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Effects of Bafilomycin A_1 and Metabolic Inhibitors on the Maintenance of Vacuolar Acidity in Maize Root Hair Cells

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Proton pumps of tonoplast membranes have been studied extensively in vitro, but data concerning their regulation in vivo are lacking. Effects of either anoxia, or the addition of KCN, 2-deoxy-d-glucose (deoxy-glucose), or bafilomycin-A_1 (BAF) on vacuolar pH of maize (*Zea mays* L.) root hair cells were followed by fluorescence microscopy after loading of 2′7′-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein. Root hair cells were able to maintain vacuolar acidity for at least 2 h in the presence of either 10 mM KCN or 50 mM deoxy-glucose or during anoxia. Treatments with either deoxy-glucose or KCN reduced total tissue ATP more than anoxia. ADP accumulated during anoxia and treatment with KCN as detected by in vivo ^{31}P-NMR spectroscopy, but not during deoxy-glucose treatment. With control roots and roots treated with deoxy-glucose, the presence of BAF, a specific inhibitor of the V-type ATPase, caused alkalization of the vacuolar pH. However, either in the presence of KCN or under anoxic conditions, BAF was relatively ineffective in dissipating vacuolar acidity. Therefore, under anoxia or in the presence of KCN, unlike the situation with air or deoxy-glucose, the V-type ATPase apparently is not required for maintenance of vacuolar acidity.

The pH of the central vacuole of higher plant cells is usually substantially more acidic than that of the cytoplasm (Sze, 1985; Kurkdjian and Guern, 1989). Maintenance of this pH gradient across the tonoplast is believed to be important to the in vivo functions of the vacuole. In vitro characterization of tonoplast membranes have identified two types of proton pumps that could maintain the acidic nature of the vacuolar lumen (Sze, 1985; Brauer et al., 1992; Rea and Poole, 1993). One of these pumps utilizes the free energy from the hydrolysis of ATP to catalyze proton transport against an electrochemical gradient, whereas the other utilizes free energy from the hydrolysis of PP. The H^+-ATPase of tonoplast membranes is a V-type transport ATPase (Nelson and Taiz, 1989), whereas the H^+-PPase pump is quite distinct from both the V-type H^+-ATPase and soluble PPase (Rea and Poole, 1993).

Although our knowledge of the in vitro characteristics of these proton pumps is increasing, little is known about their in vivo role in higher plants. Carrot (*Daucus carota*) mutants deficient in tonoplast V-type H^+-ATPase had an altered morphology but were able to complete their life cycle (Gogarten et al., 1992), suggesting that tonoplast H^+-ATPase was necessary for normal plant growth and development but not essential to the organism's survival. There have been a few reports concerning the activities of tonoplast pumps in giant algal cells (Shimmen and Mac-Robbie, 1987a, 1987b). In these experiments the integrity of the plasma membrane was disrupted surgically so that the cytoplasmic content could be manipulated by perfusion. By altering the content of the perfusate, proton transport driven by either PP or ATP was observed. Therefore, both pumps can contribute to the maintenance of vacuolar acidity in these algal cells. Data concerning which enzymes are involved in the generation of vacuolar acidity in higher plant cells have been more difficult to obtain. Recently, Ellebracht et al. (1994) concluded that only H^+-ATPase was necessary for light-stimulated acidification of the vacuole in tobacco mesophyll cells. However, this research did not address which proton pump was responsible for vacuolar acidity under other conditions and tissues.

We have recently developed a means to monitor the vacuolar pH by fluorescence microscopy after loading BCECF specifically into the vacuoles of root hair cells of maize seedlings (Brauer et al., 1995a). In this study the ability of metabolic inhibitors and BAF to dissipate vacuolar acidity has been assessed. BAF has been shown to be a specific inhibitor of V-type ATPases both in vitro (Bowman et al., 1988) and in vivo (Yoshimori et al., 1991). These results indicated that conditions that led to the accumulation of ADP in root cells caused a change in the characteristics of the maintenance of vacuolar acidity from processes sensitive to BAF to those that were relatively insensitive to BAF.

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Abbreviations: BAF, bafilomycin-A_1; BCECF, 2′7′-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein; BCECF-AM, acetoxymethyl ester of 2′7′-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein; deoxy-Glc, 2-deoxy-d-Glc; H^+-ATPase, proton-translocating ATPase; H^+-PPase, proton-translocating pyrophosphatase; K_I, apparent inhibition constant; PP, pyrophosphate.

