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Seasonal Changes in the Activities of Vacuolar H^+-pumps and their Gene Expression in the Developing Japanese Pear Fruit

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Summary

Vacuolar H^+-ATPase (V-ATPase) and vacuolar H^+-inorganic pyrophosphatase (V-PPase) generate an electrochemical gradient across the vacuolar membrane, which is indispensable for accumulating sugars into the vacuole. We investigated the enzymatic and transcriptional changes of V-ATPase and V-PPase in Japanese pear fruit to establish their roles in fruit development. As fruit developed, the V-ATPase and V-PPase activities increased on the total protein basis. The V-PPase activity was higher than that of V-ATPase throughout the developmental period. Immunoblot analyses indicated that the changes in the polypeptides for the catalytic subunit of V-ATPase and V-PPase generally paralleled the enzymatic activities. Furthermore, seasonal changes in the mRNA levels generally corresponded with those of polypeptides except at the cell division stage. Our findings suggest that V-PPase, more than V-ATPase, plays an important role throughout the development of Japanese pear fruit and that V-ATPase and V-PPase are regulated at the transcriptional level during the cell elongation stage.

Key Words: fruit development, gene expression, Japanese pear (*Pyrus serotina*), vacuolar H^+-ATPase (EC 3.6.1.3), vacuolar H^+-inorganic pyrophosphatase (EC 3.6.1.1).

Introduction

Of the various factors which make up fruit quality, size and sweetness largely govern consumer acceptance. Hence, it is important to investigate the mechanisms leading to fruit enlargement and sugar accumulation. The vacuole is strongly related with both mechanisms, because it accumulates sugars at high concentration, which in turn, serves as a space-filling component and a driving force for fruit growth. In Japanese pear fruit, cell division continues for forty days after flowering (DAF); subsequently, fruit growth depends exclusively on cell enlargement (Yamaki and Matsuda, 1977). The vacuole occupies most of the cell volume even at the late cell division stage (Shiratake et al., 1998); it occupies more than 90% of the cell volume and the mean diameter of cells reaches 200-300 μm at the mature stage. This means that the increase of cell volume mainly depends on the enlargement of the vacuole rather than an increase in cytoplasmic volume. Thus, the increase of vacuolar volume is one of the most important factors for fruit enlargement.

Sugars are accumulated to a high concentration in fruit; for example, in the mature apple fruit, sugar concentration in the vacuole reaches more than 900 mM. Consequently, the resulting high turgor pressure promotes cell expansion that leads to fruit enlargement. Sugars are transported from the cytosol into the vacuole across the vacuolar membrane by H^+/sugar antiport system (Yamaki, 1987). The electrochemical gradient for sugar transport is generated by two vacuolar proton pumps; vacuolar H^+-ATPase (V-ATPase) and vacuolar H^+-inorganic pyrophosphatase (V-PPase). Therefore, these proton pumps are essential not only for sugar accumulation but also fruit enlargement. Recently, a carrot transformant with an antisense for V-ATPase catalytic subunit caused a reduction of cell expansion that resulted in highly dissected leaves and shorter taproot (Gogarten et al., 1992), indicating that the vacuolar proton pump is important for solute accumulation and cell enlargement.

V-ATPase and V-PPase have been purified from the European pear fruit and their biochemical properties characterized (Hosaka et al., 1994; Suzuki et al., 1998; Suzuki et al., 1999). In the European pear fruit, the levels of V-PPase and V-ATPase activities paralleled those of their polypeptides. It is known that V-PPase is the main proton pump at the immature stage, whereas V-ATPase is the main one at the mature stage

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(Shiratake et al., 1997). However, changes in both protein and gene levels of V-ATPase and V-PPase during fruit development have not been investigated and there are few studies like this from the physiological viewpoint. On the other hand, studies on sugar metabolism in Japanese pear fruit have proceeded (Yamaki and Moriguchi, 1989; Moriguchi et al., 1992). Fruit development is accompanied with accumulation of sugars in the cells. It is important to investigate the changes of the vacuolar proton pumps during fruit development for understanding sugar accumulation as well as fruit enlargement. We reported the changes of V-ATPase and V-PPase on both protein and gene levels during the development of Japanese pear fruit.

