Journal of Experimental Botany, Vol. 58, No. 8, pp. 1957–1967, 2007
doi:10.1093/jxb/erm057 Advance Access publication 23 April, 2007

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Salt tolerance, salt accumulation, and ionic homeostasis in an epidermal bladder-cell-less mutant of the common ice plant *Mesembryanthemum crystallinum*

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Received 21 August 2006; Accepted 28 February 2007

Abstract

The aerial surfaces of the common or crystalline ice plant *Mesembryanthemum crystallinum* L., a halophytic, facultative crassulacean acid metabolism species, are covered with specialized trichome cells called epidermal bladder cells (EBCs). EBCs are thought to serve as a peripheral salinity and/or water storage organ to improve survival under high salinity or water deficit stress conditions. However, the exact contribution of EBCs to salt tolerance in the ice plant remains poorly understood. An *M. crystallinum* mutant lacking EBCs was isolated from plant collections mutagenized by fast neutron irradiation. Light and electron microscopy revealed that mutant plants lacked EBCs on all surfaces of leaves and stems. Dry weight gain of aerial parts of the mutant was almost half that of wild-type plants after 3 weeks of growth at 400 mM NaCl. The EBC mutant also showed reduced leaf succulence and leaf and stem water contents compared with wild-type plants. Aerial tissues of wild-type plants had approximately 1.5-fold higher Na^+ and Cl^- content than the mutant grown under 400 mM NaCl for 2 weeks. Na^+ and Cl^- partitioning into EBCs of wild-type plants resulted in lower concentrations of these ions in photosynthetically active leaf tissues than in leaves of the EBC-less mutant, particularly under conditions of high salt stress. Potassium, nitrate, and phosphate ion content decreased with incorporation of NaCl into tissues in both the wild type and the mutant, but the ratios of Na^+/K^+ and Cl^-/NO_3^- content were maintained only in the leaf and stem tissues of wild-type plants. The EBC mutant showed significant impairment in plant productivity under salt stress as evaluated by seed pod and seed number and average seed weight. These results clearly show that EBCs contribute to succulence by serving as a water storage reservoir and to salt tolerance by maintaining ion sequestration and homeostasis within photosynthetically active tissues of *M. crystallinum*.

Key words: Epidermal bladder cells, halophyte, ice plant, ion homeostasis, *Mesembryanthemum crystallinum*, salt stress, succulence.

Introduction

Halophytes have evolved numerous strategies for adaptation to growth under conditions of high salinity. Three major physiological and biochemical adaptations include (i) the accumulation of osmolytes, (ii) the control of water flux, and (iii) the maintenance of ion homeostasis (Hasegawa *et al*., 2000). In some halophytes, enzymes may also have evolved that show reduced salt sensitivity (Ghosh *et al*., 2006). The first two adaptations are

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common to both glycophytes and halophytes alike, whereas halophytes have adapted to effectively maintain steady-state ion homeostasis and metabolic activities following exposure to high salinity using the latter two mechanisms. Some halophytes have also evolved specialized epidermal cells, such as salt glands or bladder cells, for the elimination or sequestration of excess salt from metabolically active tissues (Liphschitz and Waisel, 1982; Naidoo and Naidoo, 1998*a*, *b*). There are three types of glands based on their structural organization: two-celled glands of the Poaceae; bladder cells of the Chenopodiaceae, Mesembryanthemaceae, and Oxalidaceae; and multicellular glands which are rare in the Poaceae (Johnston and Watson, 1976) but occur in several dicotyledonous families (Thomson, 1975; Oross and Thomson, 1982).

The aerial surfaces of the common ice plant, *Mesembryanthemum crystallinum* (Aizoaceae) are covered with giant (typically 500 μm in diameter) epidermal bladder cells (EBCs). These cells are characterized by a large central fluid-filled vacuole (Adams *et al*., 1992, 1998). The ice plant is a well-studied, model halophyte that undergoes a stress-inducible switch from C_3 photosynthesis to crassulacean acid metabolism (CAM), a photosynthetic adaptation that improves water use efficiency (Winter and Holtum, 2005). EBCs have been suggested to participate in the regulation of salt sequestration and water relations and to serve as a storage reservoir for water and inorganic and organic compounds such as sodium, chloride, flavonoids, and betacyanins (Haberlandt, 1904; Steudle *et al*., 1975, 1977; Adams *et al*., 1992; Vogt *et al*., 1999). EBCs may also serve to support the water relations of the mesophyll in conjunction with the osmotically important oscillations of malate levels in mesophyll cells during CAM (Rygol *et al*., 1989). Following salt stress, *M. crystallinum* accumulates sodium in a gradient from roots (about 70 mM) to shoot apices with the highest concentrations (in excess of 1 M) being present in the EBCs (Adams *et al*., 1992, 1998). The enhanced sodium accumulation in the vacuoles of EBCs correlates with tonoplast Na^+/H^+ antiport and H^+-translocating ATPase (V-ATPase) activities, which are higher in EBCs than surrounding cell types (Barkla *et al*., 1995, 2002).

In addition to sodium sequestration, EBCs may function in controlling water status by serving as a site for water storage to buffer against conditions of intermittent or diurnally changing water availability (Lüttge *et al*., 1978) or as a secondary epidermis to reduce the evaporation of water from leaves (Steudle *et al*., 1975, 1977). EBCs also serve as important storage sites for sugar alcohol compatible solutes (e.g. cyclitols). Under salt stress, pinitol accumulates to over 9% of dry matter, with concentrations exceeding 700 mM in *M. crystallinum* leaves (Paul and Cockburn, 1989; Bohnert *et al*., 1995). Inositol and ononitol, which are precursors to pinitol, accumulate significantly and simultaneously with the build-up of Na^+ (Nelson *et al*., 1998). The long-distance transport of sodium from roots to growing apices seems to be based upon sodium/inositol symporters that function in conjunction with sodium/proton antiporters (Nelson *et al*., 1998, 1999; Chauhan *et al*., 2000). These findings suggest that EBCs play an important role in the salinity tolerance of *M. crystallinum*; however, direct evaluation of the true functional contribution of EBCs to the survival of the ice plant under stress conditions has not been possible.

The first large-scale mutant collections generated from fast-neutron-irradiated *M. crystallinum* seeds were established by Bohnert and Cushman (2000). Here the isolation and phenotypic characterization of an EBC-less mutant is described. Direct comparison of wild-type and EBC-less mutant plants confirms earlier predictions that EBCs might confer an adaptive advantage to *M. crystallinum* under salt stress conditions by acting as a water storage organ and/or a reservoir for vacuolar salt sequestration and ion homeostasis.

