Fluctuations in the Protein Levels of H^+ -pumps and Water Channels of the Tonoplast and Plasma Membrane during Grape Berry Development

Katsuhiro Shiratake, Haruka Goto, Masayoshi Maeshima and Shohei Yamaki

Graduate School of Bio-agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601

Summary

Fluctuations of the protein levels of H^+ -pumps and water channels of tonoplast and plasma membrane were determined by immunoblot analysis during grape berry development. The growth pattern of a grape berry follows a double sigmoid curve during which many physiological changes are considered to occur, especially after veraison. However, the protein levels of H^+ -pumps and water channels, except V-ATPase, did not change after veraison. The level of V-ATPase, one of the tonoplast H^+ -pumps, was relatively high at 42 days after flowering (DAF) and after veraison. The level of V-PPase, another tonoplast H^+ -pump, changed more gradually than did that of V-ATPase. Two bands of plasma membrane H^+ -pump (P-ATPase) were detected; the level of each band changed differently. However, the change in total P-ATPase level was relatively small during berry development. Changes in the level of water channels of tonoplast and plasma membrane, that is, VM23G and PAQ, respectively, were similar. That is, their levels, which were lowest at 30 DAF, increased dramatically at 42 DAF, and thereafter remained nearly constant. In VM23G, the level increased 121 DAF. A previous study in pear fruit suggested that a relationship exists between tonoplast water channel and cell expansion, but such a relationship did not exist in the grape berry. Both in the pear fruit and in grape berry, V-ATPase level nearly paralleled that of the growth rate.

Key Words: grape berry, plasma membrane, proton pump, tonoplast, water channel.

Introduction

Fruit accumulate sugars at a high concentration intracellularly, while enlarging more than do other tissues. A large part of the cell volume is occupied with a central vacuole where most of the sugar is stored. While the sugar in the vacuole generates a high osmotic pressure for cell expansion, its accumulation and that of water by fruit cells lead to sweetness and growth. Therefore, the clarification of the mechanism of sugar accumulation in fruit cell is one of the important works in horticultural science.

Sugar synthesized in leaves is translocated via sieve tubes in the phloem and is unloaded in the fruit. The sugar is transported into fruit cells by sugar carriers in the plasma membrane and/or via plasmodesmata. Sugar carriers also exist on the tonoplast of fruit cells (Yamaki, 1987; Shiratake et al., 1997b) and they transport a large part of the sugar into the vacuole. Fillion et al. (1999) isolated genes encoding hexose carriers of grape and showed that one of them correlated with sugar accumulation of the berry. H^+ -pumps create a proton gradient

across the membranes; this gradient is the driving force for sugar carriers. Thus, H^+ -pumps are considered to be indispensable for sugar accumulation by fruit cells. P-type H^+ -ATPase (P-ATPase) exists in the plasma membrane and transports protons from cytosol to apoplast (Palmgren and Harper, 1999). Two different H^+ -pumps, namely, vacuolar H^+ -ATPase (V-ATPase) and vacuolar H^+ -pyrophosphatase (V-PPase) exist in the tonoplast and transport protons from cytosol to vacuole. The energy of proton gradients is used not only for sugar transport but also for other solute transports to maintain cell homeostasis. We have purified V-ATPase and V-PPase from pear fruit and characterized their properties (Hosaka et al., 1994; Suzuki et al., 1999a). Water channel proteins, which transport water across the membrane, were also found in the plasma membrane and tonoplast of a plant (Maurel, 1997). Water is loaded into the vacuole of fruit cells in response to the osmotic pressure of the vacuole. When water moves from apoplast to vacuole, water passes through the water channels of the plasma membrane and tonoplast.

We have reported the changes in H^+ -pumps and water channel of the tonoplast during fruit development of European and Japanese pears at the protein and gene levels (Shiratake et al., 1997a, 1998; Suzuki et al., 1999b, 2000). The grape berry with its double sigmoid growth pattern undergoes dramatic biochemical and physio-

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logical changes, especially around veraison, whereas the pear with its single sigmoid growth curve displays gradual changes. In this report, we determined the protein levels associated with H^+ -pumps and water channels of both the tonoplast and plasma membrane during grape berry development. The findings on grape are compared and discussed with those previously reported for pear.

Materials and Methods

Plant material

In 1998, grape (*Vitis* 'Muscat Bailey A') berries (34 or more) were harvested from a vineyard of Nagoya University at a fixed time of the day and weighted. The flesh was frozen in liquid nitrogen and stored at -80°C .

