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Effects of in Vivo Treatment with Abscisic Acid and/or Cytokinin on Activities of Vacuolar H^+ Pumps of Tonoplast-Enriched Membrane Vesicles Prepared from Barley Roots

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We investigated the effects of in vivo treatment (1 day) of barley roots with abscisic acid (ABA) and/or a cytokinin (6-benzyladenine; BA) on the ATP- and PP_i-dependent H^+ transport activities of tonoplast-enriched membrane vesicles prepared from the roots. Treatment with ABA significantly increased the two H^+ transport activities. By contrast, treatment with BA significantly decreased PP_i-dependent H^+ transport activity, while the change in ATP-dependent H^+ transport activity was small. Increases in the two H^+ transport activities caused by treatment with ABA were suppressed during treatment with ABA and BA. Changes in the NO_3^--inhibitable ATPase activity and the Na^+-inhibitable PP_iase activity of membrane vesicles after treatment of roots with phytohormone(s) (ABA, BA, ABA+BA) were similar to changes in the ATP- and PP_i-dependent H^+ transport activities of the membrane vesicles, respectively. Immunoblot analysis with antibodies raised against the functional catalytic subunits of the vacuolar H^+ pumps (H^+-ATPase and H^+-PP_iase) of mung bean revealed that only the level of the functional catalytic subunit of the H^+-PP_iase of the membrane vesicles was significantly increased by treatment with ABA alone and in combination with BA. These results suggest that treatment with ABA has a stimulatory effect on the activities of the two H^+ pumps of the vacuolar membrane of barley roots, with increase in the level of the catalytic subunit of the H^+-PP_iase, and that treatment with BA has an inhibitory effect on the two H^+ pump activities of the vacuolar membrane without changes in the levels of the catalytic subunits of either H^+ pump, with the limitation that treatment with BA has an inhibitory effect only when the activity of the H^+-ATPase has been increased by treatment with ABA.

Key words: Abscisic acid — Cytokinin — Tonoplast — H^+ pumps.

In higher plant cells, vacuoles play a fundamental role in the maintenance and regulation of cell turgor and in the transport and storage of various ions and metabolites. The electrogenic H^+-ATPase and H^+-PP_iase, located in the vacuolar membrane, are essential to the role of the plant vacuoles since the two enzymes generate an electrochemical gradient of protons, acidifying the vacuolar lumen and driving the secondary accumulation of various intracellular solutes (Sze 1985, Ward and Sze 1992). In analyses of the H^+ pumps at the molecular level, the vacuolar H^+-ATPase has been shown to be a large molecular complex consisting of several subunits (Lai et al. 1988, Moriyama and Nelson 1989, Nelson 1989, Parry et al. 1989), and the hydrolytic subunit of the vacuolar H^+-PP_iase has been purified (Maeshima and Yoshida 1989, Sarafian and Poole 1989, Britten et al. 1989).

Evidence from recent studies, described below, suggests that the actions of the vacuolar H^+ pumps may also have physiological significance in the adaptation of plants

Abbreviations: ATPase, adenosine triphosphatase; BA, 6-benzyladenine; BSA, bovine serum albumin; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; DTT, dithiothreitol; EGTA, ethyleneglycol bis(β-aminoethylether)*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; PP_iase, pyrophosphatase; TBS, Tris-buffered saline.

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to environmental stresses. For example, in *Mesembryanthemum crystallinum* L., the CAM state which is induced under water-deficit conditions (Schmitt and Piepenbrock 1992) has higher ATP-dependent H^+ transport activity of tonoplasts than when the plant is in the C_3 state (Struve et al. 1985, Bremberger et al. 1988). Moreover, the ATP-dependent H^+ transport activity of tonoplast-enriched membrane vesicles prepared from barley roots increases when the plants are grown under sodium stress (Matsumoto and Chung 1988), and stress of aluminum, whose ions are toxic to plant growth, increases the ATP- and PP_i-dependent H^+ transport activities of tonoplast-enriched membrane vesicles prepared from barley roots (Kasai et al. 1992). These suggest that higher activity of plant vacuolar H^+ pumps may contribute to the maintenance of higher cellular osmolarity and water potential via transport of various ions and solutes into the vacuoles, and such activity may enhance the sequestration of toxic ions into the vacuoles via cation/H^+ antiporter systems located in the membrane. As a result, the inhibition of growth of plants exposed to drought, salinity or toxic ions may be alleviated. The physiological significance of the vacuolar Na^+/H^+ antiporter (Blumwald and Poole 1985, Garbarino and DuPont 1988) and the Al/H^+ antiporter (Matsumoto 1991), which is still putative, in the adaptive response of plants to sodium or aluminum stress has been proposed.

