ELSEVIER

Biochimica et Biophysica Acta 1414 (1998) 265–272

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Short sequence-paper

Cloning and expression analysis of vacuolar H^+-ATPase 69-kDa catalytic subunit cDNA in citrus (*Citrus unshiu* Marc.)[1]

Yuko Takanokura [a], Akira Komatsu [a,*], Mitsuo Omura [b], Tomoya Akihama [a]

[a] *Faculty of Agriculture, Meiji University, Kawasaki, Kanagawa 214, Japan*
[b] *Dept. of Citriculture, National Institute of Fruit Tree Science (NIFTS) Okitsu, Shimizu, Shizuoka 424-0292, Japan*

Received 17 April 1998; received in revised form 20 July 1998; accepted 4 August 1998

Abstract

To investigate the mechanism of sugar accumulation in fruit vacuoles, a full length cDNA (CitVATP-A) encoding the vacuolar H^+-ATPase 69-kDa catalytic subunit was isolated from a cDNA library constructed from citrus fruit (*Citrus unshiu* Marc.). A 2304-bp insert of CitVATP-A was coded for a 623 amino acid polypeptide with a predicted molecular mass of 68.68 kDa. The deduced amino acid sequence for CitVATP-A showed a 96.5% homology with the carrot homologue. Genomic Southern blot analysis suggested that CitVATP-A is a low-copy number gene. Northern blot analysis of leaves and fruits during the developing stages showed that the level of expression is high in young leaves and is low in mature leaves, and that it increased in both the edible parts and the peel, during fruit growth and maturity.

Keywords: Vacuolar H^+-ATPase catalytic subunit; Reverse transcription-polymerase chain reaction; Gene expression; Fruit development; (*Citrus*)

In citrus fruit, one of the most important functions of the vacuole is sugar accumulation; sugar is synthesized as a photoassimilate in mature leaves (source), and is exported in the form of sucrose to accumulate in fruit tissues (sink), especially in vacuoles. Following initial cell division, the fruit of most citrus species develops by sugar accumulation in the vacuoles of individual cells [1]. The mechanism of sugar accumulation is unclear, but transport of sucrose and glucose up a gradient into the vacuole is believed to require a proton driving force produced by two proton pumps, H^+ translocating ATPase and pyrophosphatase [2].

The vacuolar H^+-ATPase (V-type ATPase) is a large, multimeric enzyme composed of a hydrophilic V_1 complex on the cytosolic face of the tonoplast and a membrane-bound V_0 complex. Two subunits ($\sim$69 and $\sim$60 kDa) associated with the V_1 complex and a $\sim$16-kDa proteolipid of the V_0 complex have been identified as the three major subunits contained in all V-type ATPases [3]. From a typical sink organ, such as pear fruit, 10 different polypeptides were isolated to clarify the efficiency of allocating the assimilated products into fruit tissue [4].

* Corresponding author. Present address: Bio-oriented Technology Research Advancement Institution (BRAIN), Dept. of Citriculture, National Institute of Fruit Tree (NIFTS) Science Okitsu, Shimizu, Shizuoka 424-0292, Japan.
Fax: +81 (543) 69-2115;
E-mail: akomatsu@okt.affrc.go.jp

[1] The nucleotide sequence data of CitVATP-A reported will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under accession number AB004247.

0005-2736/98/$ – see front matter
PII: S0005-2736(98)00148-5

The cDNA sequences of the catalytic subunit were reported for carrot plants [5], cotton [6] and *Brassica napus* [7]. The expression of this gene was investigated to explain the tolerance mechanism toward salinity [8,9] and low temperatures [7,10]. Furthermore, when the expression of a tonoplast-specific H^+-ATPase 69-kDa catalytic subunit is inhibited by antisense mRNA, transgenic carrot plants show altered leaf morphologies resulting from defective cell expansion [11]. These results suggest that the role of the catalytic subunit is related to cell expansion.

The relation between the vacuolar H^+-ATPase activity of lemon fruit and the mechanism of hyperacidification has been investigated [12]. However, few studies exist of the molecular analysis of the vacuolar H^+-ATPase in *Citrus*. To better understand the role and regulation of vacuolar H^+-ATPase in citrus fruit, we isolated and analyzed the expression of the catalytic subunit gene in citrus during fruit growth and maturity. The results of our experiment suggest that the catalytic subunit gene in citrus fruit has an important role not only in cell growth of leaves, but also in fruit development.

