Plant Physiology, July 1999, Vol. 120, pp. 811–819, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Abscisic Acid Induction of Vacuolar H^+-ATPase Activity in *Mesembryanthemum crystallinum* Is Developmentally Regulated[1]

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Abscisic acid (ABA) has been implicated as a key component in water-deficit-induced responses, including those triggered by drought, NaCl, and low- temperature stress. In this study a role for ABA in mediating the NaCl-stress-induced increases in tonoplast H^+-translocating ATPase (V-ATPase) and Na^+/H^+ antiport activity in *Mesembryanthemum crystallinum*, leading to vacuolar Na^+ sequestration, were investigated. NaCl or ABA treatment of adult *M. crystallinum* plants induced V-ATPase H^+ transport activity, and when applied in combination, an additive effect on V-ATPase stimulation was observed. In contrast, treatment of juvenile plants with ABA did not induce V-ATPase activity, whereas NaCl treatment resulted in a similar response to that observed in adult plants. Na^+/H^+ antiport activity was induced in both juvenile and adult plants by NaCl, but ABA had no effect at either developmental stage. Results indicate that ABA-induced changes in V-ATPase activity are dependent on the plant reaching its adult phase, whereas NaCl-induced increases in V-ATPase and Na^+/H^+ antiport activity are independent of plant age. This suggests that ABA-induced V-ATPase activity may be linked to the stress-induced, developmentally programmed switch from C_3 metabolism to Crassulacean acid metabolism in adult plants, whereas, vacuolar Na^+ sequestration, mediated by the V-ATPase and Na^+/H^+ antiport, is regulated through ABA-independent pathways.

The involvement of the plant growth regulator ABA in the responses of plants to water deficit has long been recognized. Endogenous levels of ABA have been shown to be elevated when plants are stressed with drought and/or NaCl, and application of ABA to unstressed plants results in the induction of numerous water-deficit-related activities. These may range from immediate responses, including the triggering of stomatal closure to reduce transpirational water loss by posttranslational modulation of ion channels in guard cells (Grabov and Blatt, 1998), to alterations in gene expression through the induction of ABA-responsive genes encoding for structural, metabolic, or transport proteins, as well as protein kinases and phosphatases (for reviews, see Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996, 1997; Bray, 1997). A role for ABA as a universal water-deficit-sensitizing signal, however, is clearly not the case. With the characterization of ABA-deficient and -insensitive mutants from Arabidopsis, evidence for water-stress-inducible genes that do not require accumulation of endogenous ABA have been identified (Gosti et al., 1995). Moreover, identification of a *cis*-acting dehydration-responsive element, distinct from ABA-responsive elements, in water-deficit-induced genes that does not respond to ABA treatment provides further support (Yamaguchi-Shinozaki and Shinozaki, 1994). These findings led to the conclusion that both ABA-dependent and -independent pathways transduce the water-deficit signal. However, more recently, a greater level of complexity to ABA signaling has been added with the findings that these ABA-dependent and -independent pathways actually cross-talk and converge to activate genes involved in the stress response to cold, drought, and salinity (Ishitani et al., 1997).

Mesembryanthemum crystallinum L. is a halophytic plant that responds to NaCl and drought stress by shifting its pathway of carbon assimilation from C_3 metabolism to the more water-conserving CAM. This metabolic transition involves the transcriptional induction of genes encoding key enzymes in the CAM pathway (for review, see Cushman and Bohnert, 1997). Several of these genes have also been shown to be up-regulated by exogenous ABA. These include the CAM-specific isoform of PEPC, 2-phospho-D-glycerate hydrolase (enolase), and phosphoglyceromutase (Chu et al., 1990; McElwain et al., 1992; Forsthoefel et al., 1995a, 1995b). Promotor analysis of the PEPC gene (*Ppc1*) has identified a putative ABA-responsive element that may be involved in ABA-responsive expression of this gene (Schaeffer et al., 1995). Correlated with these findings are the increased levels of endogenous ABA measured in the drought-stressed plant (Thomas et al., 1992).

Although environmentally triggered, CAM induction in *M. crystallinum* is developmentally regulated (Cushman et al., 1990). CAM cannot be elicited in NaCl-, drought-, or ABA-treated juvenile plants, and CAM inducibility by these factors coincides with the transition to mature growth (Adams et al., 1998). In contrast, NaCl-induced synthesis of compatible solutes and vacuolar Na^+ accumulation, mechanisms of adaptation also considered essential for growth

[1] This work was supported by Consejo Nacional de Ciencia y Tecnología grant no. 3281PN to B.J.B.

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Abbreviations: *F*, fluorescence intensity; IMT, *myo*-inositol *O*-methyltransferase; PEPC, PEP carboxylase; V-ATPase, tonoplast H^+-translocating ATPase.

of *M. crystallinum* under these conditions, are observed independently of the age of the plants (Adams et al., 1998). This would indicate distinct signaling pathways involved in the induction of these three major responses.

Well characterized is the synthesis of compatible solutes in NaCl-stressed plants. In *M. crystallinum* the sugar alcohol pinitol accumulates in stressed plants to become the major compatible solute (Adams et al., 1998). Like enzymes involved in CAM induction, this response is also controlled at the level of gene expression. Both *myo*-inositol-1-phosphate synthase and IMT, two enzymes in the pathway leading to pinitol synthesis, are transcriptionally induced by salt stress (Ishitani et al., 1996; Nelson et al., 1998). However, transcripts are induced neither by drought nor by exogenous application of ABA, although transcript accumulation for IMT can be elicited by cold stress (Vernon et al., 1993).

Compared with CAM induction and compatible solute synthesis relatively little is known about the vacuolar accumulation of Na^+ in *M. crystallinum* and of the means by which this adaptive response is regulated. Secondary active transport of Na^+ across the tonoplast would be energized by the proton gradient generated by the activity of the V-ATPase. Several groups have reported increases in V-ATPase activity in tonoplasts isolated from leaves of NaCl-treated *M. crystallinum* plants (Rockel et al., 1994; Barkla et al., 1995; Tsiantis et al., 1996). Furthermore, the NaCl-induced increase in V-ATPase activity can be correlated with changes in the amount of protein or transcripts for specific V-ATPase subunits. Dietz and Arbinger (1996) showed slight increases in antibody recognition of the V-ATPase E-subunit in leaves from NaCl-stressed plants, and transcript amounts for the 16-kD proteolipid c subunit of the enzyme have been shown to be specifically elevated under NaCl stress (Löw et al., 1996; Tsiantis et al., 1996). Recently, it was suggested that NaCl-induced proteolytic processing of the V-ATPase B-subunit may serve to regulate the activity of the enzyme (Zhigang et al., 1996). This NaCl-stimulated increase in V-ATPase activity has been associated with a parallel increase in Na^+/H^+ antiport activity in adult *M. crystallinum* plants exposed to NaCl stress (Barkla et al., 1995).

