

rank departments. There is continual pressure to fill gaps and improve academic rating schemes. Despite, or even because of controversy, the US News and World Report, and its counterparts, carry great weight and drive changes to meet their standards. The modest proposal above could recognize departments and schools that have succeeded in lifting the 'triple burden'.

Our analysis confirms the findings of various authors on women in science in different countries, that there is gender-related discrimination and stress in the practice of science. It is apparent that the stress is a product of similar reasons: a lack of universalism in science, undue burden of domestic responsibilities and lack of informal networking. Among these, while domestic burden is a product of gender stereotyping, the other two arise from the social organization of science. However, social norms related to gender infiltrate the organizations where science is practised, and the three are therefore interrelated. The common triple burden proves that the culture of science and the universal gender role schism is responsible for a low position of women in science across continents.

1. Stolte-Heiskanen, V. *et al.* (eds), In *Women in Science: Token Women or Gender Equality?*, Berg Publishers, Oxford, 1991, pp. 35–62.
2. Hicks, E., In *Women in Science: Token Women or Gender Equality?* (eds Stolte-Heiskanen, V. *et al.*), Berg Publishers, Oxford, 1991, pp. 173–192.
3. Tabak, F., Women scientists in Brazil: Overcoming national, social and professional obstacles. Paper presented at the Third World Organization of Women Scientists, Cairo, January 1993.
4. Osborne, M., Status and prospects of women in science in Europe. *Science*, 1994, **263**, 1389–1391.
5. Abir-Am, P., Science policy for women in science: from historical case studies to an agenda for women in science. History of Science Meetings, Madison, Wisconsin, 2 November 1991.
6. Stolte-Heiskanen, V., (eds), *Women in Science: Token Women or Gender Equality?*, Berg Publishers, Oxford, 1991, pp. 1–8.
7. Etzkowitz, H., Kemelgor, C. and Uzzi, B., In *Athena Unbound: The Advancement of Women in Science and Technology*, Cambridge University Press, Cambridge, 2000, pp. 203–224.
8. Merton, R. K., In *The Sociology of Science*, University of Chicago Press, Chicago, [1942] 1973, pp. 267–278.
9. Fuchs, S., von Stebut, J. and Allmendinger, J., Gender, science and scientific organizations in Germany. *Minerva*, 2001, **39**, 175–201.
10. Etzkowitz *et al.*, Women's share of science and engineering Ph Ds, 1966–1997. US NSF, Survey of Earned Doctorates, 2000, p. 11.
11. Mukhopadhyay, C. C. and Seymour, S. (eds), In *Women, Education and Family Structure in India*, Westview Press, Colorado, 1994, pp. 103–132.
12. Science career for Indian women: An examination of Indian women's access to and retention in scientific careers. Report, Indian National Science Academy, New Delhi October 2004. Accessed on 30 June 2005. <http://www.insaindia.org/Scienceservice/science.htm>
13. Gupta, N. and Sharma, A. K., Women academic scientists in India. *Soc. Stud. Sci.*, 2002, **32**, 901–915.
14. Gupta, N. and Sharma, A. K., Gender inequality in work environment at institutes of higher learning in science and technology in India. *Work, Employ. Soc.*, 2003, **17**, 619–638.
15. Etzkowitz, H., Kemelgor, C. and Uzzi, B., In *Athena Unbound: The Advancement of Women in Science and Technology*, Cambridge University Press, Cambridge, 2000, pp. 152–153.

16. Guidelines and Format for Submission of Project Proposals, Science and Society Programmes, Department of Science and Technology, Ministry of Science and Technology, New Delhi, pp. 3–4.
17. Women scientists programs <http://dst.gov.in/scprog/women.htm>, accessed on 30 June 2005.
18. Etzkowitz, H. and Gupta, N., Women in science: A fair shake? *Minerva*, 2005 (in press).

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Salt stress effects on the accumulation of vacuolar H⁺-ATPase subunit c transcripts in wild rice, *Porteresia coarctata* (Roxb.) Tateoka

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The ion pump, vacuolar H⁺-ATPase (V-ATPase) plays a major role in the maintenance of cellular pH, and influences the transport of cations into the vacuoles of plant cells. A cDNA clone (*PcVHA-c1*) encoding the c subunit of V-ATPase was isolated from the salt-tolerant wild rice, *Porteresia coarctata*. The DNA sequence of *PcVHA-c1* showed significant homology with V-ATPase subunit c of rice. The deduced amino acid sequence of *PcVHA-c1* and other reported c subunits were compared, and sequence relationships have been drawn to know their genetic relatedness. Southern analysis suggested the presence of multiple coding regions for subunit c in *P. coarctata*. Northern and Western analyses of salt-treated *P. coarctata* plants revealed that subunit c of V-ATPase is upregulated by NaCl treatment at both transcriptional and translational level.

Keywords: *Porteresia coarctata*, salt tolerance, subunit c, V-ATPase, wild rice.

TONOPLAST of the plant cell separates the vacuole from the cytoplasm. Specific proteins present in the tonoplast drive the transport of ions and other metabolites across it¹. The selective transport properties of the tonoplast are central

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to the multifarious functions of the vacuole, and serve to retain toxic materials within the vacuolar sap while exchanging metabolically useful compounds with the cytosol. Therefore, in the past few years, there has been increasing emphasis on understanding the nature of transporters in the vacuolar membranes^{2,3}. The primary active transport process in the tonoplast is the accumulation of H⁺ by the action of two H⁺ translocating enzymes⁴, i.e. H⁺-ATPase and H⁺-PPase. The vacuolar H⁺-ATPase (V-ATPase) is a universal component, and it is involved in acidification of intracellular compartments of eukaryotic cells^{1,5}. The V-ATPase pumps protons from the cytoplasm to the lumen of the vacuole against the electrochemical gradient using the energy released by ATP hydrolysis, and regulates¹ the cytoplasmic pH.

