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NaCl Regulation of Plasma Membrane H^+-ATPase Gene Expression in a Glycophyte and a Halophyte[1]

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NaCl regulation of plasma membrane H^+-ATPase gene expression in the glycophyte tobacco (*Nicotiana tabacum* L. var Wisconsin 38) and the halophyte *Atriplex nummularia* L. was evaluated by comparison of organ-specific mRNA abundance using homologous cDNA probes encoding the ATPases of the respective plants. Accumulation of mRNA was induced by NaCl in fully expanded leaves and in roots but not in expanding leaves or stems. The NaCl responsiveness of the halophyte to accumulate plasma membrane H^+-ATPase mRNA in roots was substantially greater than that of the glycophyte. Salt-induced transcript accumulation in *A. nummularia* roots was localized by in situ hybridization predominantly to the elongation zone, but mRNA levels also increased in the zone of differentiation. Increased message accumulation in *A. nummularia* roots could be detected within 8 h after NaCl (400 mM) treatment, and maximal levels were severalfold greater than in roots of untreated control plants. NaCl-induced plasma membrane H^+-ATPase gene expression in expanded leaves and roots presumably indicates that these organs require increased H^+-electrochemical potential gradients for the maintenance of plant ion homeostasis for salt adaptation. The greater capacity of the halophyte to induce plasma membrane H^+-ATPase gene expression in response to NaCl may be a salt-tolerance determinant.

When plants are exposed to salinity, the ions, typically Na^+ and Cl^-, lower the external water potential, resulting in turgor reduction or loss, and accumulate excessively in the cytoplasm, leading to inhibition of plant growth and development (Greenway and Munns, 1980). Plant cells adjust to the imbalance in water relations through osmotic adjustment utilizing both organic solutes and ions (Rhodes, 1987; Maathius et al., 1992). However, osmotic adjustment must occur without undue concentration in the cytoplasm of the ions from the saline environment. This is apparently achieved by mechanisms that regulate K^+/Na^+ selectivity and Cl^- uptake across the plasma membrane and compartmentalize Na^+ and Cl^- in the vacuole (Flowers et al., 1977; Greenway and Munns, 1980; Jeschke, 1984; Binzel et al., 1988). Maintenance of Na^+ and Cl^- concentration gradients across the plasma membrane and the tonoplast is facilitated by $\Delta\mu_{H^+}$ generated by H^+-ATPases in the respective membranes (Sze, 1985; Reinhold et al., 1989). A pyrophosphatase also functions as an electrogenic H^+ pump in the tonoplast (Rea et al., 1992).

The $\Delta\mu_{H^+}$ produced by the plasma membrane H^+-ATPase is presumed to provide the driving force to regulate intracellular Na^+ and Cl^- uptake. It is the prevailing view that, under physiological conditions, Na^+ is actively transported outward across the plasma membrane, presumably by an Na^+/H^+ antiporter. Cl^- is passively effluxed across the plasma membrane down the free energy gradient established by the $\Delta\mu_{H^+}$, which the electrogenic H^+-ATPase generates. The H^+-pumping capacity of the plasma membrane H^+-ATPase has been reported to increase after salt treatment in *Atriplex nummularia* roots (Braun et al., 1986). Previously, we demonstrated that plasma membrane H^+-ATPase gene expression is also up-regulated by NaCl in cultured *A. nummularia* cells (Niu et al., 1993).

Halophytes are plants that have been naturally selected in saline environments and are distinguishable from glycophytes by their capacity to cope with excessive levels of ions with high proficiency (Greenway and Munns, 1980; Flowers et al., 1986). Clearly, some halophytes possess unique adaptations, such as salt glands or bladders, that alleviate the deleterious effects of high ion concentrations. However, intrinsically cellular processes must make the major contribution to the capacity of plants for salt adaptation. It is not clear to what extent halophytes have evolved unique cellular determinants that make these plants more salt tolerant than glycophytes. Numerous similar cellular mechanisms are utilized by both glycophytes and halophytes for adaptation to and growth in saline environments (Flowers et al., 1986; Binzel et al., 1989). However, the halophytic forms of these salt-tolerance determinants (e.g. types of osmoprotectants [Hanson et al., 1991], vacuolar compartmentation of Na^+ and Cl^- [Maathuis et al., 1992]), seem to be more specifically adapted for saline environments, and halophytes may utilize these more efficiently in response to salt stress. At the molecular level, the greater salt-adaptive capacity of halophytes may be due to constitutive expression of genes that encode salt-tolerance determinants (Casas et al., 1992) or the greater capacity to regulate the expression of these genes in response to salt (Cushman et al., 1990; Casas et al., 1992).

