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Plasma-membrane H^+-ATPase gene expression is regulated by NaCl in cells of the halophyte *Atriplex nummularia* L.

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Abstract. An *Atriplex nummularia* L. cDNA probe encoding the partial sequence of an isoform of the plasma-membrane H^+-ATPase was isolated, and used to characterize the NaCl regulation of mRNA accumulation in cultured cells of this halophyte. The peptide (447 amino acids) translated from the open reading frame has the highest sequence homology to the *Nicotiana plumbaginifolia* plasma-membrane H^+-ATPase isoform pma4 (greater than 80% identity) and detected a transcript of approximately 3.7 kb on Northern blots of both total and poly(A)$^+$ RNA. The mRNA levels were comparable in unadapted cells, adapted cells (cells adapted to and growing in 342 mM NaCl) and deadapted cells (cells previously adapted to 342 mM NaCl that are now growing without salt). Increased mRNA abundance was detected in deadapted cells within 24 h after exposure to NaCl but not in unadapted cells with similar salt treatments. The NaCl up-regulation of message abundance in deadapted cells was subject to developmental control. Analogous to those reported for glycophytes, the plasma-membrane H^+-ATPase are encoded by a multigene family in the halophyte.

Key words: *Atriplex* – Gene expression – NaCl regulation – Halophyte – Plasma-membrane H^+-ATPase

Introduction

Since Na^+ and Cl^- lower the water potential ($\Delta\varphi$) of the external environment of plants, salt adaptation must involve cellular mechanisms that facilitate osmotic adjustment, in order to re-establish turgor and growth. This is accomplished, in part, by utilization of these ions as osmotic solutes (Flowers et al. 1977; Binzel et al. 1987). Both Na^+ and Cl^- are compartmentalized in the vacuole where these ions are accumulated to concentrations above that in the external environment (Binzel et al. 1988). However Na^+ and Cl^- are largely excluded from the cytoplasm, and organic solutes accumulate in this compartment to balance the water relations in the cytoplasm and function as osmoprotectants of intracellular processes from the deleterious activities of these ions (Rhodes 1987).

Under saline conditions, it is critical that plant cells limit net ion uptake across the plasma membrane to a rate that is compatible with the capacity to compartmentalize Na^+ and Cl^- in the vacuole, thereby preventing an excessive buildup of these ions in the cytoplasm (Binzel et al. 1988). Bidirectional ion transport across the plasma membrane, necessary for re-establishing and maintaining turgor and avoiding cytoplasmic ion toxicity, is mediated by secondary transporters, i.e. carriers or channels that facilitate active or passive ion flux across the membrane (Reinhold and Kaplan 1984). With the exception of Ca^{2+}, active transport of ions is believed to be driven by an H^+ electrochemical gradient ($\Delta\mu_{H^+}$), that is generated by the plasma-membrane H^+-ATPase as a result of vectoral H^+ transport to the apoplast (Sze 1985). Under physiological conditions, evacuation of Na^+ from the cytoplasm across the plasma membrane is energy-dependent, presumably mediated by an Na^+/H^+ antiporter (Reinhold et al. 1989), while efflux of Cl^- occurs as a result of electrophoretic downhill transport, apparently through an outward rectifying channel (Tester 1990). Control of ion transport across the plasma membrane and tonoplast is thus a key factor in salt tolerance of plant cells. We have reported that in tobacco cells the gene for the 70-kDa subunit of the tonoplast H^+-ATPase is regulated by NaCl (Narasimhan et al. 1991), as is a putative endoplasmic-reticulum Ca^+-ATPase gene (Perez-Prat et al. 1992).

