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Proton Pumps of the Vacuolar Membrane in Growing Plant Cells

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Plant growth results from the division, enlargement and specialization of cells. The two processes of the enlargement and the differentiation of cells are not spatially separated in plant tissue. We focus our attention here on the enlargement and elongation of cells. In most cases, growing plant cells contain a large central vacuole. The acid growth theory is based on the space-filling function of the large vacuole. The active transport systems in the vacuolar membrane are essential for maintenance of high osmotic pressure and for the expansion of the vacuole. The secondary active transport systems of the vacuole for sugars and ions are driven by the proton-motive force which is generated by the vacuolar H^+-ATPase and H^+-translocating inorganic pyrophosphatase. In this review, the relationship between cell elongation and these enzymes of the vacuolar membrane is emphasized.

Key words: Cell elongation — H^+-ATPase — H^+-Pyrophosphatase — Proton pump — Vacuole — Vigna radiata — Water channel

Plant Growth and the Enlargement of Cells

Plant tissues consist of relatively large cells. For example, the mean diameter of cells in the parenchyma tissue is about 100-200 μm, even though "newborn" cells that are generated from meristematic tissue by cell division are as small as animal cells (about 10 μm in diameter). Such a remarkable increase in cell volume is a characteristic of plant cells. Cell division leads to a doubling of the cell number and it usually also leads to a doubling of all components of the cell, such as the amount of DNA and the number of cell organelles. The volume of the cell, however, does not increase at all during cell division. By contrast to cell division, the elongation or expansion of a plant cell leads to a large increase in cell volume, with no necessary net increase in the number of organelles or cytoplasmic proteins. Therefore, the enlargement of plant cells appears to be one of the key processes in the growth of plants. Most enlarging cells contain a large vacuole and there may be a comparatively modest increase in the actual amount of cytoplasm during cell enlargement. The plant vacuole typically occupies more than half of the total volume of a cel

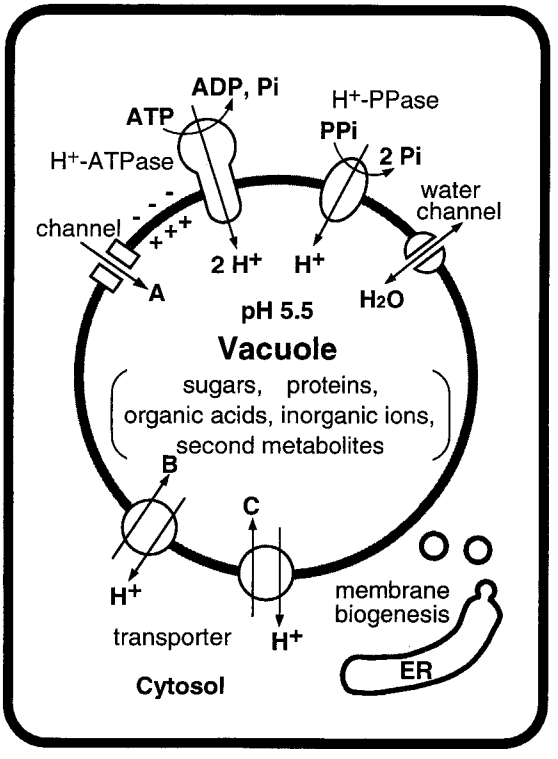

Fig. 1. Accumulation of ions and metabolites by the plant vacuole. The vacuolar membrane contains two types of proton pump: a H^+-ATPase and a H^+-transporting inorganic pyrophosphatase. Vacuolar proton pumps create both a low internal pH and an "interior-positive" membrane potential. The H^+ gradient across the membrane is used as a source of energy for secondary active transport systems, such as the sugar/H^+ antiporter, the Ca^{2+}/H^+ antiporter and NO_3^- channels. Recently, a water-specific channel was found in the membrane of the vacuole in *Arabidopsis*. ER, Endoplasmic reticulum.

Abbreviations: kD, kilodalton; H^+-PPase, proton-translocating inorganic pyrophosphatase; PP_i, inorganic pyrophosphate; TIP, tonoplast intrinsic protein; VM23, an integral membrane protein of the radish vacuole with a molecular mass of 23 kD.

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and it can occupy as much as 90% in some tissues, such as the shoots of etiolated seedlings. It must be noted that the enlargement of plant cells is accompanied by expansion of the vacuole in each cell.