In this report, NaCl regulation of plasma membrane H^+-ATPase gene expression was compared by analyses of mRNA abundance in stems, leaves, and roots of a glycophyte (*Nicotiana tabacum* L. var Wisconsin 38) and a halophyte (*A. nummularia* L.). Message accumulation was induced by

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Abbreviation: $\Delta\mu_{H^+}$, H^+ electrochemical potential gradient.

NaCl in roots (localized predominantly to the elongation zone) and expanded leaves but not in stems or expanding leaves. Plasma membrane H^+-ATPase gene expression in the halophyte *A. nummularia* was more responsive to NaCl than in the glycophyte tobacco. These results indicate that, during salt stress, the plasma membrane H^+-ATPase is active in roots, where control of Na^+ and Cl^- uptake into the plant is mediated, and in old leaves that may function as ion sinks. These data also suggest that the capacity to regulate the plasma membrane H^+-ATPase gene in response to NaCl may be a salt-tolerance determinant of the halophyte.

MATERIALS AND METHODS

Plant Material

Greenhouse-grown *Atriplex nummularia* L. (Chenopodiaceae) plants were used as the source of plant material for all experiments. Shoot-tip cuttings (<5 cm in length) with four to five leaves were rooted in peatlite on a mist bench for 3 to 4 weeks. The rooted cuttings were transferred to a growth chamber and acclimated for at least 1 week before establishing these in hydroponic solution. Conditions of the growth chamber were 26°C (day, 16 h) and 21°C (night). The cuttings were then transferred to a hydroponic culture solution (Dr. Chatelier's Plant Food; Dr. Chatelier's Plant Food Co., Inc., Oldsmar, FL). The nutrient solution was replaced every 2 to 3 d to avoid nutrient depletion.

Seeds of *Nicotiana tabacum* L. var Wisconsin 38 were germinated in vermiculite in the growth chamber. Three to 4 weeks after germination, seedlings were transferred to hydroponic culture solution. Both tobacco and *A. nummularia* plants were grown in culture solution for at least 2 weeks before initiation of experiments.

Salt Treatments

For salt treatments, NaCl (solid) was added directly to the nutrient solution and thoroughly dissolved. Similar mechanical manipulations were made to the solution of plants not treated with salt to simulate the stirring necessary to dissolve NaCl. In some experiments, NaCl was added at one time and in others the salt was added incrementally to reach the final concentrations. In all instances NaCl-treated plants were grown in solution supplied with salt until harvest. The nutrient solution was changed 24 h before the initiation of experiments and then every 2 to 3 d.

RNA Isolation and Electrophoresis

Entire plants were harvested and quickly separated into roots, leaves, and stems. The plant organs were frozen immediately in liquid nitrogen and stored at −80°C. Samples were ground in liquid nitrogen with a mortar and a pestle to a very fine powder. Total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi, 1987) as described previously (Casas et al., 1992). The RNA was further purified by washing with 4 M LiCl, another phenol-chloroform extraction, and subsequent sodium acetate-ethanol precipitation. Formaldehyde-agarose gel (1.2%) electrophoresis and capillary transfer to nitrocellulose membranes were performed following standard protocols (Sambrook et al., 1989). To ensure that equivalent quantities of RNA of equal quality were loaded onto gels and transfer was complete, ethidium bromide staining intensity of the 18S and 25S rRNA bands was evaluated on every gel before and after transfer. Transcript sizes were estimated by comparing with a 0.24- to 9.5-kb RNA ladder (BRL No. 56205A). The *Drosophila* actin 5c probe (Fyrberg et al., 1983) was used as an internal standard in certain experiments to minimize sample-to-sample mRNA variation.