The mechanisms (or factors) responsible for the changes in the activity(ies) of plant vacuolar H^+ pump(s) under environmental stresses, such as drought, salinity or aluminum stress, are still unclear. Since abscisic acid (ABA), which has been called a stress-inducible phytohormone, enhances the adaptation of plants to various environmental stresses (Zeevaart and Creelman 1988), for example, drought and salinity, enhanced levels of ABA in plant cells may perhaps be considered to be related to the mechanisms responsible for changes in the activity(ies) of the vacuolar H^+ pump(s) that were described above. Increases in levels of ABA in plant tissues occur upon exposure to drought and salinity (Cammue et al. 1989, Kefu et al. 1991). In the case of aluminum stress, only speculative and limited information is available with respect to the involvement of ABA in the responses of plants to the stress (Bennett and Breen 1991). Narasimhan et al. (1991) reported that application of ABA to tobacco cells enhanced levels of mRNA encoded the functional catalytic subunit of the vacuolar H^+-ATPase. However, the increase in the level of the mRNA was small and variable and the researchers did not analyze substrate-hydrolysis and H^+ transport activities of the vacuolar H^+-ATPase.

In the present study, we investigated the effects of in vivo treatment of barley roots with ABA on the activities of the vacuolar H^+ pumps, using tonoplast-enriched preparations of membranes from the roots of barley plants after treatment with the phytohormone. We also investigated the effects of treatment with cytokinin on the H^+ pumps since there are examples of ABA and cytokinin having opposite effects in the physiological responses of plants: for example, the negative (ABA) and positive (cytokinin) control of stomatal opening (Morsucci et al. 1991), the activity of phosphoenolpyruvate carboxylase (Schmitt and Piepenbrock 1992), the synthesis of chlorophyll *a/b* binding protein (Chang and Walling 1991), and of the activity of nitrate reductase (Lu et al. 1992). In addition, levels of the functional catalytic subunits of the vacuolar H^+ pumps of barley roots after treatment of roots with ABA or cytokinin were assessed by immonoblot analysis.

Materials and Methods

Treatment of barley roots with phytohormone(s)—Barley seeds (*Hordeum vulgare* cv. Kikaihadaka) were sown in a plastic basket filled with deionized water and germinated in the dark at 22–23°C in a growth chamber. After 3 or 4 days, plants were grown in deionized water that contained 0.1 mM $CaCl_2$ only under a regime of 12 h of light (fluorescent lamp, 4,000 lux) and 12 h of darkness. On the sixth day, the plants were cultured for one day in the same growth medium supplemented with 3 mM KCl. On the seventh day, sets of 20 barley plants were harvested and the roots of each set (fresh weight, approximately 1.5 g), after washing with deionized water (1 liter), were incubated for one day in the treatment medium (total, 65 ml) which contained 20 mM MES-BTP (pH 5.0) with phytohormone(s). A control treatment (without addition of phytohormone to the medium) was also performed in parallel. After treatment, tonoplast-enriched membrane vesicles were prepared from the roots (see below).

Preparation of tonoplast-enriched membrane vesicles—Preparation of tonoplast-enriched membrane vesicles from barley roots was performed by centrifugation of crude homogenate of roots on discontinuous Dextran T-70 gradient (2% and 10%), as described previously (Kasai et al. 1993).

Artificial pH gradient—Tonoplast-enriched membrane vesicles from the interface between two layers of Dextran T-70 were diluted with a solution that contained 250 mM sorbitol, 5 mM Tris-MES (pH 6.0) and 0.5 mM DTT, and the suspension was centrifuged at 80,000 × *g* for 40 min. The resulting pellet was suspended in the same medium. A gradient of pH (acid inside) across the membrane of vesicles was generated by adding the suspension that contained membrane vesicles (10 μg protein) to a solution (1 ml) of 250 mM sorbitol, 5 mM Tris-MES (pH 8.0) and 0.3 mM EGTA-BTP (pH 8.0) at a ratio of solution to suspension of 50 to 1 (v/v), at 28°C. Release of H^+ from the membrane vesicles was determined by monitoring the quenching of the fluorescence of acridine orange with a spectrofluorophotometer (RF-503F; Shimadzu). The excitation and emis-

sion wavelengths were 493 and 540 nm, respectively.

H^+ transport assay—The rate of transport of H^+ into the membrane vesicles was determined by monitoring quenching of quinacrine fluorescence with excitation and emission wavelengths of 420 and 495 nm, respectively. The reaction medium (0.5 ml) for the assay of the ATP-dependent H^+ transport consisted of 250 mM sorbitol, 20 mM HEPES-BTP (pH 7.5), 3 mM $MgSO_4$, 3 mM ATP-BTP, 0.3 mM EGTA-BTP, 50 mM BTP-Cl, 2 μM quinacrine and membrane vesicles (5 μg protein). The reaction medium (0.5 ml) for the assay of PP_i-dependent H^+ transport consisted of 250 mM sorbitol, 20 mM HEPES-BTP (pH 7.5), 1.5 mM $MgSO_4$, 0.1 mM PP_i-BTP, 0.3 mM EGTA-BTP, 50 mM KNO_3, 2 μM quinacrine and membrane vesicles (10 μg protein). Reactions were started by the addition of PP_i or ATP as substrate and the temperature of the reactions was 28°C.

