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ORIGINAL PAPER

Comparison of salt tolerance between *Cichorium intybus* L. transformed with *AtNHX1* or *HvBADH1*

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Abstract Drought and salinity are two major limiting factors of plant growth and agricultural productivity. An efficient way to improve salt tolerance of plants is by expression of the Na^+/H^+ antiporter gene *AtNHX1* from *Arabidopsis thaliana*, introduced via genetic transformation. Similarly, expression of the coding genes of betaine aldehyde dehydrogenase (BADH) cloned from *Gramineaceous* or *Chenopodiaceous* weeds appears to increase salt tolerance of plants. Unlike *AtNHX1* gene, the transformation of *BADH* genes additionally enhances osmotic stress tolerance to the transformants. To better understand the differences of their capacities in promoting plant salt tolerance, the *HvBADH1* gene from Hulless barley and the *AtNHX1* gene, were introduced into a glycophyte species *Cichorium intybus* L. We investigated the traits of osmotic adjustment and antioxidation ability in the transformed plants under salt stress condition. The results indicated that both *AtNHX1* and *HvBADH1*-transformed plants showed similar Na^+ and K^+ accumulations, but *HvBADH1*-transformed plants exhibited better osmotic adjustments to salt stress. And the *AtNHX1* overexpression lines exhibited superior membrane protection and relative calli growth, delivering better NaCl tolerance to the plants under conditions of severe salt stress.

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Introduction

Salinity is one of the major abiotic stresses negatively affecting plant growth and reducing agricultural productivity all over the world. Environmental salt stress can cause severe problems such as ion toxicity, water deficit and oxidative stress, resulting in cellular damage, growth reduction, and even plant death (Li et al. 2013). Higher plants have developed a set of biochemical and molecular mechanisms to deal with salt stress by selectively expressing specific stress-responding genes and accumulating compatible organic solutes such as soluble sugars, free proline and glycine betaine (GB) (Türkan and Demiral 2009).

AtNHX1, a vacuolar Na^+/H^+ antiporter in *Arabidopsis thaliana*, has been found to influence plant development and contribute to salt tolerance by mediating the transport of Na^+ and K^+ into the vacuoles (Sottosanto et al. 2007). Overexpression of *AtNHX1* in many plants observably improves their salt tolerance. Compared to wild types (WT), transgenic tomato seedlings harboring *AtNHX1* were able to grow, flower and even produce fruit under the salt stress triggered by 200 mM NaCl (Zhang and Blumwald 2001). In transgenic plants, the overexpression of *AtNHX1* also significantly enhanced the salt tolerance of transformants (Asif et al. 2011; Chen et al. 2007). The study of transgenic Buckwheat showed that transformants overexpressing *AtNHX1* were not only able to grow and flower normally, but also able to accumulate more rutin than WT in the presence of 200 mM NaCl (Chen et al. 2008). While stressed by 200 mM NaCl, transgenic

tobacco lines expressing the *AtNHX1* gene were able to grow and significantly accumulate sodium ions in their leaves (Zhou et al. 2011). The traits of enhanced salt tolerance could propagate for more than six generations in soybean lines that overexpressed *AtNHX1* (Xing et al. 2010).

Betaine aldehyde dehydrogenase (BADH) is one of the key enzymes involved in the biosynthesis of GB, a compatible organic solute synthesized in response to salt, drought and temperature stresses in a large number of plant species (Wang et al. 2013). Responding to salt and drought stresses, the endogenous BADH level is upregulated several-fold in plant species which could synthesize betaine naturally (Fan et al. 2012). Transformed by the BADH genes, many betaine-deficient plants have been confirmed to accumulate different levels of GB in cells, and shown promoted tolerance to either salt or drought stress (Singh et al. 2010).

Because of its very high altitude, the extreme environment of the Tibetan Plateau in China is believed to be one of the harshest conditions for plant growth on this planet. Tibet is also considered to be the original habitat for cultivated barley and many wild Barleys (Wang et al. 2009). Tibetan Hulless barley (*Hordeum vulgare* var.nudum.hook.f.), the most important crop in the residents' diets (Thomason et al. 2009), differs from the regular hulled barley with a bared caryopsis not covered by hulls in morphology. Unlike the cultivated barley, Tibetan Hulless barley has evolved a general resistance system to deal with all kinds of environmental stresses, and shown the strongest resistance of all plants belonging to genus Hordeum. However, the mechanism of this stress defense system is still unknown.

We have first reported the cloning of a pivotal-resistant gene *HvBADH1* (GenBank No. EF492983), which encoded an isoenzyme of BADH in Tibetan Hulless barley (Zhao et al. 2008). To test the ability of *HvBADH1* in plant stress resistance, we overexpressed *HvBADH1* gene in non-halophyte chicory (*Cichorium intybus* L.) by the method of *Agrobacterium tumefaciens*-mediated transformation, and several transformed lines with enhanced salt stress resistance were obtained (data unpublished). To make this salt stress improvement of transformants sensible, we compared the salt tolerance of *HvBADH1* or *AtNHX1* overexpression chicory (Zhao et al. 2009) by assessing the different responses of these plant lines to gradient NaCl stresses. To achieve this purpose, we measured Na^+ and K^+, malondialdehyde (MDA), soluble sugar, free proline, chlorophyll contents and the relative electrical conductivity (REC) in T_2 generation seedlings, as well as the relative growth rate of calli in different transgenic lines and WT under 0–200 mM NaCl.

