Molecular Cloning of Vacuolar H\(^+\)–ATPase A Subunit Paralogs and their Expression in Pear Fruit

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Summary

We cloned two cDNAs (PeVHA – A1, PeVHA – A2) encoding V–ATPase A subunit (catalytic subunit) in pear (Pyrus communis L. var. sativa DC, ‘La France’). They encode proteins with a calculated molecular weight of approximately 69 kDa, which consist of 623 amino acids. The expression of PeVHA – A1 mRNA was extremely high in mature fruit and young leaves, but low in flower, young fruit, mature leaves, and root, it increased with the maturation of pear fruit. On the other hand, the expression of PeVHA – A2 mRNA was relatively high at the early and late developmental stages of the pear fruit. A certain level of PeVHA – A2 expression was also observed in flowers and leaves.

Key Words: fruit development, gene expression, molecular cloning, pear (Pyrus communis L. var. sativa DC, ‘La France’), vacuolar H\(^+\)–ATPase.

Introduction

The vacuole is a multifunctional organelle in higher plants. Its important functions in fruit include sugar accumulation and fruit enlargement. In pear fruit, the vacuole occupies a large part of the cell volume even at the late cell division stage (Shiratake et al., 1998). The proton motive force, generated by two distinct proton pumps in the vacuolar membrane, i.e. vacuolar H\(^+\)–ATPase (V–ATPase) and vacuolar H\(^+\)–pyrophosphatase (V–PPase); they facilitate secondary transport of metabolites and ions across the vacuolar membrane (Marty, 1999). When the gene expression of V–ATPase A subunit in carrot was inhibited through gene transformation with antisense orientation, the transgenic plant developed serrated leaf margins and short taproots (Gogarten et al., 1992). These results imply that the vacuolar functions not only in cell expansion and storage of metabolites, directly or indirectly as affected by the two proton pumps, but that the gene transformation also altered plant morphology.

V–ATPase is found in all eukaryotic cells and is highly conserved among species (Ratajczak, 2000). It is a large complex enzyme and is composed of at least 13 different subunits (Sze et al., 2002). Eight of these subunits (A–H) form a peripheral V\(_1\) domain, which is responsible for ATP binding and hydrolysis, while another five types (a, c, c’, c\(^\ast\), and d) form a membrane integral V\(_0\) domain, which is responsible for proton transport. V–ATPase A subunit in V\(_1\) domain is also called catalytic subunit.

Recently, V–ATPase and V–PPase of pear fruit have been purified and their properties were characterized (Hosaka et al., 1994; Suzuki et al., 1999a). The changes in their activities and protein levels during pear fruit development have also been investigated (Shiratake et al., 1997). To understand the mechanisms of fruit development and sugar accumulation at the molecular level, we isolated two paralogs of V–ATPase A subunit.

Their expression patterns during fruit development and in various organs were investigated.

Materials and Methods

Plant material

Pear (Pyrus communis L. var. sativa DC, ‘La France’) fruits were harvested 18, 32, 50, 64, 78, 92, 106, 123, 137, 150 and 162 days after full bloom (DAFB) from a 17-year old tree grown at the Nagoya University orchard in 2001. Samples of flowers and leaves were collected on April 6, 2003 and July 11, 2001, respectively. Those of roots were collected from pear plants cultured in pots. These samples were stored at -80°C until used.

Molecular cloning of V–ATPase A subunit

The cDNA library constructed from premature pear fruit at 100 DAFB (Suzuki et al., 1999b) and the cDNA fragment (370 bp) of V–ATPase A subunit of Japanese pear (Suzuki et al., 2000), labeled by digoxigenin (DIG) DNA labeling kit (Roche Diagnostics Co. Ltd., Mannheim, Germany), were used for screening. Sequencing was carried out by using CEQ\(^\text{TM}\) 2000 DNA Analysis System (Beckman Coulter Inc., CA, USA).
Southern blot analysis

For total DNA was isolated from young pear leaves by using the DNeasy Plant Maxi Kit (QIAGEN, Hilden, Germany) and following the manufacturer's instruction. Ten μg of total DNA was digested by EcoRI, PstI and SpeI, electrophoresed on 0.8% (w/v) agarose gels, and blotted onto nylon membranes (Hybond-N+, Amersham Pharmacia Biotech Co., Ltd., Buckinghamshire, UK). The regions at position 1917–2189 of PeVHA-A1 and 2002–2301 of PeVHA-A2 were labeled by PCR DIG probe Synthesis Kit (Roche) and were used as probes. The membrane was hybridized with the probes (10 ng·mL⁻¹) in the hybridization buffer (5 × SSC, 1% (w/v) Blocking reagent (Roche), 0.1% (w/v) N-lauroyl sarcosine, 0.02% (w/v) SDS) at 68°C. The membrane was washed twice for 5 min with 2 × SSC, containing 0.1% (w/v) SDS, at room temperature and twice in 0.1 × SSC, containing 0.1% (w/v) SDS, at 68°C for 15 min. Detection was done by using the chemiluminescent substrate (CDP-Star, Roche) and following the manufacturer's protocol.