Assays of ATPase and PP_iase activities—ATPase activity was assayed in a reaction mixture (0.5 ml) that contained 20 mM HEPES-BTP (pH 7.5), 3 mM $MgSO_4$, 3 mM ATP-BTP, 0.3 mM EGTA-BTP, 2 μM gramicidin, 50 mM KCl or 50 mM KCl plus 50 mM KNO_3, and membrane vesicles, at 28°C. The reaction mixture for the assay of PP_i-ase activity consisted of 20 mM HEPES-BTP (pH 7.5), 1.5 mM $MgSO_4$, 0.5 mM PP_i-BTP, 0.3 mM EGTA-BTP, 2 μM gramicidin, 50 mM KNO_3 or 50 mM KNO_3 plus 50 mM $NaNO_3$, and membrane vesicles. P_i released from the respective substrates was assayed by the method of Heinonen and Lahti (1981). NO_3^--inhibitable ATPase activity or Na^+-inhibitable PP_iase activity was calculated as the difference between the activities determined in the absence and in the presence of 50 mM KNO_3 or 50 mM $NaNO_3$, respectively.

Protein determination—Protein was quantitated by the method of Bradford (1976) with bovine serum albumin (BSA) as the standard.

Gel electrophoresis and immunoblotting—Membrane vesicles were incubated for 15 min at 70°C in a solution that contained 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, and 0.5 mM PMSF, and then an aliquot of the solution was subjected to SDS/polyacrylamide gel electrophoresis (acrylamide gel; 12.5%) by the method of Laemmli (1970). The concentrations of Tris and glycine in the running buffer used in the electrophoresis were twice those used by Laemmli.

Antibodies raised against the functional catalytic subunit of the vacuolar H^+-ATPase or H^+-PP_iase from mung bean were prepared as reported previously (Maeshima and Yoshida 1989, Matsuura-Endo et al. 1992).

Immunoblotting was performed by a modified version of the method of Towbin et al. (1979). Proteins on a gel were transferred onto a nitrocellulose membrane in a semidry blotting apparatus (KS-8453; Marysol Co., Tokyo), placing the gel and the nitrocellulose membrane with five sheets of Whatman 3MM paper on each side of the anode and cathode carbon plates in the apparatus. The Whatman 3MM papers had been preimmersed in a transfer solution that contained 48 mM Tris, 39 mM glycine, 20% (v/v) methanol, and 0.02% (w/v) SDS. The transfer was performed for 50 min at 140 mA for a nitrocellulose membrane sheet of 7×10 cm^2. After the transfer, the nitrocellulose membrane was incubated in a blocking solution that contained TBS [Tris-buffered saline; 20 mM Tris-Cl (pH 7.5), 140 mM NaCl] and BSA (see below), and then it was incubated overnight in TBS that contained antibodies (see below) at room temperature. The antibodies that reacted with the antigens on the nitrocellulose membrane were detected with horseradish peroxidase-linked protein A and 4-chloro-1-naphthol (Harlow and Lane 1988, Maeshima and Yoshida 1989). The nitrocellulose membrane, after incubation in TBS that contained antibodies, was washed four times (10 min each) with TBS that contained 0.05% (v/v) Tween 20, and then it was incubated in TBS that contained horseradish peroxidase-linked protein A (second antibody; 1 : 1000 dilution) for 1 h at room temperature. After four washes (10 min each) with TBS that contained 0.05% Tween 20, followed by three washes (10 min each) with TBS, the nitrocellulose membrane was subjected to the peroxidase color-development reaction, as described above. The nitrocellulose membrane, on which colored bands developed, was subjected to scanning at 460 nm with a densitometer (CS-9000; Shimadzu).

In the procedures described above, dilutions of solutions that contained antibodies against H^+-ATPase and H^+-PP_iase in TBS were 1 : 200 and 1 : 100, respectively. The concentration of BSA in the blocking solution and the blocking times were 3% (w/v) and 1 h for detection of the catalytic subunit of H^+-ATPase, and 5% (w/v) and 2 h for that of H^+-PP_iase, respectively.

Results

H^+ transport activity—Figure 1 shows the effect of in vivo treatment of barley roots with ABA or cytokinin (6-benzyladenine; BA) on ATP- and PP_i-dependent H^+ transport activities of tonoplast-enriched membrane vesicles prepared from the roots. Compared to the control treatment, treatment with ABA increased the two activities in a concentration-dependent manner. Treatment with BA decreased PP_i-dependent H^+ transport activity in a concentration-dependent manner, while this treatment had only a slight effect on ATP-dependent H^+ transport activity.