Materials and methods

Plant materials and growth conditions

WT and T_2 generation transgenic plants of chicory containing *AtNHX1* or *HvBADH1* were grown in a greenhouse of Shaanxi Provincial Key Laboratory of Biotechnology, Shaanxi Province, China. The seeds of these plant lines were harvested, soaked in tap water for 16 h, and surface sterilized with 70 % ethanol for 30 s and 0.1 % $HgCl_2$ for 10 min. After rinsing for three times with sterile water, the seeds were placed on MS agar medium, and incubated at 25 °C under a 16/8 h L/D photoperiod (about 100 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps) for about 14 days until the cotyledons fully expanded.

The construction of plant expression vectors harboring *AtNHX1* or *HvBADH1*

Seeds of *Arabidopsis thaliana* and Hulless barley were surface sterilized using 0.1 % $HgCl_2$, and then germinated on water soaked filter paper for 7 days. The seedlings were treated with 200 mM NaCl for 24 h at room temperature, and the leaves of *Arabidopsis thaliana* or Hulless barley were frozen in liquid nitrogen and preserved at −70 °C. Total RNA was isolated from *Arabidopsis thaliana* and Hulless barley seedlings using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. The first strand cDNA was synthesized using a RevertAid H Minus Synthesis kit K1631 (Fermentas). The cDNA was then used as template DNA for PCR amplification of the *AtNHX1* (AF056190, 1,617 bp) and *HvBADH1* (EF492983, 1,512 bp) ORFs. The sequences of the primers for *AtNHX1* amplification were 5′-ggAATTCCATATgTTggATTC TCTAgTgTCgAAAC-3′ and 5′-CgggATCCTCAAgCCT-TACTAAgATCAggAggg-3′. The primers for *HvBADH1* were 5′-ggAATTCCATATggTCgCgCCggCCAAgATCC-3′ and 5′-CgggATCCCTAgTTAgCCggAgCCTTgTACCAC-3′. PCR was performed using *Pyrobest* DNA Polymerase (Takara, Dalian China). The PCR products corresponding to *AtNHX1* and *HvBADH1* were extracted from agarose gel, and then sequenced. All sequences were consistent with previously reported gene sequences of *AtNHX1* and *HvBADH1* (Zhao et al. 2008, 2009).

The amplified ORFs of *AtNHX1* and *HvBADH1* were then constructed into the multiple cloning sites of a commercial binary vector pRI101-AN using NdeI and BamHI restriction sites (Takara, Dalian China). In the final plasmid constructs, the target ORFs are under the control of the CaMV35S promoter (Fig. 1). These new plasmids, pRI101-AN-CaMV35S-*AtNHX1* and pRI101-AN-CaMV35S-*HvBADH1,* were then separately introduced

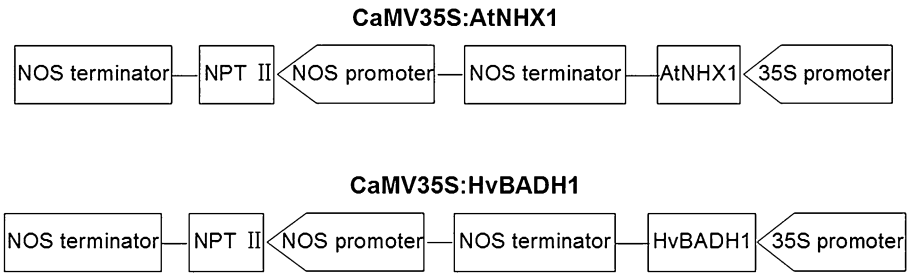

Fig. 1 Structure of the plant expression vectors, pRI101-AN-CaMV35S-*AtNHX1* and pRI101-AN-CaMV35S-*HvBADH1*

into *Agrobacterium tumefaciens* LBA4404 by electroporation using a Multiporator (Eppendorf), according to the manufacturer's instructions. The subsequent *Agrobacterium tumefaciens*-mediated plant transformation and transformant identification were performed as described previously (Zhao et al. 2008).

Determination of Na^+ and K^+ contents in transgenic chicory

The transgenic *HvBADH1* (H1, H2) and *AtNHX1* (A1, A2) lines and WT chicory seedlings were cultured on MS medium for 4 weeks after germination and then transferred to MS liquid medium containing different concentrations of NaCl (0, 50, 100, 150 or 200 mM) for 24 h. The seedlings were rinsed with distilled water to remove NaCl from the surface, and the leaves of these seedlings were harvested and dried at 45 °C overnight. Na^+ and K^+ contents were measured by an atomic absorption spectrophotometer (AA-6300C from Shimadzu).

