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The growth and accumulation of osmotic solutes of the halophyte common glasswort (*Salicornia europaea*) under salinity conditions

KEE WOONG PARK, JAE YOUNG AN, HEE JAE LEE, DAEYOUNG SON, YOUNG GEOL SOHN, CHANG-GI KIM, AND JEUNG JOO LEE*

ABSTRACT

A halophyte, common glasswort (*Salicornia europaea* L.), showed no phytotoxic or deficiency symptom at 100 and 300 mM NaCl, whereas plant growth was stunted at 0, 500, and 700 mM NaCl. In addition, the water content was lower within the shoots at 0 mM NaCl, as compared with the range of NaCl treatments. Under NaCl treatment, Na^+ levels increased in the shoot and the root, whereas the levels of other cations (K^+, Ca^{2+}, and Mg^{2+}) decreased. Na^+ accumulated to a greater amount in the shoot than it did in the root. At 0 mM NaCl, cations such as K^+, Ca^{2+}, and Mg^{2+} were highly accumulated, but Na^+ was significantly reduced. Furthermore, the total free amino-acid content in the shoots was higher non non-NaCl treatments than it was in NaCl treatments. In particular, the proline levels showed marked increase at 500 and 700 mM NaCl. The increase in glycine betaine accumulation under NaCl conditions correlated to the increase of salinity, and the pattern corresponded to the induction of betaine aldehyde dehydrogenase (BADH) activity. Even though the BADH activity was lowest, more glycine betaine accumulated under non-NaCl condition than it did under NaCl conditions. These results indicate that Na^+ is an essential element for the normal growth of common glasswort, and the accumulation of osmotic solutes, such as proline and glycine betaine, in cells occur under inhibitory NaCl concentrations.

Key words: BADH, glycine betaine, Na^+ accumulation, Proline, *Salicornia europaea*.

INTRODUCTION

Halophytes are plants that can withstand critical salt concentrations that are not normally tolerated by non-halophytes (Flowers et al. 1977). Although many halophytes grow well under low-salinity and normal-culture solutions, some species require high salt concentrations (Macke and Ungar 1971, McMillan 1974, Munns et al. 1983). Halogeton [*Halogeton glomeratus* (Stephen ex Bieb.) C.A. Mey.] and the glassworts (*Salicornia* L. spp.) are particularly notable for their poor growth under low NaCl concentrations (Williams 1960, Webb 1966, Ayala and O'Leary 1995, Glenn and Brown 1999). Nonetheless, it has been reported that common glasswort (*Salicornia europaea* L.) can survive up to 1,020 mM NaCl with no phytotoxic effect, for growth ranging from 136 to 510 mM NaCl (Macke and Ungar 1971). Other glasswort species, such as dwarf saltwort (*Salicornia bigelovii* Torr.), showed normal growth at 200 mM NaCl (Webb 1966, Ayala and O'Leary 1995).

Halophytes use controlled uptake of Na^+ into vacuoles to drive water into the plant against low external water potential (Glenn and Brown 1999). However, the accumulation of Na^+ for turgor regulation leads to toxicity at a high salt concentration. Enzymes extracted from a number of halophytes, including members of the Chenopodiaceae, are salt-sensitive (Flowers 1972, Greenway and Osmond 1972), indicating that salt balance in cells is critical to sustaining the growth of halophytes. At the cellular level, salt balance is maintained by either the effective exclusion of Na^+ and Cl^- ions or by other strategies like ion compartmentalization in the vacuole of plants (Matoh et al. 1989, Ayala et al. 1996, Parks et al. 2002). In many halophytes, ion compartmentalization seems to be a highly effective mechanism for minimizing ion toxicity. Various mineral ions, including Na^+, K^+, and Cl^-, are accumulated in plants during turgor or volume regulation (Jacoby 1999). For species such as saltbush (*Atriplex halimus* L.) (Mozafar et al. 1970), KCl elicits a growth response that is similar to that of NaCl. On the other hand, halophytes, such as herbaceous seepweed [*Suaeda maritima* (L.) Dumort.] and jojoba [*Simmondsia chinensis* (Link) C.K. Schneid.], decrease their K^+ content when exposed to increasing external NaCl concentrations without concomitant damage (Leigh and Wyn-Jones 1984). This decrease seems to be related to replacement of vacuolar K^+ with Na^+.

