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Accumulation of Vacuolar H^+-Pyrophosphatase and H^+-ATPase during Reformation of the Central Vacuole in Germinating Pumpkin Seeds[1]

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Protein storage vacuoles were examined for the induction of H^+-pyrophosphatase (H^+-PPase), H^+-ATPase, and a membrane integral protein of 23 kD after seed germination. Membranes of protein storage vacuoles were prepared from dry seeds and etiolated cotyledons of pumpkin (*Cucurbita* sp.). Membrane vesicles from etiolated cotyledons had ATP- and pyrophosphate-dependent H^+-transport activities. H^+-ATPase activity was sensitive to nitrate and bafilomycin, and H^+-PPase activity was stimulated by potassium ion and inhibited by dicyclohexylcarbodiimide. The activities of both enzymes increased after seed germination. On immunoblot analysis, the 73-kD polypeptide of H^+-PPase and the two major subunits, 68 and 57 kD, of vacuolar H^+-ATPase were detected in the vacuolar membranes of cotyledons, and the levels of the subunits of enzymes increased parallel to those of enzyme activities. Small amounts of the subunits of the enzymes were detected in dry cotyledons. Immunocytochemical analysis of the cotyledonous cells with anti-H^+-PPase showed the close association of H^+-PPase to the membranes of protein storage vacuoles. In endosperms of castor bean (*Ricinus communis*), both enzymes and their subunits increased after germination. Furthermore, the vacuolar membranes from etiolated cotyledons of pumpkin had a polypeptide that cross-reacted with antibody against a 23-kD membrane protein of radish vacuole, VM23, but the membranes of dry cotyledons did not. The results from this study suggest that H^+-ATPase, H^+-PPase, and VM23 are expressed and accumulated in the membranes of protein storage vacuoles after seed germination. Overall, the findings indicate that the membranes of protein storage vacuoles are transformed into those of central vacuoles during the growth of seedlings.

The vacuole is one of the most conspicuous compartments within a plant cell. This organelle performs numerous functions vital to cellular homeostasis, including the accumulation of amino acids, inorganic ions, and metabolic intermediates (Boller and Wiemken, 1986; Taiz, 1992). The plant vacuoles also contain a number of acid hydrolases, such as proteases, RNases, and glycosidases (Matile, 1978; Nishimura and Beevers, 1979; Van der Wilden et al., 1980). The most important aspect of plant vacuoles is their role in protein storage. The protein storage vacuoles, called protein bodies, are the site of protein deposition in plant reserve tissues such as the cotyledon and endosperm. Seed proteins including 11S globulin and 2S albumin are actively synthesized and stored in vacuoles in maturing seeds (Akazawa and Hara-Nishimura, 1985; Chrispeels, 1991; Hara-Nishimura et al., 1993). Protein storage vacuoles are generated by fragmentation of these vacuoles in the late stage of seed maturation (Hara-Nishimura et al., 1987). When seeds germinate, the proteins stored in protein storage vacuoles are hydrolyzed by acid hydrolases and utilized for growth of the embryo, and the protein storage vacuoles become fused with each other to re-form a single, central vacuole (Nishimura and Beevers, 1979; Hara and Matsubara, 1980). The transformation of protein storage vacuoles into a central vacuole is one of the dynamic aspects of vacuolar compartmentation. We are conducting a series of experiments to analyze the developmental changes in the membrane components of protein storage vacuole.

The membranes of the central vacuoles contain two distinct proton pumps, H^+-ATPase and H^+-PPase (Sze, 1985; Hedrich et al., 1989; Hedrich and Schroeder, 1989; Taiz, 1992; Rea and Poole, 1993). These pumps create a low internal pH and membrane potential, which is utilized as an energy source for the secondary transport systems of the vacuolar membrane. Among the membrane proteins in central vacuoles, these two proton pumps have been well characterized for their molecular structures and enzymatic properties (Gogarten et al., 1989; Maeshima and Yoshida, 1989; Matsuura-Endo et al., 1990; Rea et al., 1992; Sze et al., 1992). Vacuolar H^+-ATPase is a multisubunit complex, whereas the H^+-PPase is composed of a single polypeptide of 73 kD (80 kD estimated from the cDNA; Sarafian et al., 1992; Tanaka et al., 1993). These two proton pumps, particularly H^+-PPase, can be used as markers of the vacuolar membrane as described by Chanson (1990). It is known that acidic conditions of vacuoles in germinating seeds may be necessary for their physiological functions such as hydrolysis of storage protein. However,

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; ΔpH, transmembrane pH gradient; H^+-PPase, proton-translocating inorganic pyrophosphatase; MP28, 28-kD membrane protein of pumpkin protein body; PVDF, polyvinylidene difluoride; TIP, tonoplast intrinsic protein; VM23, 23-kD membrane-integral protein of radish vacuole.

there is little information concerning the H^+-ATPase and H^+-PPase in the protein storage vacuoles.

In this work we prepared vacuolar membranes from dry and etiolated cotyledons of pumpkin seeds and examined them for the presence of vacuolar-type H^+-ATPase and H^+-PPase. We found these proton pumps in the vacuolar membranes of storage cells of seeds. The levels of the two enzymes increased in cotyledons after germination. Also, this paper describes the induction of an extremely hydrophobic protein of 23 kD (designated VM23; Maeshima, 1992), which is similar to γ-TIP (Johnson et al., 1990; Höfte et al., 1992), in the membranes of protein storage vacuoles after germination.

MATERIALS AND METHODS

Materials

Pumpkin (*Cucurbita* sp. cv Kurokawa Amakuri Nankin) seeds were soaked in water for 15 h and germinated in moist vermiculite at 26°C in the dark. Cotyledons from etiolated pumpkin seedlings were used for membrane preparation. Castor bean (*Ricinus communis*) seeds were a gift from Ito Oil Co. (Yokkaichi, Japan). Seeds were grown in the dark at 30°C. Endosperms of castor bean seedlings were used for preparation of vacuolar membranes and mRNA. DCCD and valinomycin were purchased from Sigma. Bafilomycin A_1 was from Wako Pure Chemical Industries (Osaka, Japan). Oligo(dT) cellulose and protein A-Sepharose were from Pharmacia LKB. ECL western blotting detection regents, wheat germ cell-free translation mixture, and [^{35}S]Met were from Amersham. Membrane sheets of PVDF (Immobilon-P) for immunoblotting were from Millipore.