Figure 2 shows the results for fixed concentrations of ABA and BA of 50 μM and 10 μM, respectively. The effect of each phytohormone on the two H^+ transport activities was similar to that shown in Figure 1. We also treated barley roots with a combination of ABA and BA. This com-

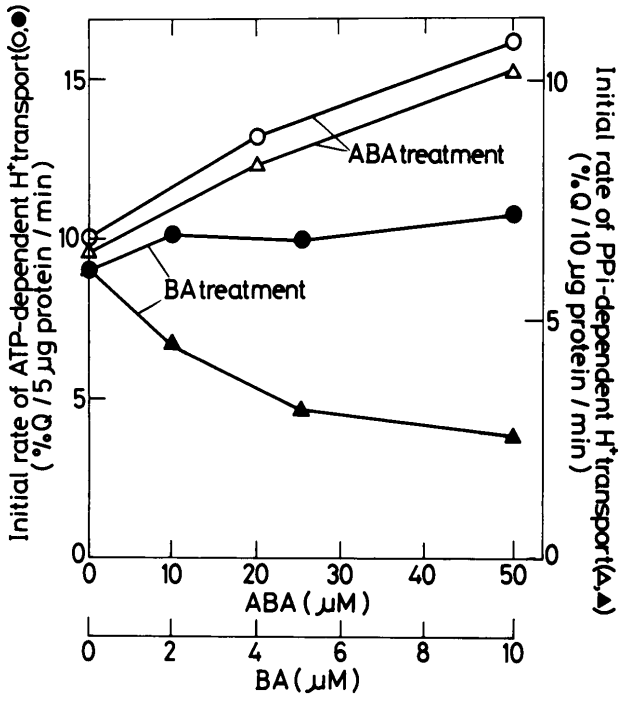

Fig. 1 Effects of treatment of barley roots with ABA or BA on ATP- and PP_i-dependent H^+ transport activities of membrane vesicles prepared from the roots. %Q, % quenching of quinacrine fluorescence.

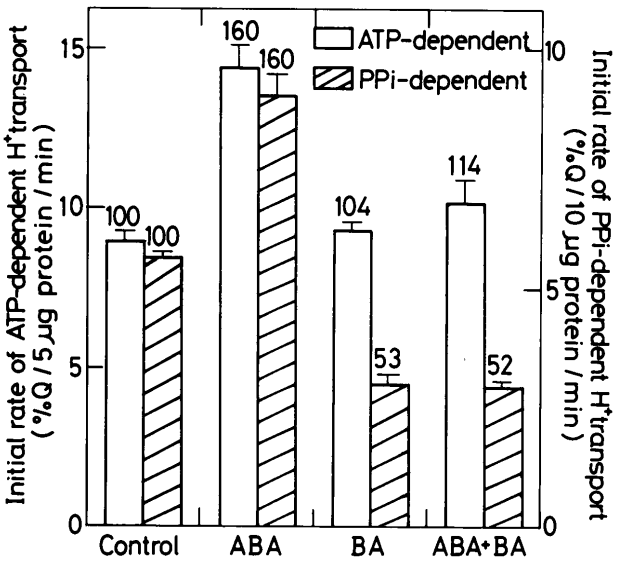

Fig. 2 Effects of treatment of barley roots with ABA and/or BA on ATP- and PP_i-dependent H^+ transport activities of membrane vesicles prepared from the roots. Concentrations of ABA and BA used for treatments were 50 μM and 10 μM, respectively. The numbers shown in this Figure are percentages of control values. They represent the average of H^+ transport activities determined in three or four independent experiments. The bars indicate S.D. of results.

bined treatment suppressed most of the increase in ATP-dependent H^+ transport activity caused by treatment with ABA alone, and the combined treatment completely suppressed the increase in PP_i-dependent H^+ transport activity caused by treatment with ABA alone to a level close to the activity observed after treatment with BA alone. Since the latter results seemed to suggest that treatment with BA at 10 μM had a strong inhibitory effect on PP_i-dependent H^+ transport activity, we also investigated the effect of BA at lower concentrations (2 and 3 μM) in treatment with ABA and BA. Results showed that the inhibitory effect at the low concentrations of BA was always lower than that at 10 μM. These results indicate that treatment with ABA has a stimulatory effect on both ATP- and PP_i-dependent H^+ transport activities, and that treatment with BA has an inhibitory effect on the two H^+ transport activities, with the limitation that treatment with BA has an inhibitory effect only when activity is increased by treatment with ABA in the case of the ATP-dependent H^+ transport.

It seemed possible that the increases in the two H^+ transport activities caused by treatment with ABA might have been due to a decrease in permeability to H^+ of the membrane since the extent of the increase in each H^+ transport activity was similar. To examine this possibility, we conducted "pH-jump" experiments. Figure 3 shows the changes in the fluorescence quenching of acridine orange, induced by an artificially generated pH gradient (acid inside), for tonoplast-enriched membrane preparations from roots treated with or without ABA. The rate of recovery of the fluorescence quenching after generation of the pH gradient, which is indicative of the rate of release of H^+ from the membrane vesicles, was almost the same in both cases.