V-ATPase is a multi-subunit complex composed of two functional domains, the peripheral V₁ cytoplasmic domain and the membrane integral V₀ domain⁶. The molecular mass of the V₁ domain is about 500 kDa, and it is responsible for ATP hydrolysis. It consists of at least eight different subunits of molecular weight 13–70 kDa (subunits A–H). The V₀ domain is a 250 kDa complex consisting of at least five different subunits of molecular weight 17–100 kDa (subunits a–d), and is involved in proton translocation⁷. The major components of V-ATPase, subunits A, B and c are found in all V-ATPases studied so far and thus seem to be essential subunits. The V₀ domain has been shown to contain the binding site for bafilomycin⁸. The 16 kDa polypeptide subunit c is particularly interesting as it is a major component of the membrane V₀ domain, and it plays a major role in forming the proton conductance pathway^{8,9}. The 16 kDa polypeptide is highly hydrophobic, initially cloned from bovine adrenal cells¹⁰ and has been widely studied in yeast¹¹ and also in higher plant systems¹².

The ability of plants to adapt and grow in a changing environment suggests that V-ATPase is subject to regulation by hormones and environmental factors², and some evidence is available for the enhancement of V-ATPase activity in response to stress due to chilling and salinity, where V-ATPase itself undergoes changes in its fine molecular structure¹³. The present investigation was therefore undertaken to understand the regulatory nature of the V-ATPase subunit c gene isolated from a salt-tolerant mangrove associated species *Porteresia coarctata*, long considered as a wild relative of cultivated rice. This communication deals with the structural and functional aspects of the V-ATPase subunit c from *P. coarctata* and its regulation under salinity stress.

P. coarctata plants growing in high saline conditions in the mangrove forests in the estuarine region of Pichavaram, Tamil Nadu, India were collected and grown in pots with clay soil in greenhouse under natural light condition. Young tillers produced in two months time were used for experimental studies.

Genomic DNA was isolated from leaves of *P. coarctata* as described previously¹⁴ and digested with different restriction enzymes according to the manufacturer's instructions. The DNA run on a 0.8% agarose gel was transferred to nylon membrane (Hybond N⁺, Amersham Biosciences) by capillary transfer method¹⁵. Membranes were incubated in pre-hybridization solution (6X SSC, 5X Denhardt's reagent, 0.5% SDS, 50% formamide and 100 µg/ml denatured Salmon sperm DNA) at 42°C. After 3 h, the pre-hybridization solution was replaced with hybridization solution (6X SSC, 0.5% SDS, 50% formamide and 100 µg/ml denatured salmon sperm DNA). Hybridization was carried out overnight at 42°C with gentle rotation. The membrane was sequentially washed in 2X SSC, 0.1% SDS and 1X SSC, 0.1% SDS at room temperature for 15 min each, followed by a high stringency wash (0.1X SSC, 0.1% SDS) at 55°C for 30 min, and exposed to X-ray film with intensifying screens at -70°C. Southern analysis was performed using the full-length *PcVHA-c1* probe.

Total RNA from leaf and root tissues of *P. coarctata* was isolated following the protocol as previously described¹⁶. Total RNA (20 µg) was separated in a 1.4% formaldehyde agarose gel and transferred to nylon membrane (Hybond N⁺, Amersham) by capillary transfer method¹⁵. Pre-hybridization was carried out for 1 h at 42°C in 50% formamide, 6X SSC (1X is 0.15 M NaCl, 0.015 M sodium citrate), 25 mM sodium phosphate buffer (pH 6.5), 10X Denhardt's solution (1X Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA) and 250 µg/ml denatured salmon sperm DNA. Hybridization was carried out overnight at 42°C in 50% formamide, 6X SSC, 25 mM sodium phosphate buffer (pH 6.5) and 250 µg/ml denatured salmon sperm DNA. The membranes were washed in medium stringency buffer (0.5X SSC, 0.1% SDS) at 55°C for 15 min, and exposed to X-ray film with intensifying screens at -70°C. Northern analysis was initially carried out with the full-length *PcVHA-c1* probe. The leaf RNA blot was then stripped and re-probed with *PcVHA-c1* UTR-specific fragment. The primers used for PCR amplification of the UTR region of V-ATPase are: V-ATPase forward- 5'-TTG CAG TAC CAA TCC GCA GTT-3'; V-ATPase reverse-5'-GGA GCA GCA AAT CCA GCC TA-3'. The program used for amplification of the UTR region using these primers included an initial denaturation step of 94°C for 1 min; 30 cycles of 94°C for 30 s, 66°C for 45 s and 72°C for 30 s; and a final extension of 72°C for 5 min. The amplified product was gel-eluted and labelled by random labelling method (Amersham Biosciences). The membranes were washed in high stringency buffer (0.1X SSC, 0.1% SDS) at 65°C for 15 min, and exposed to X-ray film with intensifying screens at -70°C. Relative transcript amount in Northern experiments was measured based on pixel intensity using a Imagemaster software (Amersham Biosciences).

Total RNA was isolated from the leaf tissue of *P. coarctata* plants treated with 0.5 M NaCl for 48 h and mRNA was purified by oligo-dT cellulose column chromatography. Double-stranded cDNA was synthesized from purified mRNA using Superscript II reverse transcriptase (Gibco-BRL cDNA synthesis kit) and ligated to pSPORT1 plasmid vector in the *NotI* and *SalI* restriction sites. The ligation product was then transformed into *Escherichia coli* DH5 α and maintained as plasmid DNA lots.

A homologous probe for V-ATPase subunit c gene from *P. coarctata* was obtained by PCR amplification using degenerate primers. The forward primer designed in the 5' coding region (5'-GGAGCTGCTTATGGWACWGC-3') and the reverse primer in the 3' coding region (5'-AAGATGAGRATSAGGATCATGCC-3') corresponded to conserved sequence domains after ClustalW alignment of V-ATPase subunit c sequences reported from other species. The double-stranded cDNA synthesized from *P. coarctata* was used as template. Thirty cycles of PCR were performed at 94°, 55° and 72°C for 1 min each. A specific amplification product of 0.35 kb fragment was gel-purified and used as a probe to screen *P. coarctata* cDNA library. A putative V-ATPase subunit c clone was obtained using screening methodology following the radioactive method of screening¹⁵.