The plasma-membrane H^+-ATPase is stimulated by K^+ and requires Mg^{2+} for catalysis (Briskin 1990). This pump is modulated by auxin, fusicoccin, inositol phospholipids, cytokinins, abscisic acid, light and is sensitive to changes in turgor (Reinhold et al. 1984; Poole 1988; Hedrich and Schroeder 1989; Briskin 1990; Chen and Boss 1991). Even though H^+-translocating activities of the pump have been determined to increase substantially

in response to NaCl treatment (Braun et al. 1986; Watad et al. 1986), it is still uncertain if the gene is also regulated by NaCl as is the tonoplast H^+-ATPase gene.

We have utilized cells of the halophyte, *Atriplex nummularia* L. (Casas et al. 1991; 1992) to ascertain if expression of the plasma-membrane H^+-ATPase gene is regulated in response to NaCl exposure and as a function of salt adaptation in this naturally salt-tolerant species. Here, we make the initial report that accumulation of plasma-membrane H^+-ATPase mRNA is induced in plant cells by NaCl exposure. However, message levels in cells adapted to and growing in NaCl-containing medium were comparable to those in cells growing in the absence of salt. Furthermore, NaCl induction of mRNA accumulation was detected in deadapted cell (cells previously adapted to NaCl) but not in unadapted cells, indicating that adaptation enhances the responsiveness of plasma-membrane H^+-ATPase gene expression to NaCl. The capacity to regulate transcript accumulation in response to NaCl was detected in cells only at certain developmental growth stages. These data provide evidence that enhanced H^+-transport activity induced by NaCl in *A. nummularia* (Braun et al. 1986) is mediated at least in part by transcriptional or post-transcriptional processes that result in higher mRNA accumulation.

Materials and methods

Cell cultures. Atriplex nummularia L. cell cultures were isolated and maintained as described by Casas et al. (1991). Three cell lines were used: unadapted cells, grown in medium without salt for over 200 cell generations; adapted cells, adapted to 342 mM NaCl for over 200 generations; and deadapted cells, first adapted to 342 mM NaCl for over 100 cell generations, then returned to medium without salt for over 100 cell generations. Cells at the early stationary phase were inoculated into fresh medium (0.02 g fresh weight · mL^{-1}) and grown to the stage indicated. In experiments that evaluated the response of cells to NaCl, control and salt-treated cells were inoculated from the same stock culture. The appropriate amount of salt was added to cultures using a stock solution of 4 M NaCl in medium. A comparable volume of medium without NaCl was added to control cultures.

Screening of the cDNA library. An *A. nummularia* λZAPII (Stratagene, La Jolla, Calif., USA) cDNA library from cells adapted to 342 mM NaCl (Casas et al. 1992) was screened using a *Nicotiana tabacum* L. cv. W38 cDNA insert encoding a plasma-membrane H^+-ATPase. Procedures for labelling the probe and conditions for screening the library have been described previously (Casas et al. 1992). The isolated λZAPII clones were converted to pBluescript phagemid by in-vivo excision with R408 helper phage (Stratagene).

Sequencing of DNA. The sequence of both strands of the cDNA insert was determined by the dideoxy-nucleotide chain-termination method (Sequenase kit; USB, Cleveland, Oh., USA) using CsCl-purified double-stranded DNA, with T3 and T7 primers. Initially, the entire clone was used as template. The complete sequence was obtained from deletions made with Erase-A-Base (No. E5750; Promega Corp., Madison, Wis., USA).

Isolation and analysis of DNA and RNA. Cultured cells were harvested by filtration on No. 4 filter paper (Whatman Lab. Products, Clifton, N.J., USA) rinsed with fresh medium and frozen in liquid nitrogen. Cells were stored at −80° C until they were used for RNA or DNA isolation. Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi 1987) as described by Casas et al. (1992). Polyadenylated RNA was isolated by affinity chromatography using oligo(dT)-cellulose. Separation of RNA by formaldehyde agarose gel (1.2%) electrophoresis and capillary transfer to nitrocellulose membranes followed standard protocols (Sambrook et al. 1989). Transcript size was estimated on ethidium-bromide-stained gels by comparing with a 0.24 to 9.5 kb RNA ladder (No. 56205A; BRL, Gaithersburg, Md., USA). Proper loading of RNA onto gels was determined by ethidium-bromide staining and complete transfer of RNA to nitrocellulose membranes by evaluating gels and membranes after transfer with a UV transilluminator. The RNA integrity was ensured by visualizing the rRNA bands and estimating the relative quantities of 18S and 25S rRNA (Sambrook et al. 1989). In some instances gel loading, transfer and message integrity were verified using the *Drosophila* actin 5C probe (Fryberg et al. 1983).