In the present study we initiated research aimed at understanding the mechanisms regulating tonoplast Na^+/H^+ antiport and V-ATPase activity to begin deciphering components of the signal transduction pathway leading to increases in activity of these transporters under NaCl stress. We have investigated the regulatory role of NaCl and ABA and studied the possibility of a developmental program controlling the response of these transporters.

MATERIALS AND METHODS

Plant Materials

Mesembryanthemum crystallinum L. plants were grown from seeds (derived from material originally collected by Dr. K. Winter, Caesarea, Israel; Winter et al., 1978) in soil (MetroMix 200 [Scotts, Marysville, OH], mixed 2:1 [v/v] with Canadian peat moss [Fatard Peat Moss, Inkerman, MB, Canada]) in a propagation tray. Ten days to 2 weeks following germination seedlings were transferred to pots with two plants per 15-cm-diameter pot or four plants per 21-cm-diameter pot. All plants were watered daily with tap water (unless under treatment), and one-half-strength Hoagland medium (Hoagland and Arnon, 1938) was applied weekly to all plants. NaCl and/or ABA treatment was initiated either 3 weeks (juvenile plants) or 6 weeks (adult plants) after germination. NaCl was applied to the plants by increasing daily the amount added to tap water or to one-half-strength Hoagland medium (increments of 50 mM/d) until the desired concentration was reached (either 200 or 400 mM NaCl). ABA (25 μM ±-*cis*,*trans*-ABA, Sigma) in 0.005% (v/v) Tween 20 was sprayed daily onto leaves of well-watered or NaCl-treated plants. Plants were grown in a greenhouse under natural irradiation and photoperiod. Minimum temperatures ranged from 12°C to 18°C, and the maximum temperature was maintained at 28°C.

Isolation of Tonoplast Vesicles

Leaves from *M. crystallinum* were harvested and sliced into small pieces following the removal of major veins. Leaf material (60 g fresh weight) was placed directly into 300 mL of ice-cold homogenization medium, and all subsequent operations were carried out at 4°C, as previously described (Barkla et al., 1995). The homogenization medium consisted of 400 mM mannitol, 10% (w/v) glycerol, 5% (w/v) PVP-10, 0.5% (w/v) BSA, 1 mM PMSF, 30 mM Tris, 2 mM DTT, 5 mM EGTA, 5 mM $MgSO_4$, 0.5 mM butylated hydroxytoluene, 0.25 mM dibucaine, 1 mM benzamidine, and 26 mM K^+-metabisulfite, adjusted to pH 8.0 with H_2SO_4. Leaf tissue was homogenized in a commercial blender, filtered through four layers of cheesecloth, and centrifuged at 10,000*g* (20 min at 4°C) using a rotor (model SS-34, Sorvall) in a superspeed centrifuge (model RC5C, Sorvall, DuPont). Pellets were discarded, and the supernatants were centrifuged at 80,000*g* (50 min at 4°C) using a fixed-angle rotor (model 55.2 Ti, Beckman) in an ultracentrifuge (model L8-M, Beckman). The supernatant was aspirated, and the microsomal pellet was resuspended using a 10-mL glass tissue homogenizer in a suspension medium consisting of 400 mM mannitol, 10% (w/v) glycerol, 6 mM Tris/Mes (pH 8.0), and 2 mM DTT. The microsomal suspension was then layered onto discontinuous Suc gradients consisting of a top layer of 9 mL of 22% (w/v) Suc over 9 mL of 34% (w/v) Suc and a bottom layer of 9 mL of 38% (w/v) Suc, all in the appropriate suspension medium.

After the samples were centrifuged at 100,000*g* (2 h at 4°C) using a swinging bucket rotor (model SW 28, Beckman) in an ultracentrifuge (model L8-M, Beckman), membranes at the 0/22% Suc interface, corresponding to the tonoplast (Struve and Lüttge, 1987; Barkla et al., 1995), were removed with a Pasteur pipette. These membranes were diluted with the suspension solution and sedimented at 80,000*g* (1 h at 4°C) using a fixed-angle rotor (model 55.2 Ti) in an ultracentrifuge (model L8-M), and the final membrane pellet was resuspended in 200 μL of the same solution. Membranes were frozen directly in liquid N_2 and stored at −80°C in 50-μL aliquots. Those used for quina-

crine fluorescence measurements were subject to only a single freeze/thaw cycle, because additional cycles increased the leakiness of the vesicles.

Extraction of Total Protein

Leaves of *M. crystallinum* were frozen and ground in liquid N_2 in a mortar and pestle to obtain a fine powder. Powdered tissue (2 g) was vortexed for 1 min with 2 mL of extraction buffer (100 mM Tris-Mes [pH 7.5], 1 mM EGTA, 5 mM DTT, 4 mM $MgSO_4$, 1 mM benzamidine, 1 mM PMSF, and 5% [w/v] insoluble PVP). The samples were filtered through one layer of Miracloth (Calbiochem), and the crude protein extracts were then centrifuged at 9000*g* (15 min at 4°C) using an SS-34 rotor (Sorvall) in an RC5C superspeed centrifuge (Sorvall) to remove cellular debris. The supernatant was recovered, and samples were frozen in liquid N_2 for later use.

Protein Determination

Tonoplast protein was measured by a modification of the dye-binding method (Bradford, 1976) in which membrane protein was partially solubilized with 0.5% (v/v) Triton X-100 for 5 min before the addition of the dye reagent concentrate. BSA was used as the protein standard.