The expanding vacuole must actively accumulate solutes and protons to maintain the osmolarity and acidity of its contents. The vacuolar membrane includes components of several transport systems, such as proton-translocating enzymes and active transporters of sugars and inorganic ions, as shown in Fig. 1. The enzyme that actively transports protons (H^+) across the membrane in an energy-requiring process is referred to as the proton pump. The vacuolar membrane contains two types of proton pump: a H^+-ATPase and a H^+-translocating inorganic pyrophosphatase (H^+-PPase). These proton pumps acidify the contents of the vacuole and generate an "inside positive" electrical potential across the vacuolar membrane by the inward pumping of positively charged protons. The proton pumps are called the primary active transporters. The H^+ gradient generated by the proton pumps powers the secondary active transporters of sugars, organic acids and inorganic ions. Among the various transporters and channels in the vacuolar membrane of plant cells, the proton pumps have been particularly well characterized at the molecular level. In this review, we shall summarize recent work on the relationship between cell growth and the transport systems of the vacuolar membrane.

Physiological Roles of the Plant Vacuole

Plant cells contain one or several very large, fluid-filled vacuoles. In most plant cells, the vacuoles typically occupy as much as 90% of the total volume of the cell. Vacuoles are functionally related to the lysosomes of animal cells, containing a variety of hydrolytic enzymes, but their functions are remarkably diverse (Boller and Wiemken 1986, Taiz 1992). Some of the physiological roles of plant vacuoles can be summarized as follows.

Accumulation and storage of metabolites

Vacuoles accumulate large quantities of organic acids, in particular, malate, citrate, and oxalate. Some plant tissues can store large quantities of sugars in their vacuoles. Seed proteins are actively synthesized and sorted in the vacuoles in maturing seeds. These protein-storage vacuoles are called protein bodies. The vacuole is the site of a temporary and/or long-term pool of nutrients for its own cell and for new, growing organs. In addition to organic nutrients, certain pigments, such as anthocyanin and betacyanin, are also concentrated in the vacuoles.

Regulation of cytosolic levels of inorganic ions

The vacuole is important as a homeostatic device. Plant vacuoles actively accumulate inorganic ions, such as nitrate and Ca^{2+}, and re-supply these ions to the cytosol as the need arises. For example, Ca^{2+} ions in the cytosol are transported into the vacuole by active transporter and the cytosolic concentration of Ca^{2+} ions is kept at the extremely low level of about 100 nM, which allows Ca^{2+} ions to act as second messengers in the cytosol. The Ca^{2+} ions stored in the vacuole are released to the cytosol through Ca^{2+}-release channels in response to specific biochemical signals. Protons in the cytosol are also transported into the vacuole by two types of proton pump. The proton pumps maintain the pH of the content of the plant vacuole at about 5.0-5.5 and keep the pH in the cytosol constant at about 7.2.

Compartmentalization of toxic substances

Some plant tissues contain cytotoxic substances, such as cyanogenic glycosides and alkaloids. These noxious molecules, which are released from vacuoles when a plant is eaten or damaged by insects and animals, provide a defense against predators. Furthermore, Ca^{2+}, Na^+ and Cd^{2+} ions also inhibit many enzymes at high concentrations. As stated above, these inorganic ions are compartmentalized and concentrated in the vacuoles. Recently, it was shown that plant vacuole can take up glutathione *S*-conjugates from the cytosol to remove particular phytotoxic foreign compounds, such as microbial toxins and xenobiotics (Martinoia *et al.* 1993).

Hydrolysis and recycling of cellular components

Seed proteins in the protein-storage vacuole are hydrolyzed to amino acids by proteases during germination. The vacuole is the site of such degradation corresponding to the lysosome of an animal cell. The plant vacuole contains a variety of hydrolytic enzymes, such as proteases, ribonucleases, phosphodiesterases, glycosidases, phytase and acid phosphatase (Nishimura and Beevers 1979, Boller and Wiemken 1986). In yeast, during nutrient starvation, intracellular organelles and the cytosol are sequestered in autophagosomes that subsequently fuse with vacuoles. Similar phenomena have been reported in plant cells.

