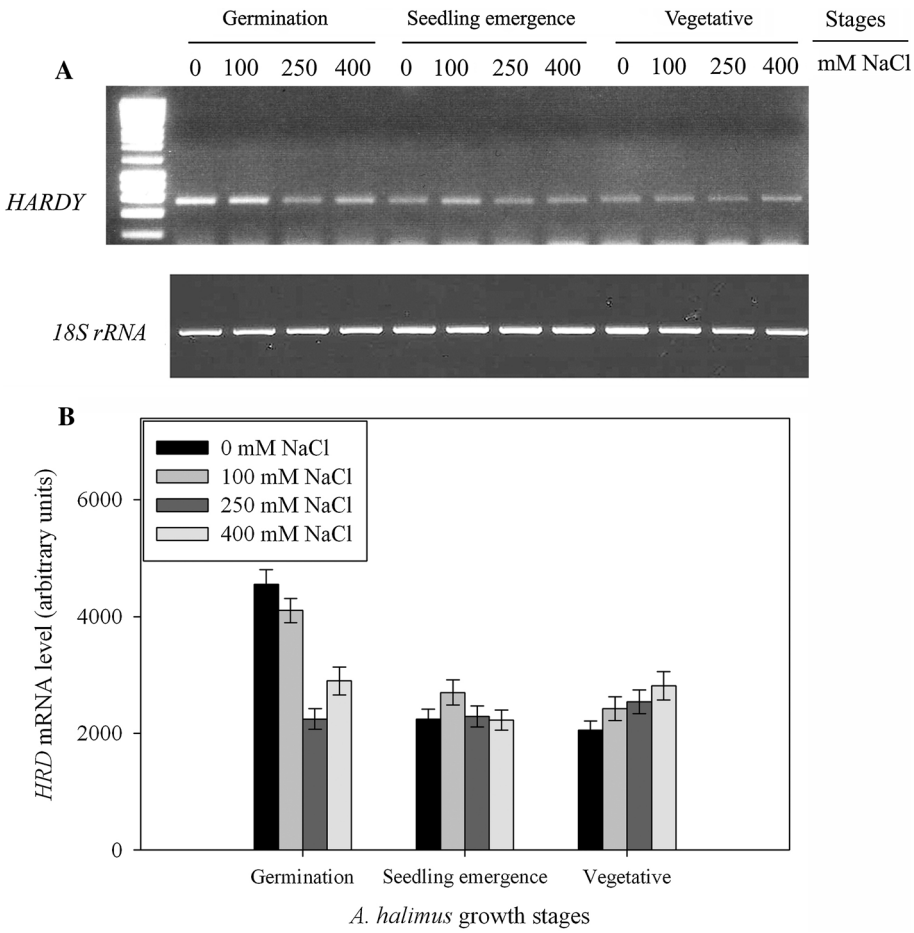

Fig. 8 Developmental response of *HARDY* to NaCl. **A** Semi-quantitative RT-PCR. **B** quantification of mRNA levels normalized with *18S rRNA* as internal control. Data is mean plus or minus SE

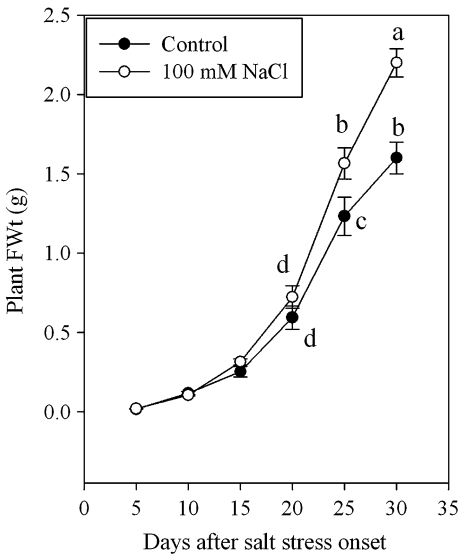

Fig. 9 Follow-up the growth rate of 5-day old seedlings under 0 or 100 mM NaCl. Data is mean plus or minus SE. Values labelled with different letters are significantly different at $p \leq 0.05$

sensitive to salt components and exhibited glycophytic features, while it was an obligate halophyte at its vegetative stage.

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