Materials and methods

Establishment of mutant seed banks

Wild-type *Mesembryanthemum crystallinum* L. seeds were collected by Klaus Winter in Israel (Winter *et al*., 1978). The plants selfed for approximately 10 generations. Seeds were irradiated by fast neutron bombardment at a range of doses from 20 Gy to 120 Gy (Gray, an SI unit of radiation dose equal to 1 joule of energy deposited in 1 kg of tissue or other material, which is an often-used unit for absorbed dose) using a ^{60}Co source at the reactor of the International Atomic Energy Agency (courtesy of Dr H Brunner, Vienna, Austria). Irradiated seeds were surface-sterilized using 50% (v/v) sodium hypochlorite containing 0.04% (v/v) Tween 20 for 5 min. After rinsing the seeds with sterile, deionized water five times, the seeds were germinated on germination medium containing 4.3 g l^{-1} MS salts (Murashige and Skoog, 1962), 1× B-5 (Gamborg *et al*., 1968), vitamins (100 mg l^{-1} myo-inositol, 10 mg l^{-1} thiamine–HCl, 1 mg l^{-1} nicotinic acid, 1 mg l^{-1} pyridoxine–HCl), 3% w/v sucrose, and 0.5% w/v type A agar (Sigma, St Louis, MO, USA), pH 5.7. The seedlings were grown in a growth chamber under 100 μmol m^{-2} s^{-1} of cool white fluorescent light on a 12 h (26 °C) light/12 h dark (18 °C) photoperiod. After 1 month the survival of the seedlings was recorded.

Plant growth and screening for EBC mutants

Seeds irradiated with 40 Gy and 50 Gy were selected for further study. Seed lots were germinated in Metromix 200 (Grace Sierra Horticultural Products, Marysville, OH, USA) in a growth chamber on a 12-h (26 °C) light/12-h dark (18 °C) cycle. Fluorescent and incandescent lighting provided a photon flux density of 450–500 μmol m^{-2} s^{-1}. Ten-day-old seedlings were transplanted to 24-well flats (~0.2 l $cell^{-1}$) containing Metromix 200 and irrigated once daily with a 0.5× Hoagland's solution No. 2 (Hoagland and Arnon, 1938). The seedlings were then transferred and grown in a greenhouse under natural sunlight with the temperature oscillating between 18 °C and 25 °C and fertilized daily with the 0.5× Hoagland's solution until the plants set seed. M_2 seeds were collected from dried seed pods and surface-sterilized, plated on agar, and transplanted to flats, and grown in greenhouse conditions

as described above. The EBC-less mutant was identified by visual inspection from the segregating M_2 population. EBC-less mutants were allowed to self-propagate and M_3 seeds were collected and cleansed for further analysis.

Plant materials and NaCl treatments

The M_3 seeds and plants were used for the experiments of long-term survival and reproductive success. The seeds of both types of plants were germinated in Metromix 200 for 10 d. The plants were transferred to 1.0 l Styrofoam® pots and grown for 4 weeks watered with 0.5× Hoagland's solution No. 2, under the same condition as those used for mutant screening. NaCl in the irrigation solution was applied to 6-week-old plants at concentrations as indicated in the figures and figure legends. After the plants had completed flowering and seed set, the pods and seeds were harvested, counted, and weighed to estimate the reproductive capability of each plant.

To measure biomass, ion content, and distribution, and make anatomical observation, M_5 seeds and plants were used. Seeds of wild type and mutant were sterilized and sown on MS agar as described above, and the plates were placed in a growth chamber (NK System Biotron; Nippon Medical & Chemical instruments Co., Ltd, Tokyo, Japan) that provided a photosynthetic photon flux of 250 μmol m^{-2} s^{-1} from cool-white fluorescent bulbs on a 16 h (22 °C) light/8 h dark (19 °C) cycle. The 7-d-old plants were transplanted and grown in the growth chamber for 10 d in 40 l tubs containing hydroponic culture solution containing a mixture of 0.075% (w/v) of Otsuka House solution No. 1 and 0.05% (w/v) of Otsuka House solution No. 2 (Otsuka Chemicals Co., Ltd, Osaka, Japan). The Otsuka House solution No. 1 contained N, 10%; P_2O_5, 8%; K_2O, 27%; MgO, 4%; MnO, 0.1%; B_2O_3, 0.1%; Fe, 0.18%; Cu, 0.002%; Zn, 0.006%; and Mo, 0.002%; and the Otsuka House solution No. 2 contained N, 11%; and CaO, 23%. The plants were then transplanted to a Hyponica hydroponic system (Kyowa, Co., Ltd, Osaka, Japan) and grown in greenhouse conditions (27 °C day and 23 °C night) at the Kyushu Electric Power Company, Inc. (Saga, Japan). NaCl was added to the culture solution when plants were 45-d-old at concentrations indicated in the figures and figure legends. The plants were harvested every 7 d for 21 d after NaCl treatment. Leaf, stem, and root fresh weights were determined immediately after harvest.

Dry weights were determined after drying tissues in an 80 °C oven for 2 d. The water content (*WC*) of leaf and stem tissues was calculated using the following formula: $WC=[(\textit{fresh weight}-\textit{dry weight})/\textit{fresh weight}]\times 100$. Statistically significant differences in fresh and dry weights were determined by Mann–Whitney *U*-test.

Ion content determination

One gram of dry material was ground with a mortar and pestle with 20 ml of deionized water and centrifuged for 15 min at 3000 *g*. The supernatant was filtered through Whatman 3MM no. 1 filter paper. The filtrates were used for ion content determination. The Na^+ and Cl^- contents of the solutions were determined using an atomic absorption spectrophotometer (Shimadzu, SPCA-626D, Kyoto, Japan) and a capillary ion analyser (Waters, Quanta 4000E, Milford, MA, USA), respectively. For ion analyses, contents of bladder cells were collected into a small glass capillary. After destroying all bladder cells of a leaf or a piece of stem, the surface of the remaining tissue was carefully rinsed and blotted dry. The cell sap was expelled from the remaining leaf or stem tissues between two stainless steel plates and collected with glass capillaries. In some cases leaf cells were broken and sap collected from the wound site with small glass capillaries. The contents of Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, NO_3^-, PO_4^{3-}, and SO_4^{2-} were determined using ion-exchange chromatography (Dionex, BioLc, Sunnyvale, CA, USA).