Space-filling function and regulation of turgor pressure

The space-filling role of the vacuole is essential to the growth of plant cells. In most of the nonphotosynthetic tissues, such as the shoot and the root, a considerable part of each cell is occupied by a large central vacuole. Many plant cells maintain an almost constant turgor pressure by changing the osmotic pressure of the cytosol and vacuole. Osmotic pressure is regulated in part by the controlled breakdown and resynthesis of polymers in the vacuole and in part by changes in rates of transport of sugars, ions, and other metabolites across the vacuolar membrane and the plasma membrane.

Properties of Vacuolar Proton Pumps

Both the vacuolar H^+-ATPase and the H^+-PPase function as proton-translocating enzymes on the same membrane, but they have markedly different biochemical and physiological properties. In this section, we summarize some properties of these enzymes. In addition, a major intrinsic membrane protein of the plant vacuole, which seems to be related to cell growth, is introduced.

Vacuolar H^+-ATPase

A vacuolar-type ATPase is found not only in the vacuolar membranes of plant and fungal cells but also in the acidic endomembranes of animal cells and in the plasma membranes of some types of animal cell. Plant vacuolar H^+-ATPases consist of several different polypeptides (subunits; Matsuura-Endo *et al.* 1990, Sze *et al.* 1992). The ATPase is composed of a hydrophilic catalytic part (V_1) and a hydrophobic membrane part (V_0), resembling the F_0F_1-type ATPases, such as the mitochondrial ATPase. Vacuolar H^+-ATPases are sensitive to low temperatures (0-4C) in the presence of Mg^{2+} ions and ATP (Moriyama and Nelson 1989). Cold inactivation in vitro results from the detachment of the peripheral catalytic part (V_1) from the vacuolar membrane. In mung bean (*Vigna radiata* L.) seedlings, selective release of the V_1 part from the membrane and partial degradation of the vacuolar ATPase complex occur *in vivo* at low temperature (Matsuura-Endo *et al.* 1992). Nitrate is an important plant nutrient and it is known as a specific inhibitor of the vacuolar ATPase. Certain chaotropic anions, such as nitrate, cause the release of the peripheral complex of vacuolar H^+-ATPase when they are present at high concentrations.

H^+-PPase

Acting in concert with the H^+-ATPase, H^+-PPase acts to acidify the content of the vacuole (Hedrich *et al.* 1989, Hedrich and Schroeder 1989, Rea and Poole 1993). The substrate for the latter enzyme is inorganic pyrophosphate (PP_i), a high-energy phosphate compound. The transport of protons is coupled with the hydrolysis of PP_i. Although vacuolar-type H^+-ATPases are found in animal cells, membrane-associated H^+-PPases have been reported only in plant vacuoles (Maeshima *et al.* 1994) and the chromatophores of photosynthetic bacteria (Baltscheffsky and Nyrén 1984, Nore *et al.* 1991). The vacuolar H^+-ATPase is a multisubunit enzyme, whereas the H^+-PPase is composed of a single polypeptide of 73 kD (Maeshima and Yoshida 1989). Proteoliposomes reconstituted with the 73-kD polypeptide purified from mung bean hypocotyls can transport protons in a PPi-dependent manner. The amino acid sequence of the enzyme has been deduced from the corresponding cDNA in the case of barley (Tanaka *et al.* 1993), *Arabidopsis thaliana* (Sarafian *et al.* 1992) and mung bean (Nakanishi and Maeshima, unpublished data), among others. The 73-kD polypeptide is a hydrophobic protein, and each polypeptide has 12 or 13 domains that seem likely to span the vacuolar membrane. The polypeptides of 73 kD include a consensus sequence found in the proteolipid subunits of F_0F_0-type and vacuolar-type ATPases. The consensus sequence serves as a binding site for *N, N'*-dicyclohexylcarbodiimide, which is an inhibitor of the transport of protons. The results confirm that the 73-kD polypeptide contains both PP_i-hydrolyzing and H^+-transporting domains and functions in the membrane as a PPi-dependent proton pump. Vacuolar membranes isolated from several green plants, including a fern and a moss, exhibit PPi-dependent H^+-transport activity and are immunoreactive with the antibodies raised against H^+-PPase from mung bean (Maeshima *et al.* 1994). These studies revealed that the vacuolar H^+-PPase is a compact proton pump and a universal enzyme in green plants.