DNA was extracted from *A. nummularia* cells, and digested with the restriction endonucleases indicated. The restricted DNA was fractionated on 0.7% agarose gels and then transferred to nitrocellulose membranes by capillary transfer (Casas et al. 1992).

The ^{32}P-labeled probes for Northern and Southern hybridizations were prepared with a random-primer labelling kit (Boehringer Mannheim, Indianapolis, Ind., USA) using α-^{32}P dCTP (ICN Biochemicals, Irvine, Calif., USA). Hybridizations were carried out as described by Casas et al. (1992). Briefly, prehybridizations were done in the solution containing 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% SDS (sodium dodecyl sulfate) and 100 μg ml^{-1} herring-sperm DNA, at 42° C, in a hybridization oven (Bellco Glass, Inc., Vineland, N.J., USA), for 3 h. The hybridizations were carried out in the same solution plus ^{32}P-labeled probes, at 42° C, overnight. Posthybridization washes were for 15 min each in 0.1% SDS plus 1 × (twice), 0.2 × and 0.1 × SSC at room temperature.

Results

An *A. nummularia* λZAPII cDNA was isolated using a 1.556-kb tobacco cDNA insert that encoded the C-terminal partial sequence (419 amino acids) of a plasma-membrane H^+-ATPase. A pBluescript phagemid was obtained that contained an insert of 2.252 kb with an open reading frame of 1.764 kb. The deduced peptide contained the KGAP, DPPR, MITGD and MTGDGVNDAPALK motifs that are proposed to be involved in ATP binding and are highly conserved in P-type ATPases (Serrano 1989). The peptide was compared to sequences in Genbank (Release 72, September 9, 1992) using the TFASTA and BESTFIT programs. The deduced amino-acid sequence is most similar to plant plasma-membrane H^+-ATPase with less homology to P-type H^+-ATPase in other organisms and to other P-type ATPases (Table 1). The *A. nummularia* cDNA insert therefore encodes a plasma-membrane H^+-ATPase. By analogy with the PMA4 isoform of the plasma-membrane H^+-ATPase from *Nicotiana plumbaginifolia,* the sequence represents 62% of the protein including the C-terminus, amino acids 364–952.

The probe used for Northern and Southern blots was a 1.829-kb insert (pAPM), that represented a 5′ 423-bp deletion of the *A. nummularia* 2.252-kb insert. The 1.829-kb insert encoded the C-terminal 447 amino acids and contained a 488-bp 3′ untranslated region. This insert detected a single mRNA band of 3.7 kb in both total and

Table 1. Homology comparison of deduced amino-acid sequences by the BESTFIT program based on Genbank Release 72 (September 9, 1992). PM, plasma membrane; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum

Species	Isoform	% identity	Reference
PM H^+-ATPases			
Nicotiana plumbaginifolia	PMA4	83.5	Moriau et al. 1992[a]
Arabidopsis thaliana	AHA2	83.3	Harper et al. 1990
Arabidopsis thaliana	AHA1	81.9	Harper et al. 1989
Arabidopsis thaliana	AHA3	78.7	Pardo and Serrano 1989
Nicotiana plumbaginifolia	PMA1	78.3	Perez et al. 1992
Nicotiana plumbaginifolia	PMA2	78.0	Boutry et al. 1989
Lycopersicon esculentum (tomato)	LHA1	77.5	Ewing et al. 1990
Nicotiana plumbaginifolia	PMA3	76.8	Perez et al. 1992
Oryza sativa (rice)	OSA1	75.3	Wada et al. 1992
Neurospora crassa		34.9	Hager et al. 1986
Schizosaccharomyces pombe (yeast)	PMA2	33.6	Ghislain and Goffeau 1991
ER Ca^{2+}-ATPases			
Lycopersicon esculentum	LCA	29.5	Wimmers et al. 1992
Drosophila melanogaster SR/ER		25.6	Magyar and Varadi 1990