Various organic solutes and mineral ions are accumulated in plants during turgor or volume regulation. Inorganic solutes at high concentrations interact directly with protein surfaces and denature the proteins. Compatible organic solutes, however, raise osmotic pressure in the cytoplasm and play an important adaptive role in stabilizing proteins and membranes when salt levels are unfavorable (McNeil et al. 1999). Accumulation of organic solutes, such as proline and glycine betaine, is associated with the plant's adaptation to osmotic stress when it is exposed to salt or water stress (Rhodes and Hanson 1993, Rhodes et al. 1999).

*First author, Department of Crop Science, Chungnam National University, Daejeon, Republic of Korea. Second, fourth, and seventh authors, Department of Applied Biology, Research Institute of Life Science, Gyeongsang National University, Jinju, Republic of Korea. Third author, Department of Horticultural Science, Seoul National University, Seoul, Republic of Korea. Fifth author, Yenong Company, Jinju, Republic of Korea. Sixth and seventh authors, Bio-Evaluation Center, KRIBB, Ochang-eup, Cheongwon-gun, Republic of Korea. Corresponding authors' E-mails: cgkim@kribb.re.kr and jjl@gaechuk.gsnu.ac.kr. Received for publication July 15, 2012 and in revised form February 19, 2013.

This study was conducted to determine the effect of NaCl on the growth, the accumulation of inorganic ions and free amino acids, the glycine betaine levels, and the betaine aldehyde dehydrogenase (BADH) activity of the halophyte, common glasswort. The results from this study can be applied to understanding the mechanisms related to maintaining the plant's existence under extreme saline conditions.

MATERIALS AND METHODS

Plant growth and NaCl treatments

Common glasswort seeds were scrubbed with sea sand and sown in an equal mixture of organic soil and sand in trays, then germinated in an incubator at 30 C (86 F) with 80% relative humidity in the dark. The seedlings were transferred to a controlled growth chamber with 30/22 C day/night, 16-h photoperiod, and 70% relative humidity with photosynthetically active radiation of 250 mol m^{-2} s^{-1} from fluorescent and metal halide lamps. Plants were watered with a 20 mM NaCl-supplemented nutrient solution. The nutrient solution, which was slightly modified from that of Cakmak and Marschner (1992), contained 0.88 mM K_2SO_4, 1 mM $Ca(NO_3)_2$, 1 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.1 mM KCl, 40 M FeEDTA, 10 M H_3BO_4, 1 M $MnSO_4$, 1 M $ZnSO_4$, 0.1 M $CuSO_4$, and 0.01 M $(NH_4)_6MoO_{24}$. The solution was adjusted to pH 5.8 with HCl. The roots of the seedlings were approximately 5 to 6 cm (1.97 to 2.36 in) long from cotyledons to shoot tip at approximately 30 d after germination. These roots were gently freed of soil by rinsing them under running tap water. The plants were then transferred to a 20 by 14 by 12-cm plastic pot containing the nutrient solution with constant aeration. After 5 d, the plants were transferred to nutrient solutions containing 0, 100, 300, 500, and 700 mM NaCl. The culture solutions were changed every day to maintain the nutrients and NaCl concentration levels.

To determine the inorganic ion concentrations, the free amino acid and glycine betaine contents, as well as the BADH activity, all sampled plants were washed thoroughly three times with ultrapure water (18.2 MΩcm) and dried with paper towels. Shoots and roots from each plant were stored at −70 C before analysis.