H^+ Transport Assays

The fluorescence quenching of quinacrine (6-chloro-9-{[4-(diethylamino)-1-methylbutyl]amino}-2-methoxyacridin edihydrochloride) was used to monitor the formation and dissipation of inside-acid pH gradients across tonoplast vesicles. Purified tonoplast vesicles (30 μg of protein) were added to 500 μL of a buffer containing 250 mM mannitol, 10 mM Tris/Mes (pH 8.0), 6 mM $MgSO_4$, 50 mM tetramethylammonium chloride, and 3 μM quinacrine. Proton translocation was initiated in vesicles by the addition of 3 mM bis Tris propane/ATP (pH 8.0). Fluorescence quenching was monitored in a thermostated cell at 25°C using a fluorescence spectrometer (model LS-50, Perkin-Elmer) at excitation and emission wavelengths of 427 and 495 nm, respectively, both with a slit width of 5 nm. For measurements of Na^+-dependent dissipation of a preformed, inside-acid pH gradient, the ATP-dependent H^+ transport activity was inhibited by the addition of 200 nM bafilomycin A_1 (Bowman et al., 1988) in 0.001% (v/v) DMSO, 250 mM mannitol, and 10 mM Tris/Mes (pH 8.0) according to the method of Barkla et al. (1995). After a constant level of fluorescence was obtained, aliquots of Na^+ (200 mM) were added to the cell, and the initial rate of Na^+-dependent fluorescence recovery was determined. As shown by Bennett and Spanswick (1983), the rate of fluorescence quench or recovery is directly proportional to proton flux. Thus, initial rates of fluorescence quenching or recovery represent initial rates of proton transport.

PEPC Activity

The activity of PEPC was measured as the oxidation of NADH in the presence of PEP, malate dehydrogenase, and total leaf protein according to the method of Chu et al. (1990) with some modifications. Twenty-five micrograms of total leaf protein was added to enzyme assay buffer (50 mM Tris/Mes [pH 8.0], 1 mM EDTA, 10 mM $MgSO_4$, 10 mM $NaHCO_3$, and 1 mM DTT), in the presence of 0.1 mM NADH and 5 units of malate dehydrogenase (porcine heart, Calbiochem), in a 1-mL quartz cuvette. The reaction was initiated by the addition of 2 mM PEP, and the change in A_{340} was measured in a diode array spectrophotometer (model 8452, Hewlett-Packard).

Chemicals

All chemicals were of standard analytical grade and were purchased from either Sigma or ICN. Na_2ATP was converted to bis Tris propane/ATP by cation exchange with Dowex 50W (Bio-Rad).

RESULTS

NaCl Regulation of V-ATPase and Na^+/H^+ Antiport Activity in Adult Plants

In *M. crystallinum* V-ATPase and Na^+/H^+ antiport activity have been shown to be coordinately up-regulated by NaCl, and it has been suggested that the combined activity of these transporters represents the principal mechanism for vacuolar Na^+ accumulation in this species (Barkla et al., 1995). To further investigate the response of these enzymes to NaCl and to the stress-induced plant growth regulator, ABA, quinacrine fluorescence quenching was used to monitor the rate of formation or dissipation of transmembrane pH gradients (inside acid) generated in sealed tonoplast vesicles by activation of the V-ATPase or Na^+/H^+ antiport, respectively. The initial rates of V-ATPase H^+ transport or Na^+/H^+ antiport activity were calculated from the rates of quinacrine fluorescence quenching or recovery taken during the first 40 s following the addition of ATP or Na^+, respectively. In all populations of vesicles used in these experiments, the initial quinacrine fluorescence level and the magnitude of the final steady-state level of quinacrine fluorescence, which reflects the pH gradient generated by the pump, were similar. Moreover, SDS-PAGE of tonoplast proteins from the different treatments showed no significant differences in protein profiles.

Tonoplast vesicles were isolated from the leaves of adult *M. crystallinum* plants (older than 6 weeks). As defined by Adams et al. (1998), this mature growth form is characterized by the emergence of side shoots and secondary leaves, which are morphologically distinct from the primary leaves observed in the juvenile phase of development. Adult plants also show progressive development of epidermal bladder cells and are competent to induce CAM (Adams et al., 1998). Leaves from 6-week-old adult plants treated for 2 weeks with 200 mM NaCl (8 weeks old at the time of tonoplast vesicle isolation) showed an increased V-ATPase H^+ transport activity of 1.7 times that of the control untreated plants of the same age (Fig. 1A; Table I), which agrees with previously reported results (Barkla et al., 1995). Growth of adult plants in 400 mM NaCl for 2

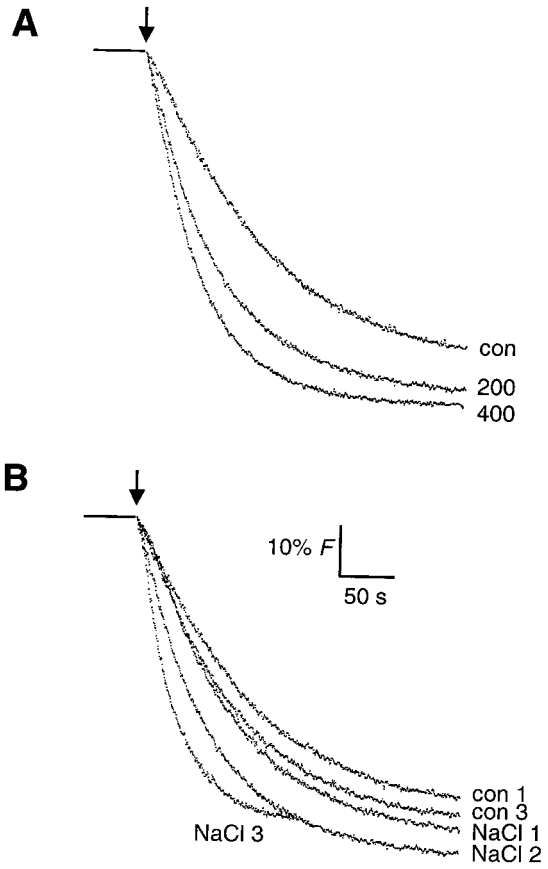

Figure 1. ATP-dependent H^+ transport into tonoplast vesicles isolated from leaves of adult *M. crystallinum* plants. Vesicle acidification was monitored by the quenching of quinacrine fluorescence, as described in "Materials and Methods." A, Tonoplast vesicles isolated from leaves of 8-week-old control (con) or NaCl-treated plants exposed to 200 mM NaCl (200) or 400 mM NaCl (400) for 2 weeks. B, Tonoplast vesicles isolated from leaves of control (con 1, 7 weeks old; con 3, 9 weeks old) and 200 mM NaCl-treated plants (NaCl 1, 7 weeks old; NaCl 2, 8 weeks old; NaCl 3, 9 weeks old) during a 3-week treatment period beginning when plants were 6 weeks old. For clarity, the trace for the control 8-week-old plant was not included in the figure. The results are original traces from one experiment representative of a total of three as detailed in Tables I and II. *F* is relative to that prior to addition of ATP (arrow).

weeks was able to elicit a further induction in the V-ATPase H^+ transport activity over that measured in plants exposed to 200 mM NaCl to a value 2.2 times that of the untreated plants (Fig. 1A; Table I).