Citrus plants (*Citrus unshiu* Marc. cvs. Miyagawa wase and Okitsu wase) used in these experiments were collected from the farm of the National Institute of Fruit Tree Science in Okitsu, Shizuoka, and from the farm of Meiji University, Kawasaki, Kanagawa. The leaves and fruits were frozen immediately in liquid nitrogen and then were stored at −80°C until used.

For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was prepared from the fruit (juice sacs/segments epidermis) of *C. unshiu* Marc. cv. Miyagawa wase as described by Komatsu et al. [13]. The sense primer (5′-CCA AGT TTA CGA AGA AAC-3′) and antisense primer (5′-CCA CAA CCA AAA GCA CC-3′) were designed on the conservative regions of vacuolar H^+-ATPase catalytic subunit genes from carrot and cotton. The PCR conditions were 94°C for 1 min, 46°C for 1 min and 72°C for 2 min, for a total of 30 cycles. The PCR products with the expected fragment length of 535 bp were cloned into the pCRII vector with a TA Cloning System Kit (Invitrogen). A candidate clone designated as pVATP-A was sequenced using the primer walking method with a Taq Dye Terminator Cycle Sequencing Kit (Applied Biosystems Instrument) and a 373S DNA sequencer. It had a 84.4% homology to the carrot catalytic subunit cDNA [5], and the 178 deduced amino acids showed a 96.0% homology with that of the carrot catalytic subunit. Therefore, pVATP-A was confirmed as an amplified fragment from the vacuolar H^+-ATPase catalytic subunit gene of citrus.

The cDNA library in λ-ZAPII derived from fruit poly(A)$^+$ RNA was screened using pVATP-A as the probe. The cDNA prepared from fruits (juice sacs/segments epidermis) of 124 days after flowering (DAF) was ligated and packaged as described by Komatsu et al. [13]. The phages were amplified in the *E. coli* XL-1-Blue MRF′ strain. Approximately 1×10^5 pfu of the fruit cDNA library were screened by the ECL direct nucleic acid labelling and detection system (Amersham). The membranes were washed twice for 20 min in 6 M urea, 0.1×SSC and 0.1% SDS at 42°C. Among the 1×10^5 pfu, one cDNA clone was screened and designated as CitVATP-A, the full length of which was 2304 bp with the translation start site (ATG) at nucleotide 45 and the stop codon (TGA) at nucleotide 1914 (Fig. 1), and was coded for a 623 amino acid polypeptide with a predicted molecular mass of 68.68 kDa. The polyadenylation signal (AATAAA) was located between nucleotides 2296 and 2301. The CitVATP-A showed a 83.9% homology with carrot cDNA in the open reading frame. Fig. 2 shows the amino acid sequences of 18 species within eukaryotes; the sequence identities to other polypeptides are shown ranging from 68.0 to 69.6% for mammals (5 species), from 67.3 to 67.4% for insects (2 species), from 61.4 to 65.1% for fungi (3 species), and from 60.6 to 68.8% for protozoans (2 species). Within the plant kingdom, it showed 73.3–74.9% of homologies for algae (2 species) and 82.9–94.2% for higher plants (4 species). The ATP-binding site motif (GAFGCGKTV) [14] between amino acid residues 251 and 260 of CitVATP-A was completely conserved (Fig. 2, Box A). The Mg^{2+}-binding region of the β subunit of F_1F_0-ATPase (VYVGCGER), the region conserved in both α and β subunit of F_1F_0-ATPase (TGITIAEYFRD), and the α and β subunit signatures (PSVNWLISYS) were also conserved in vacuolar H^+-ATPase catalytic subunit of higher