Growth measurements

Fresh weight (*FW*) and shoot length were measured 0, 5, 10, and 15 d after initiation of the NaCl treatment. Dry weight (*DW*) of shoots was determined after oven drying at 90 C for 48 h. Fresh weight and dry weight were used to calculate the water content (percentage), using the following formula:

$$\left(1 - \frac{DW}{FW}\right) \times 100. \quad [1]$$

Determination of inorganic ions

Oven-dried shoot and root samples were ground into a fine powder in a glass homogenizer. Inorganic ions were extracted by shaking the weighed powder in 1 N HCl for 24 h at room temperature and filtering through Whatman No. 2 filter paper.[1] Inorganic ion concentrations in the extracts were determined by inductively coupled plasma spectrophotometry after appropriate dilution.

Determination of free amino acids

Free amino acids were determined according to the method devised by Sanada et al. (1995). One gram (0.0353 oz) of frozen shoots was ground to fine powder in liquid nitrogen, then homogenized with 5 ml (0.16907 oz) of deionized distilled water and boiled for 5 min. The heat-treated homogenate was cooled in an ice bath and centrifuged at 15,000 × *g* for 15 min. The resulting supernatant was vortexed with trichloroacetic acid and centrifuged again. Free amino acids in the supernatant were analyzed with an automatic amino acid analyzer.[2]

Determination of glycine betaine

One gram of frozen shoots was ground to fine powder in liquid nitrogen and homogenized with 5 ml of 1 N H_2SO_4 (Wall et al. 1960). The homogenate was stirred for 12 h at 25 C and centrifuged at 2,000 × *g* for 15 min. The precipitate was resuspended in 1 N H_2SO_4 and centrifuged again under the aforementioned conditions. This step was repeated three times, and the combined supernatants were precipitated for extraction of quaternary ammonium compounds that contained glycine betaine. The glycine betaine content was quantified by proton nuclear magnetic resonance (^{1}H-NMR; Jones et al. 1986) with a Brucker AW-500 spectrometer.[3]

BADH assay

BADH was extracted from 4 to 5 g of common glasswort shoots and assayed according to the method introduced by Arakawa et al. (1990). Protein content was determined using the Bradford (1976) method with bovine serum albumin as the standard.

Statistical analysis

The experiments were completely randomized with four replicates. Especially, the experiments for growth measurement and inorganic ions were repeated twice. All the data obtained in this study were subjected to an ANOVA, and the means were compared with Tukey's Honestly Significant Difference test at the 5% level of significance.

RESULTS

Effects of salinity on growth

The growth of common glasswort was greatly affected by salinity (Figure 1). Based on the FW and the shoot length, the growth rate of common glasswort increased more at 100 and 300 mM NaCl than it did at 0 and 700 mM NaCl. Shoot water content was lower in the non-NaCl than in NaCl