MATERIALS AND METHODS

Growing Conditions and Loading Root Hair Cells with BCECF

Corn seedlings (*Zea mays* L. cv FRB73) were grown for 3 d in trays on filter paper moistened with 0.1 mM $CaCl_2$ as described by Nagahashi and Baker (1984). Root hair cells were loaded with BCECF by incubating apical root segments (2–4 cm in length) in 0.1 mM $CaCl_2$ containing 3 μM BCECF-AM for 30 min, and then rinsing in 0.1 mM $CaCl_2$ without dye for 30 min as described by Brauer et al. (1995a).

Fluorescence Microscopy

Root segments containing root hair cells loaded with BCECF were mounted with modeling clay into a sample chamber cell that was perfused at 2 mL min^{-1} with a buffer that routinely contained 50 mM Glc and 2 mM $CaSO_4$ in 10 mM Hepes (titrated to pH 7.0 with KOH). Excitation spectra of BCECF in vacuoles of root hair cells were determined using an FM-2010 fluorescence microscopy system (Photon Technology, Inc., Princeton, NJ)[1] as described in detail elsewhere (Brauer et al., 1997). Briefly, roots were illuminated with excitation light ranging from 300 to 500 nm. Emitted light passed through a 535 ± 20 nm emission filter. The fluorescence from a root hair cell was selected by an adjustable slit that was placed in the emitted light path and detected by a photomultiplier tube. Excitation spectra were compiled using OSCAR software (Photon Technology, Inc., Rockwall, TX). Excitation ratios of 490/440 nm were calculated from the excitation spectra.

Before treatments, excitation spectra were collected from cells for more than 1 h to establish the initial vacuolar pH. Excitation ratios were converted to the pH values by the calibration method reported by Brauer et al. (1997). This calibration method involved clamping the vacuolar pH at the end of the experiment first to that of the external solution by the addition of 10 mM NH_4Cl and then to that of a second perfusion buffer (10 mM Mes titrated with KOH to a pH between 5.0 and 6.5) containing 10 mM NH_4Cl. Calibration measurements obtained from these experiments are reported in Figure 1.

BAF (Sigma) was dissolved in DMSO and added to a rapidly stirring perfusion buffer. The concentration of DMSO in the perfusion buffer was 1% (v/v) of the total volume, a concentration that does not affect root metabolism (Pfeffer et al., 1987). The addition of 1% (v/v) DMSO to the pH 7.0 perfusion buffer had no effect on the fluorescence ratio (494/440) of BCECF in the vacuoles of root hair cells over a 3-h period (data not shown). Perfusion solutions containing BAF were prepared fresh from new vials of products immediately before use. For the deoxy-Glc treatment, 50 mM deoxy-Glc was substituted for the Glc in the pH 7.0 perfusion buffer. An anoxic treatment was imposed by vigorously purging the perfusion buffer with N_2 gas.

[1] Mentioning a brand or product does not constitute an endorsement by the U.S. Department of Agriculture over others of similar nature.

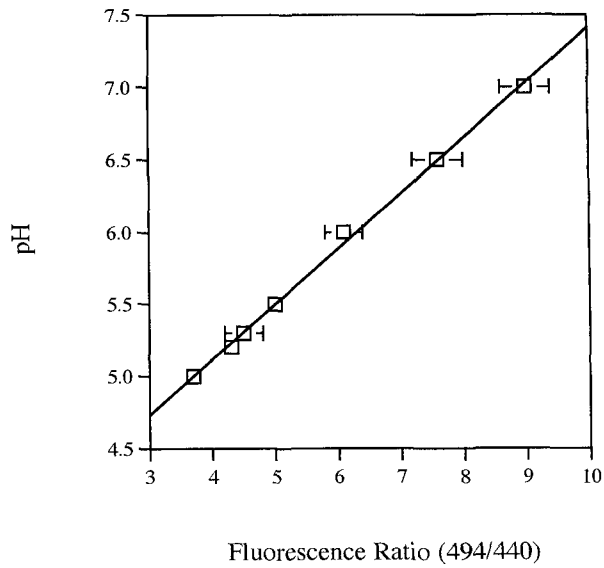

Figure 1. Relationship between fluorescence ratio (494/440) of BCECF and pH in vacuoles of root hair. Fluorescence ratio of vacuolar BCECF in root hair cells was determined at the end of the experiments as described in "Materials and Methods" after incubation in the presence of the pH 7.0 perfusion buffer containing 10 mM NH_4Cl for at least 15 min, and later after a minimum incubation of 15 min in a buffer containing 10 mM NH_4Cl at various pHs between 5 and 6.5. The bar indicates the SE of the fluorescence ratio at each pH.