Materials and Methods

Plant materials

Samples of Japanese pear fruit (*Pyrus serotina* Rehder var. *culta* Rehder cv. Housui) were collected at Anjo in Aichi Pref. in 1997. The date of full bloom was April 8th; fruit were sampled 31, 45, 59, 80, 101, 122, 143 and 157 DAF and prepared within a day of harvest for analysis of enzyme activities. For immunoblot and northern blot analyses, the flesh tissue of fruit was frozen in liquid nitrogen and stored at -80 ℃ until analyzed.

Purification of vacuolar membranes by sucrose density gradient centrifugation

Crude membranes were isolated according to the previous report with slight modifications (Shiratake et al., 1997). The flesh of Japanese pear fruit was grated into 100 mM Tris-HCl (pH 8.0) buffer, containing 250 mM mannitol, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*, tetraacetic acid (EGTA), 0.5% (w/v) bovine serum albumin (BSA), 4 mM dithiothreitol (DTT), 12.5 mM 2-mercaptoethanol, 5% (w/v) polyvinylpyrrolidone (PVP-40), 1 mM phenylmethylsulfonylfluoride (PMSF), and 2 mM $MgSO_4$, then homogenized with a glass homogenizer. The homogenate was centrifuged at 10,000 × g for 20 min and the supernatant centrifuged at 100,000 × g for 30 min. The pellet was suspended in a suspension buffer (1 mM DTT, 2 mM $MgSO_4$, 10 mM Bis-Tris-Propane (BTP)-HCl, pH 7.5), and the mixture centrifuged at 10,000 × g for 30 min. The supernatant was centrifuged again at 100,000 × g for 1 h; the pellet of crude membranes was resuspended in a suspension buffer and sonicated for 20 s. Linear sucrose density gradient was prepared with a twin-chambered gradient mixer. The chambers contained 5.3 ml of 12% (w/w) and 40% (w/w) sucrose, respectively, in a suspension buffer. The suspension of crude membranes (0.48 ml) was layered onto the sucrose density gradient and centrifuged at 100,000 × g for 18 h in a P-40 ST rotor (Hitachi, Tokyo, Japan). The sample was collected from the top with a peristaltic pump as 0.6-ml fractions. All steps were conducted at 4 ℃.

Extraction of total proteins from the flesh of Japanese pear fruit with SDS

The method of protein extraction was described previously (Shiratake et al., 1997). The flesh of Japanese pear fruit was homogenized with a mortar and pestle in a medium containing 5% (w/v) sodium dodecyl sulfate (SDS), 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM DTT, 250 mM 2-mercaptoethanol, and 50 mM Tris-HCl (pH 8.0). The mixture was further homogenized with a glass homogenizer and the homogenate centrifuged at 1,000 × g for 10 min; the supernatant was then filtered through two layers of cheese cloth. The filtrate was applied to a PD-10 column (Amersham Pharmacia) and the eluate was subjected to SDS-PAGE.

Assays of enzymatic activities

The ATPase and PPase activities were assayed according to Shiratake et al. (1997). The reaction mixture to assay ATPase activity contained 3 mM Tris-ATP, 3 mM $MgSO_4$, 50 mM KCl, 1 mM sodium molybdate, 0.02% (v/v) Triton X-100, and 50 mM BTP-HCl (pH 7.5), with or without 100 nM bafilomycin. The V-ATPase activity corresponded to the bafilomycin-sensitive ATPase activity. The reaction mixture to assay PPase activity contained 1 mM sodium pyrophosphate, 1 mM $MgSO_4$, 50 mM KCl, 1 mM sodium molybdate, 0.02% (v/v) Triton X-100, and 50 mM BTP-HCl (pH 7.5). The enzymatic activities of ATPase and PPase were colorimetrically measured by determining the rate of liberation of Pi at 30 ℃.

SDS-PAGE and immunoblotting

For immunoblotting, proteins which were separated by SDS-PAGE according to Laemmli (1970), were transferred to a PVDF membrane with a semi-dry blotting apparatus, a modified version of the method described by Towbin et al. (1979). The membrane sheets were blocked with 3% non-fat dry milk prior to reactions with antibodies against mung bean V-PPase (Maeshima and Yoshida, 1989) and pear V-ATPase subunit A (Suzuki et al., 1998), a catalytic subunit. Binding of the antibody was detected by using a chemiluminescent substrate, CSPD (Boehringer Mannheim) with alkaline phosphatase-conjugated antibody raised in a goat against rabbit and mouse immunoglobulin G.