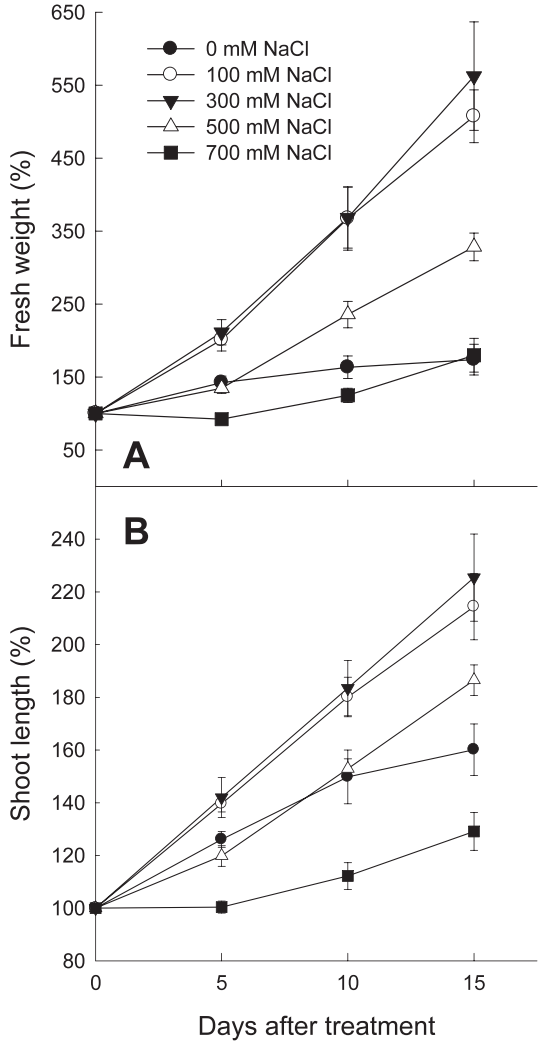

Figure 1. Effects of salinity on growth of *Salicornia europaea*. (A) Fresh weight and (B) shoot length were measured before treatment and 5, 10, and 15 d after NaCl treatment. Data represent the mean ± SE of 8 replicates from 2 independent experiments.

conditions and was not significantly different for the range of NaCl treatments (Figure 2).

Accumulation of inorganic ions

Na^+ accumulated in the shoots of common glasswort when the NaCl concentration increased for 15 d after salt treatment (Figure 3A). Most of the Na^+ content absorbed into the roots appeared to be translocated to the shoots. The levels of Na^+ at 100, 300, 500, and 700 mM NaCl in the shoots were higher by 425, 620, 644, and 813%, respectively, when compared with the Na^+ level in non-NaCl treatment. The level of K^+ decreased further within the shoot than in the root as the concentration of NaCl increased (Figure 3B). The level of Ca^{2+} and Mg^{2+} decreased as NaCl increased, and the amount of decrease was more apparent in the shoot than in the root (Figures 3C and 3D).

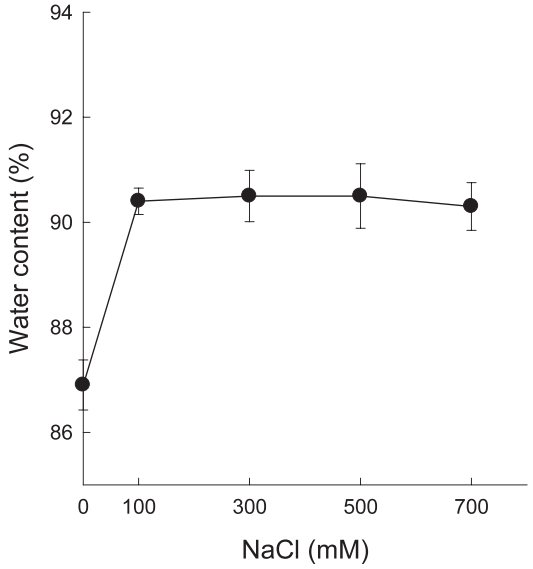

Figure 2. Water content of common glasswort shoots under different NaCl concentrations. Water contents were measured 15 d after NaCl treatment. Data represent the mean ± SE of 8 replicates from 2 independent experiments.

Changes of free amino acid content

To examine changes of free amino acids in the shoot due to salinity, the levels of 15 standard free amino acids were determined. As a result, the total content of free amino acids was measured at 13.04 μmol g^{-1} FW under non-NaCl condition but decreased to 5.07, 5.69, and 5.81 μmol g^{-1} FW in the 100, 300, and 500 mM NaCl treatments, respectively (Table 1). However, the free amino acid levels increased to 10.18 μmol g^{-1} FW at 700 mM NaCl. Under non-NaCl condition, the major free amino acids comprised glutamate, asparagine, and serine in decreasing order. At 100, 300, and 500 mM NaCl, glutamate, serine, and asparagine were major amino acids, whereas at 700 mM NaCl, glutamate, proline, asparagine, and arginine were major amino acids of common glasswort. Proline accumulated to approximately 7% of the total free amino acids at 500 mM NaCl and increased to about 19% at 700 mM NaCl.