An intrinsic protein of 23 kD (VM23) in vacuolar membranes

Plant vacuolar membranes include another integral protein of about 23 kD, tentatively designated VM23 (Maeshima 1992). VM23 accounts for 30-50% of the protein in the vacuole membranes from tap roots of radish (*Raphanus sativus* L.) and mung bean hypocotyls. The amino acid sequence of the N-terminal half, at least, of VM23 from radish is homologous to that of γ-TIP from *Arabidopsis* (unpublished data). *Arabidopsis* γ-TIP has been shown to be a water-specific channel, namely, an aquaporin (Maurel *et al.* 1993, Chrispeels and Agre 1994). Ludevid *et al.* (1992) demonstrated that the expression of γ-TIP is related to the enlargement of cells in *Arabidopsis*. VM23 is not a protein common to all green plants. VM23 was not found in membranes from *Acetabularia, Chara* or *Kalanchoë* (Maeshima *et al.* 1994). We recently purified an intrinsic membrane protein of 23 kD from vacuoles of sugar beet. The protein was different in terms of immunoreactivity from VM23 of radish (unpublished data). The protein from sugar beet might be a different molecular form of the 23-kD protein. It seems likely that there are several isoforms of VM23 in plant vacuolar membranes.

Changes in the Levels of Vacuolar Proton Pumps during Cell Growth

Shoots of etiolated seedlings grow rapidly. For example, etiolated hypocotyls of mung bean grow about 8 cm in two days at 26C (Maeshima 1990). These growing hypocotyls have both H^+-ATPase and H^+-PPase in their vacuolar membranes. The rapid elongation of cells occurs in the middle part of the hypocotyl. Cell division occurs only in the apical meristem at the top of the hypocotyl. The bottom half of the hypocotyl consists of the large mature cells. As shown in Fig. 2, there is a great difference in cell volume between "newborn" and mature cells. The volume of cells increases more than 20-fold during elongation of the hypocotyl, as judged from the size of protoplasts and the DNA content.

The growing stem is a good system for studies of the elongation of plant cells. The hypocotyls of mung bean can be separated into the dividing, elongating and mature regions. Table 1 shows the levels of H^+-ATPase and H^+-PPase in the vacuolar membranes from these three regions of mung bean hypocotyls. Although the dividing region accounts for only a small fraction of the total weight of the hypocotyl, levels of the vacuolar H^+-ATPase and H^+ PPase, on the basis of fresh weight, in the dividing region are higher than those in the elongating and mature regions. The levels of the substrates for H^+-ATPase and H^+-PPase, on the basis of fresh weight, in the dividing region are also higher than those in the mature region. Takeshige and Tazawa (1989) reported that PPi is predominantly present in the cytosol of plant cells at a concentration of 0.2 mM. Even in the cells in the dividing region of mung bean hypocotyls, vacuoles