[a] Unpublished sequence, Genbank accession number X66737

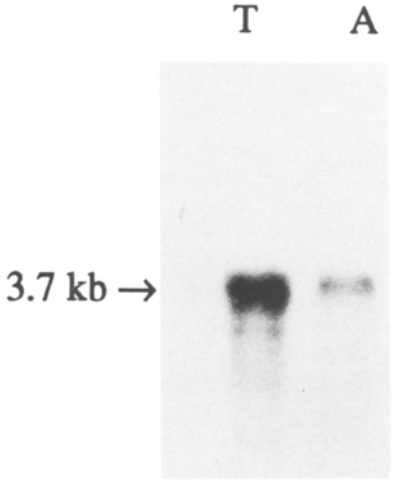

Fig. 1. The mRNA detected by *A. nummularia* cDNA probe for the plasma-membrane H^+-ATPase in both total RNA (*T*, 20 µg) and poly(A)$^+$ RNA (*A*, 1 µg), in 342-mM NaCl-deadapted cells. The approximate molecular size of the message is indicated adjacent to the band in the autoradiogram

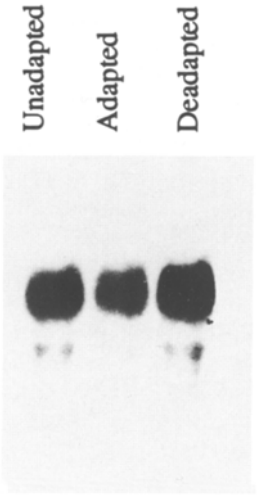

Fig. 2. Comparison of levels of plasma-membrane H^+-ATPase mRNA in unadapted, 342-mM NaCl-adapted and deadapted *A. nummularia* cells. RNA was extracted from 14-day-old unadapted cells and 10-day-old NaCl-adapted and deadapted cells. Twenty micrograms of total RNA was used for each cell line

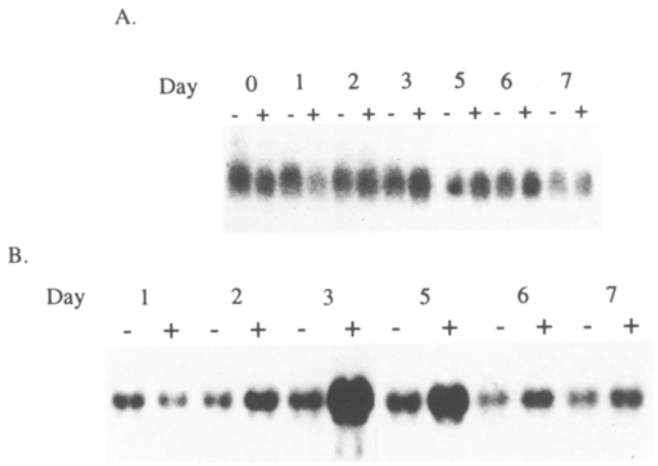

Fig. 3A, B. Levels of plasma-membrane H^+-ATPase mRNA in unadapted (**A**) and NaCl deadapted (**B**) *A. nummularia* cells 24 h after exposure to 342 mM NaCl. At the day indicated after inoculation *(Day)* the medium was adjusted to contain 342 mM NaCl by the addition of a solution of 4 M NaCl in fresh medium (+). An equal volume of fresh medium without NaCl was added to control cells at the same time (−). Illustrated are Northern blots of 20 µg of total RNA

poly(A)$^+$ RNA of NaCl-deadapted cells (Fig. 1). Similar results were observed with RNA obtained from unadapted cells and leaves, stems and roots of plants grown hydroponically (data not shown).