The BAF-mediated collapse of vacuolar acidity was fitted to first-order kinetics to facilitate comparisons between different treatments. The maximum change in the fluorescence ratio was calculated by subtracting the average fluorescence ratio during the hour before addition of BAF from the maximum fluorescence ratio after addition. The fractional change remaining at each time after the BAF addition then was calculated, and the natural logarithm of the fractional change remaining was plotted as a function of time after the BAF addition. Regression analysis was performed using those data points that constituted the linear portion of the plot. The rate constant for the dissipation of vacuolar acidity was determined from the slope. The length of the lag period was estimated by solving the regression equation for X (minutes) when Y (fraction of total change remaining) was 0.0.

ATP Extraction and Measurement

Total ATP was extracted and quantitated by a bioluminescence assay using luciferase after a modification of the procedure of Saglio and Pradet (1980). Approximately 1 g of 5-mm-long corn root segments comprising the root hair zone was harvested and frozen to the temperature of dry ice in diethyl ether. Frozen segments were transferred to a homogenizer containing 2 mL of 0.6 M TCA at 0°C. After homogenization, the extract was drained off and the residue was extracted four more times for a total volume of 10 mL. The 10 mL of homogenate was centrifuged for 10 min at 20,000*g* at 4°C. The supernatant was extracted three times with 30 mL of diethylether in a separatory funnel. The aqueous phase was collected and bubbled with air on

ice for 30 min to remove any remaining ether. The pH was adjusted to 6.0 with 0.1 M NaOH and held on ice until ATP was quantitated by bioluminescence. The quantity of ATP was determined after mixing 1 mL of firefly lantern extract (Sigma) with 1 mL of root extract and then measuring total emission with a luminescence spectrometer (model LS-5B, Perkin-Elmer). The time course of bioluminescence was recorded, and after 8 min, the emission signal had returned to the original baseline level and 4 μg of ATP was added to serve as an internal standard for quantitation. The signal was integrated after digitization using SigmaScan (Jandel, Inc., San Rafael, CA). Recovery of added ATP was found to be 90 to 95% by the addition of a known amount during the extraction procedure (data not shown).

In Vivo ^{31}P-NMR Spectroscopy

Approximately 3 g of root tissue was placed into a 10-mm NMR tube and perfused at 50 mL min^{-1} with the pH 7.0 perfusion buffer as described previously (Pfeffer et al., 1987). The perfusion buffer in a reservoir containing 200 to 1000 mL was vigorously aerated with either oxygen or oxygen-free nitrogen. ^{31}P-NMR spectra were acquired with a Unity Plus 400 MHz spectrometer (Varian, Palo Alto, CA) equipped with a 10-mm broadband probe using 70° pulses with a repetition time of 0.36 s and no decoupling.

Isolation of Tonoplast Vesicles and Proton Transport Assay

Highly purified tonoplast vesicles were obtained from 3-d-old roots of maize seedlings (cv FRB73) as described previously (Tu et al., 1987). Briefly, microsomal membranes were collected from the supernatant of a 12-min centrifugation at 7,000*g* by centrifugation at 95,000*g* for 40 min. Tonoplast vesicles were purified from the microsomal fraction by isopycnic, Suc density centrifugation. These tonoplast vesicles were essentially devoid of the ER, golgi, plasma membrane, and mitochondria based on the distribution of marker enzymes. Proton transport by the V-type H^+-ATPase and H^+-PPase was assayed by changes in the absorbance of acridine orange as described previously (Brauer et al., 1992; Brauer and Tu, 1994). Initial rates of proton transport were calculated as described previously (Tu et al., 1987). The protein concentration of tonoplast vesicles was determined by a modification of the Lowry procedure (Bensadoun and Weinstein, 1976) using BSA as a standard. Tonoplast preparations contained between 200 and 300 μg protein mL^{-1}.