PcVHA-c1 was subcloned as a *HindIII-NotI* fragment in pSPORT2 vector (CNH7), and as a *SalI-HindIII* fragment in pSPORT1 vector (VT3). The nucleotide sequence was obtained for both strands of the subclones by the Big-Dye terminator detection method using M13 forward and reverse primers on an ABI377 DNA sequencer (Applied Biosystems). The full nucleotide sequence of *PcVHA-c1* was aligned from the sequences of the subclones. BLAST algorithm was used for sequence analysis¹⁷. The protein sequence of *PcVHA-c1* was deduced using SeqAid II version 2.01(+) program. The amino acid sequence of *PcVHA-c1* and that of different subunit c protein sequences from GenBank were aligned using the multiple alignment program of CLUSTALW¹⁸. A cladogram was obtained by the tree-drawing program of DNASIS program.

Microsomal fractions were isolated from leaf tissue of control and 0.5 M NaCl-treated *P. coarctata* plants following the reported method¹⁹. *P. coarctata* plants grown in greenhouse were kept initially in nutrient solution for a week and then replaced with fresh nutrient solution (control) or nutrient solution supplemented with 0.5 M NaCl (treated) for 48 h and leaves harvested from controls and treated plants for the isolation of microsomal fractions. About 200 g fresh control and treated leaves were homogenized at 4°C with a commercial warring blender in 400 ml homogenization buffer (50 mM Hepes-bis-Tris propane, pH 7.4; 250 mM sorbitol; 0.5% BSA and 0.1 mM phenylmethylsulfonyl fluoride). The homogenate was filtered through four layers of cheesecloth and centrifuged at 14,000 g for 20 min to remove cell debris and mitochondria. The supernatant was then centrifuged at 80,000 g for 45 min to pellet microsomal

membranes. The pellet was layered onto a discontinuous sucrose gradient (16, 22, 36 and 45%) and centrifuged at 90,000 g for 5 h. The tonoplast membrane layer between 16 and 22% was collected and diluted in homogenization buffer and precipitated with 20% TCA, washed with cold acetone and finally resuspended in 25 mM Hepes-bis-Tris propane (pH 7.4), 250 mM sorbitol and 0.1 mM phenylmethylsulfonyl fluoride. Protein concentration was determined using the manufacturer's instructions (Bangalore Genei, India).

The open reading frame of *PcVHA-c1* was cloned into pET 28a(+) expression vector in the *SalI* and *NotI* restriction site (pETSc 9). The recombinant clone pETSc 9 was transformed into *E. coli* BL 21 (DE3), and induced with 1 mM IPTG. The over-expressed protein was purified through nickel affinity binding column²⁰. The purified protein (250 μ g) was injected into rabbit as a first sensitization dose. After 20 days, 125 μ g protein was injected as challenge dose. Three challenge doses were given at 20 days intervals. Antiserum was collected 20 days after the third challenge dose, and used to detect *P. coarctata* subunit c. Monoclonal antibodies for subunits A and B were obtained from Heven Sze, University of Maryland, USA. Western analysis and immunostaining were carried out following methods described earlier¹⁹.

In this study, we have constructed a cDNA plasmid library from *P. coarctata* and maintained in *E. coli* DH5 α . The cDNA library consisted of 1×10^5 clones represented in 50 lots of 2000 colonies each. An analysis performed with randomly selected colonies from the cDNA library showed that the size distribution of cDNA inserts in the library ranged from 0.5 to 2.5 Kb. The range and size-distribution of clones was a good indicator that the *P. coarctata* cDNA library could be a good source for isolation of genes differing in size and function.

A full-length cDNA clone designated *PcVHA-c1* (*Porteresia coarctata* Vacuolar H⁺-ATPase c-subunit gene number 1), coding for the V-ATPase subunit c was obtained from *P. coarctata* cDNA library. The insert size of *PcVHA-c1* was 876 bp. DNA sequencing of the clone revealed a translation initiation codon, ATG at 72–74 bp, and a stop codon, TAA at 567–569 bp. The open reading frame (ORF) was flanked by a 71 bp 5'-untranslated region (UTR), and 307 bp 3'-UTR. A polyadenylation signal, AATAAA was found between 794 and 799 bp. A poly (A)⁺ tail of 44 bp length was detected at the 3'-end. The longest ORF of *PcVHA-c1* translated a protein (designated PVC1 based on the corresponding cDNA *PcVHA-c1*) of 165 amino acids with a calculated molecular mass of 16.5 kDa (Figure 1). The deduced protein sequence showed significant homology with V-ATPase subunit c of *Oryza sativa* (GenBank accession no. U27098) and *Avena sativa* (GenBank accession no. M73232). The amino acid sequence was 99 and 98% identical with *O. sativa* and *A. sativa* respectively (Figures 2 and 3). However, the nucleotide sequences of the coding region showed 92% homology with *O. sativa*, and 88%

	CGACCCGGTGC	11
GAGAGAGGAGGAAGAGATCGAGCTCGCCTCAGGAGGAGGAAGAAGAAGAGGAAGAGCAAG		71
<u>ATG</u> TGTCGGTTTTTCAGCGGOGATGAGACAGCGCCCTTCTTCGGCTTCCTCGGGCCGCGC		131
<u>M</u> S S V F S G D E T A P F F G F L G A A		20
TCGGCCCTCGTCTTCTCATGCATGGGGCAGCGTACGGGACGGGGAAGAGTGGCGTGGC		191
S A L V F S C M G A A Y G T A K S G V G		40
GTGGCGTCCATGGGTGTGATCGCCCCGAGCTCGTCATGAAGTCCATCGTCCAGTCTGTC		251
V A S M G V M R P E L V M K S I V P V V		60
ATGCTGGTGTGCTCGGTATCTACGGGCTTATCATTGCGGTCATCATCAGTACCGGGATT		311
M A G V L G I Y G L I I A V I I S T G I		80
AACCCCAAGGCGAAGCCGTACTACCTCTTCGATGGATACGGGCATCTCTCCTCAGGGCTT		371
N P K A K P Y Y L F D G Y A H L S S G L		100
GCCTGTGGCCTTGCTGGTCTCGCCGAGGCATGGCCATGGCATCGTGGGTGATGCTGGT		431
A C G L A G L A A G M A I G I V G D A G		120
GTTAGGGCAARTGCACAACCAAGGCTTTTCGTGGGCATGATCCTCATCCTCATTTC		491
V R A N A Q Q P K L F V G M I L I L I F		140
GCTGAAGCTCTTGCTCTGTATGGTCTCATTGTGGGCATCATCCTCTCATCCCGTGGT		551
A E A L A L Y G L I V G I I L S S R A G		160
CAATCCCGTGCAGATTAAAGCACCTTGCAGTACCAATCCGCGATTATCCACTTGTATAT		611
Q S R A D * 165		
TCTTGAGAAAACCTAAAACCTGGGAGCTCTAGTTTTAATGTATTAAAGATCGATTATAG		671
CITAAGGAAGGTGGCACTTCCAGTCTTTTCGTTTCTTTGGTGGTGATTTCATGCAGAGT		731
TTTTTTTGGGTTAGGCTGGATTGCTGCTCCTGAGCAAATGGATTTAATCCTATTCTGG		791
<u>TGAATAAAGAACACGGCACTGTAGCAAAATAAAATTACAATAAAAAAAAAAAAAAAAAAAAA</u>		851
AAAAAAAAAAAAAAAAAAAAAAAAAAAA		876