Preparation of Protein Storage Vacuoles and Vacuolar Membranes

Protein storage vacuoles were isolated from cotyledons of 2-d-old seedlings of pumpkin by a nonaqueous isolation method as described previously (Hara-Nishimura et al., 1982). The isolated protein storage vacuoles were suspended with 10 mM Tris-Mes, pH 6.5, and sonicated. After removing crystalloid by centrifugation at 250*g* for 15 min, NaCl was added to the supernatant at a final concentration of 1 M. The membrane fraction was collected by centrifugation at 100,000*g* for 1 h. The precipitate was suspended in 10 mM Tris-Mes, pH 6.5, and used in subsequent analysis.

In many cases, vacuolar membranes were prepared directly from the tissue homogenates of pumpkin cotyledons and castor bean endosperms basically as described previously (Maeshima and Yoshida, 1989). The tissues were chopped with a razor blade, homogenized with a Polytron PT30, and ground in a mortar. The grinding medium contained 0.25 M sorbitol, 1 mM $MgCl_2$, 2 mM EGTA, 0.5 mM PMSF, 1% (w/v) PVP, 1% (w/v) ascorbic acid (neutralized with KOH), and 50 mM Tris-acetate, pH 7.5. The tissue homogenate was sonicated for 1 min to break the protein storage vacuoles, then filtered and centrifuged at 5,000*g* for 10 min. The supernatant was centrifuged at 120,000*g* for 30 min. The precipitate was suspended in 0.31 M Suc solution containing 20 mM Tris-acetate, pH 7.5, 2 mM DTT, 1 mM EGTA, and 1 mM $MgCl_2$ (Tris-DEM). The suspension was used as a microsomal membrane fraction. Further purification of vacuolar membranes was carried out by floating centrifugation. The microsomal membrane suspension was placed in a centrifuge tube and overlaid with the same volume of Tris-DEM buffer containing 0.25 M sorbitol. After centrifugation at 120,000*g* for 30 min, vacuolar membrane vesicles forming a band at the interface between the two solutions were collected and suspended in 10 mL of Tris-DEM buffer containing 0.25 M sorbitol. The suspension was centrifuged at 130,000*g* for 20 min, and the pellet was suspended in a small volume of the same buffer. All steps were performed at 0 to 4°C.

Extraction of Proteins from Cotyledons with SDS

Five cotyledons of pumpkin were homogenized with a mortar and pestle in 13 mL of grinding medium containing 5% (w/v) SDS, 2 mM EDTA, 2 mM DTT, 2% (w/v) β-mercaptoethanol, 50 mM Tris-HCl, pH 8.0. The homogenate was filtered through two layers of cheesecloth and centrifuged at 1000*g* for 10 min. The supernatant was applied to a Econo-Pac 10DG column (Bio-Rad) to remove ions and small metabolites, and the eluate was used for SDS-PAGE.

Enzyme Assay and Measurement of H^+ Transport

Enzymatic activities of H^+-PPase and H^+-ATPase were colorimetrically measured by determining the rates of liberation of Pi at 30°C. The standard reaction mixture for H^+-PPase contained 1 mM sodium PPi, 1 mM $MgSO_4$, 50 mM KCl, 1 mM sodium molybdate, 0.02% Triton X-100, and 30 mM Tris-Mes, pH 7.2 (Maeshima and Yoshida, 1989). The reaction mixture for vacuolar H^+-ATPase contained 3 mM Tris-ATP, 3 mM $MgSO_4$, 50 mM KCl, 1 mM sodium molybdate, 0.02% Triton X-100, and 30 mM Tris-Mes, pH 7.0 (Matsuura-Endo et al., 1990). Proton-transport activity was measured at 25°C as the rate of fluorescence quenching of the fluorescent monoamine acridine orange. The reaction medium for H^+-PPase contained 0.25 M sorbitol, 20 mM Tris-Mes, pH 7.2, 50 mM KCl, 0.8 μM acridine orange, 0.4 mM sodium pyrophosphate, and 1 mM $MgSO_4$. The medium for H^+-ATPase contained 3 mM Tris-ATP and 3 mM $MgSO_4$ in the same buffer. The changes in fluorescence were monitored with a Shimazu (Kyoto, Japan) RF-5000 fluorescence spectrophotometer set at 493 nm for excitation and at 540 nm for emission. The rate of H^+ translocation (percent fluorescence min^{-1}) was estimated graphically.

SDS-PAGE and Immunoblotting

SDS-PAGE in a 12% (w/v) polyacrylamide gel was carried out by the method of Laemmli (1970). Samples were heated in 50 mM Tris-HCl, pH 6.8, 2% SDS, and 1% β-mercaptoethanol at 70°C for 10 min prior to electrophoresis. Antibodies against mung bean H^+-PPase (Maeshima and Yoshida, 1989), the two subunits (68 and 57 kD) of mung bean vacuolar H^+-ATPase (Matsuura-Endo et al., 1992), and radish VM23 (Maeshima, 1992) were prepared as described previously. Antibody against a membrane intrinsic protein of pumpkin protein-body membrane (designated MP28) was raised in a rabbit (K. Inoue, A. Motozaki, Y. Takeuchi, M. Nishimura,

and I. Hara-Nishimura, unpublished data). IgG fractions were purified from antisera by protein A columns (Ampure PA, Amersham). For immunoblotting, proteins were separated by SDS-PAGE and transferred to a PVDF membrane with a semidry blotting apparatus (Transblot SD, Bio-Rad) by standard procedures (Harlow and Lane, 1988). The membrane sheet was blocked with 3% BSA prior to reaction with primary antibody and then rinsed with five changes of Tris-buffered saline containing 0.05% (w/v) Triton X-100. Binding of antibody was detected by colorimetric reaction using horseradish peroxidase-coupled protein A and commercial detection reagents (ECL western blotting detection kit, Amersham). Levels of antigen on the immunoblots were quantified by densitometric scanning with a densito-pattern analyzer (model EPA-300, Maruzen Petrochemical Co., Tokyo, Japan).