Extraction of RNA

Total RNA was extracted from the flesh of Japanese pear fruit with the phenol-SDS method (Nakajima et al., 1988) followed by the hexadecyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). RNA was precipitated by cold ethanol and suspended in TE buffer (10 mM Tris-HCl (pH

8.0) and 1 mM EDTA). RNA was then precipitated by 2 M LiCl and resuspended in TE buffer.

Preparation of cDNA probe

cDNAs were synthesized from the mRNAs with reverse transcriptase using an oligo dT-Adaptor primer (Takara). To prepare a DNA probe for V-PPase, cDNA templates were amplified by PCR using Taq polymerase and gene-specific primers (forward, 5'-ACTGGTTATGGTCTTGGTGGGT-3'; reverse, 5'-GGCAACATCTTGCACAGGGCTGT-3'). The primers correspond to the consensus nucleotide sequences of V-PPase cDNA of barley (Tanaka et al., 1993) and *Arabidopsis thaliana* (Sarafian et al., 1992). The amplification protocol was 5 min at 95 ℃ (once), 1 min at 95 ℃, 1 min at 55 ℃, 2 min at 72 ℃ (40 cycles), and 7 min at 72 ℃ (once). Amplified DNA fragment (630 bp) was cloned in pT7Blue T-vector (Novagen). DNA sequencing showed that the cloned cDNA fragment was 77 and 78% homologous with the cDNAs for V-PPase from barley and *Arabidopsis thaliana*, respectively.

A DNA probe for V-ATPase subunit A was also prepared by PCR using specific primers (forward, 5'-GGCCAGCGTGTTCTTGATGC-3'; reverse, 5'-TCTGCCATCATACTAACATTGTA-3'). The primers correspond to the consensus nucleotide sequences of V-ATPase subunit A cDNA of *Arabidopsis thaliana* (Magnotta and Gogarten, 1997), carrot (Zimniak et al., 1988), and cotton (Wilkins, 1993). The succeeding steps are as described above. The cloned cDNA fragment (370 bp) showed 86-89% homology with the cDNAs for V-ATPase subunit A from *Arabidopsis thaliana*, carrot, and cotton.

The DNA probes were DIG-labeled, following the supplier's protocol (Boehringer Mannheim).

Northern blot analysis

Total RNA which was isolated from Japanese pear fruit as described above, was separated by formaldehyde agarose gel electrophoresis by a standard procedure (Sambrook et al., 1989). The RNAs were blotted on a nylon membrane and prehybridized in DIG-easy hyb. (Boehringer Mannheim) at 52 ℃ for 30 min and hybridized with DIG-labeled probe DNA at 52 ℃ overnight. The membrane was washed twice in 2 × SSC (SSC; 150 mM NaCl and 15 mM sodium citrate) containing 0.1% SDS at room temperature for 5 min and in 0.1 × SSC containing 0.1% SDS at 68 ℃ for 15 min. For V-ATPase subunit A, the temperature of prehybridization and hybridization was 44 ℃ and that of washing in 0.1 × SSC containing 0.1% SDS was 65 ℃. Detection was done following the supplier's protocol using a chemiluminescent substrate, CSPD (Boehringer Mannheim). The membrane was exposed to X-ray film.

Quantification of protein

Protein contents of samples that have been extracted with SDS were measured by the modified method of Bensadoun and Weinstein (1976), with BSA as a standard. Each sample was diluted fifty-fold with distilled water prior to precipitation of proteins because SDS at high concentration inhibits the precipitation of protein.

Results

Developmental changes of Japanese pear fruit

Developmental changes in fresh weight and protein content per fresh weight of Japanese pear fruit are shown in Fig. 1. Fresh weight gradually increased until 80 DAF, then drastically increased until 178 DAF. The growth curve followed a typical single sigmoidal pattern. Protein content per fresh weight decreased with fruit development.