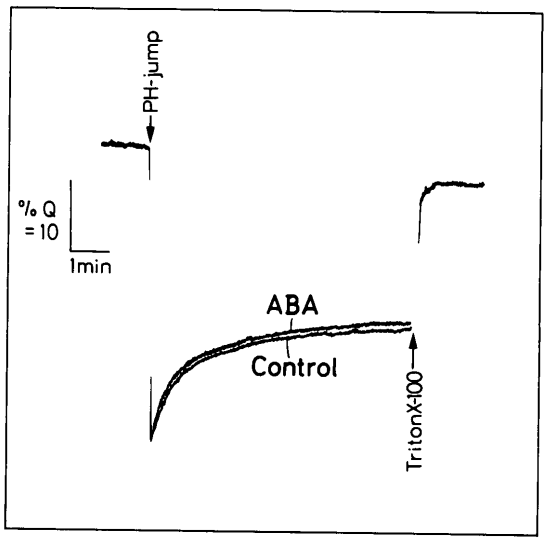

Fig. 3 Release of H^+ from membrane vesicles by a "pH-jump". Membrane vesicles prepared from barley roots that had been treated with or without 50 μM ABA were used. Triton X-100 (0.02%) was added to collapse the pH gradient across the membrane.

Membrane vesicles from other treatment (BA, ABA+BA) also released H^+ at the same rate as membrane vesicles from the control treatment. Thus, the three treatments with phytohormone(s), including treatment with ABA, did not change the permeability to H^+ of the membrane vesicles.

Activities of ATPase and PPiase—Figure 4 shows the NO_3^--inhibitable ATPase activity and the Na^+-inhibitable PP_iase activity of preparations of membrane vesicles. Our attempts to determine these activities were based on our observations that the ATP-dependent H^+ transport activity of the membrane vesicles was greatly inhibited by the presence of 50 mM NO_3^- in assay medium that contained 50 mM Cl^-, and that the PP_i-dependent H^+ transport activity of the membrane vesicles was completely inhibited by the presence of 50 mM Na^+ in assay medium that contained 50 mM K^+, as described previously (Kasai et al. 1992, 1993). Changes in the ATPase and the PP_iase activities caused by three treatments with the phytohormone(s) (ABA, BA, ABA+BA) were similar to changes in the ATP- and PP_i-dependent H^+ transport activities of the membrane preparations, respectively. Since plant vacuolar H^+-translocating ATPase and H^+-translocating PP_iase are known to be inhibited by NO_3^- and Na^+, respectively, these results suggest that changes in ATP- or PP_i-dependent H^+ transport activity of the tonoplast-enriched membrane vesicles

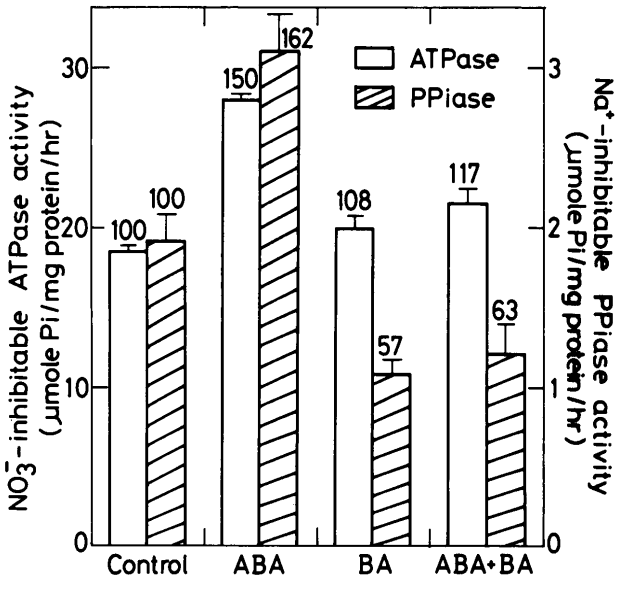

Fig. 4 Effects of treatment of barley roots with ABA and/or BA on NO_3^--inhibitable ATPase activity and Na^+-inhibitable PP_iase activity of membrane vesicles prepared from the roots. Concentrations of ABA and BA used were 50 μM and 10 μM, respectively. The numberes shown in this Figure are percentages of control values. They represent the average substrate-hydrolyzing activities determined in three or four independent experiments. The bars indicate S.D. of results.

by treatments with phytohormone(s) could be attributable to changes in the activities of the vacuolar H^+-pumping enzymes (H^+-ATPase, H^+-PP_iase) themselves in the membrane preparations.

Levels of functional subunits of H^+-ATPase and H^+-PP_iase—Considering our results from the series of experiments described above, we focused our attention on the levels of the proteins of the vacuolar H^+-ATPase and H^+-PP_iase in the membrane preparations. We performed immunoblot analysis, as described in Materials and Methods, using antibodies against the functional catalytic subunits of the vacuolar H^+-ATPase and H^+-PP_iase of mung bean.