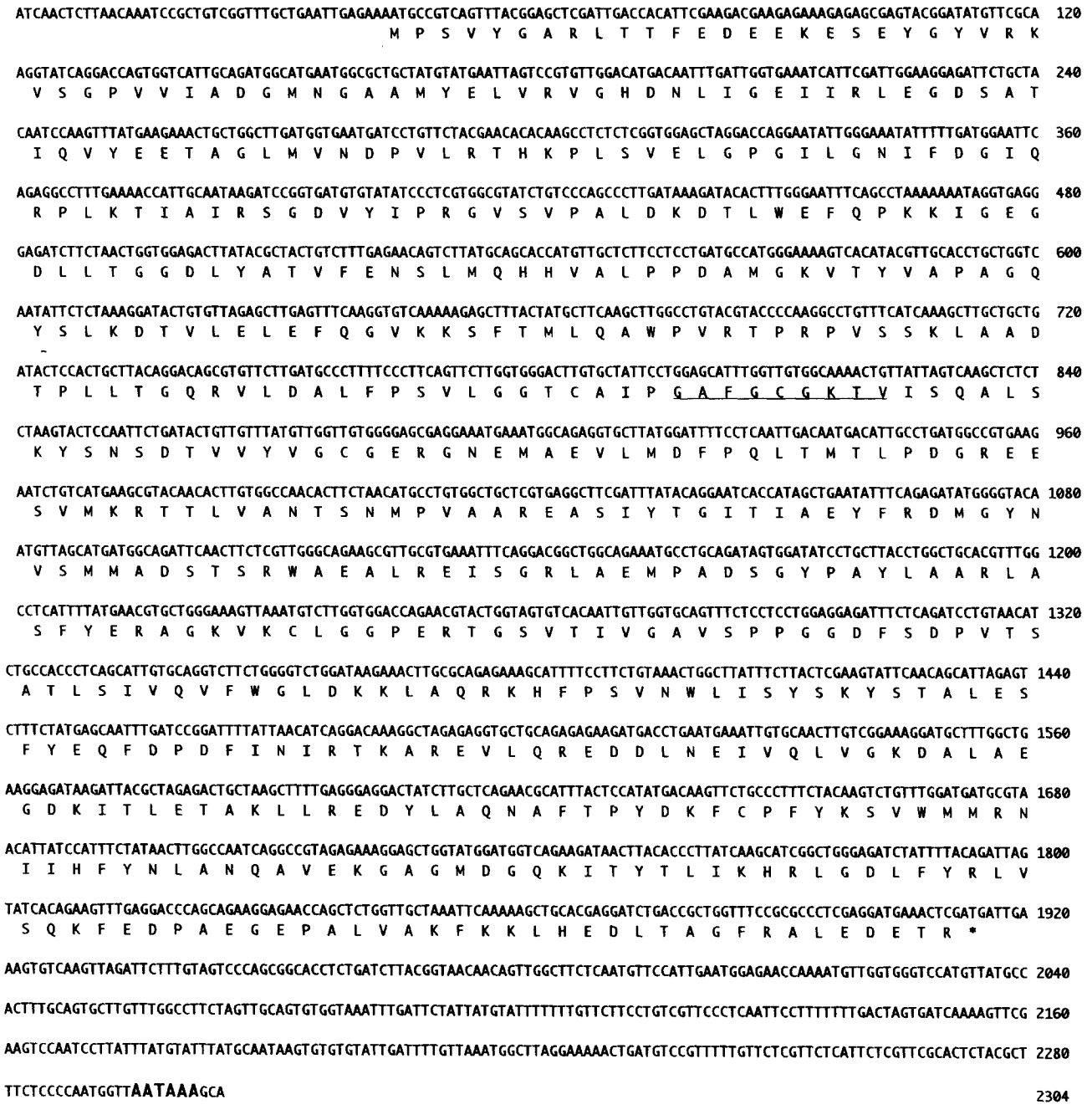

Fig. 1. Nucleotide and deduced amino acid sequences of the CitATP-A encoding the vacuolar H^+-ATPase 69-kDa catalytic subunit from *Citrus unshiu* Marc. The ATP-binding site motif is underlined, deduced polyadenylation signal in bold. The numbers on the right correspond to the nucleotide sequence.

plants (Fig. 2, Box B, C and D). Thus, citrus H^+-ATPase showed a high conservation of the vacuolar H^+-ATPase catalytic subunit gene.