Immunocytochemical Analysis

Cotyledons were harvested from 4-d-old pumpkin seedlings and vacuum infiltrated for 1 h in a fixation mixture consisting of 4% paraformaldehyde, 1% glutaraldehyde, and 60 mM Suc in 50 mM cacodylate buffer (pH 7.4). The cotyledons were then cut into slices less than 1 mm thick and treated for another 2 h with the freshly prepared fixation mixture. After washing with 50 mM cacodylate buffer, pH 7.4, samples were dehydrated in a graded dimethyl formamide series at −20°C and embedded in LR White acrylic resin (London Resin). Blocks were polymerized under a UV lamp at −20°C for 24 h. Ultrathin sections were prepared on a Reichert ultramicrotome and mounted on uncoated nickel grids. Immunocytochemical labeling with protein A gold was performed as described previously (Nishimura et al., 1993). Antibodies to H^+-PPase (dilution, 1:500) and the 68-kD subunit of H^+-ATPase (1:500) and protein A gold (1:30, Amersham) were used for the analysis. Thin sections were examined with a JEOL 1200EX transmission electron microscope at 80 kV.

RESULTS

H^+-Transport Activity of Protein Storage Vacuole Membranes

Protein storage vacuoles were isolated from the cotyledons of 2-d-old pumpkin seedlings by the nonaqueous method. The isolated protein bodies were treated with the pH indicator neutral red. The isolated protein storage vacuoles were about 5 to 9 μm in diameter: this value was the same as in the cotyledons. As shown in Figure 1, the protein storage vacuoles stained red by neutral red, indicating that the space in the vacuoles was acidic.

To determine whether or not H^+-ATPase and H^+-PPase exist in the membranes of protein-storage vacuoles of pumpkin cotyledon, transport of protons into the membrane vesicles was monitored with a ΔpH-sensitive optical probe of acridine orange. The vacuolar membranes were isolated from the cotyledons of 4-d-old seedlings by differential centrifugation followed by floating centrifugation. Mg^{2+} was added to the homogenizing medium to maintain the H^+-PPase activity (Maeshima, 1991). As shown in Figure 2, a typical quench curve was obtained upon addition of ATP or PPi.

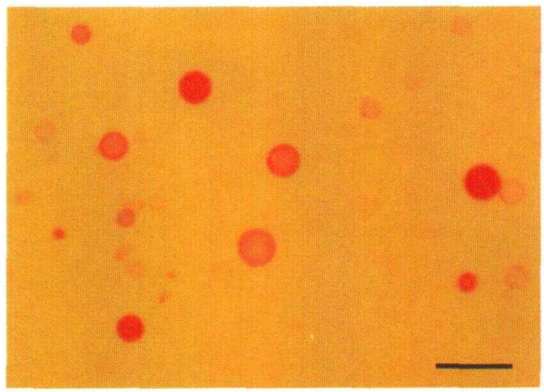

Figure 1. Photomicrographs of protein storage vacuoles of pumpkin cotyledons. Protein storage vacuoles were isolated from 2-d-old cotyledons and treated with neutral red at 0.1% (w/v). Bar = 20 μm.

When NH_4Cl was added to the transport assay at a steady-state ΔpH, the fluorescence of acridine orange rapidly returned to the initial baseline value. Furthermore, the rate of PPi-dependent H^+ transport was enhanced by the ionophore valinomycin. The results indicate that the fluorescence change was in response to ΔpH formation. The activity of PPi-dependent H^+ transport was inhibited by DCCD, an inhibitor of H^+ transport, to 40% and 3% at 50 μM and 0.5 mM, respectively (Fig. 2A). The antibody raised against mung bean H^+-PPase also inhibited the reaction of PPi-dependent H^+ transport. The activity of ATP-dependent H^+ transport was inhibited to 20% by 0.1 μM bafilomycin A_1, which is known to be a specific inhibitor of vacuolar-type H^+-ATPase (Bowman et al., 1988) (Fig. 2B). These findings showed that the membranes of protein bodies contained the typical vacuolar H^+-PPase and H^+-ATPase. The rate of proton transport depending on PPi, calculated on the basis of membrane protein, was 4 times higher than that of H^+-ATPase. The magnitude of the steady-state ΔpH established by addition of PPi was higher than that of ATP.

The specific activities of PPase and ATPase in the vacuolar membranes purified from 6-d-old cotyledons were 0.35 and 0.13 units mg^{-1}, respectively. The activity of H^+-ATPase was inhibited to less than 10% by bafilomycin A_1 at 1 μM. The specific activity of PPase on the basis of membrane protein is the same as that of red beet (0.22 units mg^{-1}; Sarafian and Poole, 1989) but less than those of hypocotyls of pumpkin (0.86 units mg^{-1}; Sato et al., 1991) and mung bean (1.1 units mg^{-1}; Maeshima and Yoshida, 1989). As shown in Table I, PPase activity was stimulated 5 times by potassium ion at 50 mM and inhibited to less than 16% by DCCD at 50 μM and Ca^{2+} at 50 μM. These properties of PPase in the membranes from cotyledons were the same as those of the vacuolar H^+-PPase purified from mung bean hypocotyls (Maeshima and Yoshida, 1989; Maeshima, 1991).