RESULTS

Effects of BAF on Vacuolar pH

The fluorescence ratio (494/440) of BCECF in the vacuoles of root hair cells was fairly constant for 300 min, when root segments were perfused with a pH 7.0 buffer containing Glc and $CaSO_4$ (Fig. 2). The fluorescence ratio (494/440) over this period averaged 4.2 ± 0.2 (SE) for untreated cells, corresponding to a vacuolar pH of 5.2 ± 0.1. Therefore, actively respiring root hair cells were able to maintain fairly acidic vacuoles.

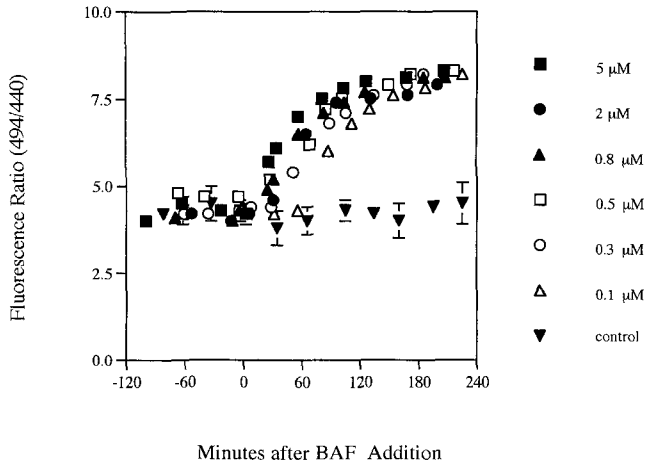

Figure 2. Effects of various BAF concentrations on the vacuolar pH of root hair cells. Vacuoles of root hair cells were loaded with BCECF, and the pH was followed by fluorescence microscopy as described in "Materials and Methods." Root hair cells were observed while being perfused with the pH 7.0 perfusion buffer and up to 240 min after the addition of BAF. At time 0, the perfusion solution was adjusted from 0 to 5 μM BAF (symbol legend is on figure). The data obtained in the presence of BAF are the average of 18 cells from 3 different experiments. The control data are from 4 experiments in which the pH 7.0 perfusion buffer was supplemented with 1% (w/v) DMSO and are the average from a total of 24 cells. SEs are presented as a bar only for the data from controls (i.e. no BAF added). The range of SEs for the control was similar to that of other treatments.

The contribution of the vacuolar H^+-ATPase to the maintenance of vacuolar acidity was assessed by monitoring the effects of BAF, a relatively specific inhibitor of V-type ATPases (Bowman et al., 1988; Yoshimori et al., 1991). The addition of micromolar concentrations of BAF led to an alkalization of the vacuolar lumen, as reflected by an increase in the fluorescence ratio of BCECF (Fig. 2). The fluorescence ratio for BCECF established a steady-state value of 8.1 ± 0.2 (SE) between 120 and 180 min after adding the inhibitor. Replacing the BAF containing the perfusion buffer with a freshly made solution containing BAF did not result in further increases in the fluorescence ratio (494/440) of BCECF (data not shown). Therefore, the steady-state fluorescence ratio of 8.1 appeared to be the maximum increase that BAF could induce. A fluorescence ratio of 8.1 ± 0.2 (SE) corresponded to a vacuolar pH of 6.7 ± 0.1 (SE). Therefore, the addition of BAF collapsed most, but not all, of the pH gradient between the vacuole and the external solution.

Decreasing the BAF concentration from 5 to 0.1 μM did not change the maximum fluorescence ratio that vacuolar BCECF attained, but did change the time dependence (Fig. 2). The time courses for BAF-induced dissipation of vacuolar acidity were analyzed using first-order kinetics to compare the effects of BAF concentration (Table I). Decreasing the BAF concentration from 5 to 0.1 μM resulted in a slight decrease in the first-order rate constant for the dissipation of vacuolar acidity from 0.024 to 0.018 min^{-1}. However, there was no direct correspondence between changes in the BAF concentration and changes in the rate constant. Progressive

Table I. *Characteristics of the dissipation of vacuolar acidity induced by varying concentrations of BAF*

The fluorescence ratio data from Figure 2 were fitted to first-order kinetics to determine the rate constant for the collapse in the pH gradient and the length of the lag between the addition of BAF and the initiation of alkalization of vacuolar pH as described in "Materials and Methods."