When NaCl induction of V-ATPase H^+ transport activity was measured weekly over a 3-week treatment period, a time-dependent increase was observed (Fig. 1B; Table II). A small factor of this increase could be attributed to the aging of the plants over the treatment period, as values for V-ATPase H^+ transport in control untreated plants increased approximately 1.3-fold from a value of 668% *F* mg^{-1} protein min^{-1} measured in tonoplast vesicles from leaves of 7-week-old plants to 844% *F* mg^{-1} protein min^{-1} in 9-week-old plants (Table II). However, following 3 weeks of treatment with 200 mM NaCl, V-ATPase H^+ transport activity in tonoplast vesicles from leaves of treated plants was 1.9 times greater than the activity measured in control plants of the same age (1614% *F* mg^{-1} protein min^{-1} and 844% *F* mg^{-1} protein min^{-1}, respectively; Table II), indicating an increase that is independent of the age of the plant but dependent on the duration of NaCl treatment.

The effect of treatment of adult plants with NaCl on the Na^+-dependent dissipation of a transmembrane pH gradient was tested in isolated tonoplast vesicles. Following the generation of a preset, inside-acid pH gradient by activation and subsequent inhibition of the V-ATPase, Na^+ (200 mM) was added to the reaction medium, and the initial rate of quinacrine fluorescence recovery was measured. The initial rate of Na^+/H^+ exchange was approximately 1.4-fold higher in tonoplast vesicles isolated from leaves of adult plants treated with 200 mM NaCl for 2 weeks, as compared with vesicles from control plants of the same age (Fig. 2A; Table I). Only a slight further induction in Na^+/H^+ exchange was observed in plants treated with 400 mM NaCl for 2 weeks, as compared with those treated with 200 mM NaCl for the same period (Fig. 2A; Table I). When studied over a 3-week period, rates of NaCl-induced Na^+/H^+ antiport activity remained relatively unchanged and were not affected by the aging of the plants (Fig. 2B; Table II).

ABA Regulation of V-ATPase and Na^+/H^+ Antiport Activity in Adult Plants

The possible involvement of ABA in the regulatory pathway leading to induction of V-ATPase activity and Na^+/H^+ antiport activity was investigated. Evidence that suggested ABA involvement in this pathway was first presented by Tsiantis et al. (1996), who demonstrated ABA induction in the level of transcripts for the 16-kD c-subunit of the V-ATPase. Foliar application of ABA (25 μM) to 6-week-old well-watered adult plants for 2 weeks stimulated V-ATPase H^+ transport activity 1.8-fold, values similar to those observed for treatment of the plants with 200 mM NaCl for 2 weeks (1241% and 1166% *F* mg^{-1} protein min^{-1}, respectively; Fig. 3; Table I), indicating that, at the level of V-ATPase activity, ABA treatment was able to mimic treatment of the plants with NaCl. When plants

Table I. *Initial rates of V-ATPase H^+ transport and Na^+/H^+ exchange activity in tonoplast vesicles isolated from leaves of 2-week-treated adult M. crystallinum plants, as indicated*

Plants were 6 weeks old at the beginning of the experiment. Quinacrine fluorescence assays were performed as described in "Materials and Methods." Values are means ± SD (n = 3 independent experiments).

Treatment	V-ATPase H^+ Transport	Na^+/H^+ Antiport
	% F mg^{-1} protein min^{-1}	
Control	693 ± 72	161 ± 12
200 mM NaCl	1166 ± 55	224 ± 15
400 mM NaCl	1496 ± 121	237 ± 12
25 μM ABA	1241 ± 105	129 ± 15
25 μM ABA + 200 mM NaCl	1534 ± 66	220 ± 31

Table II. *Initial rates of V-ATPase H^+ transport and Na^+/H^+ exchange activity in tonoplast vesicles isolated from leaves of control and 200 mM NaCl-treated adult M. crystallinum plants treated during a 3-week period, as indicated*

Plants were 6 weeks old at the beginning of the experiment. Quinacrine fluorescence assays were performed as described in "Materials and Methods." Values are means ± SD (n = 3 independent experiments).

Duration	V-ATPase H^+ Transport		Na^+/H^+ Antiport	
	Control	NaCl	Control	NaCl
week	*% F mg^{-1} protein min^{-1}*			
1	668 ± 65	904 ± 46	164 ± 15	215 ± 11
2	693 ± 72	1166 ± 55	161 ± 12	224 ± 15
3	844 ± 40	1614 ± 132	177 ± 17	239 ± 27

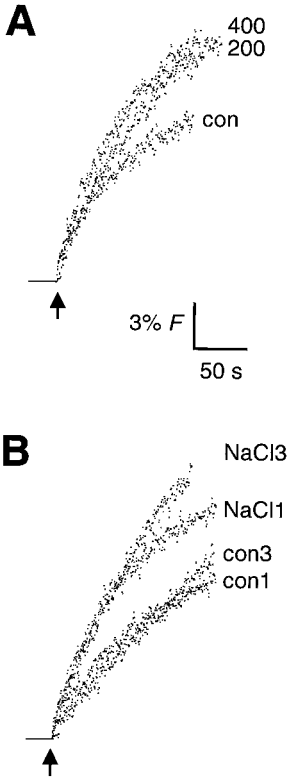

Figure 2. Na^+-dependent H^+ efflux from tonoplast vesicles isolated from leaves of adult *M. crystallinum* plants. A preset steady-state pH gradient (acidic inside) was generated in vesicles by activation of the V-ATPase, as described in "Materials and Methods." The recovery of quinacrine fluorescence, indicative of Na^+/H^+ exchange, was measured upon addition of 200 mM NaCl (arrow). A, Tonoplast vesicles isolated from leaves of 8-week-old control (con) or NaCl-treated plants treated with 200 mM NaCl (200) or 400 mM NaCl (400) for 2 weeks. B, Tonoplast vesicles isolated from leaves of control (con1, 7 weeks old; con3, 9 weeks old) and 200 mM NaCl-treated plants (NaCl1, 7 weeks old; NaCl3, 9 weeks old) during a 3-week treatment period beginning when plants were 6 weeks old. For clarity, the traces for the control and NaCl-treated 8-week-old plants were not included in the figure. The results are original traces from one experiment representative of a total of three, as detailed in Tables I and II. *F* is relative to that prior to addition of NaCl.

were treated with NaCl in addition to the application of ABA, V-ATPase activity was further stimulated to 2.2 times that of the untreated plants of the same age (1534% *F* mg^{-1} protein min^{-1}; Fig. 3; Table II), resulting in an induction of approximately 1.3-fold over treatment with either NaCl or ABA alone. These results indicate a partially additive effect of ABA and NaCl treatment on the induction of V-ATPase activity, suggesting that NaCl and ABA may act as distinct signals in independent pathways rather than components of the same regulatory pathway.