Immunological Detection of Vacuolar H^+-PPase and H^+-ATPase

Figure 3b shows the immunoblots of vacuolar membranes of pumpkin cotyledons with antibodies to H^+-PPase and the

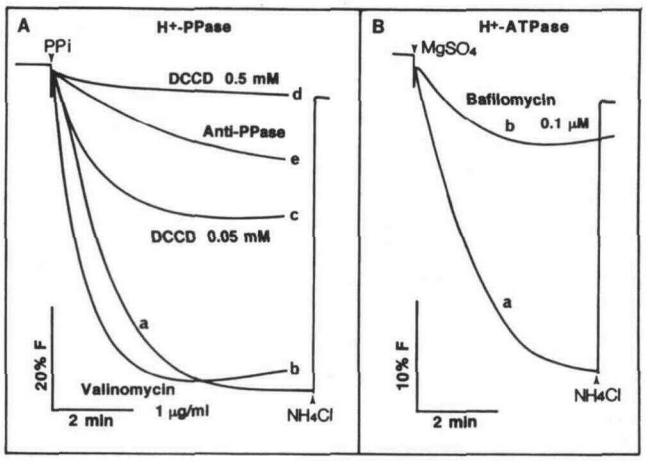

Figure 2. PPi- and ATP-dependent H^+-transport activities in vacuolar membrane vesicles of 4-d-old pumpkin cotyledon. Vacuolar membrane vesicles were isolated from cotyledons and subjected to an acridine orange fluorescence-quenching assay at 25°C. A, PPi-dependent H^+-transport activity. Membrane vesicles (51 μg of protein) were assayed in the presence of valinomycin (final concentration of 1 μg/mL, b), DCCD (0.05 and 0.5 mM, c and d, respectively), and antibody to mung bean H^+-PPase (66 μg of protein, e). Line a, Control. At the indicated time, NH_4Cl was added at a final concentration of 1.5 mM to collapse the ΔpH. In all cases, the fluorescence of acridine orange returned to the initial baseline level after addition of ammonium ion. B, ATP-dependent H^+-transport activity. Membrane vesicles (102 μg of protein) were assayed in the absence (a) or the presence (b) of 0.1 μM bafilomycin A_1. The control activities of PPi- and ATP-dependent H^+-transport were 0.61 and 0.14 percent fluorescence min^{-1} μg^{-1}, respectively.

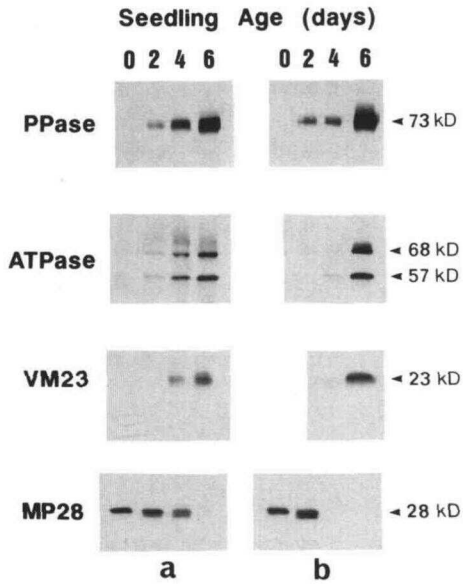

Figure 3. Developmental changes in the levels of H^+-PPase, H^+-ATPase, VM23-analogous protein, and MP28 in the pumpkin cotyledon after germination. Fractions of microsomes (a) and vacuolar membranes (b) were prepared from 70 cotyledons each of dry seeds (d 0) and etiolated seedlings grown for 2, 4, and 6 d. An aliquot of each membrane preparation was subjected to SDS-PAGE. The amount of vacuolar membranes used for each lane was equivalent to two cotyledons. The proteins in the acrylamide gels were transferred to PVDF membranes, then the membrane sheets were subjected to immunostaining using antibodies against mung bean H^+-PPase (PPase), two major subunits of mung bean vacuolar H^+-ATPase (ATPase), radish VM23 (VM23), and pumpkin MP28 (MP28). The positions of the polypeptides are marked by arrowheads.

two subunits (68 and 57 kD) of the vacuolar H^+-ATPase purified from mung bean. The 68- and 57-kD subunits of the ATPase are thought to be a catalytic and a regulatory subunit, respectively. In the case of germinated cotyledon, the immunoblots of the vacuolar membrane fractions showed immunostained bands corresponding to the H^+-PPase (73 kD) and the two subunits of H^+-ATPase (68 and 57 kD). From immunoblotting, the apparent molecular masses of the subunits were estimated to be the same as those of mung bean H^+-PPase (Maeshima and Yoshida, 1989) and H^+-ATPase (Matsuura-Endo et al., 1990). The results support the presence of the vacuolar-type H^+-PPase and H^+-ATPase in the membrane of protein storage vacuoles.

Table I. *Effect of potassium ion and inhibitors on the activity of PPase in the vacuolar membranes of pumpkin cotyledon*

Vacuolar membranes were prepared from cotyledons of 6-d-old seedlings. The assay medium contained 50 mM KCl and the indicated amount of inhibitor. To prepare stock solution (5 mM), DCCD was dissolved in ethanol. Values are presented as means ± SE for three replicates.

Inhibitor	Concentration	PPase Activity	
	μM	*units* mg^{-1}	%
None		0.350 ± 0.010	100
−KCl		0.066 ± 0.003	19
$CaCl_2$	50	0.057 ± 0.005	16
DCCD	50	0.053 ± 0.010	15

Increase in Levels of H^+-PPase and H^+-ATPase after Germination

Judging from its primary sequence H^+-PPase is a very hydrophobic protein (Sarafian et al., 1992; Tanaka et al., 1993) and it is not soluble in a detergent of low concentration (Maeshima and Yoshida, 1989). Thus, almost all H^+-PPase was expected to be recovered in the microsomal fraction before separation of vacuolar membranes. To examine the change in H^+-PPase and H^+-ATPase levels during germination of pumpkin, microsomal fractions prepared from dry and etiolated cotyledons were subjected to immunoblot analysis (Fig. 3a). In preparations of both microsome and vacuolar membrane, the level of each subunit of H^+-PPase and H^+-ATPase increased markedly after seed germination, as shown in Figures 3 and 4. Only faint bands of the subunits were observed in the preparations of dry cotyledons.