Measurement of MDA, free proline, soluble sugar and chlorophyll contents in transgenic chicory leaves

Transgenic lines and WT chicory seedlings were cultured on filter paper wetted with MS liquid medium for 28 days. Then all the plants were irrigated with MS liquid medium containing different concentrations of NaCl (0, 50, 100, 150 or 200 mM) for 24 h. 0.1 g fresh leaves from seedlings of transgenic lines and WT were used as materials for the following tests of each physiological indicator. The MDA levels were measured by a thiobarbituric acid (TBA) reaction method (Garcia et al. 2005), free proline levels were determined as described by Bates et al. (1973), and soluble sugar was measured by anthrone colorimetry as described by Yemm and Willis (1954). The same leaf samples were homogenized in a mortar and pestle containing 80 % acetone and the amount of chlorophyll was measured as the value of OD_{652} as described by Myers et al. (2013).

Determination of relative electrical conductivity

To assess membrane permeability, REC was determined according to Korkmaz et al. (2010). After being treated with NaCl as described in 2.3, leaf discs (1 cm in diameter) from transgenic lines and WT lines were taken from the middle portion of the fully developed youngest leaf. Then the discs were placed in individual test tubes containing 30 ml of distilled water. After incubating the samples at room temperature on a shaker (150 rpm) for 3 h, the electrical conductivity of the bathing solution (EC1) was determined by an electrical conductivity detector (model DDS-303A, Kangyi, Shanghai, China). The same samples were then placed in boiling water for 30 min and a second reading (EC2) was determined after cooling the solution to room temperature. The electrolyte leakage was calculated as REC = EC1/EC2 × 100 %.

Salt tolerance of transgenic calli

The hypocotyls of different transgenic lines and WT seedlings about 28 days old were cut into 0.5 cm segments and inoculated on MS medium supplemented with 6-BA 1.5 mg L^{-1}, NAA 0.2 mg L^{-1}, and ascorbic acid 100 mg L^{-1} for calli induction under 100 μmol m^{-2} s^{-1} illumination, 16/8 h L/D photoperiod and 25 ± 2 °C. The fresh calli were segregated and subcultured on the same medium for 6 weeks. After weighing, the calli were treated with gradient NaCl for 28 days. Subsequently, the calli's weight was remeasured, and the relative growth rates of calli were calculated as: Relative growth rate = final weight/primary weight. 25 calli were treated in each replicate and 3 replicates were performed for each experiment.

Determination of GB

To prepare sample solutions for GB measurement, 1.0 g fresh leaves were ground by mortar and pestle and extracted in 20 mL deionized water for 24 h at 37 °C with gentle shaking. These extracts were then centrifuged at

4,500×*g* for 15 min, and the supernatants were filtrated with waterman filter paper and 0.45 μm filter membrane. The pH of the sample solutions was adjusted to 1.0 by adding concentrated hydrochloric acid and incubated at 4 °C for 30 min. After centrifugation at 4,500×*g* for 15 min, 3 mL supernatants from sample solutions were mixed with 5 mL concentrated Reinecke's salt ($NH_4[-Cr(NH_3)_2(CNS)_4]\cdot H_2O$; China National Pharmaceutical Group Corporation), and reacted at 4 °C for 3 h. Subsequently, the solutions were centrifuged again and the supernatant was discarded. The GB crystals were dissolved in 5 mL 70 % acetone and the optical density of these solutions was measured at 365 nm.

The preparation of a standard curve was performed using GB samples from Sigma with the unit as mg/g FW (Martinez 1983).

Statistical analysis

To analyze the variance of the physiological indicators between transgenic plant strains, we used an ANOVA method of Duncan's multiple comparison procedure. In addition, paired sample *t* tests were used to evaluate the statistical significance of the difference between each transgenic line and the WT at each NaCl level. The significant levels were defined as * when $P \leq 0.05$, and ** while $P \leq 0.01$. Six plants were used for each treatment. Each experiment was repeated three times. All the data used in the statistical analysis were an average of three replicates and analyzed by the SPSS statistical software package v19.0.

Results

Determination of Na^+ and K^+ contents

The accumulation of Na^+ and K^+ in WT and T_2 generation transgenic seedlings treated with NaCl was measured. The results suggested that together with the increasing environmental NaCl, the Na^+ contents increased in leaves of all the transgenic and WT plants. Though transgenic plants could accumulate much more Na^+ than the WT at every stress levels, no observable differences were found between CaMV35S:*HvBADH1* and CaMV35S:*AtNHX1* lines (Fig. 2a).

Corresponding to increased NaCl stresses from 0 to 200 mM, the K^+ contents in the transgenic and WT plants rose rapidly. Similar to the situation of Na^+ contents, significant differences of K^+ content were found between transgenic and WT plants at each level of salt stress, but there were no noticeable differences between transgenic lines (Fig. 2b). In addition, the K^+/Na^+ ratios in transgenic plants were also higher than those of WT at each stress level, but the differences between transformed *HvBADH1* and *AtNHX1* lines were not significant (Table 1).