Light and electron microscopic analysis

The stem and leaves of 6-week-old plants were excised and prepared for light stereomicroscopic observations using a stereomicroscope (SZX12, Olympus, Tokyo, Japan). For scanning electron microscopy, epoxy replicas of epidermal cells were prepared as described (Green and Linstead, 1990). Dental impression material (Extrude Wash, Kerr Corp., Orange, CA, USA) was applied to the leaf surface, incubated for 5 min, and then peeled off. The negative moulds were filled with epoxy resin (2-Ton Epoxy S-31; Devcon Corp., USA), polymerized for 1 h at 70 °C, critical-point dried in liquid CO_2, coated with gold and palladium at 10–15 nm thickness, and analysed at 10–20 kV acceleration voltage using a scanning electron microscope (model S-2050, Hitachi, Tokyo, Japan).

Results

Mutant screening and phenotype

By visual inspection, 368 lines (7236 plants) and 1149 lines (18 317 plants) of M_2 lines irradiated with 40 Gy and 50 Gy of the fast neutron, respectively, were screened. One line (78E1) with a nearly complete absence of EBCs on its leaf and stem surfaces was isolated. When grown in soil or hydroponically (Fig. 1), the EBC-less mutant appeared similar to wild-type plants in all respects, including the ability to perform CAM, except that its leaves had a glossy appearance. The mutant exhibited less growth than the wild type, particularly following high salinity stress (800 mM) for 2 weeks (Fig. 1B). The leaf tips of the mutant turned brown within 1 week of treatment with 800 mM NaCl (Fig. 1B).

Fine structural analysis of the EBC-less mutant by light and electron microscopy revealed a lack of EBCs on both the adaxial and abaxial surfaces of leaves and stem. Wild-type plants typically exhibited large circular or elliptical ('sausage-like'; Adams *et al*., 1998) EBCs on the surface of leaves (Fig. 2A, C) and stems (Fig. 2E), respectively, whereas, these cells were absent in the mutant (Fig. 2B, D, F). In wild-type plants, EBCs on the abaxial surfaces of leaves appeared larger (~10%) than those of the adaxial surfaces (Fig. 2A–C). EBCs located on the stem were much larger and more conspicuous than those on leaves and, therefore, the difference between wild type and mutant was more obvious (Fig. 2E, F). Scanning electron microscopy revealed similar results to those observed by light stereomicroscopy (Fig. 3). On the adaxial surfaces of the mutant, some epidermal cells had a rudimentary bladder shape, although these cells appeared much smaller and were arranged irregularly compared with wild-type leaves (Fig. 3A, B). EBCs were completely absent from the abaxial surface of the mutant (Fig. 3C, D).

EBC-less mutant growth performance under salinity stress

Growth performance of wild-type and EBC-less mutant plants was compared by growing plants hydroponically,

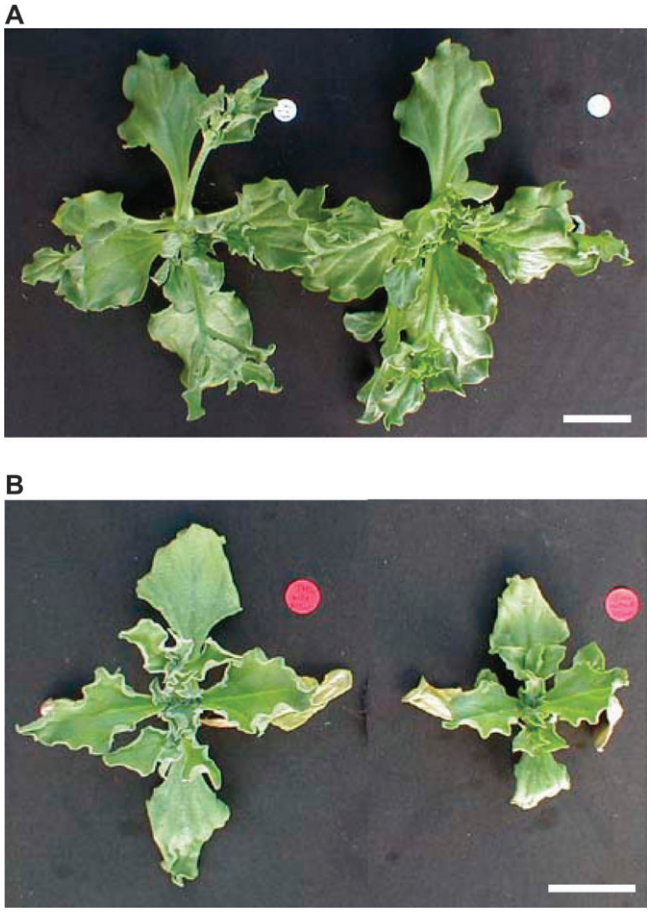

Fig. 1. Comparison of the gross appearance of 6-week-old wild-type and EBC-less mutant plants. Wild-type (left) and mutant (right) plants were grown with (A) 0 mM NaCl or (B) 800 mM NaCl for 2 weeks. Bars indicate 5 cm.

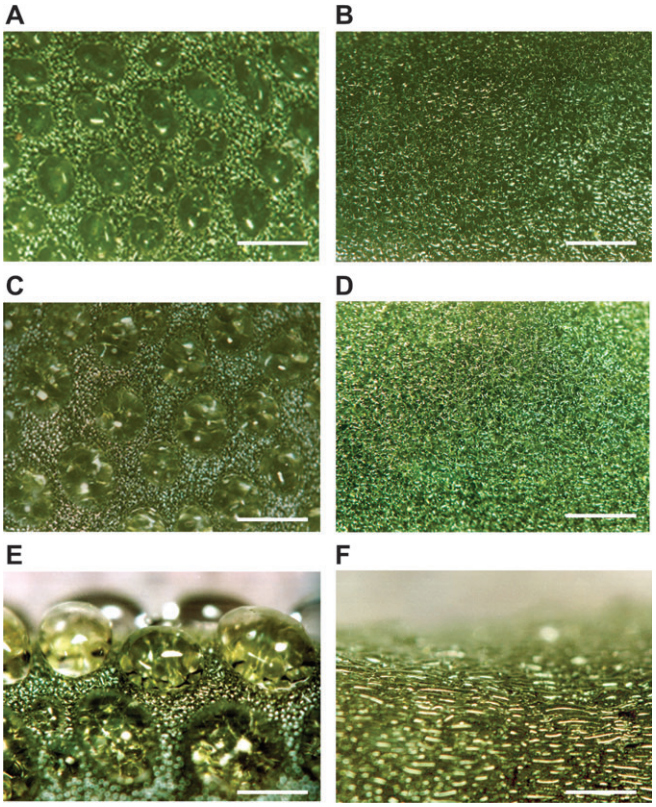

Fig. 2. Light microscopy images of epidermal bladder cells on leaves and stems of wild-type and EBC-less mutant plants. Plants were grown with 300 mM NaCl for 25 d. (A, C, E) Wild-type; (B, D, F) EBC-less mutant; (A, B) adaxial surface of leaves; (C, D) abaxial surface of leaves; (E, F) stem surfaces. Bars indicate 500 μm.

exposing them to different salt concentrations ranging from 0 to 800 mM, and measuring dry weights of aerial portions of the plants (Fig. 4). The dry weights plotted did not include the weight of NaCl. Optimal growth for both wild-type and EBC-less mutant plants occurred between 100 mM and 200 mM NaCl (Fig. 4). However, higher concentrations of NaCl (400 mM and 800 mM NaCl) significantly impaired the growth of the mutant as indicated by dry matter accumulation (Fig. 4). After 3 weeks of salt stress with 400 mM NaCl, the dry weight of wild-type plants was almost 2-fold that of mutant plants.