The microsomal and vacuolar membrane fractions from pumpkin cotyledons were assayed for the activities of H^+-PPase and nitrate-sensitive H^+-ATPase. The activities of the two enzymes in both fractions increased in parallel to the amounts of the subunits. The specific activities of H^+-PPase

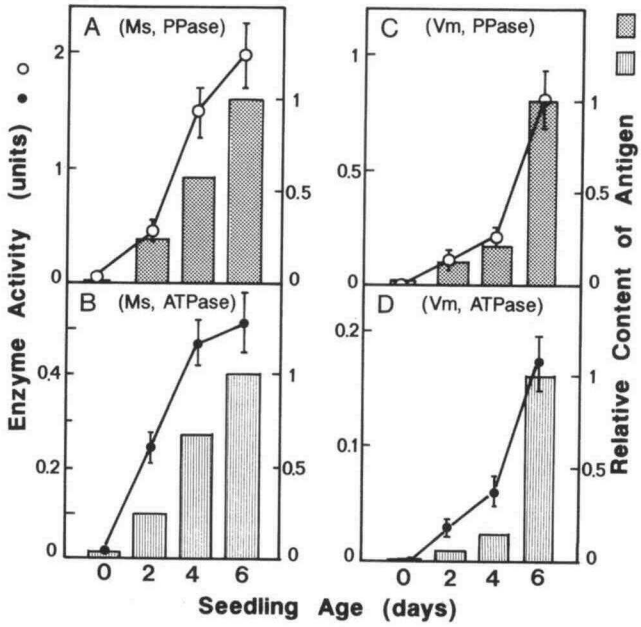

Figure 4. Increases in activities of vacuolar H^+-PPase and H^+-ATPase and in levels of their subunits after germination of pumpkin seeds. Microsomes were prepared from 100 cotyledons of dry seeds and etiolated seedlings. Vacuolar membranes were isolated from the microsomal suspension as described in "Materials and Methods." Microsomes (A and B) and vacuolar membranes (C and D) were assayed for H^+-PPase (○) and nitrate-sensitive ATPase (●). Enzyme activities are expressed as units per 100 cotyledons. The data shown are means ± SD for two experiments, each with triplicate assays. To quantify the amount of the subunits of the enzymes, aliquots of the microsomal and vacuolar membrane suspensions were subjected to SDS-PAGE and immunostaining with antibodies to H^+-PPase (stippled bars, A and C) and the two major subunits of vacuolar H^+-ATPase (hatched bars, B and D). The levels of antigen on the immunoblots were quantified by densitometric scanning of the blots. Values shown are amounts of the polypeptides per cotyledon relative to that of 6-d-old seedling.

(0.35 units mg^{-1}) and H^+-ATPase (0.13 units mg^{-1}) in the vacuolar membranes from 6-d-old cotyledons were lower than those (1.25 and 0.27 units mg^{-1}, respectively) in the vacuolar membranes from hypocotyls of 6-d-old pumpkin seedlings. The levels of the activities of H^+-PPase and nitrate-sensitive H^+-ATPase in the dry cotyledons were low.

These results suggest that the increases in activities of H^+-PPase and H^+-ATPase are due to their de novo syntheses. This was also supported by immunoblot analysis of the total cotyledon protein with SDS. The seed storage proteins, such as 7S globulin, 11S globulin, and 2S albumin, account for more than 90% of total amount of proteins in dry cotyledon. These seed proteins were hydrolyzed after germination and several polypeptides with molecular masses of more than 30 kD appeared on SDS-PAGE, as shown in Figure 5A. In contrast to the seed proteins, the subunits of H^+-PPase and H^+-ATPase increased after germination on the immunoblots of the SDS extracts (Fig. 5, B and C). This result suggests the de novo synthesis of the two enzymes in cotyledon after germination.

Induction of VM23 in Pumpkin Cotyledon after Germination

The membranes of plant vacuoles possess another integral protein with molecular mass of 20 to 30 kD, γ-TIP (Johnson et al., 1989; Höfte et al., 1992), or VM23 (Maeshima, 1992). We examined the immunoreactivity of the vacuolar membranes from cotyledons with antibody against the membrane-intrinsic protein of the central vacuole, VM23. As shown in Figure 3, the antibody against VM23 clearly reacted with both microsomes and vacuolar membranes from germinated cotyledons, but not with either microsomes or vacuolar membranes from dry cotyledons. The intensity of immunostaining of VM23 increased after germination in a pattern similar to those of H^+-PPase and H^+-ATPase. Recently, we demonstrated that the membranes of protein storage vacuoles of pumpkin cotyledons possess a membrane-intrinsic protein of

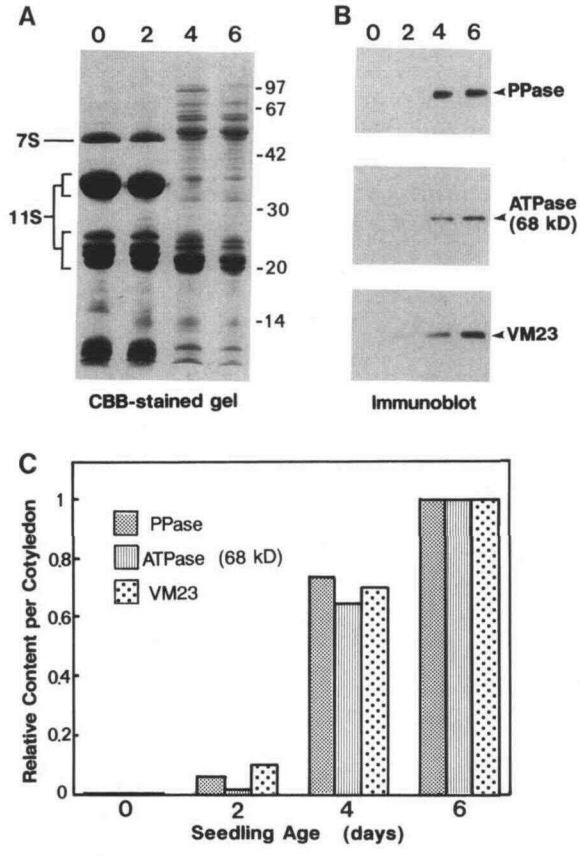

Figure 5. Immunological detection of H^+-PPase, H^+-ATPase, and VM23 in the SDS extracts of pumpkin cotyledons. Five pairs of cotyledon from dry seeds and etiolated seedlings were homogenized in the SDS solution as described in "Materials and Methods." Aliquots of the SDS extracts were subjected to SDS-PAGE (A, Coomassie blue-stained gel) and immunoblotting with antibodies to H^+-PPase, the 68-kD subunit of vacuolar H^+-ATPase, and VM23 (B). 7S globulin and 11S globulin of the major seed proteins are marked on the left of A. Levels of polypeptides in the extracts were determined by densitometric scanning of the immunoblots. Values are expressed as amounts relative to those of 6-d-old cotyledon (C).