Figure 1. Nucleotide sequence and deduced amino acid sequence of *PcVHA-c1* cDNA. The deduced amino acid sequence is shown in single letter code below the first nucleotide of each codon. The putative translation start site ATG is indicated in bold and underlined. The TAA termination codon is indicated by an asterisk and underlined. The polyadenylation signal AATAAA is also underlined.

homology with *A. sativa*. The nucleotide sequence of *PcVHA-c1* was submitted to GenBank under the accession number AF286464.

Southern analysis showed the presence of more than one hybridizing fragment with all the restriction enzymes, even after stringent hybridization washes using full-length *PcVHA-c1* probe (Figure 4). The fragments ranged in size from 0.63 to 24 Kb. It produced seven fragments with *EcoRI*, *SacI*; six with *DraI*, *HindIII*; five with *PvuII*, *BglIII*. These results indicated the possibility that a multi-gene family encodes the subunit c V-ATPase in *P. coarctata*.

Transcript level for V-ATPase subunit c in leaves and roots of *P. coarctata* was determined by Northern blot analysis. *PcVHA-c1* probe used for preliminary Northern analysis correspond to the entire coding region of subunit c, and therefore, the resulting transcript signal reflected the sum of all isoforms of V-ATPase subunit c. Northern analysis on RNA isolated from leaves indicated that there

is no significant increase in the subunit c transcript level until 5 h after salt treatment. However, increase in the transcript level was two-fold after 24 h and three-fold after 48 h. The subunit c transcript level declined after 10 h of salt withdrawal (Figure 5). In roots, the transcript level of V-ATPase subunit c increased two-fold after 5 h, indicating the response in roots was immediate and more pronounced than in leaf tissues. After salt withdrawal, the transcript levels started decreasing. The level of transcript declined to 2.5-fold after 10 h of withdrawal from salt stress (Figure 5). Furthermore, we wanted to analyse the expression with *PcVHA-c1* 3'-UTR-specific primers as probe, and we observed a similar trend. Again, we observed that the transcript levels did not increase until 5 h of salt stress leaf in tissues (Figure 5). Our results with leaf RNA blot confirmed that the upregulation of subunit c transcripts observed upon NaCl stress in *P. coarctata* leaf and root tissues was specific for *PcVHA-c1* and also salt-specific.