Succulence and water content

Leaf succulence was evaluated by specific leaf weight (fresh weight per leaf area) and was found to be higher for wild-type compared with EBC-less mutant plants particularly after 2 weeks of 400 mM NaCl treatment (Fig. 5A). The relative percentage water content in the leaves and stems of the mutant was also lower than that of wild-type plants, especially following stress with 400 mM NaCl for 1–2 weeks (Fig. 5B).

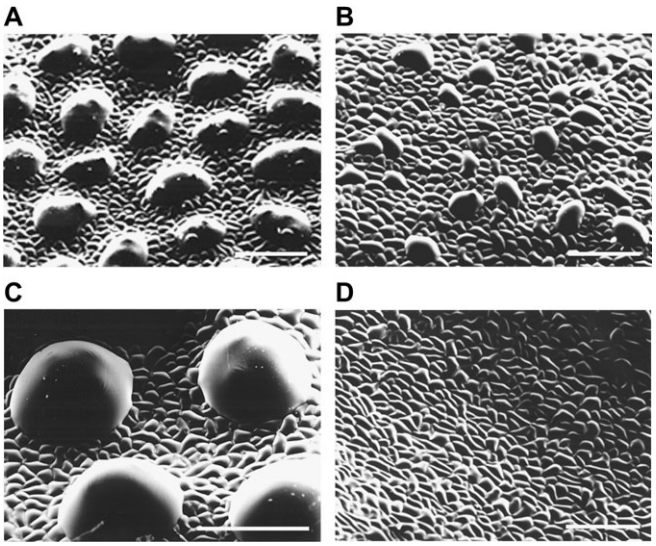

Fig. 3. Scanning electron microscopy images for epidermal bladder cells on leaves of wild-type and EBC-less mutant plants. Plants were grown with 300 mM NaCl for 25 d. (A, C) Wild type; (B, D) mutant; (A, B) adaxial surface; (C, D) abaxial surface. Bars indicate 500 μm.

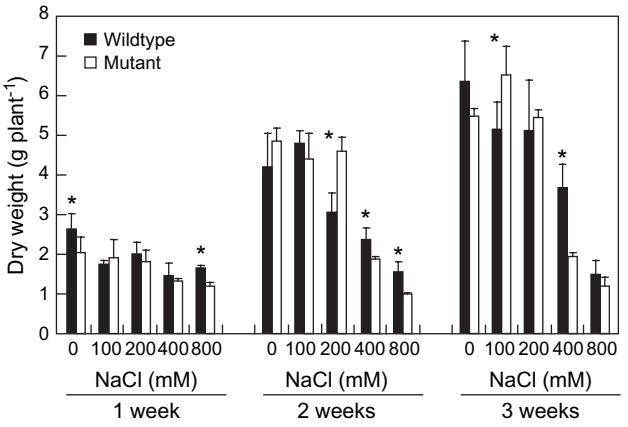

Fig. 4. Effects of NaCl on the growth of wild-type and EBC-less mutant plants. The dry weight minus NaCl weight of the aerial parts of hydroponically grown plants was measured following exposure from 0 to 800 mM NaCl for 1, 2, and 3 weeks. Measurements were from four plants ±standard deviation. Asterisks indicate statistical differences at P <0.05 as determined by analysis of variance with a Mann–Whitney U-test.

Sodium and chloride content of leaves and stems

To investigate the contribution of EBCs to salt accumulation within the aerial portions of the plant, Na^+ content was measured following 1–3 weeks of salt stress treatment using 0–800 mM NaCl. Under severe salt stress (400 mM or 800 mM NaCl), wild-type plants absorbed a significantly higher content of Na^+ (Fig. 6A) and Cl^- (Fig. 6B) than EBC-less mutant plants. The general trends of Cl^- content for both wild-type and EBC-less mutant plants were similar with the EBC-less mutant accumulating about 2-fold less Cl^- at 400 mM NaCl (Fig. 6B).

Ionic concentration in the leaves and stems

To investigate the role that EBCs have on the accumulation of other cations and anions, the content of these ions was measured in the leaves of wild-type and EBC-less mutant plants grown with 0, 100, and 400 mM NaCl for 2 weeks and expressed on a dry weight basis (Fig. 7A–C). These values were calculated on the basis of dry weight without NaCl. Upon incorporation of Na^+ and Cl^- into tissues, the relative content of other anions and cations was reduced. In the mutant grown with 400 mM NaCl for 2 weeks, the content of K^+, NO_3^-, and PO_4^{3-} were significantly lower than in the wild type. The mutant accumulated Na^+ and Cl^- inside leaves at concentrations similar to the sum of those ions partitioned between bladder cells and underlying leaf tissue of wild-type plants. Photosynthetically active leaf tissues of wild-type plants carried a relatively lower ion load than leaves of the EBC-less mutant, particularly under conditions of high salt stress (Fig. 7C). A similar tendency was observed in the stems (data not shown).