28 kD, tentatively named MP28 (K. Inoue, A. Motozaki, Y. Takeuchi, M. Nishimura, and I. Hara-Nishimura, unpublished data). In this experiment, a clear immunostained band of MP28 was observed in membranes from both dry cotyledons and 2- and 4-d-old etiolated cotyledons. Thus, our preparations were regarded as membranes of protein storage vacuoles to be suitable for this study. A faint band was observed at 27 kD in the immunoblots of the membranes of 2-d-old cotyledon (Fig. 3). It may be a proteolytic product of MP28. The vacuolar membranes of the 6-d-old cotyledon lost MP28.

The relative amount of VM23 in the vacuolar membranes of cotyledon was compared with that of hypocotyl by immunoblotting. The vacuolar membranes were isolated from hypocotyls and cotyledons of 6-d-old seedlings, and portions of the membrane fractions that contained equal activity of H^+-PPase were subjected to immunoblotting. As shown in Figure 6, the intensity of immunostained bands of H^+-PPase and the two subunits of H^+-ATPase did not differ between the membrane preparations of hypocotyl and cotyledon (lanes 4–6). However, the content of VM23 in the membranes from cotyledons was lower than that from hypocotyls (Fig. 6, lanes 4 and 5). Judging from the intensity of the immunostained band of VM23 and the protein amount applied, the specific content of VM23 in the membranes of cotyledon was less than 10% of that of hypocotyl.

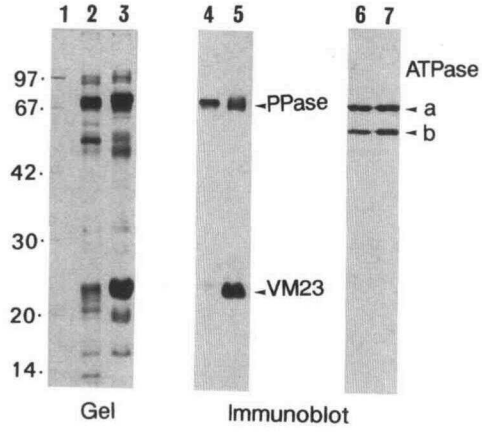

Figure 6. Comparison of the content of VM23 in the vacuolar membranes between cotyledon and hypocotyl of pumpkin seedlings. Vacuolar membranes were prepared from cotyledons and hypocotyls of 6-d-old seedlings and the proteins were separated by SDS-PAGE. Lane 1, Molecular mass standards (kD); lane 2, vacuolar membranes from cotyledons (3 μg protein/lane); lane 3, vacuolar membranes from hypocotyls (3 μg); lanes 4 to 7, immunoblot. Proteins of vacuolar membranes of cotyledons (lanes 4 and 6, 8.2 μg of protein) and hypocotyls (lanes 5 and 7, 2.2 μg of protein) with equal activity of H^+-PPase (2.7×10^{-3} units) were separated by SDS-PAGE and transferred to a PVDF membrane to compare the amount of VM23. The membrane sheets were probed with a mixture of antibodies to H^+-PPase and VM23 (lanes 4 and 5) and a mixture of antibodies to 68- and 57-kD subunits of vacuolar H^+-ATPase (lanes 6 and 7).

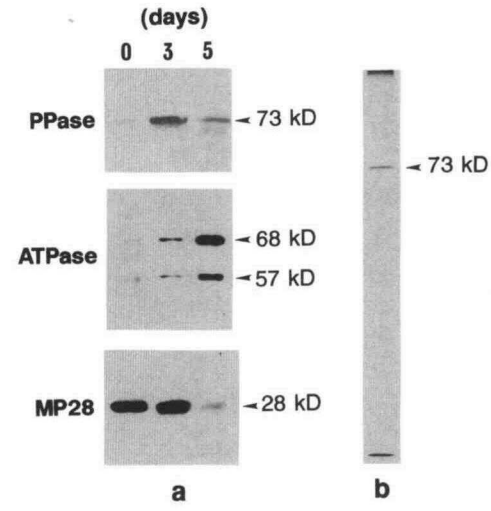

Figure 7. Immunodetection of H^+-PPase, H^+-ATPase, and MP28 in the membrane of protein storage vacuoles (a) and cell-free translation of H^+-PPase of castor bean endosperm (b). Vacuolar membranes were prepared from 40 endosperms each of dry seeds (d 0) and 3- and 5-d-old seedlings and subjected to SDS-PAGE. The amount of membranes applied to SDS-PAGE corresponds to two endosperms. Immunodetection was done with antibody to H^+-PPase (a). Poly(A)$^+$ RNA was isolated from endosperms of 2-d-old castor bean seedlings and translated in a wheat germ cell-free protein synthesis system with [^{35}S]Met. Vacuolar H^+-PPase was immunoprecipitated from the translation product with the antibody and protein A-Sepharose. Labeled polypeptides were analyzed by SDS-PAGE and fluorography (b).