Measurement of MDA contents

MDA is the product of lipid peroxidation and relates closely to the oxidative degradation of polyunsaturated fatty acids of cell membrane. Changes in cellular MDA levels during abiotic stress are coordinated with the process of membrane damage and reflect the antioxidative capacity of plant cells (Tian et al. 2011). In our study, the contents of MDA increased dramatically in all the tested plants corresponding to the increase of environmental NaCl levels. Under each stress level, the MDA contents of transgenic lines were lower than the WT, but no noticeable differences were observed between CaMV35S:*HvBADH1* and CaMV35S:*AtNHX1* lines when stressed with 50–150 mM NaCl. However, the CaMV35S:*AtNHX1* lines showed a significant higher accumulation of MDA than *HvBADH1* lines until the NaCl stress reached 200 mM (Fig. 3a).

Determination of free proline contents

As a physiological response to hostile environment, plant cells usually produce abundant free proline to raise their cellular osmotic potential. Triggered by NaCl stress, the contents of free proline in leaves of transgenic and WT plants increased rapidly, and the proline levels of both transgenic lines were higher than the WT at each stress level. In addition, CaMV35S:*HvBADH1* lines displayed a significantly higher ability to accumulate more free proline than the CaMV35S:*AtNHX1* lines at all the tested stress levels (Fig. 3b).

Determination of soluble sugar contents

The young leaves of transgenic and WT plants grown under different NaCl stresses were used to measure soluble sugar contents. When no additional NaCl was supplied, all the plants contained nearly the same soluble sugar contents. Similar to the MDA and proline measurements, soluble sugar contents also increased with NaCl concentration in both transgenic lines. The soluble sugar content of CaMV35S:*HvBADH1* leaves showed 1.67-fold higher values than that of CaMV35S:*AtNHX1* when exposed to 150 mM NaCl and 1.47-fold to 200 mM NaCl (Fig. 3c).

Measurement of chlorophyll contents

To estimate the damage on the photosynthetic system caused by the salinity stress, the amounts of chlorophyll content were measured in transgenic lines and WT treated with different concentrations of NaCl. With increased salt,

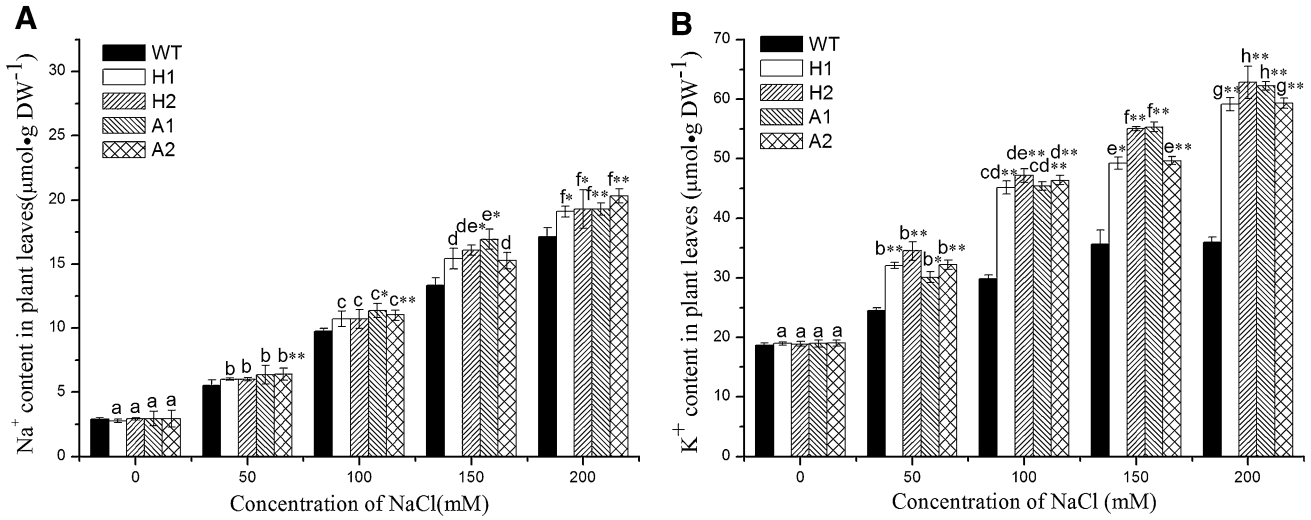

Fig. 2 Na^+ and K^+ contents in leaves of transgenic and WT chicory. *WT* wild type; *H1*, *H2* transgenic *HvBADH1* plant No. 1 and No. 2; *A1*, *A2*: transgenic *AtNHX1* plant No. 1 and No. 2. These *symbols* present the same plant lines in all the figures and table in this paper