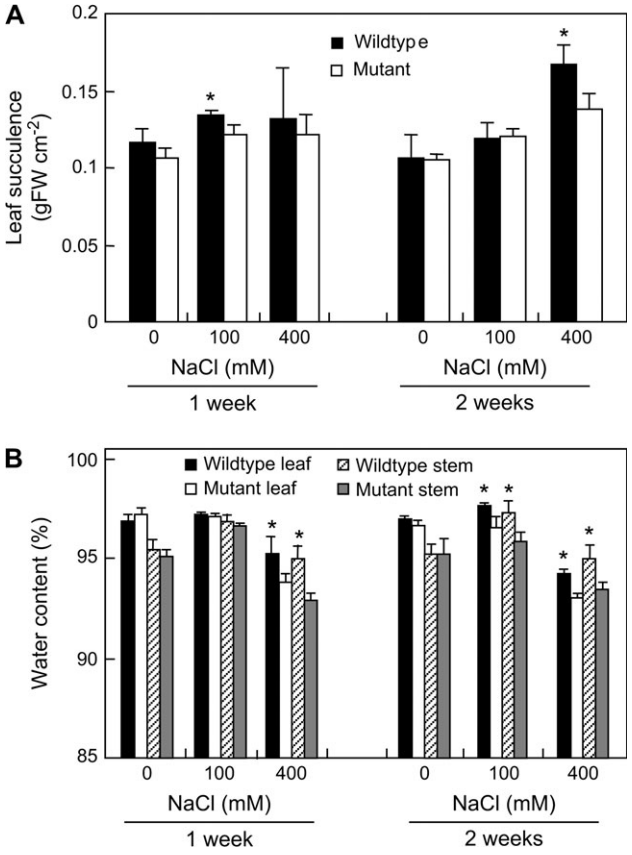

Fig. 5. Effects of NaCl on leaf succulence and the water content of leaves and stem in wild-type and EBC-less mutant plants, grown with different salt concentrations, ranging from 0 to 400 mM for 1 and 2 weeks: (A) leaf succulence; (B) water content of leaves and stems. Measurements were from four plants ±standard deviation. Asterisks indicate statistical differences at P <0.05 as determined by analysis of variance with a Mann–Whitney U-test.

Salt distribution between epidermal bladder cells and underlying photosynthetically active tissues

To better illustrate the sequestration of Na^+ and Cl^- within leaf and stem tissues of wild-type and EBC-less mutant plants and between leaf and EBCs, the concentration of Na^+ and Cl^- in EBC and leaf and stem tissues of wild-type and EBC-less mutant plants was determined (Fig. 8). Na^+ and Cl^- accumulated to higher concentrations in the underlying tissues of wild-type plants than in the EBCs or leaf tissue of the EBC-less mutant, with the exception of leaves of plants grown with 100 mM NaCl in which case these ions were higher in the EBCs. Na^+ and Cl^- were not concentrated preferentially into EBCs at 400 mM NaCl treatment. The salt storage capacity of the EBC-less mutant was impaired in both leaves and stems under high salinity stress conditions.

Ionic balance in different types of cells

In order to assess the possible role of EBCs in differential ion sequestration, the Na^+ and K^+ concentrations, or Cl^-

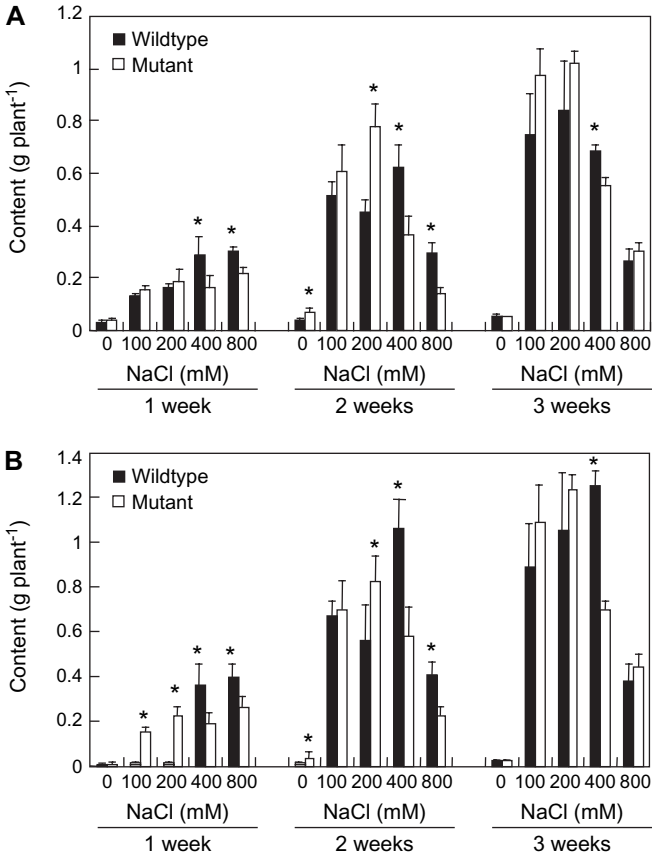

Fig. 6. Total amounts of Na^+ and Cl^- in the aerial parts of wild-type and EBC-less mutant plants grown with different salt concentrations, ranging from 0 to 800 mM for 1, 2, and 3 weeks: (A) Na^+; (B) Cl^-. Measurements were from four plants ±standard deviation. Asterisks indicate statistical differences at P <0.05 as determined by analysis of variance with a Mann–Whitney U-test.

and NO_3^- concentrations within EBCs, leaves and stems of wild-type and EBC-less mutant plants grown with 0, 100, and 400 mM NaCl for 1 and 2 weeks were plotted (Fig. 9). In leaves, EBCs and underlying tissues of wild-type and EBC-less mutant plants, with increasing $[Na^+]$ from about 50 mM to 200 mM, $[K^+]$ was reduced from about 150 mM to 70 mM. The underlying tissues of wild-type leaves accumulated Na^+ to above 700 mM and also incorporated K^+ up to 200 mM. By contrast, EBCs of the wild-type leaves and leaf tissues of the EBC-less mutant accumulated Na^+ up to only 600 mM and $[K^+]$ did not increase (Fig. 9A). Similar trends were observed in stems (Fig. 9C). $[NO_3^-]$ showed reduced accumulation with increasing $[Cl^-]$ in EBCs and underlying tissues of wild-type and mutant plants when they accumulated $[Cl^-]$ at lower concentrations (~200 mM in leaves and 300 mM in stems) (Fig. 9B, D). However, as $[Cl^-]$ was increased further by ~500 mM in leaves (B) and ~800 mM in stem (D), $[NO_3^-]$ increased in the underlying leaf and stem tissues of wild-type plants, but the relative increase in EBCs and the leaf/stem tissues of the EBC-less mutant was not as great as in wild-type plants (Fig. 9B, D).