Accumulation of glycine betaine and increase in BADH activity

The glycine betaine content in the shoots of common glasswort was 82.6 μmol g^{-1} FW under the non-NaCl condition but decreased to 34.3 and 33.5 μmol g^{-1} FW at 100 and 300 mM NaCl, respectively; and 49.2 and 47.6 μmol g^{-1} FW at 500 and 700 mM NaCl, respectively (Figure 4). The amount of glycine betaine increased slightly 15 d after 300 mM NaCl treatment yet increased rapidly after 15 d under non-NaCl condition (Figure 5).

The BADH activity in the shoots of common glasswort was lowest in nonsalinity conditions but increased with increasing NaCl concentrations (Figure 6). At 700 mM NaCl, the enzyme activity increased about 4.8 times when

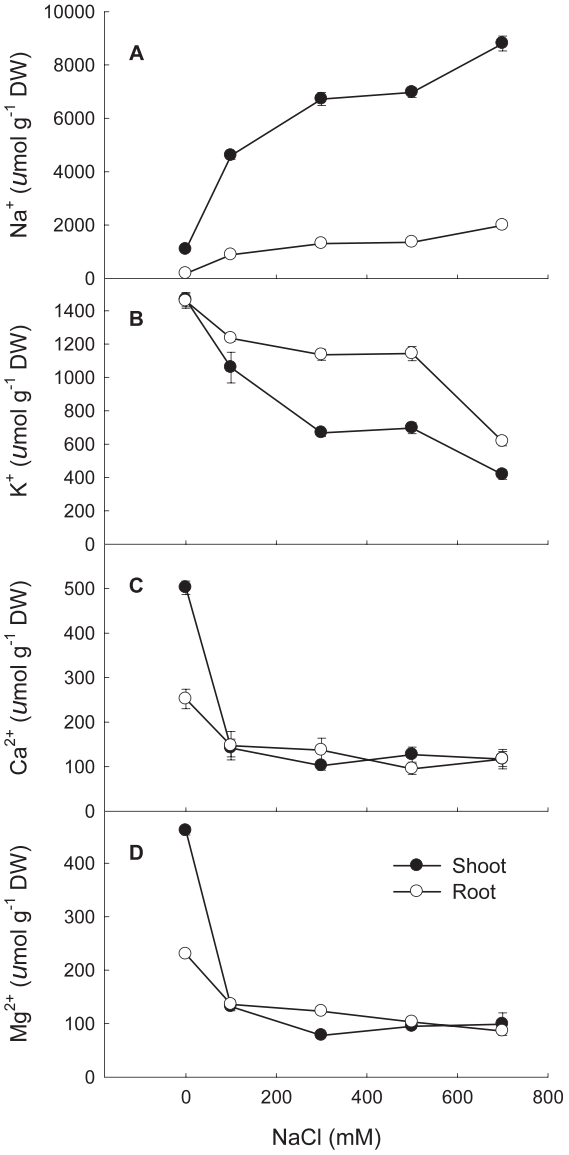

Figure 3. Effects of NaCl on inorganic ion contents in the shoots and roots of common glasswort. Inorganic ion content was measured 15 d after NaCl treatment. Data represent the mean ± SE of 8 replicates from 2 independent experiments. In some cases, the error bar is obscured by the symbol.

compared with the activity observed under non-NaCl condition.