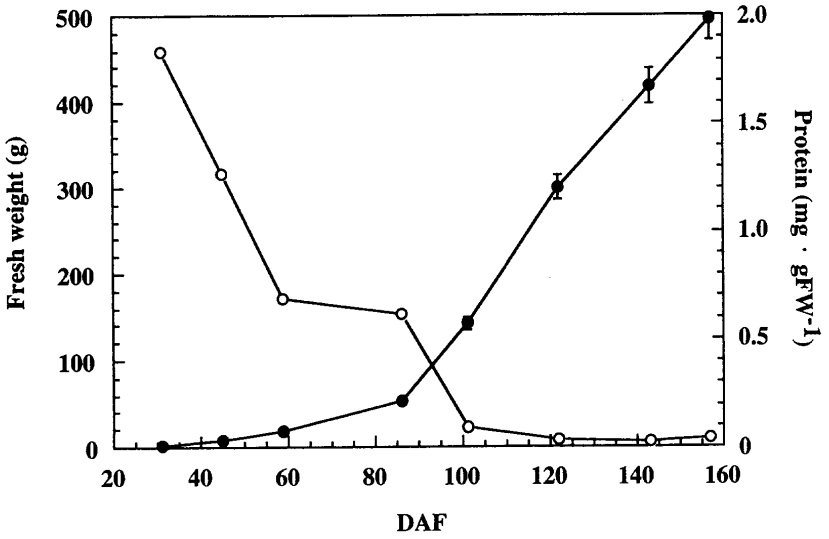

Fig. 1. Seasonal changes in fresh weight (●) and protein content per fresh weight (○) during the development of Japanese pear fruit.

Seasonal changes in the activities of V-ATPase and V-PPase

To clarify the roles of the vacuolar proton pumps during the development of Japanese pear fruit, vacuolar membranes were prepared with a linear sucrose density gradient centrifugation of microsome. Both the activities of V-ATPase and V-PPase on the basis of fresh weight decreased with fruit development on account of dilution accompanying rapid cell expansion (data not shown); their patterns of fluctuation were almost similar to those in European pear fruit (Shiratake et al., 1997). Their activities on the basis of fresh weight were converted on the basis of total protein to assess the physiological importance of the vacuolar proton pumps during fruit development (Fig. 2A). The V-ATPase activity remained low till 80 DAF, then increased rapidly till 122 DAF; it remained high but decreased at 143 DAF. The trends of the V-PPase activity followed the same pattern as that of V-ATPase, although the increase after 80 DAF was smaller. The ratio of the V-PPase to V-ATPase activities revealed that V-PPase activity was higher than that of V-ATPase (Fig. 2B); the ratio decreased gradually with fruit development.

Changes in the levels of polypeptide and mRNA for V-ATPase with development

The levels of polypeptide and mRNA for V-ATPase subunit A, which is a catalytic subunit, were determined at each harvest date to seek its regulation in Japanese pear fruit. Immunoblot analysis with the antibody against European pear V-ATPase subunit A revealed that the level of polypeptide on the basis of total protein was low until 80 DAF; it then increased rapidly to 122 DAF and remained high (Fig. 3A). Changes in the level of mRNA were shown in Fig. 3B. The mRNA level was low till 80 DAF except for 31 DAF; it then increased till 122 DAF and stayed high although decreased slightly.