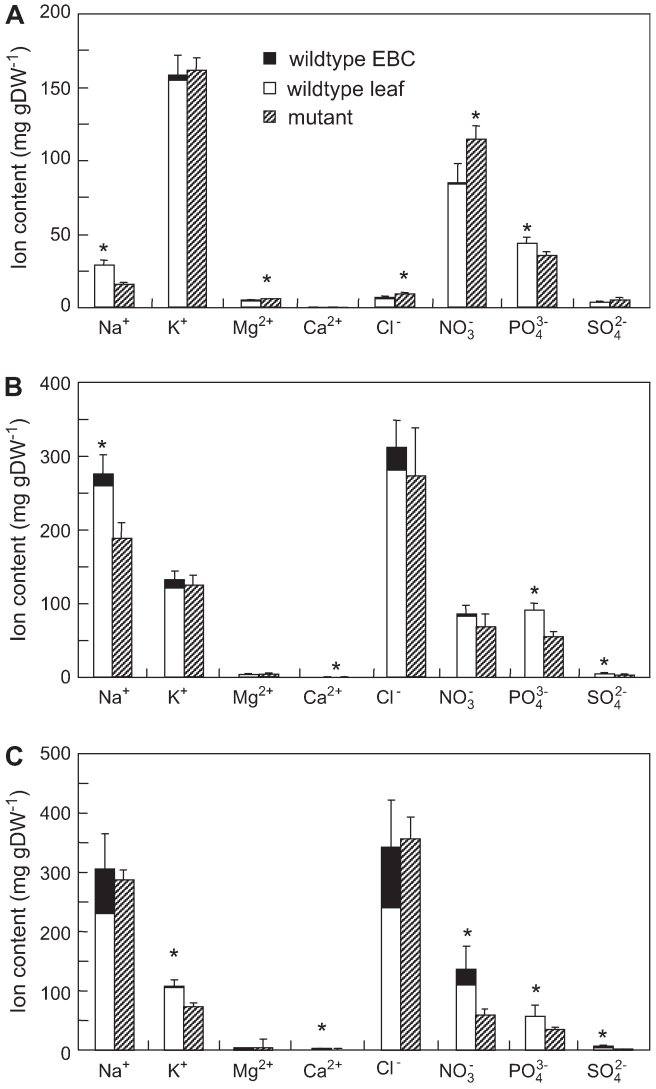

Fig. 7. Cation and anion content in epidermal bladder cells and leaves of wild-type and EBC-less mutant plants grown with different NaCl concentrations for 2 weeks: (A) 0 mM; (B) 100 mM; (C) 400 mM NaCl. Measurements were from four plants ±standard deviation. The contents were calculated on the basis of dry weight without NaCl. Asterisks indicate statistical differences at P <0.05 as determined by analysis of variance with a Mann–Whitney U-test.

Reproductive success following salt stress treatment

In order to test the contribution of EBCs to the reproductive success of the ice plant in the face of high salinity stress, both wild-type and EBC-less mutant plants were subjected to a series of different salt concentrations ranging from 0 mM to 800 mM NaCl over the lifetime of the plant from age 6 weeks to the completion of flowering and seed set. The effect of salt stress was measured by counting the number of seeds produced, the total seed weight, the average weight of individual seeds, and the number of flowers counted as seed pods (Fig. 10). The wild-type plants showed optimal seed production at

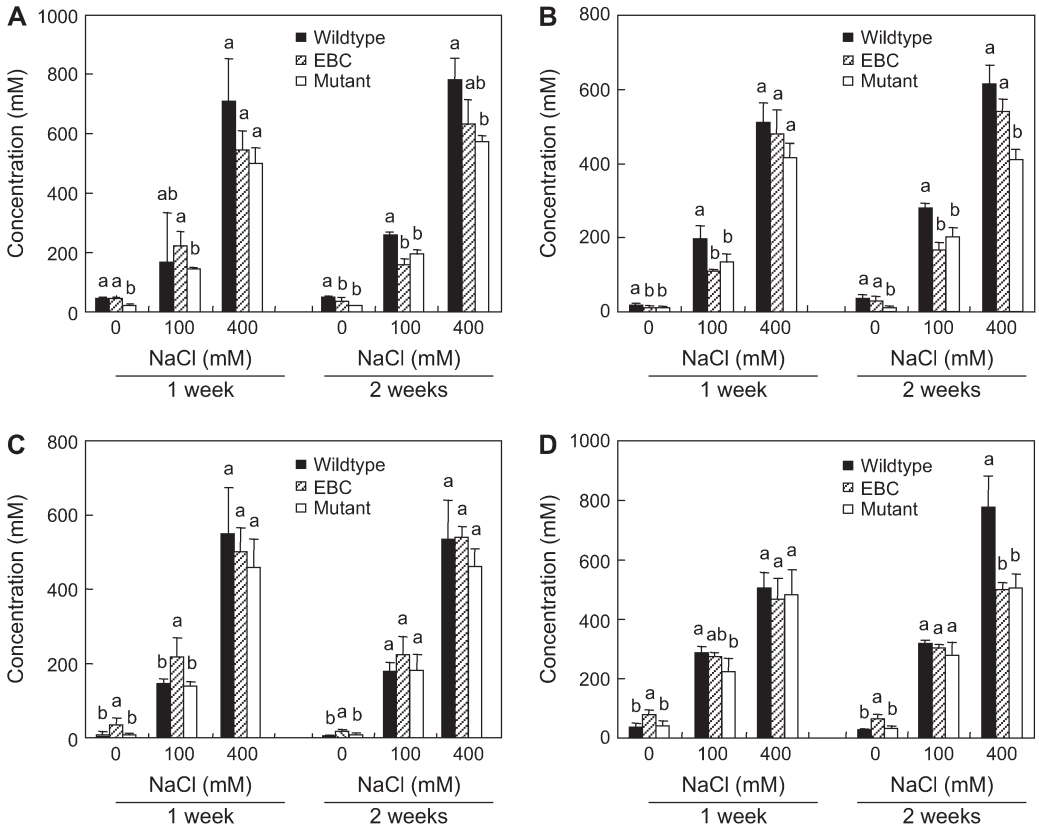

Fig. 8. Comparison of Na^+ and Cl^- concentrations in epidermal bladder cells of wild-type plants (EBC) and leaves and stems of wild-type and EBC-less mutant plants grown with 0, 100, and 400 mM NaCl for 1 week and 2 weeks: (A, C) leaf; (B, D) stem; (A, B) Na^+ concentration; (C, D) Cl^- concentration. Measurements were from four plants ±standard deviation. Letters (a, b) indicate statistical differences at $P < 0.05$ as determined by analysis of variance with Scheffe's test.

100–200 mM NaCl as assessed by seed number and total seed weight (Fig. 10A, B). Seed production decreased with increasing NaCl stress in both wild-type and mutant plants. However, EBC-less mutant plants showed only one-tenth the seed production of wild-type plants with optimal production occurring between 100 mM and 200 mM NaCl and production falling off dramatically above 500 mM NaCl (Fig. 10A, B). The average weight of individual seeds was similar for both wild-type and the EBC-less mutant plants at salt concentrations ranging from 0 mM to 500 mM NaCl; however, the average seed weight decreased dramatically at higher concentrations in the EBC-less mutant (Fig. 10C). Flower/pod production for both wild-type and EBC-less mutant plants was maximal at 0 mM NaCl, and it decreased with increasing NaCl concentration (Fig. 10D).