In contrast to these results, ABA treatment of adult *M. crystallinum* plants had no stimulatory effect on Na^+/H^+ antiport activity. Treatment of 6-week-old plants with 25 μM ABA for 2 weeks resulted in a slightly lower level of Na^+-dependent H^+ transport when compared with control untreated plants of the same age (Fig. 4; Table I). Treatment with ABA in the presence of 200 mM NaCl gave rates of Na^+/H^+ exchange equivalent to those obtained from treatment with NaCl alone (Fig. 4; Table I). These results indi-

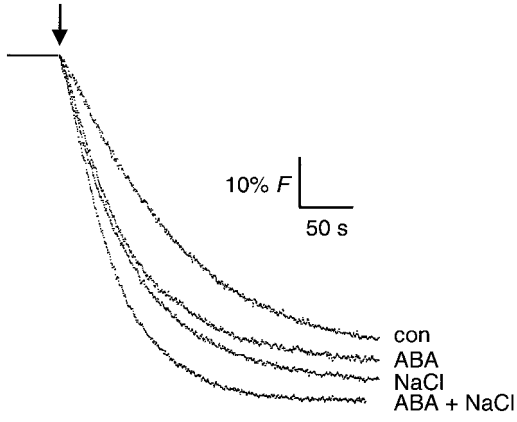

Figure 3. ATP-dependent H^+ transport into tonoplast vesicles isolated from leaves of ABA-treated adult *M. crystallinum* plants. Vesicle acidification was monitored by the quenching of quinacrine fluorescence, as described in "Materials and Methods." Tonoplast vesicles were isolated from leaves of 8-week-old control (con) *M. crystallinum* plants or plants treated for 2 weeks with either 200 mM NaCl (NaCl) or foliar applications of 25 μM ABA (ABA) or with both (ABA + NaCl). The results are original traces from one experiment representative of a total of three, as detailed in Table I. *F* is relative to that prior to addition of ATP (arrow).

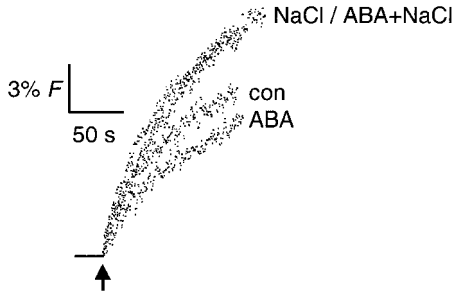

Figure 4. Na^+-dependent H^+ efflux from tonoplast vesicles isolated from leaves of ABA-treated adult *M. crystallinum* plants. A preset steady-state pH gradient (acidic inside) was generated in vesicles by activation of the V-ATPase, as described in "Materials and Methods." The recovery of quinacrine fluorescence, indicative of Na^+/H^+ exchange, was measured upon addition of 200 mM NaCl (arrow). Tonoplast vesicles were isolated from leaves of 8-week-old control (con) *M. crystallinum* plants or plants treated for 2 weeks with either 200 mM NaCl (NaCl) or foliar applications of 25 μM ABA (ABA) or with both (ABA + NaCl). The results are original traces from one experiment representative of a total of three, as detailed in Table I. *F* is relative to that prior to addition of NaCl.

cate that, unlike NaCl, ABA treatment does not result in parallel increases in V-ATPase and Na^+/H^+ antiport activites; rather, ABA appears to regulate only V-ATPase activity.

The ability of ABA to regulate V-ATPase activity with no stimulatory effect on Na^+/H^+ antiport activity would suggest that ABA induction of V-ATPase activity is not linked to vacuolar Na^+ accumulation. However, it is possible that the ABA-dependent increase in V-ATPase activity is necessary to drive the passive uptake of malate into the vacuole, which occurs during CAM induction. Moreover, many of the genes up-regulated during the establishment of CAM are also induced by ABA (Cushman and Bohnert, 1997). To investigate whether this could be an explanation for the increase in V-ATPase activity observed in the adult plants treated with ABA, it was necessary to attempt to separate regulatory processes involved in CAM induction from those involved in vacuolar Na^+ accumulation in *M. crystallinum*. One way to do this was to study the regulation by NaCl and ABA of the V-ATPase in juvenile plants in which, in this facultative CAM plant, stress-inducible CAM activity is absent (Adams et al., 1998). Whereas CAM cannot be elicited in juvenile plants, other NaCl-adaptive mechanisms, including vacuolar accumulation of Na^+, are still present (Adams et al., 1998).

Table III. *PEPC activity in total protein extracts from leaves of 2-week-treated juvenile and adult M. crystallinum plants*

Plants were treated as described in "Materials and Methods." Total protein extracts were prepared from N_2 frozen leaf samples obtained from the third and fourth leaf pair of adult plants and the second and third leaf pair of juvenile plants taken 2 h into the day-light period (6 and 3 weeks old at initiation of treatment, respectively). PEPC was measured as the oxidation of NADH in the presence of PEP, malate dehydrogenase, and 25 μg of total leaf protein. Values are means of three independent experiments with SD not exceeding 10%. ND, No data.

Treatment	PEPC Activity	
	Juvenile plants	Adult plants
	μmol NADH mg^{-1} protein h^{-1}	
Control	1.85	1.82
200 mM NaCl	1.74	3.16
25 μM ABA	1.44	2.36
25 μM ABA + 200 mM NaCl	ND	3.49

PEPC Activity in Adult and Juvenile *M. crystallinum* Plants

The activity of PEPC, a key enzyme in CAM, has been widely used as a marker to monitor the presence or absence of CAM in juvenile and adult *M. crystallinum* plants. During the NaCl-induced transition from C_3 metabolism to CAM in *M. crystallinum*, PEPC transcripts, protein, and activity have been show to be significantly increased within 24 h of the initiation of NaCl stress (Vernon et al., 1993), and application of ABA can mimic this response (Dai et al., 1994). In 6-week-old adult plants treated for 2 weeks with 200 mM NaCl, PEPC activity increased 1.7-fold over that measured in control plants of the same age (Table III). Treatment of plants with 25 μM ABA for the same time was also able to induce enzyme activity (1.3-fold increase), and treatment of plants with ABA in combination with NaCl resulted in increases in PEPC activity similar to those measured in the presence of NaCl (Table III). In contrast, neither NaCl nor ABA treatment of juvenile plants resulted in induction of PEPC activity (Table III). These results show the expected NaCl and ABA induction of CAM in adult plants and the absence of CAM induction in juvenile plants.