Changes in Levels of H^+-PPase and H^+-ATPase in Castor Bean Endosperm

Endosperms of castor bean also contain many protein bodies in their cells (Nishimura and Beevers, 1979). The vacuolar membranes were prepared from endosperms of dry seeds and germinated seedlings and subjected to immunoblotting with antibodies to H^+-PPase, the two subunits of H^+-ATPase, VM23, and MP28. The specific activity of H^+-PPase was 0.13 units mg^{-1}. As shown in Figure 7a, the subunits of H^+-PPase and H^+-ATPase were detected in the membranes from endosperms of germinated seedlings. On immunoblots, the levels of the subunits were increased in the etiolated endosperms, although the amount of MP28 was decreased in the membranes of 5-d-old endosperms. The immunoblot of the membrane preparation from 5-d-old endosperms with antibody to H^+-PPase showed only a faint band. This band may be due to a proteolytic degradation of the enzyme during membrane preparation, because the immunoblot of the extract showed a more intense band. When poly(A)$^+$ RNAs prepared from 2-d-old endosperms were translated in a cell-free translation system containing [^{35}S]-Met and the translation products were immunoselected with antibody to H^+-PPase, a major band of 73 kD was detected on the polyacrylamide gel (Fig. 7b). Judging from the radioactivity of the immunoprecipitate, the content of mRNA for H^+-PPase accounted for 0.15% of total translatable mRNA

in etiolated endosperms. These findings suggest active synthesis and accumulation of the proton pumps in the germinating endosperms. In contrast to the reaction with pumpkin cotyledon, the antibody against radish VM23 did not react with the microsomal or vacuolar membrane fraction of the etiolated castor bean endosperm (not shown).

Immunocytochemical Analysis of H^+-PPase in Cotyledon

To examine the localization of H^+-PPase, we analyzed pumpkin cotyledon cells by electron-microscopic immunochemistry. Thin sections of cotyledon from 4-d-old seedlings were treated with antibody to H^+-PPase prior to treatment with colloidal gold linked to protein A. As shown in Figure 8, colloidal gold labeling for H^+-PPase was detected on the vacuolar membrane in the parenchyma cells of cotyledon, whereas no gold particles were observed in other organelles such as the mitochondria. Some vesicles in protein-storage vacuoles were labeled with the antibody to H^+-PPase. From studies on soybean protein body by electron-microscopic immunocytochemistry with antibody to the α-type TIP, Melroy and Herman (1991) reported that internalized autophagic vesicles in the protein storage vacuole were labeled with the antibody. They proposed that the mechanism of α-TIP removal involves autophagic sequestering of membrane inside the protein-storage vacuole. There is a possibility that the vesicles labeled with antibody to H^+-PPase in protein storage vacuoles of pumpkin cotyledon may be autophagic vesicles. Another possibility is that the vesicles are derived from the process of membrane fusion during transformation of protein storage vacuoles into a vacuole. It must be determined whether or not these internalized vesicles of germinating cotyledon contain both the α-type TIP and the vacuole-specific components, such as H^+-PPase and VM23.

DISCUSSION

This study was done to examine the presence of H^+-PPase and H^+-ATPase and changes in their levels in the membranes of protein storage vacuoles of germinating seed. The two enzymes are markers of the central vacuole and function as proton pumps that acidify the vacuoles and generate membrane potentials. The vacuolar membranes of etiolated pumpkin cotyledon showed not only PPi-hydrolyzing activity but also PPi-dependent H^+ transport activity. The PPase activity was stimulated 5-fold by K^+ and inhibited by Ca^{2+}, like the H^+-PPase purified from the central vacuole (Rea and Poole, 1986; Maeshima and Yoshida, 1989; Maeshima, 1991). The vacuolar membranes of pumpkin cotyledons contain the 73-kD polypeptide that reacted with antibody against mung bean H^+-PPase. These findings indicate that H^+-PPase exists in the membrane of protein storage vacuoles as a functional proton pump. Its presence on the vacuolar membranes of pumpkin cotyledon was confirmed by immunogold labeling.

The present study also demonstrated the presence of H^+-ATPase in the protein storage vacuoles. It was shown to be a typical vacuolar H^+-ATPase, since its activity was inhibited by nitrate and bafilomycin A_1, a specific inhibitor of vacuolar H^+-ATPase (Bowman et al., 1988), but not by azide or vanadate. This was supported by the results of immunoblot tests with the specific antibodies to the subunits of vacuolar H^+-ATPase. Among the several subunits of vacuolar H^+-ATPase, the two larger ones (approximately 70 and 60 kD) have been demonstrated to be a catalytic and a regulatory subunit, respectively (Gogarten et al., 1989; Sze et al., 1992). The specific antibodies against the 68- and 57-kD subunits of mung bean enzyme cross-reacted to the corresponding polypeptides in the membranes of cotyledon.

The activities of H^+-PPase and H^+-ATPase in the vacuolar membranes of pumpkin cotyledon increased during seedling growth. High activities of the enzymes in the etiolated cotyledons are due to a net increase in the amounts of enzyme proteins. This was shown by the results of immunoblots of the purified vacuolar membranes, microsomes, and SDS extracts with the antibodies to the enzyme subunits (Figs. 3–5). Also, the increase in the amounts of H^+-PPase and H^+-

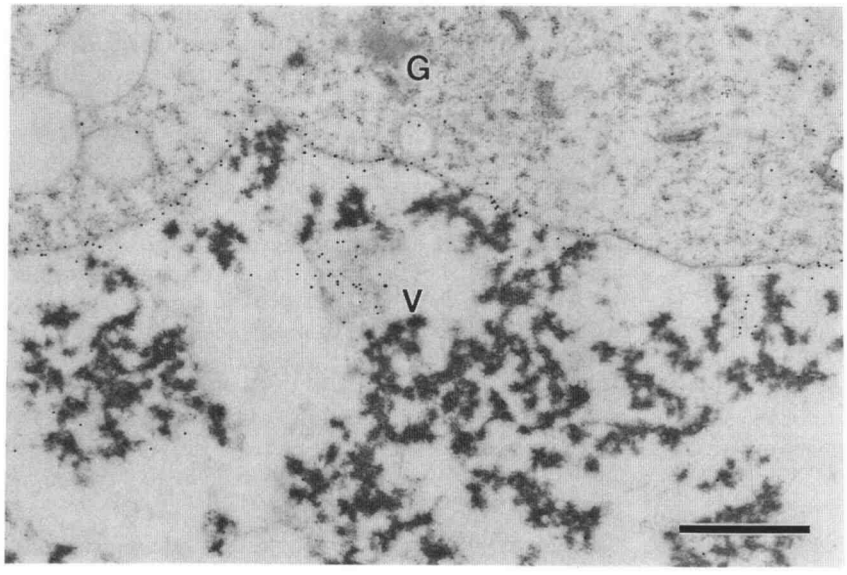

Figure 8. Immunogold labeling of vacuolar H^+-PPase in pumpkin cotyledon. A thin section of a cotyledon of a 4-d-old pumpkin seedling was treated with antibody to H^+-PPase, and bound antibody was visualized with protein A coupled to 15 nm of colloidal gold. G, Golgi apparatus; V, protein-storage vacuole. Bar = 1 μm.