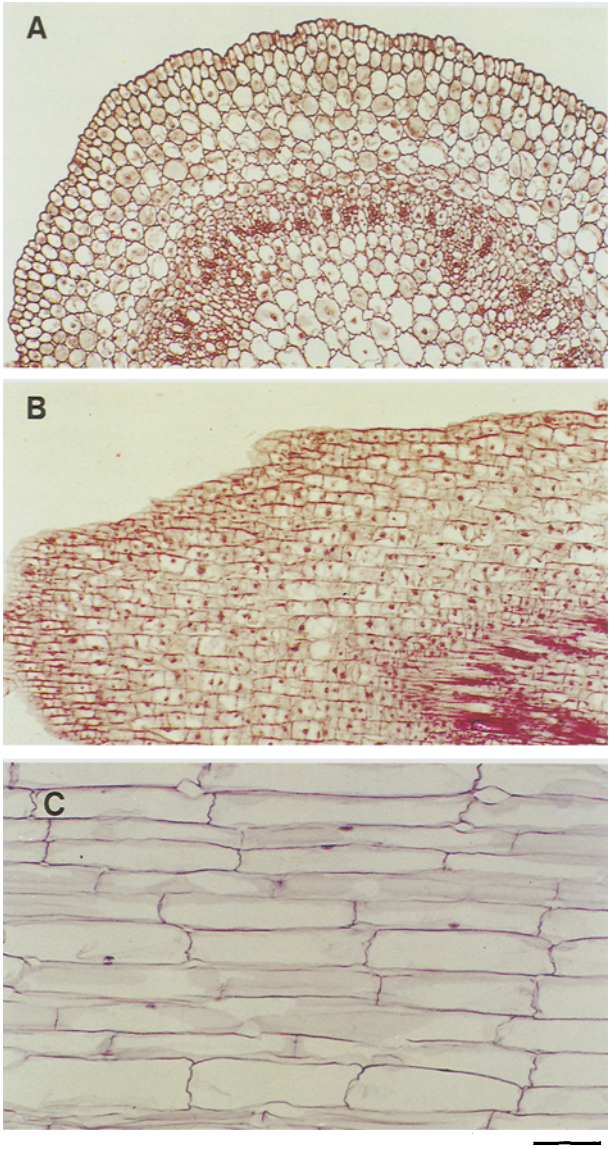

Fig. 2. The dividing and mature regions of the hypocotyl of a mung bean seedling. A, Cross section of the dividing region, namely, the top part of the hypocotyl, of 3-day-old etiolated seedling. B, Median longitudinal section of the dividing region of the hypocotyl, C, Median longitudinal section of a mature region of the same hypocotyl. Bar= 100 μm.

occupy more than 50% of the volume of each cell. Therefore, the cytoplasmic concentrations of ATP and PPi can be estimated to be about a few mM and a few hundred μM, respectively. The values are sufficient to support the maximal activities of H^+-ATPase and H^+-PPase. In the case of mung bean H^+-PPase, the *K*m value for PPi of PPi-dependent H^+-transport activity is 15 μM. The H^+-PPase and H^+-ATPase proteins and their substrates are present at relatively high levels in the growing cells of the dividing and elongating regions of the hypocotyl. The proton pumps are essential for maintenance of the proton gradient across the vacuolar membrane in the elongating cells.

If the vacuole were to act exclusively as a storage compartment for waste products, young cells in the dividing and elongating regions would not need large central vacuoles. It seems likely that vacuoles in growing tissues function as a space-filling compartment and support the enlargement of cells. For maintenance of the turgor pressure required for the continuous elongation of cells, solutes must be accumulated actively in the growing vacuole so that the osmolarity of its contents remains high, and the growing vacuole must actively take up protons at a much higher rate than does the small vacuole. Nakahori *et al.* (1991) measured intracellular pressure in intact hypocotyls of *Vigna unguiculata* by the pressure-probe technique. The intracellular pressure in the elongating region was 627 kPa (about 6 atmospheres). It was showed that treatment of the seedlings with auxin changed some physicochemical properties of the cell wall, such as the yield threshold of the cell wall, but not the intracellular pressure (Nakahori *et al.* 1991, Katou and Okamoto 1992). They reported that auxin enhanced the rate of elongation of the hypocotyl by increasing the effective turgor pressure. Although the details of the effect of auxin cannot be discussed here, it is clear that the intracellular pressure was maintained at high level during rapid elongation of cells. These observations suggest that the active incorporation of solutes into cells and vacuoles drives the growth of the cells.

The activity of H^+-PPase in mung bean hypocotyls is four times that of the H^+-ATPase, as shown in Table 1. H^+-PPase is the main proton pump in the vacuolar membrane of the hypocotyl. The molecular activity of the H^+-ATPase, calculated on the basis of the ATP-hydrolyzing activity of the completely purified H^+-ATPase, is about 45 sec^{-1}, and this activity is similar to that of the H^+-PPase (about 30 sec^{-1}). The high activity of the H^+-PPase in the vacuolar membrane of mung bean hypocotyl is due to the high level of H^+-PPase molecule. The physiological significance of the high level of H^+-PPase molecules is discussed in the next section.

The cells in the mung bean hypocotyl are young, being about 3 days old. We have investigated the changes in the levels of the vacuolar proton pumps during maturation of root tissues of radish. The radish root requires about three months to mature. We prepared vacuolar membranes from root tissues at various stages of growth and equal amounts of the membranes were subjected to immunoblot analysis. Figure 3 shows the results of immunoblotting with antibodies against the vacuolar H^+-ATPase, H^+-PPase and VM23. As mentioned above, the vacuolar H^+-ATPase consists of several subunits and the catalytic part of the enzyme is composed of the major two subunits (68-kD and 57-kD subunits). The relative levels of the major two subunits did not change during the growth of radish roots. By contrast, the H^+-PPase was present at high levels in the vacuolar membranes prepared from young, small roots, but the relative level of the enzyme, on the basis of total vacuolar membrane proteins, decreased during the maturation of roots. We want to emphasize two points here. First, as is the case in the mung bean hypocotyl, the H^+-PPase is the main proton