The sequence analysis of the catalytic subunit gene and its characteristic in the genome have been analyzed to determine its evolution. In a study of the copy number of the genome, Starke and Gogarten [15] found that land plants contain two catalytic subunit genes, from the direct sequencing of two discrete PCR products. Each sequence would comprise a

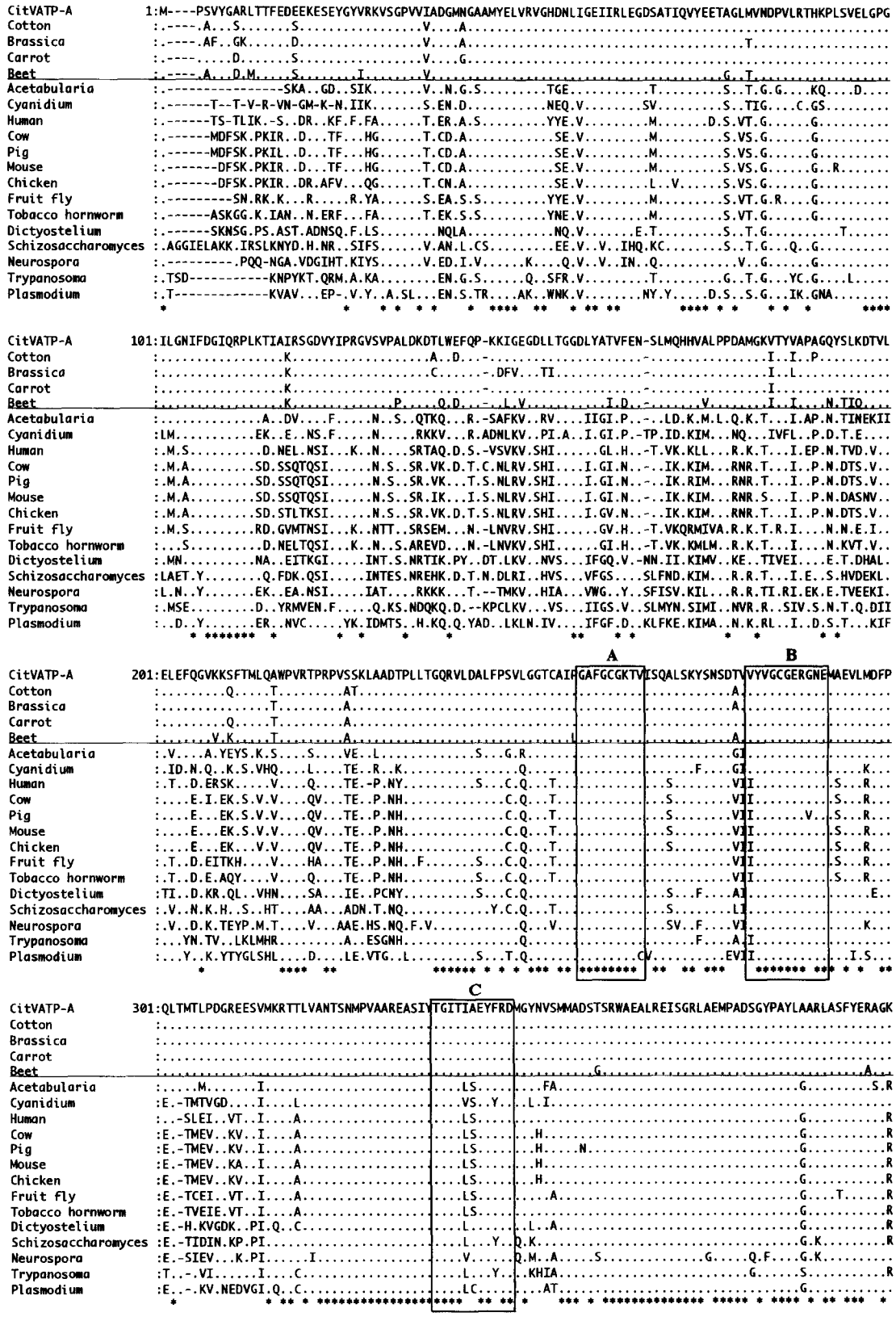

Fig. 2.