DISCUSSION

In this study, common glasswort showed no phytotoxic or deficiency symptom at 100 and 300 mM NaCl (Figure 1), and the growth was significantly inhibited at 0, 500, and 700 mM NaCl. The plants grown under non-NaCl condition contained less water than the plants grown under saline conditions did (Figure 2). Ayala and O'Leary (1995) reported that the growth of *S. bigelovii*, another species of halophyte, was reduced under low NaCl concentrations. In the case of common glasswort, the Na^+ accumulation was low but the accumulations of K^+, Ca^{2+}, and Mg^{2+} were high under non-NaCl condition (Figure 3). This result suggests that if Na^+ deficiency occurs when common glasswort needs a large amount of Na^+, the plant absorbs inorganic ions, such as K^+, Ca^{2+}, and Mg^{2+}, as a substitute for Na^+ from the nutrient solution, and then translocates them to the shoots. Considering the growth and water-content reduction of common glasswort under non-NaCl condition, it seems that K^+, Ca^{2+}, and Mg^{2+} accumulated in the shoots are not used for inducing osmotic driving forces. These inordinately accumulated cations might have contributed to reduced growth in the plants. The growth of some halophytes was substantially reduced by high K^+ instead of Na^+ (Flowers et al. 1977, Munns et al. 1983). Also, high exogenous levels of Ca^{2+} inhibited plant growth by raising the pH of the Donan free space and by inhibiting cell wall–loosening enzymes with acidic pH optima (Cleland et al. 1990). Common glasswort needs Na^+ accumulation for the growth (Figures 1 and 3). The Na^+ could be compartmentalized in the vacuole and used to provide an osmotic driving force. Ayala et al. (1996) and Parks et al. (2002) reported that, under NaCl condition, efficient vacuolar sequestration of Na^+ in *S. bigelovii* shoots occurred, and the $Na^+ : H^+$ exchange for vacuolar sequestration of Na^+ was clearly stimulated when the plants were grown in the presence of high NaCl concentration.

Flowers et al. (1977) reported that halophytes biosynthesize amino acids, mainly glutamate and aspartate, as the primary products of dark fixation in photosynthesis. In this study, the content of glutamate was the highest under NaCl conditions (Table 1). Total content of free amino acids was minimal at 100 and 300 mM NaCl and was highest under non-NaCl condition, suggesting that the free amino acids

Table 1. Effects of NaCl on the content of free amino acids in the shoots of common glasswort.

	Amino Acid (mol g^{-1} FW)[1]							
NaCl (mM)	Ala	Arg	Asn	Asp	Glu	Gly	His	Ile
0	0.77 ± 0.04	0.41 ± 0.21	2.85 ± 0.33	1.13 ± 0.10	4.08 ± 0.34	—	0.24 ± 0.004	0.22 ± 0.01
100	0.39 ± 0.06	0.10 ± 0.02	0.62 ± 0.22	0.30 ± 0.06	1.32 ± 0.36	0.06 ± 0.01	0.15 ± 0.02	0.09 ± 0.03
300	0.39 ± 0.05	0.16 ± 0.04	0.96 ± 0.03	0.26 ± 0.03	1.53 ± 0.38	0.07 ± 0.01	0.14 ± 0.03	0.11 ± 0.02
500	0.36 ± 0.01	0.15 ± 0.02	0.92 ± 0.12	0.26 ± 0.01	1.46 ± 0.22	—	0.15 ± 0.08	0.12 ± 0.01
700	0.49 ± 0.05	1.29 ± 0.60	1.34 ± 0.15	0.28 ± 0.01	2.15 ± 0.30	—	0.42 ± 0.10	0.17 ± 0.01

Free amino acid content was measured 15 d after NaCl treatment. Data represent the mean ± SE of 4 replicates.
[1]Abbreviations: Ala = alanine; Arg = arginine; Asn = asparagine; Asp = aspartic acid; FW = fresh weight; Glu = glutamic acid; Gly = glycine; His = histidine; Ile = isoleucine; Leu = leucine; Lys = lysine; NaCl = sodium chloride; Phe = phenylalanine; Pro = proline; Ser = serine; Thr = threonine; Val = valine.