Table 1. Levels of vacuolar H^+-ATPase, H^+-PPase, ATP and PP_i in the three regions of the mung bean hypocotyl (Maeshima 1990)

Enzyme or metabolite	Region of hypocotyl		
	Dividing	Elongating	Mature
Fresh weight (mg/seedling)	7.70	41.2	193
DNA (μg/g fresh weight)	59.9	16.0	5.16
H^+-ATPase (units/g fresh weight)	0.12	0.12	0.067
H^+-PPase (units/g fresh weight)	0.87	0.46	0.28
(μg/μg of DNA)	3.3	7.2	13
ATP (μmol/g fresh weight)	0.93	0.30	0.18
PP_i (nmol/g fresh weight)	84	70	64
P_i (μmol/g fresh weight)	4.9	4.2	4.5

Vacuolar membranes were prepared from each regions of hypocotyls and assayed for the activities of the enzymes. The amount of H^+-PPase was determined by the immunochemical method with the antibody against the enzyme. The amount of H^+-PPase on the basis of DNA content correspond to the relative amount of the enzyme per cell. The activity of H^+-ATPase is expressed as the activity of nitrate-sensitive ATPase.

pump of the vacuolar membranes of young cells in radish roots. Second, the levels of three components of the vacuolar membrane seem to be controlled independently of one another.

The Physiological Significance of the Vacuolar H^+-PPase in Growing Cells

Plant growth is accompanied by the expansion of cells, and cell expansion is supported by both hydrostatic and osmotic forces. In most cases, the increase in cell volume is accounted for by enlargement of the vacuole rather than by an increase in the volume of the cytoplasm. The enlargement of the vacuole cannot occur without an increment of the contents of the vacuole and the active biogenesis of the vacuolar membrane. In order to maintain the high osmotic pressure of the contents of the expanding vacuole, the vacuole must actively incorporate solutes since the osmotic pressure depends on concentrations of the solutes incorporated into the vacuole. Both the vacuolar H^+-ATPase and the vacuolar H^+-PPase are primary active transporters that provide the power for the secondary active transporters. Therefore, we propose the hypothesis that the vacuolar proton pumps are essential not only for the enlargement of vacuoles but also for the growth of cells.

From measurements of enzymatic activities and the immunochemical quantification of the vacuolar proton pumps, we showed that the H^+-PPase is present at higher levels than that of H^+-ATPase. This difference is reasonable from the perspective of the cell's energetics. In growing tissue, RNAs, proteins and polysaccharides are actively synthesized for construction of cells and, as a result, a lot of PP_i is produced as a byproduct of these metabolic processes. The β-oxidation of fatty acids also generates PP_i. PP_i accumulated in the cytosol at high concentrations inhibits these reactions. The vacuolar H^+-PPase scavenges the PP_i in the cytosol and utilizes it as a source of energy for the active transport of protons and the acidification of the expanding vacuole. In mature cells, metabolic activity decreases and PP_i may not be available in such large amounts. In addition, the rate of transport of solutes into the vacuole also decreases and expansion of the vacuole ceases. Indeed, we found that H^+-PPase activity was about half that of the H^+-ATPase in the mature tap roots of radish, even though its activity was four times that of the H^+-ATPase in the young roots. The existence of the H^+-PPase in the plant vacuolar membrane helps to conserve ATP, which is a universal energy source of many cellular activities, such as the synthesis and transport of cellular components. To test our hypothesis, we must examine the effects of genetic suppression of the expression of H^+-PPase and of biochemical inhibition of H^+-PPase in growing tissues.