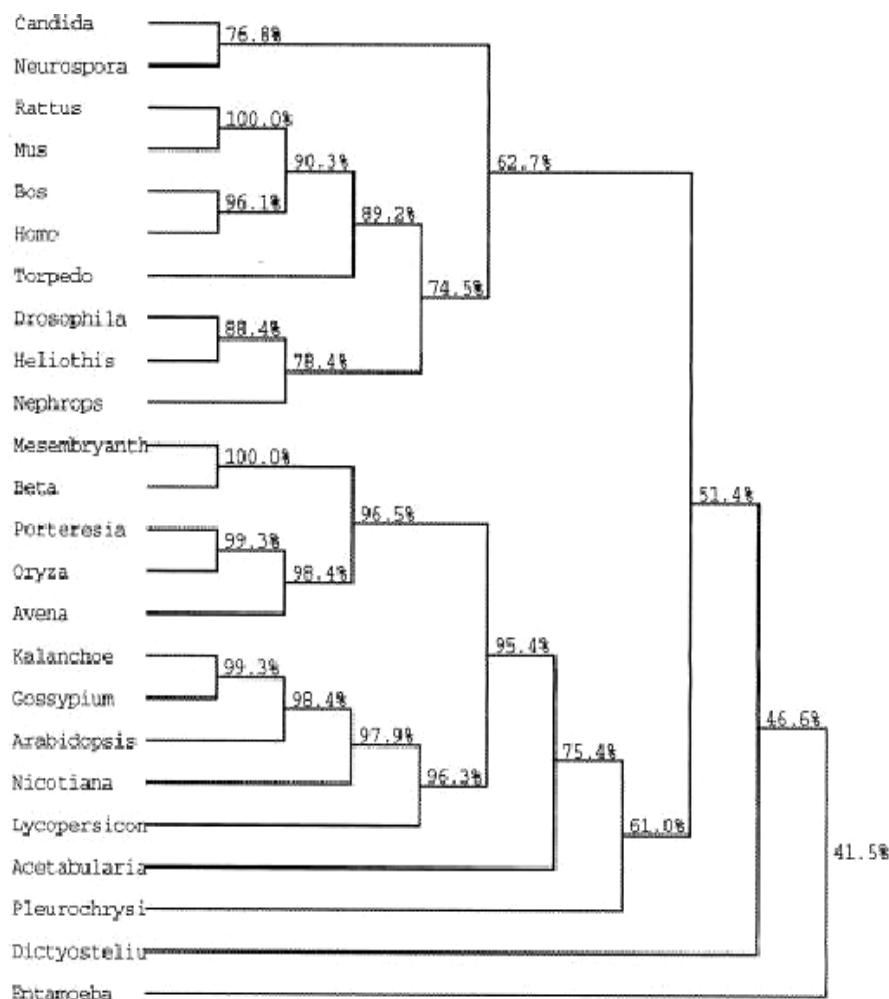


Figure 2. Phylogenetic relationships deduced from the protein sequences of V-ATPase subunit c from *Porteresia coarctata* (present study) and other species. The protein sequences were aligned using CLUSTALW program and the output from this program was used to generate a cladogram using the tree-drawing program of DNASIS. *Entamoeba histolytica* (GenBank accession no. U01057), *Dictyostelium discoideum* (X90516), *Pleurochrysis carterae* (U81519), *Acetabularia acetabulum* (AB003938), *Lycopersicon esculentum* (AF010228), *Nicotiana tabacum* (X95751), *Arabidopsis thaliana* (L44585), *Gossypium hirsutum* (U13669), *Kalanchoe daigremontiana* (U16244), *Avena sativa* (M73232), *Oryza sativa* (U27098), *P. coarctata* (present study), *Beta vulgaris* (X98851), *Mesembryanthemum crystallinum* (X94999), *Nephrops norvegicus* (S40059), *Heliothis virescens* (L16884), *Drosophila melanogaster* (X77936), *Torpedo marmorata* (X52002), *Homo sapiens* (M62762), *Bos taurus* (P23956), *Mus musculus* (M64298), *Rattus norvegicus* (D10874), *Neurospora crassa* (P31413), *Candida tropicalis* (U02877).

Equal quantity of protein (10 µg) from microsomal fractions of control and NaCl-treated *P. coarctata* was transferred to PVDF membrane. The immunostaining experiment showed that the monoclonal antibodies for subunits A and B strongly hybridized with a 70 and a 60 kDa in protein *P. coarctata*. Increase in the intensity of the 70 and 60 kDa proteins was observed in salt-treated plants. Western blot and immunostaining experiments using both polyclonal and monoclonal antibodies of V-ATPase with *P. coarctata* indicated increase of translational product of 70 kDa (subunit A), 60 kDa (subunit B) and 16.5 kDa (subunit c) of V-ATPase in 0.5 M NaCl-treated *P. coarctata*. Polyclonal antibodies raised against PVC1 protein showed cross-reaction with a 16.5 kDa protein, and significant in-

crease in its expression was observed in salt-treated plants (Figure 6).

The multimeric enzyme V-ATPase plays a major role in the maintenance of cellular pH, and it influences the transport of cations into the vacuoles of plant cells. The proteolipid domain of vacuolar H⁺-ATPase plays a major role in H⁺-transport in microvesicles and other acidic organelles. A full-length cDNA clone (*PcVHA-c1*) corresponding to V-ATPase subunit c was isolated from the *P. coarctata* cDNA library. Deduced amino acid sequence revealed that *PcVHA-c1* cDNA clone encoded 165 amino acids and the calculated molecular mass was 16.5 kDa. These results are in correspondence with that of V-ATPase subunit c isolated from other species such as *O. sativa*²¹ and *A. sativa*¹².

PcVHA-c1	1	MSSVFSGDEETAPFFGFLGAASALVFSMGAAYGTAKSGVGVASMGVMRPELVMKSIQPVV	6
Q40635	1I.....	6
P23957	1A.....	6
Q96473	1	...T.....A.....	6
BAA75515	1	..A.....A.....	6
Q39437	1	..T.N.....A.....	6
Q43434	1	...T.....A.....	6
BAA75516	3	..TL.....A.....	6
AAA82977	1	..TT.....A.....	6
Q39039	1	..A.T.....A.....	6
O22552	4A.....	5
Q40585	1	..P.T.....A.....	6
AAF04597	4A.....	5
CAA65063	1	..A.T.....A.....C.....	6
O24011	2	..N.A.....A.....	5
AAA99934	1A.....	5
AAC12797	1A.....	4
PcVHA-c1	61	MAGVLGIYGLIIAVIISTGINPKAKPYYLFDGYAHLSSGLACGLAGLAAGMAIGIVGDAG	1
Q40635	61F.....	1
P23957	61F.....	1
Q96473	61S.....S.....	1
BAA75515	61S.....S.....	1
Q39437	61S.....S.....	1
Q43434	61S.....S.....	1
BAA75516	62S.....S.....	1
AAA82977	61S.....S.....	1
Q39039	61S.....S.....	1
O22552	60S.....S.....	1
Q40585	61T.S.....S.....	1
AAF04597	60S.....S.....	1
CAA65063	61L.....T.S.....S.....	1
O24011	60T.S.....S.....	1
AAA99934	53S.....S.....	1
AAC12797	41S.....S.....	1
PcVHA-c1	121	VRANAQPKLFLVGMILILIFAEALALYGLIVGIIILSSRAGQSRAD	165
Q40635	121	165
P23957	121	165
Q96473	121	165
BAA75515	121	165
Q39437	121	165
Q43434	122E	165
BAA75516	122	166
AAA82977	121E	165
Q39039	121E	165
O22552	120	164
Q40585	121E	165
AAF04597	120	164
CAA65063	121E	165
O24011	120E	164
AAA99934	113E	157
AAC12797	101	145

Figure 3. Comparison of amino acid sequence of plant V-ATPase subunit c aligned using BLAST multiple alignment program (NCBI). Dots indicate amino acid residues that are identical with the deduced amino acid sequence of *PcVHA-c1*. The deduced amino acids of *PcVHA-c1* show similarities with the following plant species. GenBank accession numbers are given in brackets. *O. sativa* (Q40635); *A. sativa* (P23957); *K. daigremontiana* (Q96473); *Citrus unshiu* (BAA75515, BAA75516); *M. crystallinum* (Q39437); *G. hirsutum* (Q43434, AAA82977), *A. thaliana* (Q39039, AAA99934); *N. tabacum* (Q40585, CAA65063); *Dendrobium crumenatum* (AAF04597), *L. esculentum* (O24011); *Vigna radiata* (AAC12797, O22552).

The CLUSTALW algorithm showed significant identities among V-ATPase subunit c sequences from different plant species; fungal species, insects, animals, yeast and amoebae. The difference between the proteolipid from plants, fungi and animals resides within the carboxyl-terminus domain. The proteolipid carboxyl terminus in animal species is characterized by a short stretch of three to four hydrophilic amino acids. However, this hydrophilic region is extended by an additional seven amino acids in plants²².