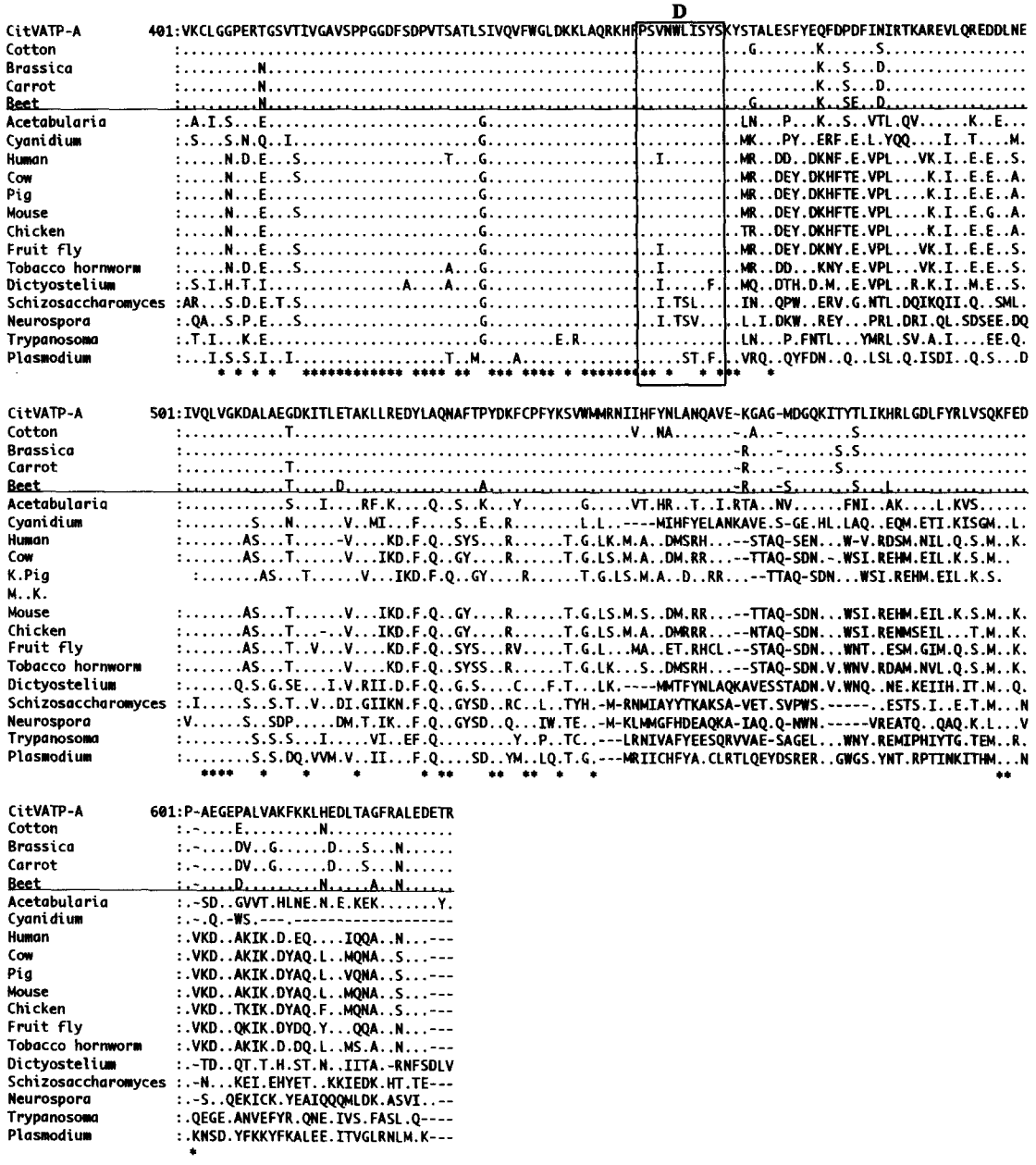

Fig. 2. Comparison of the deduced amino acid sequence encoded by CitVATP-A with sequences from cotton 91.0% (L03186), brassica 91.0% (U15604), carrot 94.2% (J03769), beet 82.9% (X98767), *Acetabularia acetabulum* 74.9% (D50528), *Cyanidium caldarium* 73.3% (U17100), human 68.0% (L09234), cow 68.7% (X58386), pig 69.6% (X62338), mouse 69.3% (U13837), chicken 69.0% (U22077), fruit fly 67.3% (U19742), tobacco hornworm 67.4% (S21107), *Dictyostelium discoideum* 65.1% (U49169), *Saccharomyces pombe* 62.3% (X68580), *Neurospora crassa* 61.4% (J03955), *Trypanosoma congolense* 68.8% (Z25814) and *Plasmodium falciparum* 60.6% (L08200). The ATP-binding site motif is shown in box A, the Mg^{2+}-binding region of the β subunit of F_1F_0-ATPase is shown in box B, the region conserved in both the α and β subunit of F_1F_0-ATPase is shown in box C, and the α and β subunit signatures are shown in box D. The vacuolar H^+-ATPase catalytic subunit from higher plants is shown above the line.

gene family; a superfamily composed of two gene families has been reported for cotton [16].