Plasma Membrane H^+-ATPase Probes and RNA Hybridization

Preliminary experiments indicated that heterologous probes could not be used to detect plasma membrane H^+-ATPase mRNA. In the majority of experiments, the *A. nummularia* mRNA probe was the insert (1.829 kb) in pAPM (Niu et al., 1993), which contains a sequence that encodes the C-terminal 447 amino acids of the plasma membrane H^+-ATPase and 488 bp of the 3′ untranslated region. In some experiments the insert from a truncated pAPM subclone (pAPME) was used to detect plasma membrane H^+-ATPase mRNA. The pAPME insert is 830 bp and contains 792 bp of the open reading frame encoding the C-terminal 264 amino acids (corresponding to amino acid positions 689–952 in PMA4 of *Nicotiana plumbaginifolia* [GenBank accession No. X66737] with 81.0% identity) and 38 bp of the 3′ untranslated region that was cloned into the pGEM7Zf(−) vector (Promega). For tobacco, a 1.529-kb cDNA insert cloned into the pTZ18U vector (pG1) was used as the probe for plasma membrane H^+-ATPase mRNA (Perez-Prat et al., 1993). The pG1 clone was isolated from a tobacco cDNA library of ABA-treated cells, and the translated peptide of 419 amino acids is 98.8% identical with PMA2 of *N. plumbaginifolia* (Boutry et al., 1989).

The ^{32}P-labeled probes were prepared with a random primer labeling kit (United States Biochemical) and [α-^{32}P]-dCTP (ICN). Procedures for RNA hybridizations were as described by Niu et al. (1993). Briefly, prehybridizations and hybridizations were carried out in the same solution, which contained 50% formamide, 6× SSC (1× SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 5× Denhardt's solution (Sambrook et al., 1989), 0.1% (w/v) SDS, and 100 μg mL^{-1} of herring sperm DNA, in a hybridization oven at 42°C. Prehybridizations were for 3 h, and hybridizations were done overnight with ^{32}P-labeled probes. Sequential posthybridization washes were for 15 min each in 0.1% (w/v) SDS plus 1× (twice), 0.2×, and 0.1× SSC at room temperature.

In Situ Hybridization

Whole-mount in situ hybridization in *A. nummularia* roots was performed according to the procedure of Ludevid et al. (1992). Roots from plants grown in hydroponic solution without or with 400 mM NaCl (24 h) were fixed in PBS, 67 mM EGTA, and 6% formaldehyde for 25 min at room temperature. Fixed roots were stored in ethanol at −20°C for 3 d.

Digoxigenin-labeled RNA probes were obtained from the pAPME insert following the manufacturer's instructions (Boehringer Mannheim Biochemica No. 1277073) using T7 (sense) or SP6 (antisense) RNA polymerases. Hybridizations were carried out at 55°C for 18 h. Anti-digoxigenin coupled to alkaline phosphatase (Boehringer Mannheim Biochemica No. 1207733) was preabsorbed to root tips (previously fixed) before use on experimental material. Root tips were incubated with the preabsorbed anti-digoxigenin conjugate (1:2000 dilution) overnight at 4°C. The chromogenic reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium was carried out for 30 min.

RESULTS

Organ-Specific Induction of Plasma Membrane H+-ATPase Gene Expression by NaCl

The inserts from pAPM and pG1 detected single transcripts of 3.7 and 3.4 kb in *A. nummularia* and tobacco, respectively, in both poly(A)+ and total RNA (Niu et al., 1993; data not shown). The constitutive levels of plasma membrane H+-ATPase mRNA in the absence of NaCl treatment were comparable in leaves, roots, and stems of both *A. nummularia* and tobacco (Fig. 1). These results indicate that the ATPase probes isolated from cDNA libraries of cultured cells do not exhibit specificity for the transcripts produced in these different organs. However, differences in the level of mRNA were detected in leaves, stems, and roots during development (data not shown).