Changes in the levels of polypeptide and mRNA for V-PPase with development

Changes in the levels of polypeptide and mRNA for V

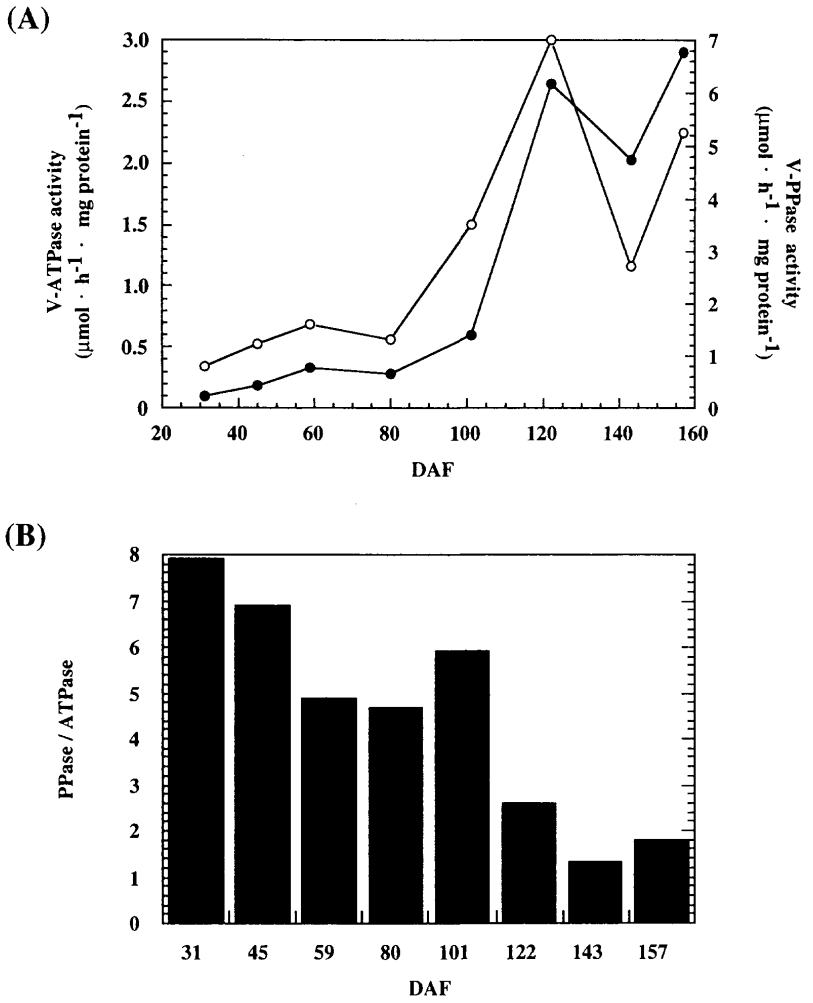

Fig. 2. Seasonal changes in the activities of V-ATPase (●) and V-PPase (○) on the basis of total protein (A) and the ratio of V-PPase to V-ATPase (B) in developing Japanese pear fruit. Crude microsomes from fruit flesh on each harvest date were subjected to continuous sucrose density gradient centrifugation. The V-ATPase activity corresponded to that of bafilomycin-sensitive ATPase.

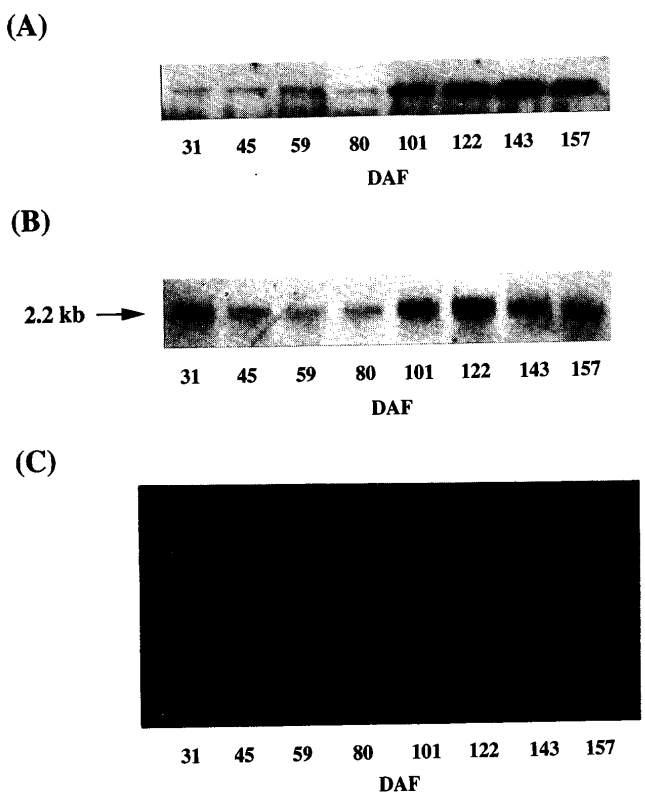

Fig. 3. Developmental changes in the levels of protein and mRNA for V-ATPase subunit A in Japanese pear fruit. The same samples as those in Fig. 2 were used. (A), Immunoblot analysis. Aliquots (5 μg) of proteins extracted with SDS were subjected to SDS-PAGE and blotted onto a PVDF membrane. The membrane was immunostained with antibody against pear V-ATPase subunit A. (B), Northern analysis. Aliquots (20 μg) of the total RNA were electrophoresed and blotted onto a nylon membrane. The membrane was subjected to northern analysis with a DIG-labeled cDNA fragment of V-ATPase subunit A. (C), Ethidium bromide staining of rRNAs.