This heptapeptide terminates with an Asp residue (D) in moderately salt-tolerant plant species such as *O. sativa* and *A. sativa*; and salt-tolerant species *Kalanchoe daigremontiana*, *Mesembryanthemum crystallinum* and *P. coarctata*. It terminates with a glutamate residue (E) in salt-sensitive species such as *Arabidopsis thaliana*, *Gossypium hirsutum*, *Lycopersicon esculentum* and *Nicotiana tabacum* (Figure 3). How much of this difference in amino acid residues contributes to functional differences in V-ATPase

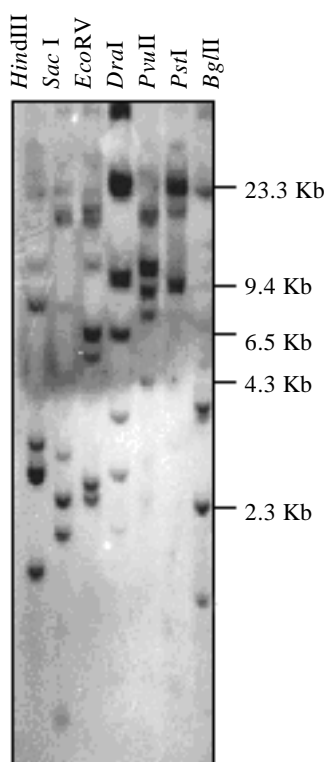


Figure 4. Genomic DNA gel blot analysis for *PcVHA-c1*. Genomic DNA of *P. coarctata* digested with different restriction enzymes and probed with *PcVHA-c1*.

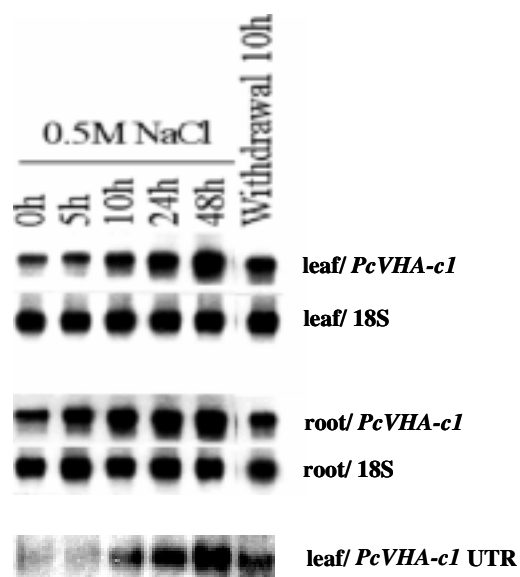


Figure 5. Effect of salt treatment (500 mM NaCl) on the expression of *PcVHA-c1* in leaves and roots of *P. coarctata*. Total RNA isolated from leaf and root tissue of *P. coarctata* plants treated with 0.5 M NaCl for different duration was probed with *PcVHA-c1* coding region. Total RNA from leaf and root tissue of *P. coarctata* plants treated with 0.5 M NaCl for 48 h and then transferred to normal growth medium (withdrawal of salt stress) for 10 h (shown in the figure). Blots were probed with 18S to determine uniformity in RNA loading. The leaf RNA blot was then stripped and re-probed with *PcVHA-c1* UTR region.

subunit c activity in plants remains to be elucidated; nevertheless, this was an interesting observation from our study. Apart from the amino acid differences at the N- and C-terminal ends of the coding regions, the plant and fungal sequences were consistently longer at the C-terminus compared to those reported from animals (figure not shown). The position of the membrane-spanning α -helices predicted by TMAP analysis²³ on deduced amino acid sequences from *PcVHA-c1* agreed with detailed structural models²⁴ of V-ATPase subunit c. Glu-142 in *P. coarctata* proteolipid IV hydrophobic domain, like *O. sativa*²¹, oat¹², *K. daigremontiana*²⁵, *M. crystallinum*²⁶ or Glu-139 in bovine¹⁰ or Glu-137 in *Saccharomyces cerevisiae*¹¹ is the site for DCCD inhibition. The sensitivity of the V-ATPase to DCCD has been documented. It has been reported that exposure to this inhibitor leads to covalent modification of the 16 kDa proteolipid^{27,28}.

Southern analysis of *P. coarctata* genomic DNA using *PcVHA-c1* as probe suggests that a multigene family may encode the V-ATPase subunit c of *P. coarctata*. The complex genomic organization of this gene could be due to existence of more than one copy of this gene or less likely due to the tetraploid nature of *P. coarctata* ($2n = 4x = 48$). Multigene family has been reported for the c subunit of the V-ATPase in *A. sativa*¹², *A. thaliana*²⁹, *Beta vulgaris*³⁰, *K. daigremontiana*²⁵, *M. crystallinum*²⁶, and from a polyploid species *G. hirsutum*²². In contrast, *S. cerevisiae*¹¹, *Drosophila melanogaster*³¹, *Neurospora crassa*³² all possess a single gene copy for the V-ATPase subunit c polypeptide. This suggests that there may be greater degree of specificity

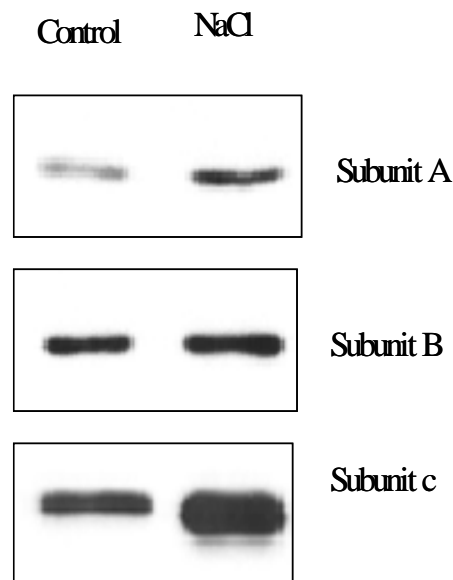


Figure 6. Western blot analysis of different V-ATPase subunits. Proteins prepared from microsomal fractions of leaf tissue from control and salt-treated (0.5 M NaCl for 48 h) *P. coarctata* plants were probed with monoclonal antibodies for subunits A and B, and polyclonal antibody for subunit c.

and/or regulation of expression of this polypeptide in plants compared to other organisms²⁵.