NaCl and ABA Regulation of V-ATPase Activity in Juvenile Plants

In 3-week-old juvenile *M. crystallinum* plants treated for 10 d with 200 mM NaCl, V-ATPase H^+ transport activity

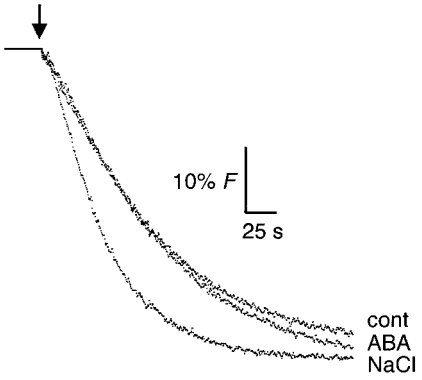

Figure 5. ATP-dependent H^+ transport into tonoplast vesicles isolated from leaves of juvenile *M. crystallinum* plants. Vesicle acidification was monitored by the quenching of quinacrine fluorescence, as described in "Materials and Methods." A, Tonoplast vesicles isolated from leaves of 5-week-old control plants (cont) or plants treated with either 200 mM NaCl (NaCl) or 25 μM ABA (ABA) for 2 weeks. The results are original traces from one experiment representative of a total of three. *F* is relative to that prior to addition of ATP (arrow).

was 1.7-fold greater than that measured in untreated plants of the same age (534% and 882% $F\ mg^{-1}$ protein min^{-1}, respectively; Fig. 5). This level of induction is similar to that observed in adult plants treated with 200 mM NaCl for the same time (Fig. 1A; Table I). However, treatment of juvenile plants with ABA (25 μM) did not result in a concomitant increase in V-ATPase activity. The initial rate of V-ATPase H^+ transport was similar to that observed in untreated plants of the same age (571% $F\ mg^{-1}$ protein min^{-1}; Fig. 5). These results suggest that ABA induction of V-ATPase activity is dependent on the plant reaching its adult phase of development.

DISCUSSION

The mechanisms by which *M. crystallinum* senses changes in the environment and transduces these signals into physiological responses resulting in drought and salinity tolerance, including CAM induction, compatible solute synthesis, and vacuolar Na^+ accumulation, have only recently begun to be investigated. It is unlikely that there exists a single signaling pathway that coordinately regulates these key adaptive responses; rather, it is becoming increasingly clear that the response to environmental factors is complex, requiring several distinct pathways to explain the intricate patterns of gene expression and enzyme responses elicited by a range of environmental stimuli (Bohnert et al., 1995). To better understand the signaling pathways involved in *M. crystallinum* stress tolerance we have investigated the role played by both NaCl and ABA in initiating changes in the V-ATPase activity in both adult and juvenile plants.

Independently of the age of the plant, treatment with NaCl resulted in substantial increases in V-ATPase H^+ transport activity to a maximum induction of 1.7- and 1.9-fold in juvenile and adult plants, respectively (Figs. 1 and 5; Table I), in agreement with previously reported results (Rockel et al., 1994; Barkla et al., 1995; Tsiantis et al., 1996). Similar treatment periods of adult and juvenile plants resulted in a comparable increase in V-ATPase activity (1.7-fold following 2 weeks of treatment), indicating, despite developmental differences, that plant age does not affect the response of the V-ATPase to salinity. In adult plants the maximal level of induction was reached after a treatment period of 3 weeks, suggesting a sustained effect of NaCl treatment on enzyme activity. This NaCl-induced increase in V-ATPase activity in adult plants was paralleled by increases in activity for the Na^+/H^+ antiport (Fig. 2A; Table I), providing a link between increased tonoplast energization and increased vacuolar Na^+ accumulation via Na^+/H^+ exchange. However, maximal levels of Na^+/H^+ antiport activity were reached after 1 week of treatment with 200 mM NaCl, suggesting that increases in NaCl concentration or age of plant had no effect on antiport activity (Fig. 2B; Table II).

In adult plants application of exogenous ABA to the leaves of well-watered plants also induced V-ATPase H^+ transport activity by 1.8-fold, a level similar to that observed for plants treated with 200 mM NaCl (Fig. 3; Table I). This increase was not due to direct effects of ABA on enzyme activity, because V-ATPase activity in tonoplast vesicles isolated from leaves of control *M. crystallinum* plants preincubated in the presence of ABA showed no such induction (data not shown). ABA-induced V-ATPase H^+ transport activity has also been reported for barley roots treated with ABA (Kasai et al., 1993), and transcripts for the V-ATPase A-subunit have been shown to be up-regulated by ABA application to tobacco cell-suspension cultures and leaves of *Brassica napus* (Narasimhan et al., 1991; Orr et al., 1995). ABA application to leaves of *M. crystallinum* increased transcript levels of the V-ATPase c-subunit (Tsiantis et al., 1996).

In contrast to the stimulation of V-ATPase activity, ABA application to leaves of *M. crystallinum* failed to induce Na^+/H^+ antiport activity in either adult or juvenile plants (Fig. 4; Table I; also data not shown). These results suggest that ABA is not involved as a signal mediating NaCl-induced antiport activity.