Table 1 Effects of NaCl stress on the K^+/Na^+ in the leaves of chicory

Concentration of NaCl (mM)	K^+/Na^+ in the leaves of chicory				
	WT	H1	H2	A1	A2
0	6.44 ± 0.10	6.83 ± 0.23^{fg}	6.45 ± 0.08^{f}	6.68 ± 0.21^{f}	7.17 ± 0.19^{g**}
50	4.44 ± 0.26	5.32 ± 0.04^{d*}	5.72 ± 0.14^{e**}	4.76 ± 0.40^{cd}	5.04 ± 0.26^{d}
100	3.06 ± 0.02	4.22 ± 0.14^{b**}	4.41 ± 0.20^{bc**}	4.00 ± 0.14^{b**}	4.21 ± 0.09^{b**}
150	2.66 ± 0.07	3.20 ± 0.10^{a**}	3.42 ± 0.07^{a**}	3.27 ± 0.10^{a**}	3.26 ± 0.10^{a*}
200	2.11 ± 0.04	3.10 ± 0.01^{a**}	3.26 ± 0.14^{a**}	3.22 ± 0.04^{a**}	2.92 ± 0.06^{a**}

Means of 3 replicates ± SD

WT wild-type chicory, *H1* transgenic *HvBADH1* plant No. 1, *H2* transgenic *HvBADH1* plant No. 2, *A1* transgenic *AtNHX1* plant No. 1, *A2* transgenic *AtNHX1* plant No. 2

* Presents significantly different at $P \leq 0.05$, ** presents significantly different at $P \leq 0.01$, according to the paired sample *t* test. Those with different letters are significantly different according to One-way ANOVA test at $P \leq 0.01$

the chlorophyll contents decreased in all tested plants. Not surprisingly, more dramatic decline in chlorophyll content was found in WT leaves compared to both transgenic lines under each stress level. Contents of chlorophyll in CaMV35S:*HvBADH1* lines were slightly higher than that of *AtNHX1* transformants when environmental NaCl level was lower than 150 mM, but significant differences were observed between these two lines at NaCl concentrations of 200 mM (Fig. 3d).

Determination of relative electrical conductivity

For the purpose of evaluating the protective function of *HvBADH1* and *AtNHX1* on the cell membrane under osmotic stress, REC was measured in two transgenic lines and WT. Without salt stress, the values of REC in all tested plants were similar. Under conditions of 50–200 mM NaCl, however, the REC data from each plant strain showed dramatic differences. The values of REC in all plant strains increased with rising NaCl concentration, and the level of REC in WT was always significantly higher than that of transgenic lines. Again no significant differences in REC were detected between the CaMV35S:*AtNHX1* and CaMV35S:*HvBADH1* lines when stressed by the 50–150 mM NaCl. However, the REC of CaMV35S:*HvBADH1* plants was much higher than the CaMV35S:*AtNHX1* plants under 200 mM of NaCl stress (Fig. 4a).

Salt tolerance of transgenic calli

The relative growth rates of transgenic and WT calli were measured to evaluate the salt tolerance of different transgenic lines at the cellular level. In the control group (without NaCl), the relative growth rates of transgenic calli were