Induction of plasma membrane H+-ATPase mRNA accumulation occurred after NaCl treatment in roots but not in leaves (young and old combined) or stems, with substantially greater induction detected in roots of *A. nummularia* than of tobacco (Fig. 1). Similar results were obtained when the pAPME insert was used as a probe for the *A. nummularia* blots (Fig. 1A). Because this insert encodes essentially all of the open reading frame of the C-terminal portion of the ATPase, in which there is a high degree of conservation among plant plasma membrane H+-ATPases, it is unlikely that this probe would distinguish isoform-specific transcripts (Roldán et al., 1991). Thus, it is not probable that NaCl-induced stem- or leaf-specific isoforms had not been detected in these organs of *A. nummularia*.

The NaCl-induced increase in plasma membrane H+-ATPase mRNA in *A. nummularia* roots was localized to cells predominantly in the elongation zone and to some extent in the differentiation zone (Fig. 2). A low level of message was also detected in the cells of these zones in roots not treated with NaCl, and presumably this was due to requirements for the ATPase associated with housekeeping functions not related to salt adaptation. Little, if any, mRNA was detected in the root cap or meristem.

Constitutive plasma membrane H+-ATPase mRNA levels (Fig. 3) were substantially lower in mature (expanded) than in young (expanding) leaves of *A. nummularia*. No NaCl induction of mRNA accumulation occurred in expanding leaves. Salt treatment stimulated the accumulation of plasma membrane H+-ATPase message in expanded leaves. However, this induction increased the plasma membrane H+-ATPase mRNA from a level that was barely detectable to a level equivalent to that in expanding leaves not treated with NaCl. These data indicate that plasma membrane H+-ATPase levels in expanded leaves are low, presumably because osmotic adjustment and growth have ceased, thus minimizing the $\Delta\mu_{H^+}$ requirement. NaCl treatments result in an induction of the H+-ATPase, which is in part mediated by an increase in the message level, to facilitate energy-dependent transport across the plasma membrane to establish homeostasis in the new osmotic and ionic environment.

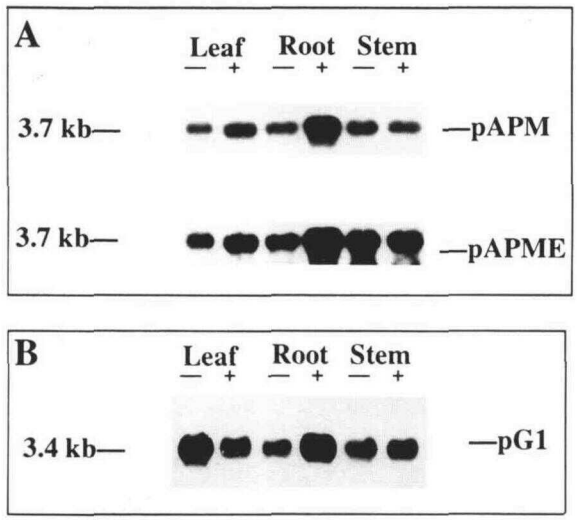

Figure 1. Organ specific induction of plasma membrane H+-ATPase mRNA accumulation by NaCl. Roots, stems, and leaves (young and old combined) of: A, *A. nummularia* plants grown without (−) or with (+) 513 mM NaCl in the nutrient solution added in 171 mM increments every 3 d starting at 171 mM with the plants being harvested 7 d after initiation of salt treatment, or B, tobacco plants grown without (−) or with (+) 428 mM NaCl in the nutrient solution added in 85.5 mM increments every 3 d starting at 85.5 mM with the plants being harvested 10 d after initiation of salt treatment. Each lane was loaded with 10 μg (*A. nummularia*) or 20 μg (tobacco) of total RNA. Probes used in hybridizations (refer to "Materials and Methods") and the approximate sizes of transcripts are indicated.