When adult plants were treated with ABA in the presence of NaCl, a further stimulation in V-ATPase H^+ transport activity was observed, demonstrating a partially additive effect of treatments on enzyme activity (Fig. 3; Table I). These data, and the lack of effect of ABA on Na^+/H^+ antiport activity, suggest that NaCl and ABA do not act through the same signal transduction pathway. However, it is possible that two distinct pathways exist: (a) the first mediated by NaCl and independent of ABA, responsible for up-regulation of V-ATPase and Na^+/H^+ antiport activity to energize Na^+ accumulation, and (b) the second dependent on water-deficit-induced increases in ABA, which stimulates V-ATPase enzyme activity to drive malate accumulation into the vacuole during the establishment of CAM. Additional support for this view comes from results of studies of juvenile *M. crystallinum* plants. In contrast to adult plants, ABA treatment of juvenile plants failed to induce V-ATPase H^+ transport activity, whereas NaCl elicited a response similar to that observed in the adult plants (Figs. 3 and 5; Table I). This age-dependent ABA response is reminiscent of the developmentally programmed, stress-induced switch from C_3 metabolism to CAM in *M. crystallinum*, in which only adult plants are competent in CAM induction (Table III; Cushman et al., 1990). Therefore, in juvenile plants, in which NaCl-stress-induced Na^+ accumulation is also observed, increases in V-ATPase activity would be required. However, because CAM is not triggered in young plants, V-ATPase energization of vacuolar malate accumulation is not necessary. To justify this hypothesis, it must be possible to elicit two distinct pathways initiating from the recognition by the plant of the salinity signal. For salinity stress, with which a water deficit is also imposed, drought-related responses would be expected (including CAM induction in older plants to increase water-use efficiency). However, there is also the added ionic effects related to the presence of high concentrations of Na^+ and Cl^-, which could require survival responses by the plant different from drought stress alone. Evidence for specific responses to the ionic component of NaCl stress, with the ability to differentiate the osmotic component, has recently been shown for an Arabidopsis calcineurin B homolog (Liu and Zhu, 1998) and in

M. crystallinum for the induction of IMT and *myo*-inositosol-1-phosphate synthase, enzymes in the pathway leading to synthesis of the compatible solute pinitol (Nelson et al., 1998). To further support this hypothesis it will be necessary to identify specific components of each signaling pathway.

Recent advances in this field have been made in the glycophyte Arabidopsis, for which NaCl-stress adaptive responses were found to trigger changes in cytoplasmic Ca^{2+} (Knight et al., 1997), with the possible involvement of calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase (Liu and Zhu, 1998; Pardo et al., 1998). However, this pathway is similar to that characterized in yeast, in which the pathway was shown to primarily regulate ion homeostasis through the coordination of Na^+ influx and efflux systems (Mendoza et al., 1994) and may not be relevant when studying adaptive mechanisms of halophytes such as *M. crystallinum,* which selectively accumulate Na^+ rather than exclude this ion. The different responses of halophytes and glycophytes to NaCl stress strengthens the need to study signaling pathways in NaCl-tolerant plants and to focus research on understanding those mechanisms that are unique to each group. Furthermore, the results presented in this study highlight the importance of interpreting the adaptive responses to salinity of *M. crystallinum* specifically and plants in general within the context of existing developmental programs.

Whether the NaCl- or ABA-induced increase in V-ATPase activity observed is due to increased pump density or control of existing pump activity is unknown. We do know that there is no coordinate transcriptional upregulation of genes for V-ATPase subunits in NaCl-stressed *M. crystallinum* (Löw et al., 1996) and so far, only the c-subunit of the membrane sector has been shown to be transcriptionally regulated by NaCl in both juvenile and adult plants (Tsiantis et al., 1996). At the protein level, antibodies against V-ATPase subunits A, B, E, and Ac39 (yeast *vma6* gene product) do not identify NaCl-induced increases in protein for these subunits in tonoplast isolated from *M. crystallinum* leaves and cell suspensions (Maldonado-Gama, 1997; Vera-Estrella et al., 1999). However, an increase in pump density may not require upregulation of all subunits; rather, key subunits may direct assembly of the holoenzyme (Stevens and Forgac, 1997). Alternatively, increases in a particular subunit may not increase pump density but, instead, alter the coupling efficiency of H^+ transport and ATP hydrolysis. Although posttranslational modifications of V-ATPases have not been described in plants, in yeast V-ATPases have been shown to be regulated by Glc through rapid disassembly and reassembly in vivo (Kane, 1995), and evidence has been provided for regulation of V-ATPases by heterotrimeric G-proteins and protein kinase C (for review, see Merzendorfer et al., 1997).

ACKNOWLEDGMENT

We thank Henk Miedema for critical review of the manuscript.

Received January 21, 1999; accepted April 2, 1999.

LITERATURE CITED

Adams P, Nelson DE, Yamada S, Chmara W, Jensen RG, Bohnert HJ, Griffiths H (1998) Growth and development of *Mesembryanthemum crystallinum* (Aizoaceae). New Phytol **138:** 171–190

Barkla BJ, Zingarelli L, Blumwald E, Smith JAC (1995) Tonoplast Na^+/H^+ antiport activity and its energization by the vacuolar H^+-ATPase in the halophytic plant *Mesembryanthemum crystallinum.* Plant Physiol **109:** 549–556

Bennett AB, Spanswick RM (1983) Optical measurements of ΔpH and $\Delta\Psi$ in corn root membrane vesicles: kinetic analysis of Cl^{-1} effects of proton-translocating ATPase. J Membr Biol **71:** 95–107

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. Plant Cell **7:** 1099–1111

Bowman EJ, Siebers A, Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc Natl Acad Sci USA **85:** 7972–7976

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci **2:** 48–54

Chu C, Dai Z, Ku SBM, Edwards G (1990) Induction of Crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* by abscisic acid. Plant Physiol **93:** 1253–1260

Cushman JC, Bohnert HJ (1997) Molecular genetics of Crassulacean acid metabolism. Plant Physiol **113:** 667–676

Cushman JC, Michalowski CB, Bohnert HJ (1990) Developmental control of Crassulacean acid metabolism inducibility by salt stress in the common ice plant. Plant Physiol **94:** 1137–1142

Dai Z, Ku MSB, Zhang D, Edwards GE (1994) Effects of growth regulators on the induction of Crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* L. Planta **192:** 287–294

Dietz KJ, Arbinger B (1996) cDNA sequence and expression of subunit E of the vacuolar H^+-ATPase in the inducible Crassulacean acid metabolism plant *Mesembryanthemum crystallinum.* Biochim Biophys Acta **1281:** 134–138

Forsthoefel NR, Cushman MA, Cushman JC (1995a) Posttranscriptional and posttranslational control of enolase expression in the facultative Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. Plant Physiol **108:** 1185–1195

Forsthoefel NR, Vernon DM, Cushman JC (1995b) A salinity-induced gene from the halophyte *Mesembryanthemum crystallinum* encodes a glycolytic enzyme, cofactor-independent phosphoglyceromutase. Plant Mol Biol **29:** 213–226

Gosti F, Bertauche N, Vartanian N, Giraudat J (1995) Abscisic acid-dependent and -independent regulation of gene expression by progressive drought in *Arabidopsis thaliana*. Mol Gen Genet **246:** 10–18