In this study, Southern blot analysis of genomic DNA was made to estimate the number of vacuolar H^+-ATPase catalytic subunit genes in the citrus genome. Total DNA was isolated from mature leaves

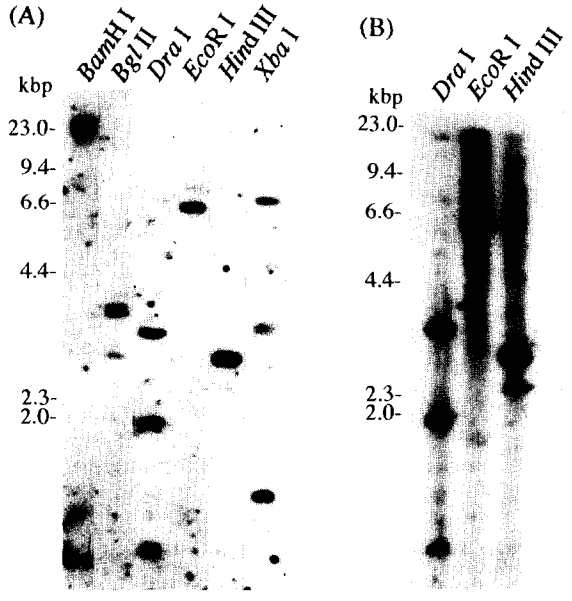

Fig. 3. Southern blot analysis of total DNA of *C. unshiu* using pATP-A as a probe. Blots were washed in high stringency: 68°C, 0.1×SSC (A) and in more moderate stringency: 50°C, 1×SSC (B).

of *C. unshiu* using the method of Dellaporta et al. [17]. Fifteen μg of total DNA was digested with *Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII and *Xba*I, electrophoresed on 0.8% agarose gels, and was blotted onto a nylon membrane (Hybond N, Amersham). The membranes were hybridized using a pVATP-A insert labeled with Dig-11-dUTP and a random primer DNA labeling kit (Boehringer Mannheim). From one to three bands were detected in *Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII and *Xba*I digests when the blots were washed under high stringency conditions of 68°C, 0.1×SSC (Fig. 3A). Almost identical results were obtained with more moderate stringency conditions of 50°C, 1×SSC with *Dra*I, *Eco*RI and *Hin*dIII (Fig. 3B). However, some additional weak bands appeared. Therefore, these fragment patterns suggest that the vacuolar H^+-ATPase catalytic subunit gene is organized as a low-copy number with a somewhat low identity with the citrus genome.

We investigated the expression of the vacuolar H^+-ATPase catalytic subunit gene using Northern blot analysis. Total RNA was isolated from the leaves and fruits of *C. unshiu.* cv. Okitsu wase. Leaves at different stages of development, 2.5, 4–5, 6, 7–8 and 10 cm long, were collected as samples. Fruits were harvested at 89, 120, 148, 187, 223 and 297 DAF and were divided into two tissues, juice sacs/segments epidermis (edible parts) and albedo/flavedo (peel). Five μg of total RNA were electrophoresed on 1.0% agarose gels containing formaldehyde and were blotted onto nylon membranes (Hybond N, Amersham). Hybridization was carried out using the same procedures as for Southern blot analysis, except for stringent washing conditions of 0.2×SSC and 0.1% SDS at 65°C.

In all samples of this study, a single band with approximately 2.5-kb transcript was observed as the expected transcription size (Figs. 4 and 5). From the results of Southern blot analysis and the size of transcripts, these single bands derived from pATP-A or other vacuolar H^+-ATPase catalytic subunit genes. The transcription levels were high in the youngest leaves (2.5 cm long) and low in the other leaves (4–5, 6, 7–8, 10 cm long) (Fig. 4). The transcription levels of the fruit increased in the juice sacs/segments epidermis (Fig. 5A) and in the albedo/flavedo peel (Fig. 5B) until fruit maturity (DAF 89–223), but decreased only after harvest time (DAF 297) in the juice sacs/segments epidermis (Fig. 5A).