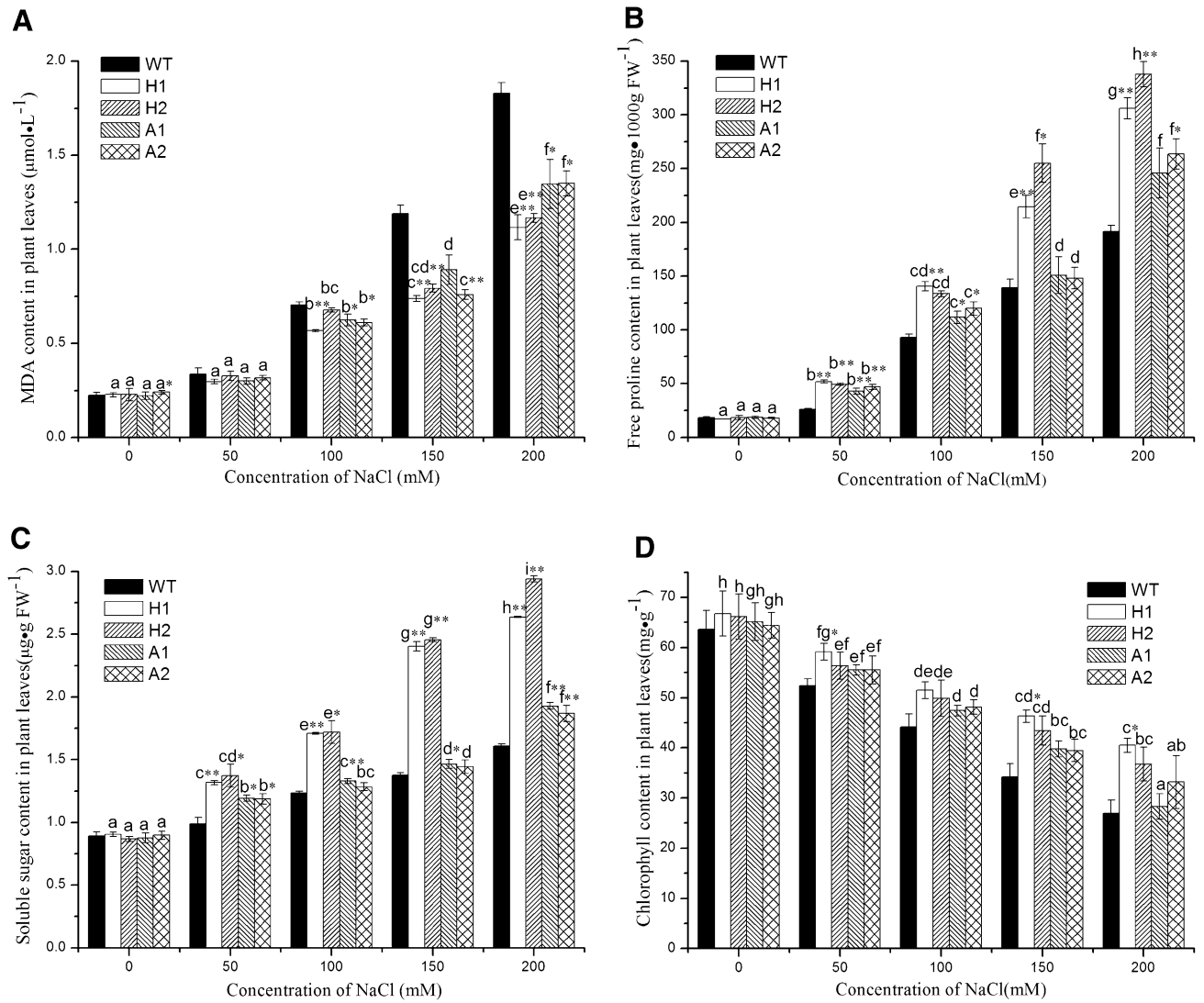

Fig. 3 MDA (**a**), free proline (**b**), soluble sugar (**c**) and chlorophyll (**d**) in transgenic and WT chicory

similar to WT. With increased NaCl concentration, relative growth rates declined rapidly in all the tested calli lines, though that of the transgenic lines was significantly higher than WT at each stressed level. By comparing the data from different transgenic calli grown under 100–200 mM NaCl, we confirmed that overexpression of *AtNHX1* could promote the survival rates of calli to a greater extent than the *HvBADH1* gene. At 200 mM of salt stress, the WT calli turned brown and only kept 2.97 % growth rates whereas most of the transgenic calli survived and even maintained growth rates of 10.7–22.3 % (Fig. 4b).

Determination of GB

The accumulation of GB in WT and T_2 generation transgenic seedlings was measured. Compared to the values of WT and CaMV35S:*AtNHX1* lines under regular growth conditions, a 6.77-fold higher GB content was observed in *HvBADH1* lines, but no noticeable accumulation of GB was found in WT or CaMV35S:*AtNHX1* lines (Fig. 5). Growing under NaCl-stressed condition, *HvBADH1* lines, as well as the WT and *AtNHX1* lines, showed the same GB content level with their control plantlets which grew under non-stressed condition.

Discussion

Many efforts have been made to promote the tolerance of cultivars by transgenic methods, and quite a lot of genes involved in stress responses have been cloned for this purpose (Yu et al. 2012). Na^+/H^+ anti-transporter genes function in a specific salt stress-resistant pathway by transporting excessive cytosolic Na^+ into vacuoles (Apse