The growth rate (GR) between T_2 and T_1 stage was calculated from the following formula: $GR = (W_2 - W_1) / (T_2 - T_1)$, where W and T represent the fresh weight and days after flowering (DAF) on the respective sampling dates.

Protein extraction

Total proteins were extracted by a modified method of Maeshima et al. (1994). The flesh was homogenized with a mortar and pestle in a medium that contained 5% (w/v) sodium dodecyl sulfate (SDS), 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol, 2% (w/v) β -mercaptoethanol, and 50 mM Tris-HCl, pH 8.0, and it was further homogenized with a glass homogenizer. pH of the homogenate was adjusted to pH 7.0 with 1 M Tris and centrifuged at $1,000\times g$ for 10 min; the supernatant was filtered through two layers of nylon (300 mesh). The filtrate was applied to a PD-10 column (Amersham Pharmacia Biotech) and the eluate was subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE and immunoblotting

SDS-PAGE was performed as described by Laemmli (1970) and the separated proteins were transferred to a cellulose nitrate membrane with a semi-dry blotting apparatus according to Towbin et al. (1979). Prior to reactions with the primary antibody, the membrane sheet was blocked with 3% (w/v) nonfat dry milk in a Tris-buffered saline solution that contained 0.05% (w/v) Tween 20 (TBS-T) and followed by three rinsings with TBS-T. Antibodies against pear V-ATPase (Shiratake et al., 1997a), mung bean V-PPase (Maeshima and Yoshida, 1989), radish VM23 (Maeshima, 1992), maize P-ATPase (Nagao et al., 1987) and radish PAQ (plasma membrane aquaporin, Maeshima et al., unpublished) were used. PAQ antibody was raised against the peptides of the first transmembrane domain of the plasma membrane water channel of radish. The binding of these antibodies was detected by a colorimetric reaction with alkaline phosphatase-conjugated antibodies, raised in goat against mouse or rabbit immunoglobulin G (Promega).

Immunoblotting was performed at least three times for each protein. The depth of bands on the immunoblotting was quantified by densitometric scanning with a densitometer (model AE-6920, ATTO).

Quantification of protein

Protein contents of samples that had been extracted with SDS were measured by a modified method of Bensadoun and Weinstein (1976) that contains the precipitation step by trichloroacetic acid. A high concentration of SDS inhibits this step but at 0.125% (w/v), quantification by bovine serum albumin was unaffected (data not shown). Thus, each sample was diluted fifty-fold with distilled water to achieve the 0.125% (w/v) SDS prior to protein precipitation.

Results

Changes in fresh weight, growth rate and protein content of grape berry

The double sigmoid growth curve of grape berry is more clearly shown by the growth rate curve (Fig. 1). The growth rate curve peaks at 50 and 90 DAF.

The high recovery rate of total proteins, especially membrane proteins, was obtained from berry flesh with the simple SDS method. The initial protein content of berry, which was $1.8 \text{ mg} \cdot \text{g fresh weight}^{-1}$, decreased to $0.66 \text{ mg} \cdot \text{g fresh weight}^{-1}$ by 42 DAF, and then remained nearly constant (Fig. 2).

Cross-reactivity of antibodies

To determine the levels of H^+ -pumps and water channels by immunoblot analysis, we checked cross-reactivity and specificity of antibodies against H^+ -pumps and water channels of other plant species (Fig. 3). V-ATPase is composed of about 10 different subunits (Hosaka et al., 1994). Antibodies against the 55 and 33 kDa subunits of pear V-ATPase clearly reacted with ca. 55 and 33 kDa peptides of grape berry, respectively. The antibody against mung bean V-PPase also reacted with

Fig. 1. Developmental changes in fresh weight (●) and growth rate (○) of grape berry. Vertical bars represent SE ($n \geq 34$).

Fig. 2. Seasonal changes in total protein content (fresh weight basis) during grape berry development. Vertical bars represent SE ($n \geq 6$).

Fig. 3. Immunoblotting tests for determining specificity of antibodies against the 33 and 55 kDa subunits of pear V-ATPase (lanes 1 and 2, respectively), mung bean V-PPase (lane 3), radish VM23 (lane 4), maize P-ATPase (lane 5) and radish PAQ (lane 6) with total protein fraction from grape berry. Total protein fraction (0.5 μ g for VM23G and 2 μ g for others) was subjected to SDS-PAGE and immunoblot analysis.