The increase in the mRNA levels by Northern blot analysis may be strongly related to cell expansion

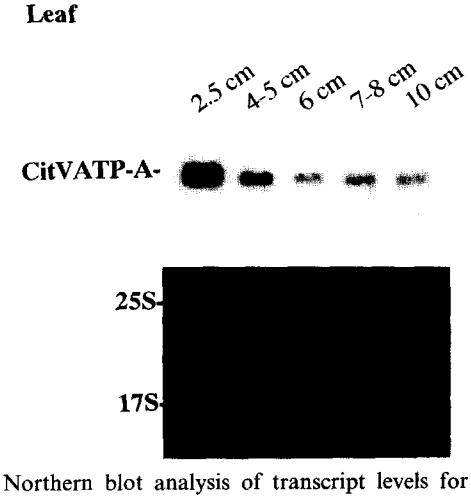

Fig. 4. Northern blot analysis of transcript levels for vacuolar H^+-ATPase catalytic subunit during leaf expansion. Total RNA was isolated from leaves of 2.5, 4∼5, 6.0, 7∼8 and 10 cm long. Five-microgram samples of total RNA were fractionated by electrophoresis on 1.0% agarose gel including 2.2 M formamide and transferred to Hybond-N membrane. In the lower panel, total RNA on the gel was stained with ethidium bromide.

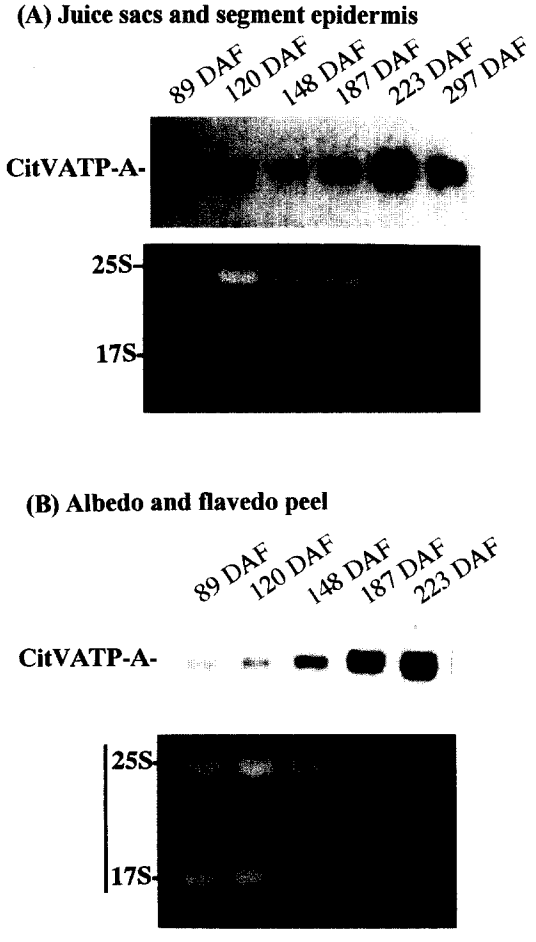

Fig. 5. Northern blot analysis of transcript levels for vacuolar H^+-ATPase catalytic subunit during fruit expansion. Total RNA was isolated from edible parts (juice sacs/segment epidermis) of 89, 120, 148, 187, 223 and 297 DAF (A), and peel parts (albedo/flavedo) of 89, 120, 148,187 and 223 DAF (B). Five-microgram samples of total RNA were fractionated by electrophoresis on 1.0% agarose gel including 2.2 M formamide and transferred to Hybond-N membrane. In the lower panel, total RNA on the gel was stained with ethidium bromide.

and sugar accumulation. When the expression of a tonoplast-specific H^+-ATPase 69-kDa catalytic subunit is inhibited by antisense mRNA, transgenic carrot plants show altered leaf morphologies resulting from defective cell expansion [11]. The constituent levels of accumulation mRNA for the 70-kDa subunit in unexpanded tomato leaves are much higher than those in expanded leaves [9]. In citrus, Northern blot analysis in this study of leaves at various stages of development agreed with these previous reports. The fact that the expression level of the youngest leaves is higher than at any other stage is interesting, so further investigation is needed to clarify the relationship between cell expansion and the H^+-ATPase catalytic subunit gene. However, the relative levels of immunoblotting with antibodies against two major subunits (68 and 57 kDa) of the vacuolar H^+-ATPase do not change during the growth of radish root [18].