NaCl Induction of Plasma Membrane H+-ATPase mRNA Accumulation Is Greater in Roots of the Halophyte than the Glycophyte

Induction of plasma membrane H+-ATPase gene expression by NaCl was substantially more pronounced in roots of the halophyte *A. nummularia* than in those of the glycophyte tobacco (Figs. 1 and 4). At comparable NaCl concentrations, the difference in mRNA abundance between control and treated plants is substantially greater in *A. nummularia* than in tobacco roots (Fig. 4). In some instances, NaCl induction of mRNA accumulation in *A. nummularia* roots was so great that exposure of northern blots to x-ray film resulted in saturation of the mRNA signal before the transcript could be detected in the root RNA of untreated plants. The rate of induction of the plasma membrane H+-ATPase mRNA accumulation was much more rapid in *A. nummularia* than in

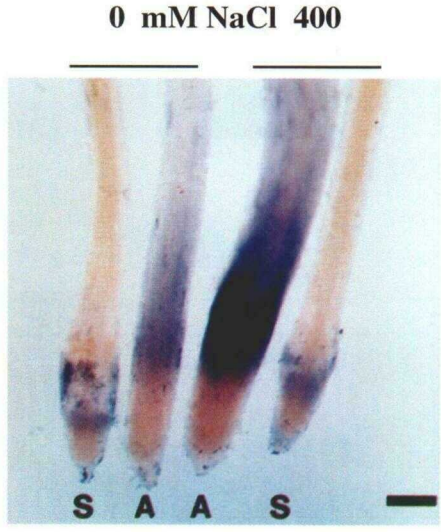

Figure 2. Localization of plasma membrane H^+-ATPase mRNA in the elongation and differentiation zones of *A. nummularia* roots. Roots were obtained from plants grown without or with 400 mM NaCl (which was added at one time) 24 h after the start of the salt treatment. Fixed roots were hybridized with antisense (A) or sense (S) RNA probes produced from the pAPME insert. The plasma membrane H^+-ATPase message location is visualized by the purple alkaline phosphatase reaction product, with greater intensity detected in salt-treated roots. Scale, 1 mm.

tobacco roots (Figs. 4 and 5). From 8 to 24 h, accumulation of plasma membrane H^+-ATPase mRNA in *A. nummularia* roots was dependent on time and the salt concentration; however, at 24 h and after, the message level was saturated at all NaCl concentrations (Figs. 4 and 5). In *A. nummularia*, plasma membrane H^+-ATPase mRNA accumulated in roots in response to extreme NaCl treatments that resulted in no induction or reduced message levels in tobacco roots (data not shown).

Although induction of the plasma membrane H^+-ATPase

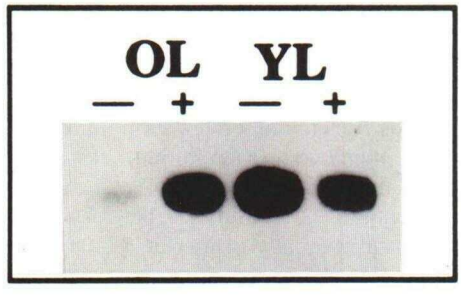

Figure 3. Developmental differences in NaCl induction of mRNA accumulation in *A. nummularia* leaves. Leaves were obtained from plants grown in hydroponic solutions without (—) or with 400 mM (+) NaCl for 24 h (salt was added to the solution at one time). YL, Expanding leaves; OL, expanded leaves. Each lane was loaded with 10 μg of total RNA.

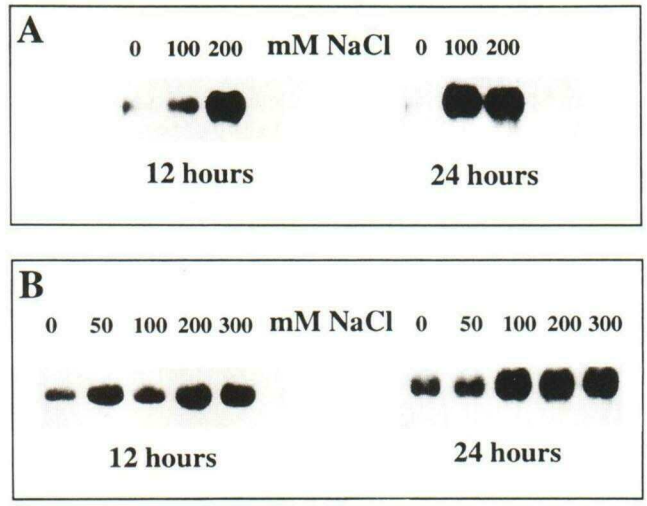

Figure 4. NaCl induction of plasma membrane H^+-ATPase mRNA accumulation in halophyte and glycophyte roots. Plants of *A. nummularia* (A) and tobacco (B) were grown in hydroponic solution without and with NaCl, and roots were harvested at 12 and 24 h after salt treatment. Each lane has 10 μg of total RNA.