Grabov A, Blatt MR (1998) Co-ordination of signalling elements in guard cell ion channel control. J Exp Bot **49:** 351–360

Hoagland DR, Arnon DI (1938) The water culture method for growing plants without soil. Calif Exp Stn Circ **347:** 1–39

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 377–403

Ishitani M, Majumder AL, Bornhouser A, Michalowski CB, Jensen RG, Bohnert HJ (1996) Coordinate transcriptional induction of *myo*-inositol metabolism during environmental stress. Plant J **9:** 537–548

Ishitani M, Xiong L, Stevenson B, Zhu J-K (1997) Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. Plant Cell **9:** 1935–1949

Kane PM (1995) Disassembly and reassembly of the yeast vacuolar H^+-ATPase *in vivo*. J Biol Chem **270:** 17025–17032

Kasai M, Yamamoto Y, Maeshima M, Matsumoto H (1993) Effects of in vivo treatment with abscisic acid and/or cytokinin on activities of vacuolar H^+ pumps of tonoplast-enriched membrane vesicles prepared from barley roots. Plant Cell Physiol **34:** 1107–1115

Knight H, Trewavas AJ, Knight MR (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. Plant J **12:** 1067–1078

Liu J, Zhu J-K (1998) A calcium sensor homolog required for plant salt tolerance. Science **280:** 1943–1945

Löw R, Rockel B, Kirsch M, Ratajczak R, Hörtensteiner S, Martinoia E, Lüttge U, Rausch T (1996) Early salt stress effects on the differential expression of vacuolar H^+-ATPase genes in roots and leaves of *Mesembryanthemum crystallinum*. Plant Physiol **110:** 259–265

Maldonado-Gama M (1997) Regulación de la H^+-ATPasa del tonoplasto por salinidad y ABA en *Mesembryanthemum crystallinum*. Thesis. Facultad de Ciencias Biológicas, Universidad Autónoma del Estado de Morelos, México

McElwain EF, Bohnert HJ, Thomas JC (1992) Light moderates the induction of phospho*enol*pyruvate carboxylase by NaCl and abscisic acid in *Mesembryanthemum crystallinum*. Plant Physiol **99:** 1261–1264

Mendoza I, Rubio F, Rodriguez-Navarro A, Pardo JM (1994) The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*. J Biol Chem **269:** 8792–8796

Merzendorfer H, Gräf R, Huss M, Harvey WR, Wieczorek H (1997) Regulation of proton-translocating V-ATPases. J Exp Biol **200:** 225–235

Narasimhan ML, Binzel ML, Perez-Prat E, Chen Z, Nelson DE, Singh NK, Bressan RA, Hasegawa PM (1991) NaCl regulation of tonoplast ATPase 70-kilodalton subunit mRNA in tobacco cells. Plant Physiol **97:** 562–568

Nelson DE, Rammesmayer G, Bohnert HJ (1998) Regulation of cell-specific inositol metabolism and transport in plant salinity tolerance. Plant Cell **10:** 753–764

Orr W, White TC, Iu B, Robert L, Singh J (1995) Characterization of a low-temperature-induced cDNA from winter *Brassica napus* encoding the 70 kD subunit of tonoplast ATPase. Plant Mol Biol **28:** 943–948

Pardo JM, Reddy MP, Yang S, Maggio A, Huh G-H, Matsumoto T, Coca MA, Paino-D'Urzo M, Koiwa H, Yun D-J, and others (1998) Stress signaling through Ca^{2+}/calmodulin-dependent protein phosphatase mediates salt adaptation in plants. Proc Natl Acad Sci USA **95:** 9681–9686

Rockel B, Ratajczak R, Becker A, Lüttge U (1994) Changed densities and diameters of intra-membrane tonoplast particles of *Mesembryanthemum crystallinum* in correlation with NaCl-induced CAM. J Plant Physiol **143:** 318–324

Shaeffer HJ, Forsthoefel NR, Cushman JC (1995) Identification of silencer and enhancer regions involved in salt responsive gene expression of Crassulacean acid metabolism (CAM) genes in the facultative halophyte *Mesembryanthemum crystallinum*. Plant Mol Biol **28:** 205–218

Shinozaki K, Yamaguchi-Shinozaki K (1996) Molecular responses to drought and cold stress. Curr Opin Biotechnol **7:** 161–167

Shinozaki K, Yamaguchi-Shinozaki K (1997) Gene expression and signal transduction in water-stress response. Plant Physiol **115:** 327–334

Stevens TH, Forgac M (1997) Structure, function and regulation of the vacuolar (H^+)-ATPase. Annu Rev Cell Dev Biol **13:** 779–808

Struve I, Lüttge U (1987) Characteristics of $MgATP^{2-}$-dependent electrogenic proton transport in tonoplast vesicles of the facultative Crassulacean-acid-metabolism plant *Mesembryanthemum crystallinum* L. Planta **170:** 111–120

Thomas JC, McElwain EF, Bohnert HJ (1992) Convergent induction of osmotic-stress responses. ABA and cytokinin and the effects of NaCl. Plant Physiol **100:** 416–423

Tsiantis MS, Bartholomew DM, Smith JAC (1996) Salt regulation of transcript levels for the c subunit of a leaf vacuolar H^+-ATPase in the halophyte *Mesembryanthemum crystallinum*. Plant J **9:** 729–736

Vera-Estrella R, Barkla BJ, Bohnert HJ, Pantoja O (1999) Salt-stress in *Mesembryanthemum crystallinum* cell suspensions activates adaptive mechanisms similar to those observed in the whole plant. Planta **207:** 426–435

Vernon DM, Ostrem JA, Bohnert HJ (1993) Stress perception and response in a facultative halophyte: the regulation of salinity induced genes in *Mesembryanthemum crystallinum*. Plant Cell Environ **16:** 437–444

Winter K, Lüttge U, Winter E, Troughton JH (1978) Seasonal shift from C_3 photosynthesis to Crassulacean acid metabolism in *Mesembryanthemum crystallinum* growing in its natural environment. Oecologia **34:** 225–237

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6:** 251–264

Zhigang A, Löw R, Rausch T, Lüttge U, Ratajczak R (1996) The 32 kDa tonoplast polypeptide D_i associated with the V-type H^+-ATPase of *Mesembryanthemum crystallinum* L. in the CAM state: a proteolytically processed subunit B? FEBS Lett **389:** 314–318