Semi–quantitative RT–PCR Southern blot analysis

Total RNA was extracted according to Suzuki et al. (1999b). A single-stranded cDNA was synthesized by using RNA PCR kit (Takara Shuzo Co., Ltd., Shiga) with 1 μg of total RNA and oligo (dT) primer. The cDNA (1/20 of the reaction mixture) was amplified semi–quantitatively by PCR. The primers and optimal cycle numbers for the PCR were as follows: for PeVHA-A1, 5′-TGCTCTTGAAGATGAAACC-3′ and 5′-CTA-CACAATAAAGGAATGGC-3′, 11 cycles; for PeVHA-A2, 5′-TGCTCTTGAAGATGAAACC-3′ and 5′-CACAATAAAGGAATGGC-3′, 11 cycles; and for actin, 5′-CATCGTCGTCATGTTTGG-3′ and 5′-CATCGTCGTCATGTTTGG-3′, 13 cycles. Actin of pear (DBJ accession number: AB190176) was used as a control gene. The resulting PCR products were separated on 1% (w/v) agarose gel, and blotted onto the nylon membrane (Hybond-N+). Hybridization, washing, and detection were executed as described above.

Results and Discussion

About 240000 phages of the cDNA library of pear fruit were screened by DIG-labeled partial cDNA of V-ATPase A subunit. Of the 15 positive clones that were sequenced, 14 clones were confirmed to contain identical sequences. The longest one, named PeVHA-A1 (accession number: AB189963), was fully sequenced, while the remaining clone was named PeVHA-A2 (AB189964), was also fully sequenced, it possesses the entire coding region. Both PeVHA-A1 and PeVHA-A2 had an open reading frame of 1872 nucleotides that encode putative 623 amino acids. They contain the P-loop required for ATP binding and the regulatory Cys residues at position 250–261. The residues, indispensable for catalytic function, such as F435 (MacLeod et al., 1998), and for regulation, such as C535 (Feng and Forcag, 1994), were also present in PeVHA-A1 and PeVHA-A2.

They have extremely similar coding regions. Their nucleotide sequences and deduced amino acid sequences showed 97% and 99% identities, respectively. However, 5′- and 3′- UTRs showed lower identities, that is 61% and 66%, respectively. The deduced amino acid sequence of both clones showed 93%, 95%, 95%, 95%, 97%, 93%, and 93% identities with V-ATPase A subunit, previously isolated from Arabidopsis thaliana, Citrus unshiu, Daucus carota, Gossypium hirsutum-1, Gossypium hirsutum-2, Prunus persica, Lycopersicon esculentum-1 and Lycopersicon esculentum-2, respectively. A phylogenetic tree, based on the deduced amino acid sequences that was constructed with the UPGMA program (Fig. 1) shows that V-ATPase A subunit is highly conserved in plants.

To distinguish PeVHA-A1 and PeVHA-A2, we used the specific probe for 3′-UTR region of each clone and carried out Southern blot analysis (Fig. 2). The specific probe detected one to four restriction fragments whose band patterns differentiated PeVHA-A1 from PeVHA-A2, indicating that the two are transcribed from different genes.
Fig. 3. Gel plates derived from RT-PCR Southern blot analysis of PeVHA-A1 and PeVHA-A2. (A) The mRNA expression in various organs: F: Flowers, Yf: Young fruit (18 DAFB), Mf: Mature fruit (162 DAFB), Yl: Young leaf, Ml: Mature leaf, R: Root. (B) Changes in level of mRNAs with fruit development. Actin was used as a standard control (lowest panel).

Semi-quantitative RT-PCR Southern blot analysis was performed to investigate the expression of PeVHA-A1 and PeVHA-A2 (Fig. 3). The expression of PeVHA-A1 mRNA was extremely high in mature fruit and young leaves, but low in other tissues, whereas the expression of PeVHA-A2 mRNA in flowers, young leaves, and mature leaves was almost the same as in fruits; its expression in roots was considerably low. Furthermore, the expression of PeVHA-A1 mRNA that was relatively low in young fruit increased with fruit maturation. Although the expression of PeVHA-A2 mRNA was relatively high in fruitlets at 18 and 32 DAFB, it decreased until 106 DAFB; thereafter it increased again with fruit maturation. In pear fruit, although V-PPase is the main vacuolar proton pump in young fruit, the levels of subunit proteins and the activity of V–ATPase increased with fruit development; it became the main proton pump at the mature stage (Shiratake et al., 1997). Although the expression of PeVHA-A1 and PeVHA-A2 at protein level is unclear, it is suggested that these two genes have their own physiological functions.

During fruit maturation, the accumulation of sugars at a high concentration in the vacuoles requires a proton motive force for active sugar transport into the vacuoles. To maintain the high activity of V-ATPase for sugar accumulation, PeVHA-A1 and PeVHA-A2 expressions should increase during fruit maturation. To clarify the physiological role and the importance of V-ATPase in fruit development, further investigations are needed.

**Literature Cited**


**セイヨウナシ果実の液胞型H⁺-ATPase A subunit cDNAパラログのクローニングとその発現解析**

**要約**

セイヨウナシ (Pyrus communis L. var. sativa DC.) および2つのV-ATPase A subunit cDNA (PeVHA -A1, PeVHA -A2)をクローニングした。両者はともに243アミノ酸からなる推定分子量約69 kDaのタンパク質をコードすと考えられた。PeVHA-A1 mRNAの発現は成熟果実や幼果では高かったが、花、未熟果実、成熟葉および根での発現は低く、果実では成熟に伴って高まる傾向が認められた。これに対しPeVHA-A2 mRNAの発現は成熟果実だけでなく幼果で高く、花や葉でも認められた。