mRNA accumulation could be detected as early as 8 h after NaCl treatment in *A. nummularia* roots (Fig. 5), the difference in message level between untreated and salt-treated roots persisted after 2 weeks and 1 week for *A. nummularia* (Fig. 6A) and tobacco (Fig. 6B), respectively. It is assumed that these results indicate that salt adaptation continues beyond these periods at the NaCl concentrations used in these experiments. In cultured *A. nummularia* cells, it was determined that NaCl-induced mRNA accumulation occurred during the period of adaptation; however, after cells established a new steady-state growth in the saline environment, the message levels were lower and similar to those in cells growing in medium without salt (Niu et al., 1993). Presumably, this means that there is increased requirement for ATPase production initially after salt treatment to establish ion homeostasis; however, maintenance of ion concentration gradients in the adapted state is dependent on secondary transport adaptations.

DISCUSSION

We have established that NaCl induces the accumulation of plasma membrane H^+-ATPase mRNA in cultured cells of *A. nummularia* (Niu et al., 1993) and tobacco (Perez-Prat et al., 1993). It was suggested that these cells, which must be salt tolerant through intrinsically cellular mechanisms, regulate the activity of this ATPase in response to NaCl to accommodate solute accumulation necessary for osmotic adjustment. Some of these solutes, including Na^+ and Cl^-, are toxic to cytosolic metabolisms; thus, uptake of these ions must be coordinated with the intracellular capacity for vacuolar compartmentation. In this report, we provide evidence of the organ-specific induction of plasma membrane H^+-ATPase gene expression in response to NaCl, principally in roots and expanded leaves. Although our probes apparently

do not differentiate transcripts of different isoforms, it is possible that unique plasma membrane H+-ATPase genes exist that are regulated by NaCl or by changes in the osmotic environment (DeWitt et al., 1991).

NaCl-induced plasma membrane H+-ATPase mRNA accumulation in the roots of *A. nummularia* and tobacco indicates a requirement for this pump in these organs during salt adaptation. The higher message accumulation in *A. nummularia* roots provides an indication that increased H+ pump activity detected in this organ after salt treatment (Braun et al., 1986) is at least partially due to transcriptional regulation. A requirement for increased pump activity in this situation is consistent with the fact that, with few exceptions, the root is the primary barrier to the uptake of Na^+ and Cl^- into the plant (Pitman, 1984; Flowers et al., 1986; Clarkson, 1991). Ion entry into the root symplasm is controlled at the plasma membrane of the epidermal, cortical, or endodermal cells (Clarkson, 1991), because the Casperian strip in the endodermis presumably prevents apoplastic transport into the xylem, from where ions are transported to the shoot (Pitman, 1984). Immunocytochemical localization has indicated that the plasma membrane H+-ATPase is concentrated in the epidermis and endodermis of oat roots (Parets-Soler et al., 1990). Contrary to one previous distinction made about glycophytes and halophytes, it is now known that both types of species can limit Na^+ uptake into the shoot, i.e. salt tolerance in halophytes is not necessarily dependent on high levels of Na^+ accumulation in the shoot (Glenn et al., 1992).

The high degree of NaCl induction of plasma membrane H+-ATPase mRNA accumulation in *A. nummularia* roots as determined by analyses of the entire organ was apparently the result of increased transcript levels in cells of the elongation and differentiation zones. Histochemical evaluation has identified high plasma membrane H+-ATPase contents in cells associated with the differentiation zones of oat and pea roots (Parets-Soler et al., 1990). The pattern of increased mRNA levels in the elongation and differentiation zones may be indicative of regions in the roots that are functioning substantially in the regulation of plant ion homeostasis during salt adaptation.