Plasma-membrane H^+-ATPase mRNA levels and NaCl induction of message accumulation in unadapted, deadapted and NaCl-adapted A. nummularia cells. Similar amounts of plasma-membrane H^+-ATPase mRNA were detected in unadapted, deadapted and NaCl-adapted cells (Fig. 2). Little variation in mRNA level was detected during the growth cycle from lag to early/mid-stationary phase for any of the cell lines.

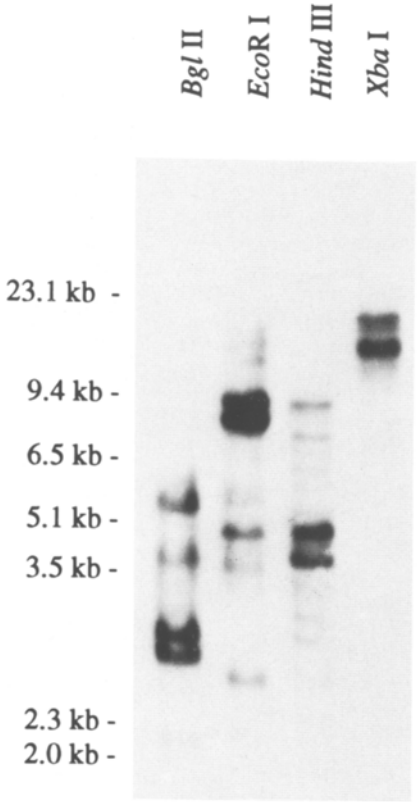

Fig. 4. Southern blot analysis of *A. nummularia* genomic DNA. Each lane was loaded with 15 μg *A. nummularia* genomic DNA digested with the indicated restriction endonucleases and separated on a 0.7% agarose gel

Plasma-membrane H^+-ATPase message was induced by 24-h exposure to 342 mM NaCl in deadapted cells but not in unadapted cells (Fig. 3A, B). These data indicate that previous adaptation to salt increases the responsiveness of cells to re-exposure to NaCl. However, our data do not preclude the possibility that message accumulation occurs in unadapted cells after longer periods of exposure to NaCl or in the presence of different salt concentrations. Since results from cells in culture would seemingly not be complicated by differential expression of organ- or tissue-specific isogenes as occurs in cells of plants, it seems unlikely that the NaCl-regulated transcript could not be detected in unadapted cells. It is not clear that the probe used in the experiments is unique enough to detect differentially any specific isoforms. Also, since a high proportion of cells in a culture population are actively growing, it is likely more difficult to identify an increase in a specific H^+-ATPase isoform transcript, i.e. salt-induced mRNA, because of the background of message encoding the isoform that is active during cell growth.

The induction of mRNA accumulation within 24 h after re-exposure to NaCl may indicate a functional role for the plasma-membrane H^+-ATPase in the generation of $\Delta\mu_{H^+}$ necessary for osmotic adjustment and salt adaptation. This is in contrast to results reported for *Helianthus annuus* (Roldán et al. 1991). These contrasting results may be explained by differences in NaCl responsiveness of the glycophyte sunflower and the cells of the halophyte *A. nummularia*. The capacity of *A. nummularia* cells to accumulate H^+-ATPase mRNA in response to NaCl was developmentally regulated, with cells in the transition from lag to exponential growth phase being most inducible (Fig. 3B). Previously, we have determined that NaCl induces the accumulation of mRNA for two other ATPases, the 70-kDa subunit of the tonoplast ATPase (Narasimhan et al. 1991) and a putative endoplasmic Ca^{2+}-ATPase (Perez-Prat et al. 1992), in deadapted cells of the glycophyte tobacco.