Changes in the transcript levels for the different subunits of V-ATPase have been reported when the plants are exposed to salinity stress^{30,33}. In this study, increase in V-ATPase subunit c transcript levels after salt treatment was consistently observed in both leaves and roots of *P. coarctata* plants with full-length cDNA and later confirmed with gene-specific *PcVHA-c1* UTR probe. Transcripts for *PcVHA-c1* were found to be specific to NaCl treatment, as transcript levels declined after withdrawal from NaCl stress. Studies have been made to understand salt effects on V-ATPase transcript expression in plants. In *M. crystallinum*, transcript levels of V-ATPase subunits, particularly of subunit c, are markedly increased by salinity stress in mature leaves³³.

Earlier studies have shown the coordinate upregulation of V-ATPase subunits upon imposed salt stress. For example, studies have revealed a significant increase in levels of V-ATPase subunits A and B in salt-treated sugar beet plants using polyclonal antiserum directed against the V-ATPase holoenzyme of *K. daigremontiana*³⁰. There are also reports which suggest that the increase in transcripts for V-ATPase subunit c in *Tortula ruralis* upon salt stress is not reflected in abundance in protein level using polyclonal antibodies³⁴. We therefore wanted to study the effect of salt stress on the individual subunits, i.e. on PVC1 subunit protein using polyclonal antibody and on A and B subunits using monoclonal antibodies. We also observed an increase in the translational product of subunit A, subunit B and PVC1 subunit protein in leaves of *P. coarctata* plants treated with 0.5 M NaCl using Western blot and immunostaining experiments. However, only a modest increase in subunits A and B was observed in this study. On the contrary, induction of PVC1 was more pronounced upon salt stress. Though we did not rule out the possibility of an additive effect of the isoforms of subunit c in our Westerns, our study clearly shows that PVC1 protein accumulates with salt stress. This is corroborated by studies which show the intra membrane particles build-up of subunit c in salt-stressed plants³⁵. These studies support the idea that induction of V-ATPase is involved in osmotic adjustment in plant cells.

The present communication therefore suggests that there is a contributory role for the V-ATPase subunits in the salt stress response in *P. coarctata*. Furthermore, the present study and other studies³⁶ provide a basis for analysing the regulatory/functional role of transporters in a salt-tolerant species like *P. coarctata*.

- Sze, H., Ward, J. M. and Lai, S., Vacuolar H⁺-ATPase from plants: Structure, function and isoforms. *J. Bioenerg. Biomembr.*, 1992, **24**, 371–381.
- Sze, H., Li, X. and Palmgren, M. G., Energization of plant cell membranes by H⁺ pumping ATPase: Regulation and biosynthesis. *Plant Cell*, 1999, **11**, 677–689.
- Maeshima, M., Tonoplast transporters: Organization and function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 2001, **52**, 469–497.
- Rea, R. A. and Sanders, D., Tonoplast energization: Two H⁺ pumps, one membrane. *Physiol. Plant.*, 1987, **77**, 131–141.
- Bowman, B. J., Allen, R., Wechsler, M. A. and Bowman, E. J., Isolation of the genes encoding the *Neurospora* vacuolar ATPase: analysis of vma 2 encoding the 57 kDa polypeptide and comparison to vma-1. *J. Biol. Chem.*, 1988, **263**, 14002–14007.
- Ratajczak, R., Structure, function and regulation of the plant vacuolar H⁺-translocating ATPase. *Biochim. Biophys. Acta*, 2000, **1465**, 17–36.
- Stevens, T. H. and Forgac, M., Structures, function and regulation of the vacuolar H⁺-ATPase. *Annu. Rev. Cell Dev. Biol.*, 1997, **13**, 779–808.
- Crider, B. P., Xie, X.-S. and Stone, D. K., Bafilomycin inhibits proton flow through the proton channel of vacuolar proton pumps. *J. Biol. Chem.*, 1994, **269**, 17379–17381.
- Zhang, J., Feng, Y. and Forgac, M., Proton conduction and bafilomycin binding by the V₀ domain of the coated vesicle V-ATPase. *J. Biol. Chem.*, 1994, **269**, 23518–23523.
- Mandel, M., Moriyama, Y., Hulmes, J. D., Pan, Y. C., Nelson, H. and Nelson, N., cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of H⁺-ATPase. *Proc. Natl. Acad. Sci. USA*, 1988, **85**, 5521–5524.
- Nelson, H. and Nelson, N., The progenitor of ATP synthetase was closely related to the current vacuolar H⁺-ATPase. *FEBS Lett.*, 1989, **247**, 147–153.
- Lai, S., Watson, J. C., Hansen, J. N. and Sze, H., Molecular cloning and sequencing of cDNAs encoding the proteolipid subunit of the vacuolar H⁺-ATPase from a higher plant. *J. Biol. Chem.*, 1991, **266**, 16078–16084.
- Luttge, U. and Ratajczak, R., The physiology, biochemistry and molecular biology of the plant vacuolar ATPase. *Adv. Bot. Res.*, 1997, **25**, 251–296.
- Saghai-Marouf, M. A., Soliman, K. M., Jorsenson, R. A. and Allard, R. W., Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 8014–8018.
- Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
- Chomczynski, P. and Sacchi, N., Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.*, 1987, **162**, 156–159.
- Altschul, S. F. *et al.*, Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.*, 1997, **25**, 3389–3402.
- Thompson, J. D., Higgins, D. G. and Gibson, T. J., CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, 1994, **22**, 4673–4680.
- Li, X., Hsu, H.-T., Su, R. T. and Sze, H., The molecular chaperone calnexin associates with the vacuolar H⁺-ATPase from oat seedling. *Plant Cell*, 1998, **10**, 119–130.
- Smith, M. C., Furman, T. C., Ingolia, T. D. and Pidgeon, C., Chelating peptide-immobilized metal affinity chromatography. *J. Biol. Chem.*, 1988, **263**, 7211–7215.
- Xiao, C., Sequencing of the cDNA encoding the 16-kDa proteolipid subunit of vacuolar H⁺-ATPase from rice and homology searching. Ph D dissertation, Fudan University, China, 1995.
- Hasenfratz, M. P., Tsou, C. L. and Wilkins, T. A., Expression of two related vacuolar H⁺-ATPase 16 kilo-Dalton proteolipid genes is differentially regulated in a tissue-specific manner. *Plant Physiol.*, 1995, **108**, 1395–1404.
- Persson, B. and Argos, P., Prediction of transmembrane segments in protein utilizing multiple sequence alignments. *J. Mol. Biol.*, 1994, **237**, 182–192.