The induction of plasma membrane H+-ATPase mRNA accumulation in old (fully expanded) but not in young (expanding) *A. nummularia* leaves might be linked to the function of these organs as Na^+ and Cl^- sinks in a salt episode

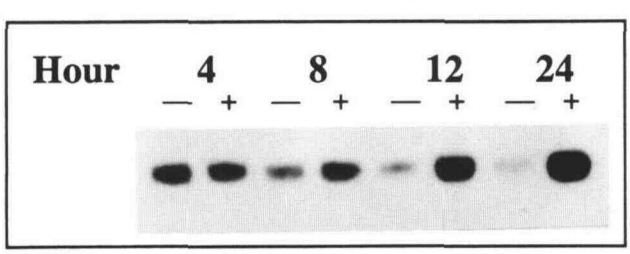

Figure 5. Kinetics of NaCl induction of plasma membrane H+-ATPase mRNA accumulation in *A. nummularia* roots. Plants were grown in the absence (−) or presence (+) of 400 mM NaCl (added at one time) for the time indicated before harvest. Total RNA (10 μg) was loaded for each sample.

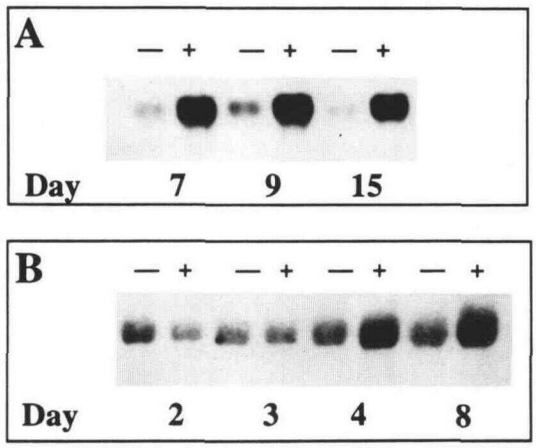

Figure 6. Maintenance of elevated plasma membrane H+-ATPase mRNA levels in roots after induction by NaCl. Roots of plants growing without (−) and with (+) NaCl in the nutrient solution were harvested on the days indicated. A, NaCl was added to the hydroponic solution of *A. nummularia* plants in 171 mM increments every 3 d starting at 171 mM; the final concentration was 513 mM. B, Tobacco plants were treated with 200 mM NaCl (added at one time). Time after initiation of salt treatment is indicated. Each lane was loaded with 10 μg of total RNA.

(Flowers et al., 1986). Despite the fact that the ions would be compartmentalized in the vacuole (Jeschke, 1984; Binzel et al., 1988), control of uptake across the plasma membrane is critical to the maintenance of ionic homeostasis in the cytosol (Jeschke, 1984). Our capacity to detect the induction of plasma membrane H+-ATPase mRNA accumulation, or perhaps that of a specific isoform, by NaCl was perhaps facilitated by the quiescent state of the cells in old leaves that resulted in relatively low levels of the message before salt treatment. The low mRNA level is indicative that energy-dependent solute accumulation for osmotic adjustment in these nonexpanding cells is minimal, requiring little pump activity. However, perturbation by NaCl induces the pump activity, in part mediated by increased message accumulation, to reestablish Na^+ and Cl^- homeostasis.

Enhanced H+-translocating activity of the plasma membrane H+-ATPase was detected in *A. nummularia* roots but not in the roots of the glycophyte cotton after NaCl treatment (Braun et al., 1986; Hassidim et al., 1986). Our data indicate that the response to NaCl of the halophyte *A. nummularia* for induction of plasma membrane H+-ATPase gene expression is substantially faster and relative message accumulation is greater than in the glycophyte tobacco. The significance of rapid mRNA accumulation is not yet understood, although a rapid turnover of the plasma membrane H+-ATPase, an apparent half-life of 12 min, has been reported in maize (Hager et al., 1991). The greater capacity of the halophyte to induce the H+-ATPase in response to NaCl, presumably due to transcriptional activation, indicates the presence of unique regulatory elements and/or transcription factors that are highly responsive to salt.

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