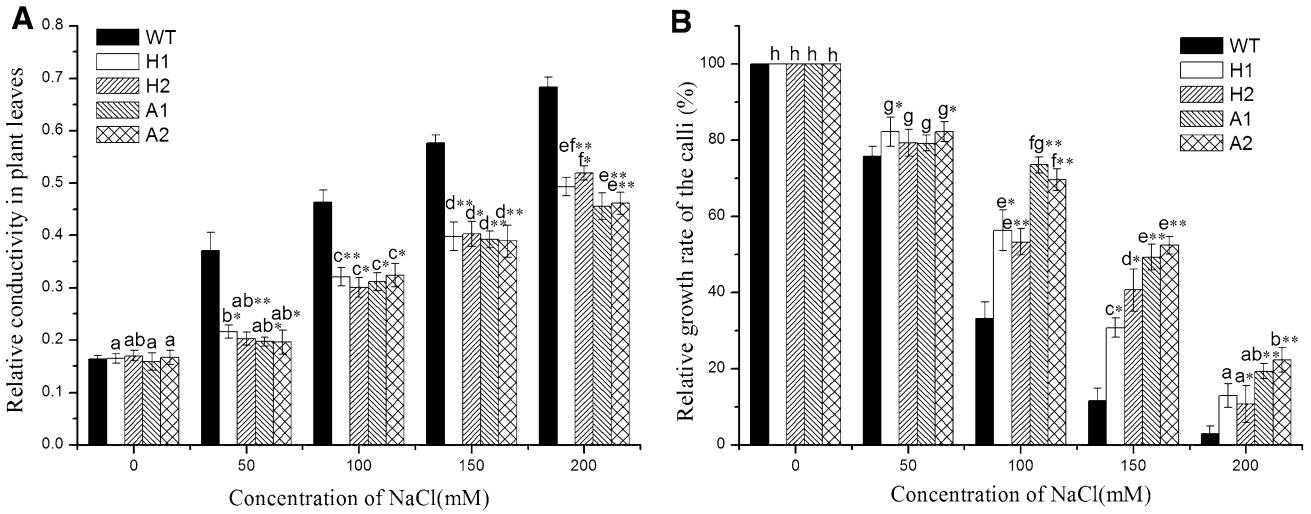

Fig. 4 REC (**a**) and relative growth rate (**b**) in transgenic and WT chicory

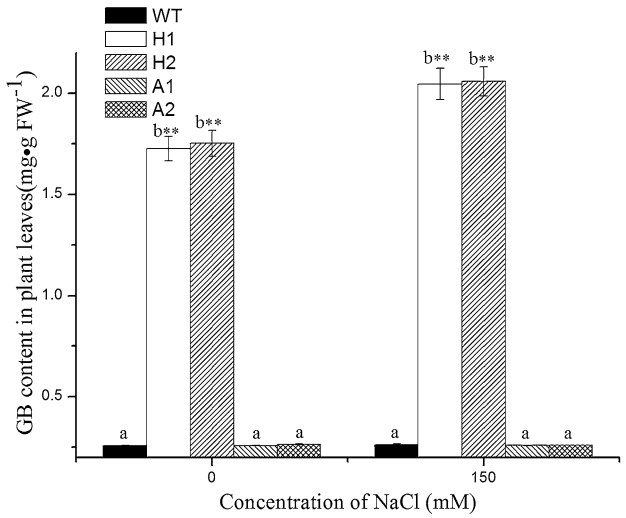

Fig. 5 GB contents in transgenic and WT chicory

et al. 1999), while BADH genes are believed to play a key role in a general osmotic stress-resistant pathway. Genetic transformations of both anti-transporter genes and BADH genes have already been used in many attempts to promote the salt stress resistance of plants (Wang et al. 2013). High altitude plants have evolved over millions of years to develop a complex defense system to adapt to environmental stresses, such as lower O_2, high UV radiation and extreme temperature, and understanding the mechanisms of the specific stress-resistance system in these plants may provide deep insight into how to promote the salt stress resistance of other major crops. In this work, we evaluated the salt stress tolerance in transgenic plants of *HvBADH1* from highland Hulless barley (*Hordeum vulgare* var.nudum.hook.f.) and *AtNHX1* from *Arabidopsis thaliana.*

A resting state with intracellular higher [K^+] and lower [Na^+] is important to maintain the activities of many cytosolic enzymes, as well as a regular membrane potential. When salt stress occurs, the efficiency of Na^+/H^+ anti-transporters is affected by excessive Na^+ in cytosol and expression of genes encoding Na^+ transporters, such as *AtNHX1*, is upregulated to increase the amount of these anti-transporters on the membrane of vacuoles (Zhu 2003). Little difference was found between CaMV35S:*AtNHX1* and CaMV35S:*HvBADH1* lines in this study for Na^+, K^+ contents or K^+/Na^+ ratio, although the data from both transgenic lines were much higher than WT under stress conditions. By overexpressing these two genes, the transgenic cells could maintain cytosolic enzyme activity and plasma membrane integrity against the ion toxic effect and prevent the inhibition of K^+ uptake of cells caused by salt stress. Compared to the effect of Na^+ compartmentalization caused by AtNHX1 overexpression, the osmotic potential promoting process caused by overaccumulation of GB made a similar contribution to increase the salt tolerance by modulating both the levels of K^+ and Na^+ and keeping the K^+/Na^+ balance mainly in the leaves.

As a by-product of the membrane damage process triggered by reactive oxygen species (ROS), MDA is released to the cytosol soon after stress (Wang et al. 2011). In the present study, the MDA levels suggested that the overexpression of *HvBADH1* gene and subsequent over-accumulation of GB in the cytosol raised the osmotic potential and decreased the generation of ROS in cytosol. This effect allowed the *HvBADH1* lines to show a superior ability than *AtNHX1* strains to minimize membrane damage under high salt stress. However, because of its ability to transport redundant Na^+ into the vacuoles, the overexpressed *AtNHX1* exhibited a similar antioxidative capacity

to *HvBADH1* at salt concentrations less than 150 mM NaCl. Thus, both transgenic lines showed protective activities against oxidative damage to the cell membrane, though *HvBADH1* had higher protection under severe stress conditions.