24. Holzenburg, A. *et al.*, Evidence for a common structure for a class of membrane channels. *Eur. J. Biochem.*, 1993, **213**, 21–30.
25. Bartholomew, D. M., Rees, D. J. S., Rambaut, A. and Smith, J. A. C., Isolation and sequence analysis of a cDNA encoding the c subunit of a vacuolar-type H⁺-ATPase from the CAM plant *Kalanchoe daigremontiana*. *Plant Mol. Biol.*, 1996, **31**, 435–442.
26. Tsiantis, M. S., Bartholomew, D. M. and Smith, J. A. C., Salt regulation of transcript level for the c subunit of a leaf vacuolar H⁺-ATPase in the halophyte *Mesembryanthemum crystallinum*. *Plant J.*, 1996, **9**, 729–736.
27. Arai, H., Berne, M. and Forgac, M., Inhibition of the coated vesicle proton pump and labeling of a 17,000 Dalton polypeptide by DCCD. *J. Biol. Chem.*, 1987, **262**, 11006–11011.
28. Kaestner, K., Randa, S. and Sze, H., N,N'-Dicyclohexyl carbodiimide-binding proteolipid of the vacuolar H⁺-ATPase from oat. *J. Biol. Chem.*, 1988, **263**, 1282–1287.
29. Perera, I. Y., Li, X. and Sze, H., Several distinct genes encode nearly identical to 16 kDa proteolipids of the vacuolar H⁺-ATPase from *Arabidopsis thaliana*. *Plant Mol. Biol.*, 1995, **29**, 227–244.
30. Kirsch, M., Zhigang, A., Viereck, R., Low, R. and Rausch, T., Salt stress induces an increased expression of V-type H⁺-ATPase in mature sugar beet leaves. *Plant Mol. Biol.*, 1996, **32**, 543–547.
31. Meagher, L., McLean, P. and Finbow, M. E., Sequence of a cDNA from *Drosophila* coding for the 16 kDa proteolipid component of the vacuolar H⁺-ATPase. *Nucleic Acids Res.*, 1990, **18**, 6712.
32. Sista, H., Wechsler, M. A. and Bowman, B. J., The proteolipid subunit of the *Neurospora crassa* vacuolar ATPase: Isolation of the protein and the *vma 3* gene. *Mol. Gen. Genet.*, 1994, **243**, 8290.
33. Low, R. *et al.*, Early salt stress effects on the differential expression of vacuolar H⁺-ATPase genes in roots and leaves of *Mesembryanthemum crystallinum*. *Plant Physiol.*, 1996, **110**, 259–265.
34. Chen, X., Kanokporn, T., Zeng, Q., Wilkins, T. A. and Wood, A. J., Characterization of the V-H⁺-ATPase in the resurrection plant *Tortula ruralis*: accumulation and polysomal recruitment of the proteolipid c subunit in response to salt-stress. *J. Exp. Bot.*, 2002, **53**, 225–232.
35. Rockel, B., Ratajczak, R., Becker, A. and Lutge, U., Changed densities and diameters of intra-membrane tonoplast particles of *Mesembryanthemum crystallinum* in correlation with NaCl-induced CAM. *J. Plant Physiol.*, 1994, **143**, 318–324.
36. Barkla, B. J., Zingarelli, L., Blumwald, E. and Smith, J., Tonoplast Na⁺/H⁺ antiport activity and its energization by the vacuolar H⁺-ATPase in the halophytic plant *Mesembryanthemum crystallinum* L. *Plant Physiol.*, 1995, **109**, 549–556.

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Differential expression of tomato ACC oxidase gene family in relation to fruit ripening

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The enzyme ACC oxidase (ACO) catalyses the terminal step in the ethylene biosynthesis pathway and is encoded by a small multigene family in tomato. We studied the expression of the ACO gene family during various stages of fruit ripening using the multiplex RT-PCR approach in three tomato cultivars differing in their rate of ripening. The ACO1 gene expression, compared to other isoforms of the ACO gene family, showed a strong correlation with the rate of fruit ripening and ethylene evolution. The results revealed LEACO1 as the predominant ripening-regulated isoform, and demonstrated the potential of the multiplex RT-PCR technique to analyse the rate of ripening in diverse tomato genotypes.

Keywords: ACC oxidase, fruit ripening, multiplex RT-PCR, tomato.

THE gaseous phytohormone ethylene orchestrates many aspects of plant growth and development, including fruit ripening, leaf and flower senescence and abscission, seed germination and plant responses to environmental stimuli^{1,2}. Fruit ripening is a genetically programmed specialized phase of plant senescence and requires differential gene expression that is triggered, modulated and coordinated by ethylene. The terminal ethylene-forming enzyme (EFE), now confirmed as ACC oxidase, is encoded by at least four members of a multigene family in tomato³ and appears to be primarily located in the cell wall of ripening fruits⁴. The enzyme apparently is a dioxygenase belonging to the superfamily of iron-ascorbate oxidases, expressed constitutively in most vegetative tissues¹ and found to be induced during fruit ripening⁵. In view of the mounting evidence for major changes in ACC oxidase transcript abundance in the ripening tomato fruit, the present study was undertaken to investigate the differential expression pattern of ACC oxidase gene family during fruit ripening in three selected Indian tomato cultivars, which differed in their rate of ripening.

The level of ACC oxidase gene expression during fruit ripening was monitored by multiplex RT-PCR⁶ analysis in three commercially important Indian tomato cultivars, Pusa Ruby, Pusa Sheetal and Pusa Uphar, selected on the basis

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