Discussion

Earlier reports suggested that EBCs served as an external water reservoir for metabolically active mesophyll cells (Haberlandt, 1904; Steudle *et al.*, 1975, 1977) and that they may support the water relations of the mesophyll in conjunction with the osmotically important oscillations of malate levels in mesophyll cells during CAM (Rygol *et al.*, 1989). Consistent with these earlier reports, comparison of the water content and leaf/stem succulence of the wild type and the EBC-less mutant showed that the presence of EBC resulted in higher leaf succulence and stem water content (Fig. 5). Greater leaf and stem succulence could ameliorate the ionic and osmotic stress effects of high salinity treatment and could also provide a long-term reservoir of water storage to facilitate improved reproductive capacity under salinity stress conditions (Fig. 10).

Another important potential role for EBCs is in osmotic adjustment and salt sequestration. EBCs store osmolytes including Na^+, Cl^-, compatible solutes such as polyols (e.g. D-ononitol and D-pinitol), and amino acids (e.g. proline). The accumulation of such compounds generates a turgor gradient that can accelerate the growth of new cells, which can, in turn, accommodate more NaCl (Lüttge, 1993). Adams *et al.* (1998) showed that *M. crystallinum* acquired salt tolerance only when organized tissues that included bladder cells were present. In their study, juvenile leaves having fewer and smaller EBCs did

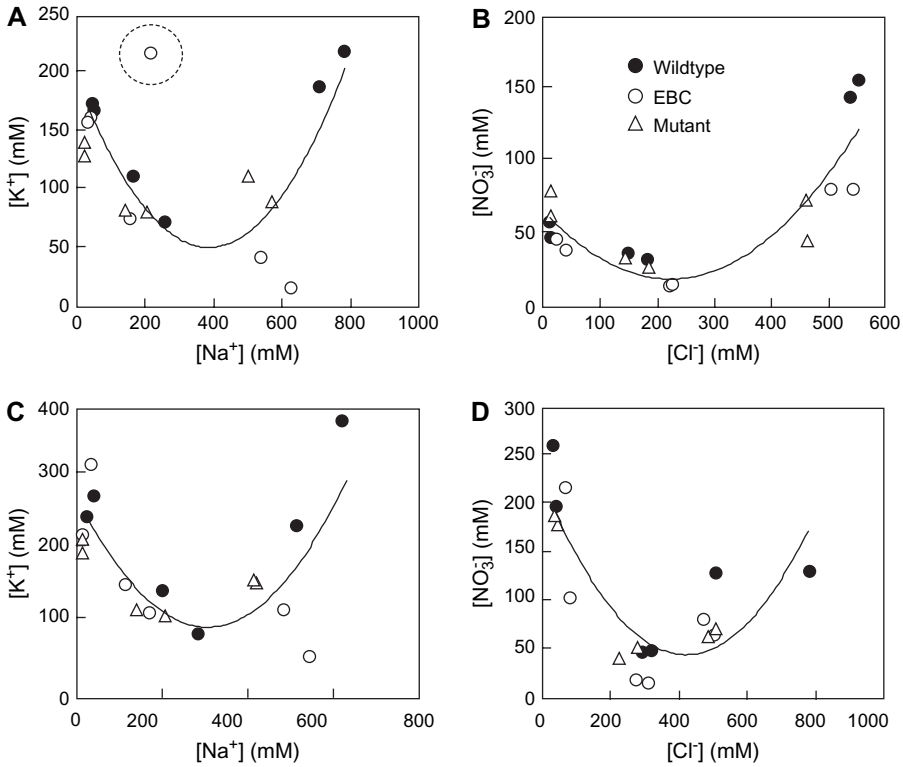

Fig. 9. Comparison of Na^+ and K^+ concentrations or Cl^- and NO_3^- concentrations in epidermal bladder cells of wild-type plants (EBC) and leaves and stems of wild-type and EBC-less mutant plants grown with 0, 100, and 400 mM NaCl for 1 week and 2 weeks: (A, B) leaf; (C, D) stem; (A, C) Na^+ concentration versus K^+ concentration; (B, D) Cl^- concentration versus NO_3^- concentration. The curves shown were approximated with a quadratic regression to describe the trends. The data point circled with dot-dashed line in (A) was excluded from the calculation.

not accumulate compatible solutes to the same concentrations present in mature leaves, suggesting perhaps that these younger leaves were subjected to greater salt toxicity. In a comparison of wild-type and EBC-less mutant plants, the total concentration of NaCl accumulated was similar in both plants, but the mutant was unable to partition part of the salt load into EBCs (Fig. 7). Therefore, NaCl accumulation in the cytosol of mesophyll cells is likely to be more toxic than in wild-type plants, resulting in greater salt damage (Fig. 1), lower growth rates as reflected in dry weight accumulation (Fig. 4), and poorer reproductive capacity (Fig. 10). This greater salt toxicity may disrupt other ion homeostasis functions and the ability to synthesize osmoprotectants, as observed in tissue-cultured cells and juvenile leaves. Although the rates of osmoprotectant biosynthesis were not compared in this study, the EBC-less mutant isolated here could serve as a useful model to elucidate the contribution of the bladder cells to osmotic adjustment in the common ice plant.

Some *Atriplex* species have bladders with a salt gland-like stalk cell, which can actively excrete NaCl from inside tissues into the bladders. *Mesembryanthemum crystallinum*, however, appears to incorporate NaCl into EBCs without the associated activity of such gland stalk cells (Fig. 8). Adams *et al*. (1998) suggested that NaCl was actively incorporated into EBCs, but they analysed the concentrations of Na^+ in bladder cell sap collected from the external surfaces of seed pods, not leaves. Barkla *et al*. (2002) showed that in the cell sap of leaves from plants treated with 200 mM NaCl for 2 weeks, the Na^+ concentration was higher in EBCs than in other cells: 458, 380, and 371 mM in EBCs, whole leaf, and mesophyll tissues, respectively. This tendency was consistent with the present results (Fig. 8), but not in the case of leaves of plants that contained NaCl at higher concentrations. When the leaves accumulated Na^+ to concentrations in excess of 600 mM, EBCs located on the leaf surface or stem accumulated less Na^+ than the underlying photosynthetic tissues (Fig. 8). It appears unlikely that Na^+ is being concentrated into EBCs against the NaCl concentration gradient established across the EBCs and underlying photosynthetic tissues (Fig. 8).