To protect themselves from various abiotic stresses, plants responsively synthesize and accumulate some organic osmolytes in the cells, such as soluble sugar, free proline and GB (Rodríguez-Calcerrada et al. 2011), which helps plants maintain cell turgor during salt and osmotic stresses (Zheng et al. 2009). Overaccumulated soluble sugar and free proline in protoplast can protect macromolecules from being hurt by various abiotic stresses such as dehydration and salinity; proline also takes part in the endogenous radical scavenging system. In our research, increased soluble sugar and free proline triggered by salt stress modulated the maintenance of osmotic equilibrium in protoplasts of the transgenic lines. The values of these two osmolytes showed a jump in the *HvBADH1* lines, but not in *AtNHX1* lines at 150–200 mM NaCl stresses, suggesting that overexpressing *HvBADH1* may attribute to the salt tolerance of transformants by maintaining the osmotic potential in cells to severe NaCl stresses.

The contents of chlorophyll reflect the physiological status of plants, which are also used to estimate the tolerance of plants to salinity (Gitelson et al. 2003). Lower chlorophyll contents in leaves directly limit both the photosynthetic activity and efficiency of plants encountered with salt stress. The redundant Na^+ and Cl^- ions permeating into cytoplasm reduce the chlorophyll levels by interfering the activity of chlorophyll-synthesizing enzymes (Garriga et al. 2014). In the present study, the contents of chlorophyll in *HvBADH1* lines were slightly higher than that of *AtNHX1* lines, which suggested that transformation of *HvBADH1* was better to protect the plant photosynthesis system from salt damage.

REC is another important physiological status indicator that directly reflects the extent of cell membrane damage under osmotic stress. At levels of NaCl stress lower than 150 mM, no noticeable differences were detected between the transgenic plant strains. The significant lower level of REC in *AtNHX1* lines at 200 mM NaCl indicated that the overexpression of *AtNHX1* provided the cell membrane with a better protection than *HvBADH1* lines.

Plant tissue tolerance is thought to be a major component to determination of overall plant response to salinity (Getnet Dino Adem et al. 2014). The measurement of relative growth rate of calli showed that the *AtNHX1* lines could endure severe salinity stress better than the *HvBADH1* lines. But under slight salt stress conditions, the two lines responded more similarly. These phenotypes may be explained by a hypothesis that the toxic effect of overaccumulated ions, such as Na^+ and Cl^- might be major obstacles to physiological metabolism and cell growth under severe salt stress conditions, while osmotic stress inhibits plant growth under slight salt stress. The precise segmentation point between severe and slight stress conditions might vary for different plant species or different physiological indicators, but in this study, this segmentation point was 100 mM NaCl.

GB is a common osmolyte that accumulates in many plant species, and the levels of GB in the plant tissues are correlated with salt tolerance. Genetic evidence that GB improves salinity tolerance has been obtained in barley and maize (Zhang et al. 2009). In the present study, CaMV35S:*HvBADH1* lines exhibited an over accumulation of GB in leaves under stressed conditions, which may correspond to enhanced salt stress tolerance. Our results are consistent with the theory that osmoprotection caused by osmolyte accumulation may play a pivotal role in natural stress environments where stress does not affect plant survival (Neha Gupta et al. 2014).

A recent report found that *NHX1* and *NHX2* genes, the homologues of *AtNHX1* in barley, may not necessarily confer the same protein(s) function as *AtNHX1* in Arabidopsis, though they have similar nucleotide sequence (Getnet Dino Adem et al. 2014). Therefore, we chose *AtNHX1* from Arabidopsis as a reference to evaluate the contribution of *HvBADH1* from Hulless barley to the ionic and osmotic stress parameters determining salinity tolerance.

In conclusion, we evaluated how overexpressing *AtNHX1* and *HvBADH1* affected the salt tolerance of transgenic plants by measuring eight different physiological indicators in CaMV35S:*AtNHX1* and CaMV35S:*HvBADH1* chicory lines. The determination of calli growth rate and REC revealed that the overexpression of *AtNHX1* gene promoted the tolerance of transgenic calli, and increased the ability of transgenic plants to maintain homeostasis against membrane injury under severe salinity condition. In contrast, the *HvBADH1* overexpressing plants exhibited better capacity to maintain the osmotic potential of cytoplasm by synthesizing and accumulating more soluble sugar and free proline. The MDA and chlorophyll levels showed that the overexpression of *HvBADH1* also enhanced the ability of transgenic plants to protect the integrity of membrane system and photosynthesis system when threatened by NaCl stress. In summary, our results suggest that *AtNHX1* is more suitable for severe salinity, while *HvBADH1* seems superior for dealing with general abiotic stresses. However, further field tests will be required to determine the long-term productivities of *HvBADH1* and *AtNHX1* lines. These results support the idea that a successful progress in the breeding of crops with enhanced NaCl stress tolerance can only be achieved by targeting several complementary traits together (Getnet Dino Adem et al. 2014). Because these two genes

confer different responses to stress, it is a logical next step to assess the salt-resistant phenotype of plants that expresses both *HvBADH1* and *AtNHX1* genes.

Author contribution statement Yuwei Zhao designed the research. Fang Zhang, Xiaolong Li and Pan Lai performed experiments. Fang Zhang, Xiaolong Li and Pengfei Li analyzed data. Fang Zhang wrote most of the article.

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Conflict of interest The authors declare that they have no conflict of interest.

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