Repeated experiments using membrane vesicles from control plant roots showed that the molecular mass of the subunit of the membrane protein that reacted with the antibodies against the functional catalytic subunit of the vacuolar H^+-ATPase of mung bean was 70–72 kDa, while two different membrane protein subunits (77–79 kDa and 23–24 kDa) reacted with antibodies against the catalytic subunit of the vacuolar H^+-PP_iase of mung bean (see Fig. 6A). We wish to emphasize here that cross-reaction of the protein subunit of 23–24 kDa with the H^+-PP_iase antibody is attributable to contamination of antibodies against the vacuolar membrane protein subunit of 23–24 kDa in the preparation of H^+-PP_iase antibodies (Maeshima 1992). To examine whether the two immunodetected protein subunits, apart from the subunit of 23–24 kDa, were the functional catalytic subunits of the vacuolar H^+-ATPase and H^+-PP_iase of barley roots, we investigated the effects of the antibodies on the H^+ transport activities of membrane vesicles at ratios of membrane protein to antibodies that had been shown to be effective in antibody-inhibition experiments with tonoplast from mung bean (Maeshima and Yoshida 1989, Matsuura-Endo et al. 1992). The results showed that antibodies against the vacuolar H^+-ATPase and H^+-PP_iase of mung bean inhibited ATP- and PP_i-dependent H^+ transport activities of tonoplast-enriched membrane vesicles from barley roots, respectively (see Table 1). The extent of inhibition was similar in each case to that of the inhibition of ATP- and PP_i-dependent H^+ transport activities of tonoplast from mung bean by the two species of antibodies (Matsuura-Endo et al. 1992, Maeshima and Yoshida 1989). These results indicate that two protein subunits detected in our immunoblot analysis, apart from the subunit of 23–24 kDa, were the functional catalytic subunits of the vacuolar H^+-translocating ATPase and H^+-translocating PP_iase of barley roots, respectively. In similar experiments with tonoplast membrane vesicles from maize and tobacco cells, the inhibition of H^+ transport activity by antibodies against the functional catalytic subunit of the tonoplast H^+-ATPase of red beet was interpreted to indicate the immunological specificity of the antibodies (Rausch et al. 1987, Reuveni et al. 1990).

Comparisons of levels of the catalytic subunits of the

Table 1 Inhibition of ATP- and PP_i-dependent H^+ transport activities of membrane vesicles by antibodies

Antibody	H^+ transport (%Q/10 μg protein/min[a])	
	ATP-dependent	PP_i-dependent
None	14.5±0.5 (100%)	4.7±0.3 (100%)
anti-H^+ATPase	5.5±0.3 (38%)	—
anti-H^+PP_iase	—	1.5±0.2 (32%)

Tonoplast-enriched membrane vesicles from a control treatment were used. H^+ transport activities were determined after incubation (20 min, room temperature) of the membrane preparations (10 μg protein) with or without antibodies against the functional catalytic subunit of H^+-ATPase (70 μg, protein) or H^+-PP_iase (20 μg, protein) of the vacuolar membrane of mung bean. Values are expressed as means±S.D. calculated from results of three individual measurements.

[a] %Q, % quenching of quinacrine fluorescence.

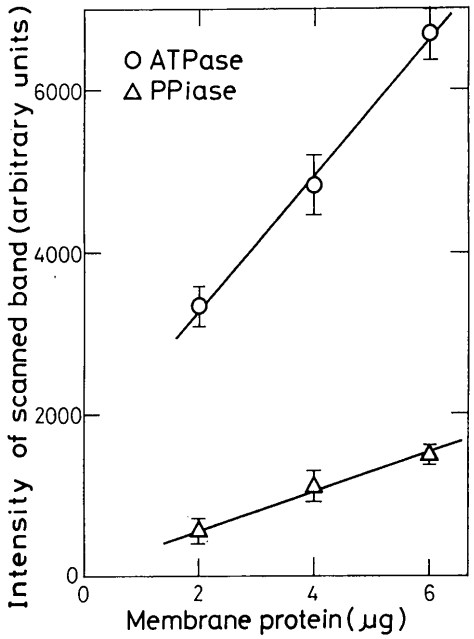

Fig. 5 Binding of the antibodies against the functional catalytic subunit of H^+-ATPase or H^+-PP_iase of mung bean tonoplast as a function of increasing amount of protein of membrane vesicles prepared from barley roots. Membrane vesicles from control treatment were subjected to SDS-PAGE, with subsequent immunoblot analysis, as described in "Materials and Methods". The immunodetected bands on the nitrocellulose membrane were subjected to densitometric scanning and the values obtained were plotted as a function of the amount of protein of membrane vesicles. The relationship between the value obtained by densitometry (V_D) and the amount of membrane protein was linear, giving the linear regression lines: for H^+-ATPase, $y(V_D)=842\times$(amount of membrane protein)+1,586 ($r=0.998$); for H^+-PP_iase, $y(V_D)=238\times$(amount of membrane protein)+73 ($r=0.996$). The bars indicate S.D. of results from immunoblot analysis that was carried out four times using membrane vesicles from two independent control treatments.