Let us now consider the physiological meaning of the existence of the H^+-ATPase. The amount of PP_i produced in a cell depends mainly on the rates of synthesis of proteins and RNAs. ATP is constantly supplied by oxidative phosphorylation and glycolysis. The concentration of cytosolic ATP is kept strictly at the millimolar level. Therefore, vacuolar H^+-ATPase can operate as a fundamental proton pump in plant cells at any physiological stage. The level of the vacuolar H^+-ATPase remains relatively constant during the growth of mung bean hypocotyls and radish roots. Furthermore, the actions of the two proton pumps seem to compensate for each other under abnormal conditions. For example, the H^+-PPase is markedly inhibited by relatively low levels of Ca^{2+} ions while the H^+-ATPase is insensitive to Ca^{2+} ions (Maeshima 1991). The vacuolar H^+-ATPase is sensitive to low temperatures and nitrate (Matsuura-Endo *et al.* 1990, 1992) while the H^+-PPase, with its simple structure, is stable under such conditions.

The third component of the plant vacuolar membrane, VM23, also seems to participate in the enlargement of the vacuole and the growth of cells. The specific content of VM23 on the basis of the vacuolar membrane protein was found to be low in young cells and to increase markedly

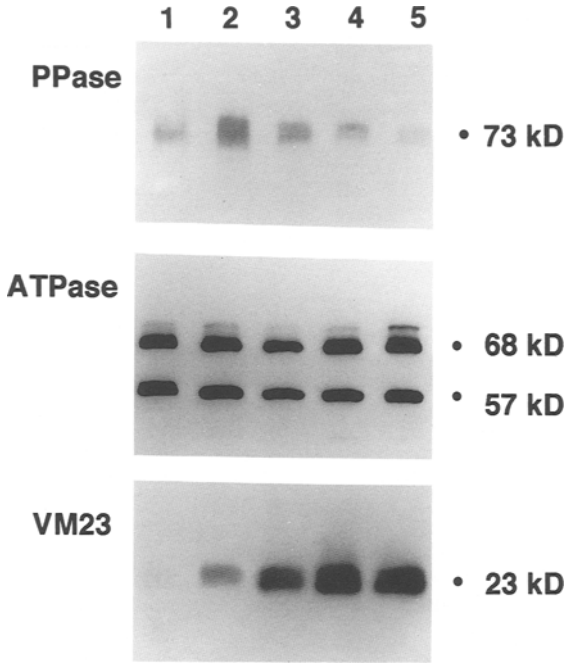

Fig. 3. Changes in the levels of H^+-PPase, H^+-ATPase and VM23 in vacuolar membranes during the growth of radish tap roots. Japanese radish (*Raphanus sativus* L.) plants were grown at the university farm, and the vacuolar membranes were purified from the roots or leaves harvested at stated times. The same amount of membranes was used for immunoblot analysis with antibodies against H^+-PPase from mung bean, the two major subunits of the H^+-ATPase from mung bean and VM23 from radish. Lane 1, vacuolar membranes from leaves harvested on 7 June; lanes 2-5, vacuolar membranes from roots harvested on 7 June, 20 June, 15 July and 22 August, respectively. The weight of radish roots increased from about 1 g (7 June) to about 600 g (22 August).

during the growth of radish roots (Fig. 3) and mung bean hypocotyls (Maeshima 1990). From the primary sequence of VM23 and its immunochemical reactions, VM23 was deduced to be a member of the γ-TIP family and to function as a water channel. The elongation of a cell is driven largely by the uptake of water into the cell and the vacuole. Thus, the water channel may be a key element in cell growth. Small vacuoles in young cells probably do not need many water channels. In the vacuoles of young cells, the simple diffusion may be sufficient for trans-membrane movement of water. In the case of large vacuoles, a lot of water must pass across the membrane during enlargement of the vacuole. VM23 and γ-TIP might support the rapid transport of water. In preliminary experiments, we have identified some isoforms of VM23 in the vacuolar membranes from radish roots and mung bean hypocotyls. It is not clear that all of the isoforms function as water-specific channels.

Plant cell expansion is explained by "acid growth theory" (Hager *et al.* 1971, Taiz 1994). Auxin induces the elongation of plant cells by causing an activation of proton pump of the plasma membrane and a reduction in the cell wall pH. The low pH activates a class of wall proteins termed "expansins" which disrupt the hydrogen bonding between cellulose microfibrils (Taiz 1994). As a consequence, the rigidity of the wall is reduced, and the cell can expand. This acid-growth theory is based on the internal pressure of cells. Osmotic pressure is maintained at high level by the vacuole in each cell. Expansion of plant cells requires the vacuolar proton pumps and water channel. Further study of these molecules at the molecular and cell biological level may make our hypothesis clear.

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