a peptide of grape berry. VM23, a γ TIP-type water channel of radish tonoplast (Higuchi et al., 1998), recognized the predominant ca. 23 kDa peptide of grape berry, designated VM23G, and another peptide of a higher molecular mass. The antibody against maize P-ATPase yielded two bands of ca. 100 kDa in grape berry proteins. Several isoforms of P-ATPase exist in plants (Palmgren and Harper, 1999; Mito et al., 1996). Therefore, this antibody might have reacted with two isoforms of P-ATPase of grape berry. PAQ, a water channel of the plasma membrane of radish (accession nos. AB012044, AB012045), reacted with an antibody predominant ca. 30 kDa peptide of grape berry and another peptide to yield a fainter band. The fainter bands in the VM23G and PAQ are considered to be their dimer or tetramer based on the findings of Maeshima (1992) who

Fig. 4. Developmental change in the levels of V-ATPase subunits (33 and 55 kDa), V-PPase and VM23G in grape berry. Total protein fractions from berry harvested at 30, 42, 56, 71, 85, 98 and 121 DAF were subjected to SDS-PAGE and immunoblot analysis. Each sample is equivalent 0.5 μ g protein for VM23G or 2 μ g for others.

found that hydrophobic proteins, such as VM23, tend to form a dimer or tetramer during sample preparation for SDS-PAGE. The six antibodies (Fig. 3) could recognize specific polypeptides of grape berry, so that we used them for immunoblot analysis in further experiments.

Developmental changes in the levels of H^+ -pumps and water channel of tonoplast

The fluctuations in the levels of two H^+ -pumps and water channel of tonoplast during grape berry development were detected by the immunoblotting technique (Fig. 4) and their relative amounts determined with a densitometer (Fig. 5).

Highest levels of these tonoplast proteins were observed at 121 DAF, although their levels changed differently. The levels of the 33 and 55 kDa subunits of V-ATPase, changed similarly, although they differed slightly at 85 DAF. The levels fell below 35% of the maximum level at 30 DAF and increased dramatically at 42 DAF; they decreased at 56 DAF and increased again after 85 DAF. The level of the water channel of grape tonoplast, VM23G, fluctuated similarly to V-ATPase, but the decreases at 56 and 71 DAF did not occur. Although V-PPase level was relatively high in the later stage of berry development, it changed more gradually than did that of V-ATPase.

Developmental changes in the levels of H^+ -pump and water channel of plasma membrane

The changes in levels of H^+ -pump and water channel of plasma membrane were also detected by immunoblot analysis (Fig. 6). An antibody against P-ATPase recognized two isoforms of P-ATPase among the grape berry proteins. The isoform representing the upper band was predominant at 30 and 42 DAF; the two isoforms had almost the same levels during the other stages. Relative

amounts of P-ATPase and PAQ were calculated with a densitometer (Fig. 7), but the two bands of P-ATPase could not be discriminated by the densitometric analysis. Thus, the relative amount of P-ATPase was calculated from the total depth of the two bands.

Fig. 5. Relative amounts of V-ATPase subunits (33 and 55 kDa), V-PPase and VM23G during grape berry development. The amount of each protein was quantified by densitometric scanning of the immunoblotting. Vertical bars represent SE ($n \geq 3$).

The changes in H^+ -pump and water channel of plasma membrane differed from those in tonoplast ones. That is, P-ATPase and PAQ levels were highest at 42 DAF, whereas the levels of V-ATPase, V-PPase and VM23G attained their highest levels at 121 DAF. The P-ATPase level ranged between 63–79 % of the level at 42 DAF, except at 42 and 98 DAF. On 98 DAF, it was 45% of the maximum (100%) level. The PAQ level was 42% of the maximum level at 30 DAF, then increased dramatically at 42 DAF, and thereafter its level remained above 70% of the maximum level.

Discussion

Our results confirmed that the growth curve of 'Muscat Bailey A' is double sigmoid (Fig. 1). The

Fig. 6. Developmental changes in the levels of P-ATPase and PAQ in grape berry. Total protein fractions from berry harvested at 30, 42, 56, 71, 85, 98 and 121 DAF were subjected to SDS-PAGE and immunoblot analysis. Each sample was equivalent to 2 μ g protein.