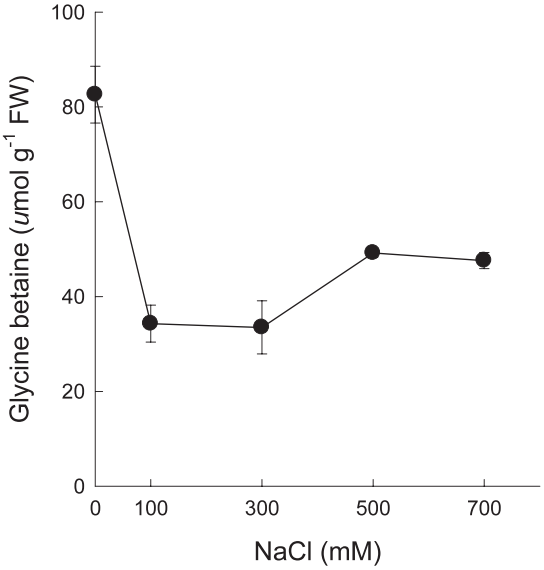

Figure 4. Effects of NaCl on glycine betaine content in *Salicornia europaea* shoots. Glycine betaine content was measured 15 days after NaCl treatment. Data represent the mean ± S.E. of four replicates. In some cases, the error bar is obscured by the symbol.

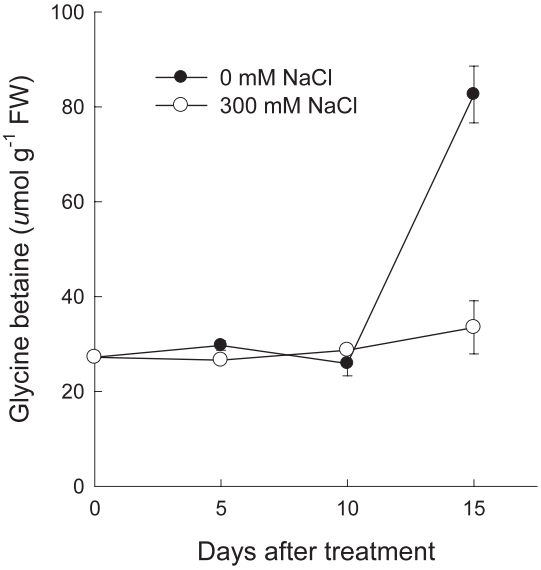

Figure 5. Effects of NaCl on glycine betaine accumulation with time in common glasswort shoots. Glycine betaine content was measured before treatment and 5, 10, and 15 d after NaCl treatment. Data represent the mean ± SE of four replicates. In some cases, the error bar is obscured by the symbol.

are not used for the biosynthesis of organic acids or proteins in the plants. Excessively accumulated Na^+ in the plant cell can cause ion toxicity, ion imbalance, water stress or a combination of these injurious factors (Glenn and Brown 1999). Halophytes usually absorb large amounts of Na^+, which is thought to be sequestered in the vacuole (Heuer 1999); otherwise, numerous essential enzymes and metabolic processes would be impaired (Flowers et al. 1977, Jacoby 1999). To keep the cytoplasm osmotically balanced with the vacuole, plants usually accumulate compatible solutes, such as proline (Rhodes et al. 1999), glycine betaine (Matoh et al. 1987, McNeil et al. 1999), polyols (Rhodes and Hanson 1993, Stoop et al. 1996), and some sugars (Muralitharan et al. 1992), which are correlated with salinity tolerance of plants. In this study, common glasswort accumulated proline at 500 and 700 mM NaCl, which inhibited its growth (Table 1). Similar results were obtained with the halophyte crystalline iceplant (*Mesembryanthemum crystallinum* L.) (Demming and Winter 1986). Common glasswort accumulated more glycine betaine under non-NaCl conditions than it did under NaCl conditions (Figures 4 and 5). Glycine betaine content decreased at 100 and 300 mM NaCl, which promoted its growth, and increased at 500 and 700 mM NaCl. In general, the level of glycine betaine is known to rise with exposure to stress, such as salinity, water deficiency, and low temperature, because BADH, a biosynthetic enzyme, is activated under such stressful conditions (Rhodes and Hanson 1993). Also in this study, the increase in BADH activity generally coincided with a rise in glycine betaine accumulation under NaCl conditions (Figure 6). The accumulated proline and glycine betaine could help to balance inordinate osmotic potential in the cytoplasm under the inhibitory NaCl concentrations. In this study, BADH activity was lowest under non-NaCl condition and more glycine betaine accumulated under non-NaCl condition than accumulated under NaCl conditions. Further studies should be conducted to understand the discrepancy of BADH activity and glycine betaine accumulation in this study.