ATPase was observed in endosperms of germinating castor beans. In dry cotyledons of pumpkin, the membranes of protein storage vacuoles showed activities of both H^+-PPase and H^+-ATPase and had polypeptides that reacted with antibodies to H^+-PPase and the subunits of H^+-ATPase. However, both the activities and the amounts of the enzymes were extremely low. There may be no possibility of activation or reassembly during germination of the enzyme proteins that existed in dry cotyledons. The present observations demonstrate that the amounts of proton pumps increased during germination, which means that they were de novo synthesized and added to the protein body membranes.

The levels of ATP and PPi in cotyledon are thought to be adequate for the H^+-ATPase and H^+-PPase, although the substrate concentrations were not accurately determined. In cells of germinating seeds, ATP is supplied mainly by mitochondrial oxidative phosphorylation. A marked increase in respiratory activity in storage tissues during seed germination is brought about by active biogenesis of mitochondria. Since RNAs and proteins are actively synthesized in germinating seeds, high levels of PPi are produced as a byproduct in these metabolic pathways.

In conclusion, the H^+-PPase and H^+-ATPase are present in the protein storage vacuoles and function as proton pumps, utilizing the substrates provided by an active metabolism in the storage tissues of germinating seeds. The acidic condition maintained by the proton pumps may be essential to the hydrolytic enzymes in vacuoles. For example, a pH of 5 was found by Nishimura and Beevers (1979) to be optimal for hydrolysis of storage proteins in both intact and lysed protein-storage vacuoles of castor bean endosperm. The pH of extracts of castor bean vacuoles was 5.7 (Nishimura, 1982). Many other active hydrolases with acid pH optima are located in the protein-storage vacuoles (Nishimura and Beevers, 1978). In seedlings of pumpkin, where the cotyledon regreens and has the function of a leaf, the vacuole is likely to have different roles, such as accumulation of newly assimilated photosynthetic products. The vacuolar proton pumps that increase after germination may support not only the hydrolysis of stored components in protein-storage vacuoles but also the accumulation of metabolites, as in the central vacuoles of a leaf.

VM23 was also accumulated in the membranes of protein-storage vacuoles after germination of pumpkin seeds, although the content of VM23 was low compared with that in vegetative tissue such as hypocotyl. Recently, another vacuolar membrane protein, TIP, was isolated and characterized by Johnson et al. (1989, 1990). Höfte et al. (1992) demonstrated that α- and γ-TIPs are expressed specifically in seeds and vegetative cells, respectively. γ-TIP (Höfte et al., 1992) and VM23 (Maeshima, 1992; Maeshima et al., 1994) are detected in the vegetative tissues, such as root, stem, and leaf, and the expression of their proteins seems to be related to the enlargement of cells, namely the enlargement of vacuoles (Maeshima, 1990; Ludevid et al., 1992). VM23 isolated from radish vacuolar membranes may belong to the γ-TIPs, because its amino-terminal amino acid sequence has sequence identity with *Arabidopsis* γ-TIP (Höfte et al., 1992). Furthermore, the antibody against radish VM23 cross-reacted to a 23-kD polypeptide in vacuolar membranes of *Arabidopsis* leaves (Maeshima et al., 1994).

In this study, we found that VM23-analogous protein exists in the membranes of protein-storage vacuoles from germinated cotyledons, although the protein was absent in dry cotyledons. This means that a γ-type TIP is induced and added to the membranes of protein-storage vacuoles during seedling growth. There is a possibility that VM23 may function as a water channel like γ-TIP, as proposed by Maurel et al. (1993). The condition of osmotic pressure changes drastically in cells of germinating seeds. It results from influx of water into cells, and from increase in the transport of small molecules derived from storage substances. Since the vacuole is an acidic compartment containing several hydrolases, physical damage of the vacuolar membrane by osmotic stress must result in the death of the cells. The physiological function of VM23 and the regulatory mechanism of the amount of VM23 in cotyledon cells remains to be determined at the levels of biochemistry and molecular biology.

In contrast, α-TIP is accumulated in the protein-body membranes during seed maturation and disappears during seedling growth of soybean (Melroy and Herman, 1991; Höfte et al., 1992). We recently detected α-TIP homologs, MP28, and a 23-kD protein in the membranes of protein storage vacuoles from dry seeds of pumpkin (K. Inoue, A. Motozawa, Y. Takeuchi, M. Nishimura, and I. Hara-Nishimura, unpublished data). The levels of these membrane-intrinsic' proteins decreased during seed germination. Indeed, MP28 disappeared from the membranes of pumpkin cotyledons and castor bean endosperms of germinating seeds (Figs. 3 and 7). From consideration of this observation and the present results, the α-TIP may be replaced by VM23 (γ-TIP) during transformation of the protein-storage vacuole into the central vacuole in pumpkin cotyledon. Merloy and Herman (1991) also demonstrated that the α-type TIP disappeared from the protein-body membranes of soybean cotyledon after germination. The present finding shows that some proteins specific to the central vacuole, such as H^+-PPase, H^+-ATPase, and VM23, are newly synthesized and added to the membranes of protein-storage vacuoles after germination. Exchange of MP28 for VM23 in germinating cotyledons may suggest the difference in cellular function between the seed-specific α-TIP and the vegetative-cell γ-TIP.

In germinating seeds, the membrane proteins in the protein-storage vacuoles were substituted with those of vacuolar membranes. The removal of some components of the vacuolar membrane and the introduction of several proteins into the membrane were coordinated events. The present findings raise a question about how the membrane proteins are exchanged during the transformation of the protein-storage vacuole into the central vacuole. Further studies on the biosynthesis and intracellular trafficking of H^+-PPase, H^+-ATPase subunits, and VM23 should provide valuable information on the mechanism of protein-body vacuole transformation.

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