Comparison of ion concentrations in EBCs and underlying tissues of leaves and stems suggested that EBCs have a role in ion homeostasis within mesophyll tissues. In wild-type plants that accumulated Na^+ to high concentrations in stems (~500–600 mM) and in leaves (~700–800 mM), $[K^+]$ increased in the underlying tissues up to or slightly above the levels found in unstressed plants, but not in EBCs of wild-type plants or in leaves of the EBC-less mutant (Fig. 9A, C). Na^+ disturbs K^+

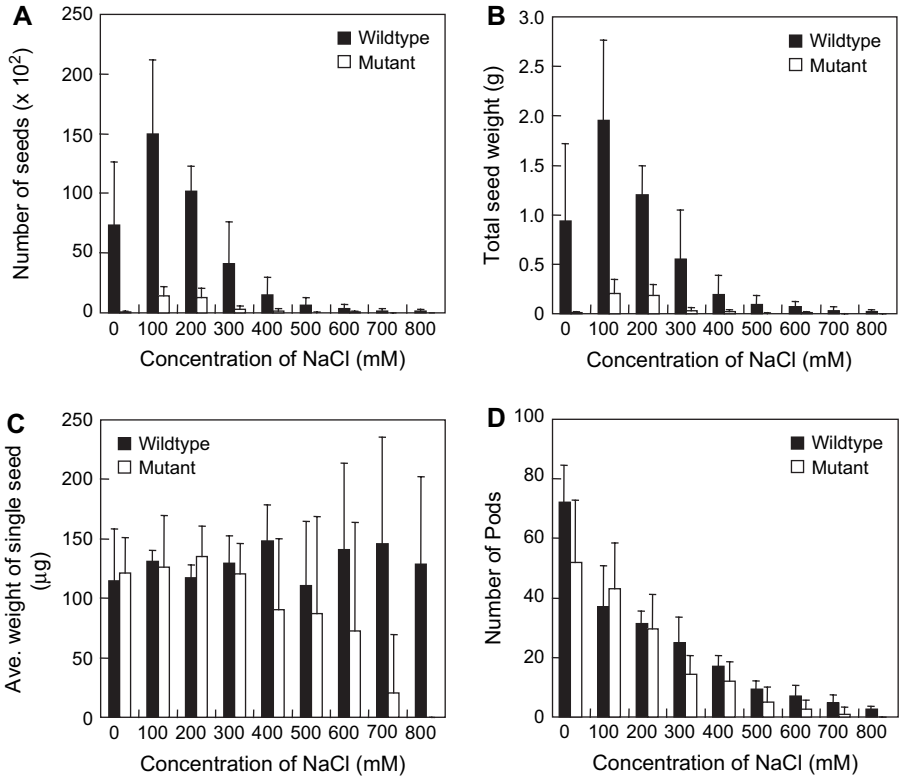

Fig. 10. Reproductive capacity of wild-type and EBC-less mutant plants under salt stress conditions: (A) total number of seeds per plant; (B) total seed weight per plant; (C) average weight of single seed; (D) total number of pods per plant. Values are means ±SD from six individual measurements.

homeostasis in plants because the cytotoxic Na^+ ions compete for binding sites in transport systems that mediate K^+ uptake (Niu *et al*., 1995; Hasegawa *et al*., 2000). Several K^+ transporter genes responsible for K^+ uptake have been isolated and characterized. In the common ice plant, the expression of KMT1 (a AKT/KAT family member) (Su *et al*., 2001) and various HAK/KUP (high affinity K^+ transporter/K^+ uptake transporter)-type genes (Su *et al*., 2002), and SKD1 (suppressor of K^+ transport growth defect) of the AAA (ATPase associated with a variety of cellular activities)-type ATPase family genes have shown increased steady-state transcript abundance in response to salt stress treatment. HAK transcripts are abundantly expressed in EBCs as well as in leaf vascular bundles, mesophyll, and epidermal cells (Su *et al*., 2002). SKD1, a full-length salt-induced transcript homologous to SKD1, was also preferentially expressed in the epidermal bladder cells of leaves and the outer cortex of roots and stems (Jou *et al*., 2004). These genes may function in maintaining optimal cytoplasmic K^+:Na^+ ratios in high salinity environments.

Changes in the concentrations of NO_3^- with increasing Cl^- showed a similar tendency to those of K^+ and Na^+ (Fig. 9B, D). Increased concentrations of chloride competed with NO_3^- uptake and accumulation (Liu and Shelp, 1996). Proline, a compatible solute, is synthesized *de novo* from glutamate (Nanjo *et al*., 1999; Hasegawa *et al*., 2000), thus maintenance of nitrogen homeostasis and amino acid uptake and transport systems is important in salt-stressed plants. The members of two different families of nitrate transporters have been identified: the NNP family (nitrate-nitrite porters) and the PTR family (Forde, 2000). In *M. crystallinum*, steady-state transcripts of *McNRT1*, which encodes a PTR-type nitrate, increase in abundance in both leaf and root tissues in response to salt stress treatment, although this gene appears to be expressed preferentially in roots. The ratios of Na^+:K^+ and Cl^-:NO_3^- were higher in the EBC-less mutant plants than in wild-type plants, suggesting that the Na^+ concentrations may have reached cytotoxic levels, such that the machinery for maintaining the homeostasis of these ions was disturbed. However, the *in vivo* functional roles of these transporters and associated changes in mRNA abundance remain to be determined.

The present comparative analysis of the EBC-less mutant with wild-type plants clearly showed that the bladder cells contribute to salt accumulation and tolerance in *M. crystallinum*. When the wild-type plants were cultured under 400 mM NaCl for 49 d, Na^+ and Cl^- were about 10 mg and 12 mg per fresh weight, and the content of both Na^+ and Cl^- was about 5 g in the shoot of a single plant (data not shown). The capacity *M. crystallinum* to

accumulate large amounts of salt makes it a potentially useful model for the reclamation of saline soils. EBCs were responsible for accumulating ~30% and ~25% of the total Na^+ and Cl^-, respectively, in the leaves of wild-type plants. Although the genes responsible for the development of EBCs have not yet been cloned from the ice plant, the molecular characterization of these genes will certainly advance our understanding of salt tolerance and salt accumulation mechanisms in *M. crystallinum* and related succulent plants that utilize epidermal bladder cells.

Acknowledgements

This work was supported, in part, by the Salt Science Research Foundation (grant no. 0120 and 0217 to SA), the Foundation for Earth Environment (in 2001 and 2002 to SA), the National Science Foundation Integrative Plant Biology Program (IBN-0196070 to JCC), and the Nevada Agricultural Experiment Station, publication #03077009.

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