These results suggest that common glasswort needs NaCl for normal growth, and osmotic solutes, such as proline and glycine betaine, are accumulated in the plant cell under high NaCl level, to maintain the balance of osmotic potential induced by Na^+ accumulated in vacuoles.

Table 1. Extended.

Amino Acid (mol g^{-1} FW)[1]							
Leu	Lys	Phe	Pro	Ser	Thr	Val	Total
0.22 ± 0.02	0.41 ± 0.03	0.35 ± 0.06	—	1.64 ± 0.01	0.34 ± 0.04	0.38 ± 0.03	13.04
0.09 ± 0.01	0.27 ± 0.11	0.18 ± 0.07	—	1.23 ± 0.17	0.12 ± 0.02	0.15 ± 0.03	5.07
0.11 ± 0.02	0.33 ± 0.10	0.19 ± 0.02	—	1.17 ± 0.11	0.10 ± 0.02	0.17 ± 0.02	5.69
0.13 ± 0.05	0.39 ± 0.1	0.21 ± 0.03	0.40 ± 0.1	0.09 ± 0.06	0.91 ± 0.003	0.18 ± 0.01	5.81
0.20 ± 0.03	0.53 ± 0.31	0.39 ± 0.17	1.94 ± 0.08	0.69 ± 0.03	0.10 ± 0.01	0.19 ± 0.02	10.18

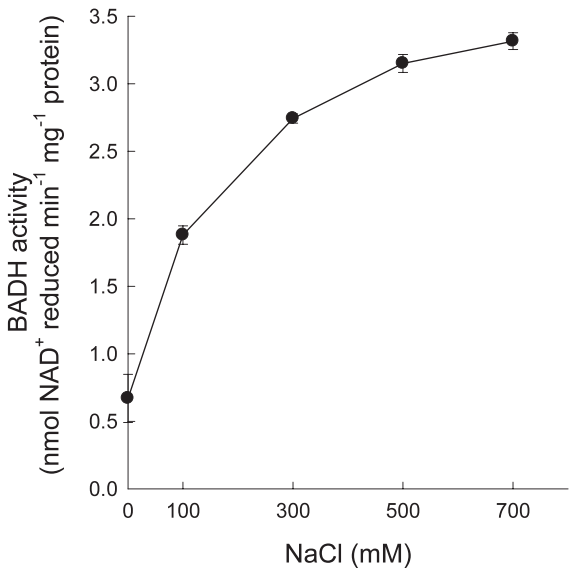

Figure 6. Effects of NaCl on specific activities of BADH in common glasswort shoots. BADH activity was measured 15 d after NaCl treatment. Data represent the mean ± SE of 4 replicates. In some cases, the error bar is obscured by the symbol.

SOURCES OF MATERIALS

[1]Whatman No. 2 filter paper, Whatman plc, James Whatman Way, Maidstone, Kent, ME14 2LE, U.K.

[2]Biochrom 20, Amersham Pharmacia Biotech, GE Healthcare Bio-Sciences AB, Björkgatan 30, SE-751 84, Uppsala, Sweden.

[3]Brucker AW-500 spectrometer, Bruker Scientific Instruments, 40 Manning Road, Billerica, MA 01821.

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