Fig. 7. Relative amount of P-ATPase and PAQ during grape berry development. The amount of each protein was quantified by densitometric scanning of the immunoblotting. Vertical bars represent SE ($n \geq 3$).

growth rate exhibited peaks at 50 and 90 DAF indicating that the berry had three growth phases as previously reported (Coombe, 1980; Opara, 2000). That is, there is an early growth stage (–56 DAF, S-I), a slow growth stage (56–71 DAF, S-II) and a final growth stage (71 DAF–, S-III). While the grape berry enlarges rapidly in S-I, it accumulates organic acids. It grows slowly in S-II but much physiological changes of grape berry occur as it ends and at the inception of S-III, called 'veraison'. The events that take place after veraison are re-acceleration of growth, softening of the berry, decrease in organic acids and a dramatic increase in sugars and anthocyanin (Kanellis and Roubelakis-Angelakis, 1993). Some of these events, such as berry enlargement and the accumulation of sugar and organic acids are highly correlated with the functions of H^+ -pumps and water channels. Therefore, their biochemical roles in berry development would be helpful in understanding its physiology. We have already determined the developmental changes in the levels of tonoplast H^+ -pumps and water channel in pear fruit (Shiratake et al., 1997a). In this study, we determined how their levels change during the development of grape berry and compared the changes with those of pear fruit.

V-ATPase and V-PPase are H^+ -pumps of the tonoplast that generate an electrochemical gradient across the membrane for the secondary transport of sugars and organic acids. In grape berry, which follows a double sigmoid growth curve, the pattern of V-ATPase level exhibited two peaks at 42 and 121 DAF. In the pear fruit, which follows a single sigmoid growth curve, V-ATPase level increased almost linearly with development (Shiratake et al., 1997a), demonstrating that V-ATPase level is correlated with the fruit growth pattern. Gogarten et al. (1992) transformed a carrot with an antisense for the catalytic subunit of V-ATPase. The plant had smaller cells and a shorter taproot, suggesting the importance of V-ATPase for cell expansion. The major organic acids of grape berry are tartaric and malic acids, which accumulate rapidly in the latter part of S-I (Kanellis and Roubelakis-Angelakis, 1993). Terrier et al. (1998) suggested that the same carrier transports the two acids across the tonoplast. Martinoia et al. (1991) showed that the malate transport across the tonoplast depends on the membrane potential driven by H^+ -pumps. Hence, the high level of V-ATPase at 42 DAF might be ascribed to the rapid cell expansion and accumulation of organic acids by a young berry. In S-III, growth rate increased (Fig. 1) concurrent with increases in sugar content after veraison (Fillion et al., 1999). Therefore, in S-III, V-ATPase may also participate in cell expansion and sugar accumulation. However, V-ATPase level was increasing at 121 DAF (Fig. 5), when the growth rate was decreasing 90 DAF (Fig. 1). Coombe (1980) reported that sugar continued accumulating in berry after berry growth ceased, so that the final increase of V-ATPase at 121DAF might be related with that process.

The level of V-PPase changed more gradually than did that of V-ATPase (Fig. 5). In pear fruit, the V-PPase level was especially high at the cell-division stage during early fruit development, and remained nearly constant thereafter (Shiratake et al., 1997a). The high level of V-PPase has also been found in young tissues, such as mung bean hypocotyls (Maeshima, 1990), pumpkin cotyledons (Suzuki and Kasamo, 1993), and young radish taproot (Maeshima et al., 1996). Consequently, V-PPase is thought to scavenge for pyrophosphate produced in young tissues. However, V-PPase level was not high in the young grape berry (Fig. 5), even though most of the cell divisions of flesh tissue of grape berry occurs before 10 DAF (Kanellis and Roubelakis-Angelakis, 1993). Berries are so small at the cell-division stage that we could not sample them. The V-PPase level of the berry at cell-division stage might be higher than that which we noted at 30 DAF.

The level of V-ATPase increased dramatically after veraison, while that of V-PPase did not. A similar tendency was observed in pear fruit (Shiratake et al., 1997a), which suggests that the increase in V-ATPase level during maturation might be common among fleshy fruits. In the pear, the major H^+ -pump of the tonoplast was V-PPase in young fruit; that role was assumed by V-ATPase as the fruit mature (Shiratake et al., 1997a). However, Terrier et al. (1998) reported that V-PPase activity was about 3 times higher than that of V-ATPase in the ripe grape berry which tend to agree with our results (Fig. 5), emphasizing the importance of V-PPase in ripening grape berry.

The level of tonoplast water channel, VM23G, was lowest at 30 DAF and remained at that level from 42 to 98 DAF; it then increased at 121 DAF (Fig. 5). γ TIP-type water channels, such as VM23, attains a high level in expanding cells (Ludevid et al., 1992; Maeshima, 1990; Higuchi et al., 1998). The protein level of the water channel of the pear tonoplast (VM23P) increased rapidly during the drastic cell-expansion stage (Shiratake et al., 1997a). Therefore, we postulate that the tonoplast water channel play an important role in the cell expansion in fruit. However, in the grape berry, change in VM23G level (Fig. 4) did not accompany with that of the growth rate (Fig. 1) nor the relative growth rate (data not shown). Maurel (1997) reported that the tonoplast is up to 100-fold more permeable to water than the plasma membrane in some plant cells. From these observations, Kjellbom et al. (1999) suggested that the water channels of plasma membrane and tonoplast play important roles in water flux of cell and the buffering of osmotic fluctuations of the cytosol, respectively. The diameter of a grape berry can fluctuate more than 10% diurnally under an arid circumstance (Kobayashi et al., 1963), indicating that a considerable amount of water is lost during the day which is replenished at night. Thus, in a grape berry, an adjustment of osmotic pressure of the cytosol is very important for maintaining cell homeo-