Genomic southern analysis. Southern blot analyses of *A. nummularia* nuclear DNA indicated the existence of a plasma-membrane H^+-ATPase multigene gene family (Fig. 4). Under the conditions used, it was possible to discern at least two copies of the ATPase gene. These data provide evidence that, analogous to glycophytes (Ewing et al. 1990; Harper et al. 1990), halophytes also contain multiple copies of the H^+-ATPase gene.

Discussion

It is generally envisaged that a primary consequence of exposing plants to a high NaCl environment is the rapid accumulation of Na^+ and Cl^- in the cytoplasm of cells that are directly in contact with the saline solution. Initially, this may be restricted to root cortical cells; however, movement of the ions to the xylem and exposure of cells in the shoot portion of the plant occur subsequently. Since the cytoplasm is very susceptible to the inhibitory effects of Na^+ and Cl^-, evacuation of these ions from this intracellular compartment is a priority for survival and growth. Efflux of these ions across the plasma membrane is mediated by secondary active transporters, presumably an Na^+/H^+ antiporter and a Cl^- channel (Braun et al. 1989; Tester 1990), that utilize the H^+ electrochemical gradient produced by the activity of the plasma-membrane H^+-ATPase. Our data indicate that the "activation" of the plasma-membrane H^+-ATPase in response to a saline environment involves mRNA accumulation that is detected within 24 h after NaCl treatment and this is likely a primary process in adaptation. These results are indicative that increased H^+-pump activities detected in *A. nummularia* roots grown with 400 mM NaCl (Braun et al. 1986) are in part due to transcriptional or post-transcriptional processes that increase the abundance of plasma-membrane H^+-ATPase mRNA. In a previous report, we have described the NaCl induction of mRNA accumulation of the 70-kDa subunit of the tonoplast H^+-ATPase in tobacco cells (Narasimhan et al. 1991).

An apparent consequence of previous NaCl adaptation is genetic changes that enhance the stress responsiveness of plasma-membrane H^+-ATPase gene expression. This is similar to observations made for mRNAs of the 70-kDa subunit of the tonoplast H^+-ATPase and a putative endoplasmic reticulum Ca^{2+}-ATPase in tobacco cells (Narasimhan et al. 1991; Perez-Prat et al. 1992). Analogous observations have been made in the studies

of habituation (Meins 1989), thermotolerance (Nagao et al. 1990), and salt tolerance (Winicov and Button 1991; Winicov and Seeman 1990), yet the genetic or epigenetic mechanisms behind the well-documented observations remain ambiguous. In principle, DNA methylation, transposition of controlling elements and gene amplification are a few possible modifications in the genome that might occur during stress adaptation (Cullis 1990), and perhaps any of these could be the basis for enhanced plasma-membrane H^+-ATPase gene expression in NaCl-deadapted cells in response to re-imposition of salt. It also would be intriguing to determine if altered responsiveness of genes to a stress result from *cis* element structure alterations or modifications in expression or effectiveness of *trans* activating complexes.

Assuming that the levels of plasma-membrane H^+-ATPase mRNA detected in unadapted, deadapted and NaCl-adapted cells are reflective of H^+-transport activities, then the data presented in this report indicate that high pump activities required in cells after salt exposure are not necessary after adaptation. It is expected that cells would utilize substantial energy resources to drive the plasma-membrane H^+ pump in order to survive the sudden and potentially catastrophic elevation of cytosolic Na^+ and Cl^- that occurs as a result of salt imposition. However, our data [comparable carbon-use efficiencies of (Schnapp et al. 1991a, b), and mRNA levels in, cells growing without NaCl and salt-adapted cells] indicate that after adaptation cells do not have high pump activities and, as a consequence, do not expend large amounts of energy to maintain ion homeostasis. This strongly implies that plasma-membrane adaptations that regulate passive ion flux contribute substantially to the maintenance of intracellular ion relations. These passive flux adaptations would seemingly be critical in order for cells to redirect energy resources from the H^+ pump to metabolic and physiological processes required for growth in the saline environment.

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