vacuolar H^+-ATPase and H^+-PP_iase in membrane preparations after treatment with and without phytohormone(s) were made in terms of extent of immunoreactivity of antibodies with the catalytic subunits, as evaluated by densitometric scanning of immunoblots. Figure 5 shows that, in both cases, the extent of immunoreactivity increased linearly with increases in the amount of membrane protein subjected to the immunoblot analysis, in which the membranes from control treatment were used. Based on this relationship between the amount of membrane protein and the extent of the immunoreactivity determined from the scanning of immunoblots, we estimated the relative levels of the catalytic subunits of the vacuolar H^+-ATPase and H^+-PP_iase in membrane preparations from treatment with phytohormon(s) (see Fig. 6B). An analogous estimation using antibodies has been made for a comparison of the amounts of the functional catalytic subunit of H^+-ATPase between tonoplast membrane preparations from unadapted and NaCl-adapted tobacco cells (Reuveni et al. 1990). Changes in levels of the catalytic subunit of the vacuolar H^+-ATPase in membrane preparations were small after all treatments. A small change in the level of the catalytic subunit was also observed in the case of the H^+-PP_iase when barley roots were treated with BA alone, but the level was increased about 1.5- to 2-fold when roots were treated with ABA or with the combination of ABA and BA. Figure 6A shows a representative result of the immunoblot analysis for the catalytic subunits of the vacuolar H^+-ATPase and the H^+-PP_iase of membrane vesicles from four treatments (control, ABA, BA, ABA+BA).

We postulated that changes in the activities of the H^+ transport and the substrate-hydrolysis in membrane preparations after treatment with phytohormon(s) might be due to changes in the extent of the vacuolation in the cells of barley roots. Such a possibility can actually be considered in the case of treatment with ABA because of the similar extents of the increases in the two H^+ transport activities and the two substrate-hydrolyzing activities in tonoplast-enriched membrane preparations. However, the small changes in the levels of the catalytic subunit of the vacuolar H^+-ATPase in membrane preparations after treatment with phytohormone(s) suggest that such a possibility is unlikely.

All the results described above, including the results from the immunoblot analysis, suggest the following: treatment with ABA has a stimulatory effect on the activities of both the H^+-ATPase and the H^+-PP_iase of the vacuolar membrane of barley roots, without a change in the level of the catalytic subunit of the H^+-ATPase, and with an in-