stasis. We postulate that VM23G plays an important role in the regulation rather than in the cell enlargement process. Although the highest level of VM23G at 121 DAF might relate with this role, its exact physiological importance at this stage is still unclear.

Cell enlargement and sugar compartmentation are said to depend on the carriers located on the plasma membrane. Hence, we tested the idea by determining the levels of H^+ -pump and water channel of plasma membrane, namely P-ATPase and PAQ, respectively (Fig. 6 and 7). An antibody against P-ATPase recognized two isoforms in grape berry proteins; the total depth of the two bands was nearly equal during berry development, although the levels at 42 and 98 DAF were slightly higher and lower, respectively. However, the change in each band differed, in that the level of the upper band, which was predominant at 30 and 42 DAF, subsequently became almost equal. These results suggest that, different isoforms of P-ATPase may take on different regulatory roles as in other plants (Michelet and Boutry, 1995). Our data show that total P-ATPase to be nearly constant during berry development.

Water channels of plasma membrane are encoded by a multi-gene family (Kjellbom et al., 1999). An antibody against PAQ was raised against the conserved region among water channel isoforms of plasma membrane, indicating that it recognized PAQ isoforms of grape berry. Thus, our result might reflect the total level of PAQ isoforms. The plasma membrane water channels are thought to control water influx and efflux of cells. However, PAQ level was almost constant during berry development, except at 30 DAF (Fig. 7), which may mean that the water permeability of the plasma membrane might depend less on the PAQ level. Perhaps another mechanism may control water permeability of the plasma membrane, such as the modification of PAQ activity by protein phosphorylation and dephosphorylation (Maurel, 1997; Kjellbom et al., 1999).

This is the first report, in which the fluctuations of the levels of proteins associated with H^+ -pumps and water channels of the tonoplast and plasma membranes were determined. In both pear and grape, the changes in V-ATPase levels paralleled the fruit growth pattern, except at the end of fruit development. Our data indicate that the role of VM23G, the tonoplast water channel of grape berry, is to buffer the osmotic fluctuations of the cytosol rather than to control cell enlargement directly, as suggested in the pear fruit.

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ブドウ果実生長における液胞膜と原形質膜のプロトンポンプおよび水チャンネルタンパク質の変動

白武勝裕・後藤陽加・前島正義・山木昭平

名古屋大学大学院生命農学研究科 464-8601 名古屋市千種区不老町

摘 要

ブドウの果実生長における液胞膜と原形質膜のプロトンポンプおよび水チャンネルタンパク質の変動を免疫ブロット法により検討した。ブドウ果実の新鮮重の変化は二重S字曲線を示し、一般的なベレゾーン期を境にした生理状態の変化が予想された。しかしながら、液胞膜と原形質膜のプロトンポンプと水チャンネルの中で、ベレゾーン期を境に蓄積量が大きく変化したものはV-ATPaseだけであった。液胞膜のプロトンポンプであるV-ATPaseの蓄積は、開花後42日とベレゾーン期以降の二度のピークがみられた。一方、もう一つの液胞膜のプロトンポンプであるV-PPaseの蓄積量の変化はV-ATPaseに比べて小さかった。原形質膜のプロトンポンプであるP-ATPaseでは、アイソフォームと考えられる2

本のバンドが検出された。2本のバンドのそれぞれの蓄積パターンは異なっていたが、両者のバンドの濃さを合計した値は、果実生長を通してそれほど大きく変化しなかった。液胞膜水チャンネル(VM23G)と原形質膜水チャンネル(PAQ)の蓄積は、開花後30日目では低く、その後増加し、他のステージではほぼ一定であった。但し、VM23Gの蓄積量は成熟期にあたる開花後121日に再度増加した。セイヨウナシ果実における結果から液胞膜の水チャンネルと細胞肥大の関連が示唆されていたが、今回のブドウ果実の結果ではその関連はみられなかった。また、ブドウとセイヨウナシにおいて、果実の生長パターンと蓄積量に最も相関がみられたのはV-ATPaseであった。