Our results of Northern blot analysis in fruits were different from the results in leaves. The levels of mRNA were high in expanded fruits and low in unexpanded fruits. Thus, we believe that other factors have a role in gene expression, including sugar accumulation. The fruit growth of this cultivar (cv. Okitsu wase) is almost completed by late September, corresponding to 120 DAF. The fruits then accumulate sugar. The sugar content of fruits, both juice sacs/segments epidermis and albedo/flavedo, increased rapidly, reaching a maximum in late December, corresponding to 223 DAF (data not shown). This change in sugar content correlated with an increase in catalytic subunit mRNA. Vacuolar H^+-ATPase catalytic subunit mRNA with salt stress increases in mature leaves that have accumulated salt in the vacuoles [8,9]. Therefore, we believe that the increase in this mRNA at fruit maturity may be involved in the function of sugar accumulation as a sink organ. Furthermore, such a high mRNA level in the youngest leaves may indicate the initial function of the leaves as the sink.

This is the first report on the isolation, characterization of the genomic organization and the transcription of catalytic subunit of vacuolar H^+-ATPase in fruit trees in relation to the vacuolar function of sugar accumulation, but the roles of vacuolar H^+-ATPase cannot be explained only by the investigation of catalytic subunit genes. More detailed investigations, such as by using Western blot analysis, measurement of enzymatic activity and analysis of other subunit genes [19], should make these roles clear during the sugar accumulation in citrus fruit.

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan (No. 06304013). We thank Professor S. Yamaki, Nagoya University and Dr. T. Moriguchi, National Institute of Fruit Tree Science for helpful advice and support.

References

[1] J.M. Bain, Austr. J. Bot. 6 (1958) 1–24.
[2] E. Martinoia, Bot. Acta 105 (1992) 232–245.
[3] M. Forgac, Physiol. Rev. 69 (1989) 765–796.
[4] Hosaka, Y. Kanayama, K. Shiratake, S. Yamaki, Phytochemistry 36 (1994) 565–567.
[5] L. Zimniak, P. Dittrich, J.P. Gogarten, H. Kibak, L. Taiz, J. Biol. Chem. 263 (1988) 9102–9112.
[6] T.A. Wilkins, Plant Physiol. 102 (1993) 679–680.
[7] W. Orr, T.C. White, L. Iu, L. Robert, J. Singh, Plant Mol. Biol. 28 (1995) 943–948.
[8] M.L. Narasimhan, M.L. Binzel, E. Perez-Prat, Z. Chen, D.E. Nelson, N.K. Singh, R.A. Bressan, P.M. Hasegawa, Plant Physiol. 97 (1991) 562–568.
[9] M.L. Binzel, J.R. Dunlap, Planta 197 (1995) 563–568.
[10] Y. Moriyama, N. Nelson, J. Biol. Chem. 264 (1989) 3577–3582.
[11] J.P. Gogarten, J. Fichmann, Y. Braun, P. Styles, S.L. Taiz, K. DeLapp, L. Taiz, Plant Cell 4 (1992) 851–864.
[12] M.L. Müller, U. Irkens-Kiesecker, D. Kramer, L. Taiz, J. Biol. Chem. 272 (1997) 12762–12770.
[13] A. Komatsu, Y. Takanokura, M. Omura, T. Akihama, Mol. Gen. Genet. 252 (1996) 346–351.
[14] N. Nelson, J. Bioenerg. Biomembr. 21 (1989) 553–571.
[15] T. Starke, J.P. Gogarten, FEBS Lett. 315 (1993) 252–258.
[16] T.A. Wilkins, C.-Y. Wan, C.-C. Lu, Theor. Appl. Genet. 89 (1994) 514–524.
[17] S.L. Dellaporta, J.L. Wood, J.B. Hicks, Plant Mol. Biol. Rep. 1 (1983) 19–21.
[18] M. Maeshima, T. Nakanishi, C. Matsuura-Endo, Y. Tanaka, J. Plant Res. 109 (1996) 119–125.
[19] R. Löw, B. Rockel, M. Kirsch, R. Ratajczak, S. Hörtensteiner, E. Martinoia, U. Lüttge, T. Rausch, Plant Physiol. 110 (1996) 259–265.