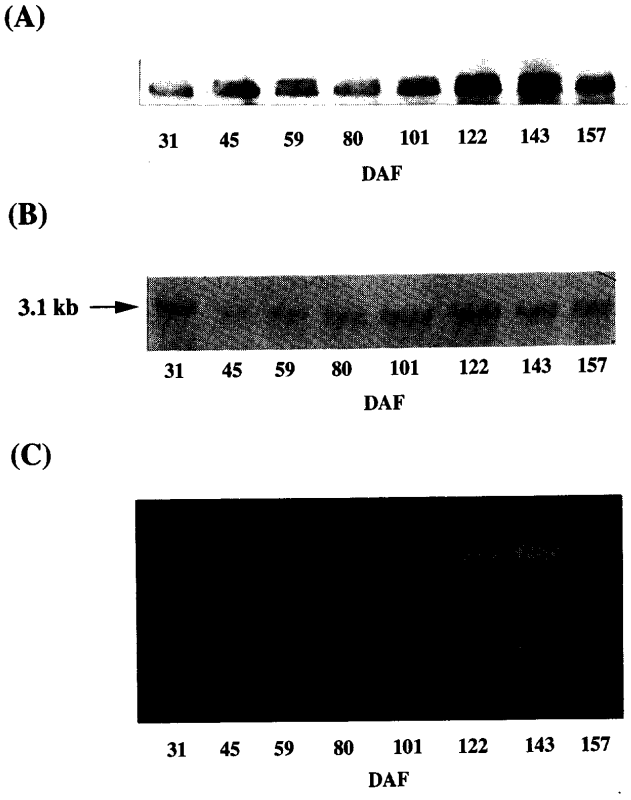

Fig. 4. Developmental changes in the levels of protein and mRNA for V-PPase in Japanese pear fruit. The same samples as those in Fig. 2 were used. (A), Immunoblot analysis. (B), Northern analysis. (C), Ethidium bromide staining of rRNAs. All of the assay methods were similar to those described in Fig. 3 except that 10 μg of the total RNA was used in (B).

-PPase, another proton pump in vacuolar membrane, during the development of Japanese pear fruit were also determined by immunoblot and northern blot analyses for the same purpose as V-ATPase. Immunoblot analysis with the antibody against mung bean V-PPase revealed two bands of V-PPase, indicating an existence of isoforms (Fig. 4A). The polypeptide levels were lowest 31 DAF, increased to a small extent till 80 DAF; they increased subsequently until 122 DAF, then remained farily constant except for a slight decrease at 157 DAF (Fig. 4A). The mRNA level was relatively high 31 DAF differing from the pattern of polypeptides; it then decreased, and reaching the lowest level at 45 DAF. Afterward, it increased until 122 DAF and remained high; a slight decrease occurred at 157 DAF (Fig. 4B).

Discussion

In apple and European pear, much sugars are accumulated in the fruit as they develop (Yamaki and Ino, 1992; Yamaki et al., 1993). The accumulation causes high turgor pressure within the vacuole which leads to fruit enlargement. Vacuolar proton pumps are necessary to translocate sugars into the vacuole. In European pear fruit, V-PPase was the main vacuolar proton pump at the immature stage and V-ATPase at the mature stage (Shiratake et al., 1997). Thus, we investigated the changes of the vacuolar proton pumps in Japanese pear fruit, which develops accompanied with the accumulation of much sugars in cells, to clarify their role in fruit development. With Japanese pear fruit growth, the V-ATPase activity increased and the extent of its increase was much high (Fig. 2A). This result suggests that V-ATPase plays important roles at the mature stage in Japanese pear fruit as in the case of European pear fruit. On the other hand, the V-PPase activity in Japanese pear fruit also increased with fruit enlargement (Fig. 2A), but not in European pear fruit (Shiratake et al., 1997). At the cell division stage, the ratio of V-PPase to V-ATPase was about 7.9 (Fig. 2B). As fruit grew, the ratio decreased and finally it was 1.8. The H^+ /substrate stoichiometries for V-ATPase and V-PPase was reported to be 2 and 1, respectively (Schmidt and Briskin, 1993), indicating that V-PPase seems to have important roles not only at the immature stage but also at the mature stage and be essential throughout the

developmental period of Japanese pear fruit.