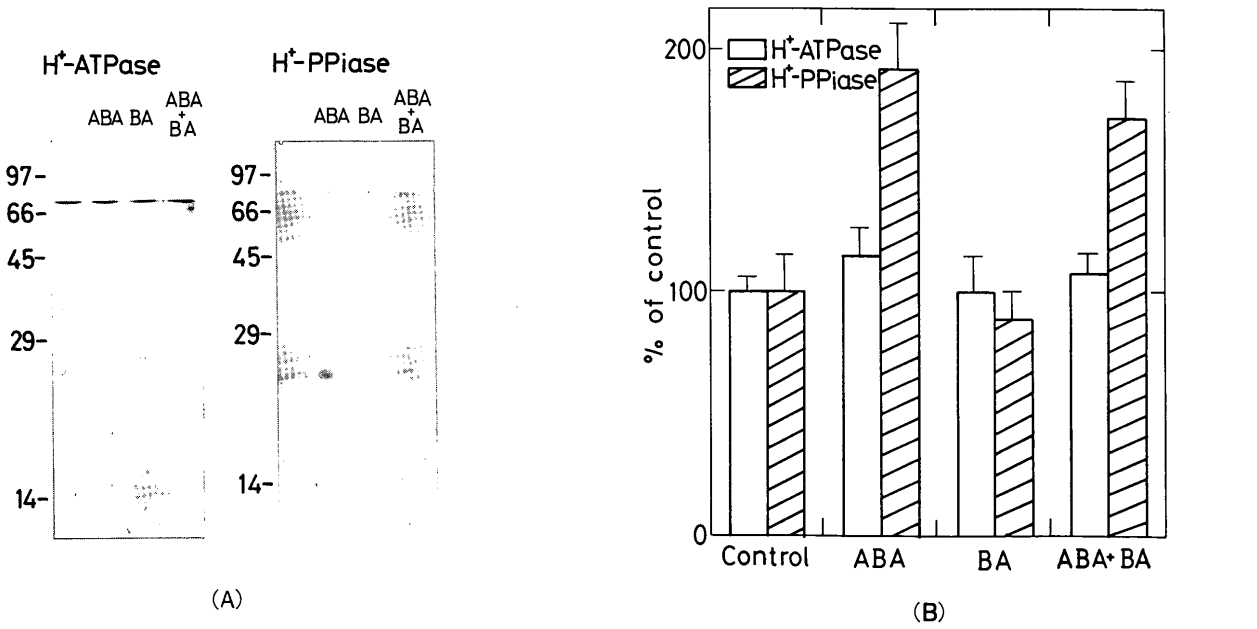

Fig. 6 Immunoblot analysis of the functional catalytic subunit of the vacuolar H^+-ATPase or the H^+-PP_iase of membrane vesicles prepared from barley roots treated with or without phytohormone(s). (A) Immunoblots. The amount of protein of membrane vesicles subjected to SDS-PAGE, followed by immunoblot analysis was 2.5 μg in all cases. The number in this Figure indicates the size (kDa) of the molecular markers. (B) Relative levels of the functional catalytic subunit. Using the values obtained by densitometric scanning of immunoblots, as shown in (A), amounts of protein were calculated from the equations in the legend to Fig. 5. In the control treatment, the calculated amount of protein was 2.6 ± 0.1 μg (mean $\pm$ S.E.) in the analysis of the catalytic subunit of the vacuolar H^+-ATPase, and it was 2.5 ± 0.4 μg in the case of H^+-PP_iase. In this Figure, the values from treatments with phytohormone(s) are shown as percentages of the control values. The bars indicate S.E. of results from three independent experiments.

crease in the level of the catalytic subunit of the H^+-PP_i-ase; treatment with BA has an inhibitory effect on the activities of both the H^+-ATPase and the H^+-PP_iase of the vacuolar membrane without changes in the levels of the catalytic subunits of either enzyme, with the limitation that the treatment has an inhibitory effect only on the increased activity caused by treatment with ABA in the case of H^+-ATPase.

Discussion

This study was performed to investigate whether ABA and a cytokinin might be involved in the regulation of the activities of plant vacuolar H^+ pumps (H^+-ATPase and H^+-PP_iase). Using preparations of tonoplast-enriched membrane vesicles from barley roots that had been treated with or without phytohormone(s), we analyzed the H^+ transport and substrate-hydrolysis activities of the membrane preparations. We also analyzed the levels of the functional catalytic subunits of the two vacuolar H^+ pumps in the membrane preparations by immunoblotting. The analyses were carried out with attention paid to possible changes in the permeability to H^+ of the membrane preparations, and considering also the vacuolation of root cells. Our data suggested the following. (1) In vivo treatment with ABA has a stimulatory effect on the activities of both the H^+-ATPase and the H^+-PP_iase of the vacuolar membrane of barley roots with an increase in the level of the catalytic subunit of the H^+-PP_iase. (2) In vivo treatment with BA, a cytokinin, has an inhibitory effect on the two activities without changes in the levels of the catalytic subunits of either H^+-pump, with the limitation that the treatment has an inhibitory effect only on the H^+-ATPase activity when it has been increased by treatment with ABA. These provide evidence for the possibility that ABA in plant cells may work as a regulatory (stimulatory) factor that induces qualitative and quantitative changes in the vacuolar H^+-ATPase and H^+-PP_iase to increase the activities of the two H^+-pumps, and that cytokinin in plant cells may work as a regulatory (inhibitory) factor that induces qualitative changes in the two vacuolar H^+-pumps to decrease their activities, in which the vacuolar H^+-ATPase may be affected by cytokinin, perhaps, at the effective levels of ABA in plant cells. A qualitative alteration in the vacuolar H^+-ATPase has been shown to occur upon the adaptation of tobacco cells to a highly saline environment, in which the ATP-hydrolysis and H^+ transport capacities of the H^+-ATPase increase (Reuveni et al. 1990).

The biochemical basis for changes in substrate-hydrolysis and H^+ transport capacities of the two vacuolar H^+ pumps by in vivo treatment with ABA or BA is not yet defined. Plant plasma membrane H^+-ATPase is regulated by phospholipids (Kasamo and Nouchbi 1987) and protease-treatment (Palmgren 1991). It had been proposed that kinase-mediated phosphorylation of the plasma membrane H^+-ATPase may be involved in regulation of its activity (McDonough and Mahler 1982, Palmgren 1991). Plant vacuolar H^+-PP_iase has been reported to be regulated by a lysolipid (Bille et al. 1992), and the vacuolar H^+-ATPase from bovine kidney has been reported to be regulated by regulatory proteins existing in the cytosol (Zhang et al. 1992). An alteration in the subunit composition of the vacuolar H^+-ATPase has been shown to occur upon the adaptation of *M. crystallinum* L. to salts (Bremberger et al. 1988). Erythrocyte Ca^{2+}-ATPase has been shown to be activated by self-association (Kosk-Kosicka and Bzdega 1988). It is likely that the altered levels of ion-pumping activity are a result of transcriptional regulation. Further studies, based on the observations described above, will be required if we are to characterize the detailed mechanisms responsible for the regulation of the vacuolar H^+ pumps by ABA and/or cytokinin. In such studies, the quantitative analysis of intracellular levels of ABA and cytokinin will also be necessary as part of the effort to assess the physiological significance of the phytohormones in the regulation of the plant vacuolar H^+ pumps.

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