The fresh weight of Japanese pear fruit increased dramatically after 101 DAF (Fig. 1) and the activities of the vacuolar proton pumps (the sum of the V-ATPase and V-PPase activities) also increased drastically after 101 DAF (Fig. 2A). Thus, drastic enlargement of Japanese pear fruit might need high activities of the vacuolar proton pumps. In the case of European pear fruit, the fresh weight increased gradually even after 101 DAF with a corresponding increase in the activities of the vacuolar proton pumps; but the increase in the V-PPase activity was not prominent (Shiratake et al., 1997). These results suggest that the growth pattern of fruit might be directly related to the fluctuation in the vacuolar proton pump activities.

In *Kalanchoë daigremontiana*, isoforms of V-PPase were detected with SDS-PAGE and immunoblot; their molecular masses were 72,000 and 70,000 (Becker et al., 1995). In Japanese pear fruit, isoforms of V-PPase were also detected with immunoblot and might be regulated differently because only a lower band 157 DAF became conspicuous (Fig. 4A). Further studies on isoforms, for example, their regulation mechanism, isolation of their cDNAs, etc. need.

Immunoblot analyses revealed that the levels of polypeptides for V-ATPase subunit A and V-PPase were low at the immature stage but increased with fruit maturity (Fig. 3A, Fig. 4A); their patterns of fluctuations were generally similar to those of their activities. Northern analyses revealed that two vacuolar proton pumps were actively transcribed at the mature stage, especially 122 DAF (Fig. 3B, Fig. 4B). Changes in the mRNA levels generally paralleled with those of the polypeptide, except at the cell division stage. This indicates that the vacuolar proton pumps are seemingly regulated differentially at the gene level between the cell division and cell elongation stages. These findings suggest that the vacuolar proton pumps are regulated at transcriptional level during cell elongation in Japanese pear fruit. Whereas, in European pear fruit, the polypeptide levels for V-ATPase and V-PPase varied (Shiratake et al., 1997), in Japanese pear fruit the expression pattern of V-ATPase was quite similar to V-PPase, but the regulatory mechanism of V-ATPase and V-PPase remains unknown.

Our findings show that the vacuolar proton pumps are regulated at the transcriptional level during the cell elongation stage and that V-PPase contributes more than V-ATPase as the vacuolar proton pump throughout the developmental period of Japanese pear fruit. Furthermore, the activities of the vacuolar proton pumps correspond closely to cell enlargement. Additional investigations on the mechanism of gene expression of V-ATPase and V-PPase and the construction of transgenic plants that lack either V-ATPase or V-PPase should reveal their contribution to fruit development.

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ニホンナシ果実の生長過程での液胞膜プロトンポンプの活性の変動と遺伝子発現

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摘　要

液胞膜プロトン輸送性ATPase(V-ATPase)と液胞膜プロトン輸送性ピロホスファターゼ(V-PPase)は，液胞膜を介した電気化学勾配を形成するが，この電気化学勾配は糖を液胞内に輸送する上で極めて重要である．そこで果実生長におけるV-ATPaseとV-PPaseの役割を明らかにするために，ニホンナシの果実生長に伴うそれらの酵素の変動を調べた．その結果，果実の生長に伴い，全タンパク質量当たりのV-ATPaseとV-PPaseの活性は上昇し，生長期間全般を通じてV-PPase活性はV-ATPase活性より高かった．イムノブロット解析から，V-ATPaseの触媒サブユニットとV-PPaseのポリペプチドレベルの変動が活性の変動とおおむね一致することが明らかとなった．また，それらのmRNAレベルの変動も細胞分裂期を除いてポリペプチドレベルの変動とおおむね一致していた．これらの結果から，ニホンナシ果実の生長過程ではV-PPaseが主要なプロトンポンプとして働いていること，そして細胞肥大期におけるV-ATPaseとV-PPaseの活性は主に転写レベルで制御されていることが示唆された．

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