BAF	Rate Constant	Lag Period	r (*n*)[a]
μM	min^{-1}	min	
5	0.024	6	0.99 (7)
2	0.026	6	0.97 (5)
0.8	0.019	9	0.99 (6)
0.5	0.018	23	0.98 (5)
0.3	0.018	31	0.99 (6)
0.1	0.018	53	0.99 (6)

[a] r and (*n*) refer to the correlation coefficient and number of observations, respectively, in the regression analysis used to calculate the rate constant and length of the lag.

decreases in the BAF concentration resulted in progressive increases in the length of the lag between the addition of BAF and initiation of alkalization of vacuolar pH. Decreasing the BAF concentration from 5 to 0.1 μM resulted in about a 9-fold increase in the lag period from 6 to 53 min. These analyses indicated that differences in the time course of the alkalization of vacuolar pH over the tested range of BAF concentrations resulted primarily from changes in the lag phase, and lowering the BAF to 0.1 μM did not reduce its ability to dissipate vacuolar acidity. In addition, the use of 2 μM BAF appeared to be sufficient to inhibit the vacuolar H^+-ATPase in living root hair cells as rapidly as possible.

Effects of Inhibitors and Anoxic Treatment on Tissue ATP and ADP

Because the data in Figure 2 indicated that the vacuolar H^+-ATPase was responsible for a majority of the proton transport into the vacuolar lumen, the ability of various inhibitors to alter the ATP content of root tissue was determined as a first step to assess the ATP dependence of vacuolar acidity in vivo. The ATP content of root segments comprising the root hair zone averaged 57.4 ± 2.8 μg g^{-1} fresh weight over 120 min of incubation in the pH 7.0 perfusion buffer (Fig. 3). The addition of 2 μM BAF had no effect on total ATP during the first 60 min of incubation, suggesting that BAF did not grossly disturb the metabolism. By the end of 120 min of BAF treatment, total ATP had declined by about 30%. The decrease in total ATP at 120 min may not reflect a direct effect of BAF on respiration, but rather a depression of metabolic activity due to acidification of the cytoplasm in response to the alkalization of the vacuole (Fig. 2).

Total ATP content of root segments was assessed in the presence of two different metabolic inhibitors, deoxy-Glc and KCN, and under an anoxic condition induced by purging the perfusion buffer with N_2 gas. The addition of 50 mM deoxy-Glc led to a progressive decline in total ATP over 180 min of treatment (Fig. 3). The addition of KCN resulted in a rapid decrease in ATP during the first 120 min followed by a slight increase between 120 and 180 min of treatment. The rate of decrease in total ATP over the first 2 h of treatment was less in the presence of deoxy-Glc, as compared with that induced by KCN. After 60 and 120 min, the ATP content of root segments treated with KCN was 24 ± 2 and 21 ± 2% of control, respectively, as compared with 42 ± 3 and 30 ± 2% of control, respectively, for segments treated with deoxy-Glc. After 180 min, root segments treated with deoxy-Glc had lower levels of ATP than roots treated with KCN, 9 ± 2 versus 33 ± 2% of control. Therefore, the addition of either 50 mM deoxy-Glc or 10 mM KCN was able to lower total ATP significantly over the course of a few hours.

The anoxic treatment decreased tissue ATP less than either KCN or deoxy-Glc (Fig. 3). Under anoxia tissue ATP had declined to 57 ± 6 and 49 ± 3% of control values after 60 and 120 min, respectively. There was a slight increase in ATP content between 120 and 180 min of anoxic treatment to 57 ± 2% of control values.

Because ADP has been shown to be a potential inhibitor of proton transport by the vacuolar H^+-ATPase in isolated maize root tonoplast vesicles (Brauer and Tu, 1994), the presence of ADP in response to either the addition of deoxy-Glc and KCN or the anoxic treatment was determined by in vivo ^{31}P-NMR spectroscopy (Fig. 4). When corn root tissue was incubated in the pH 7.0 perfusion buffer (Fig. 4, spectra A), the presence of ADP could not be detected. Spectra of ^{31}P metabolites gathered during the 2nd h of anoxic treatment indicated the presence of ADP (Fig. 4B). The intensity of the β-P ADP signals was greater with the KCN treatment (Fig. 4D) than during the anoxic

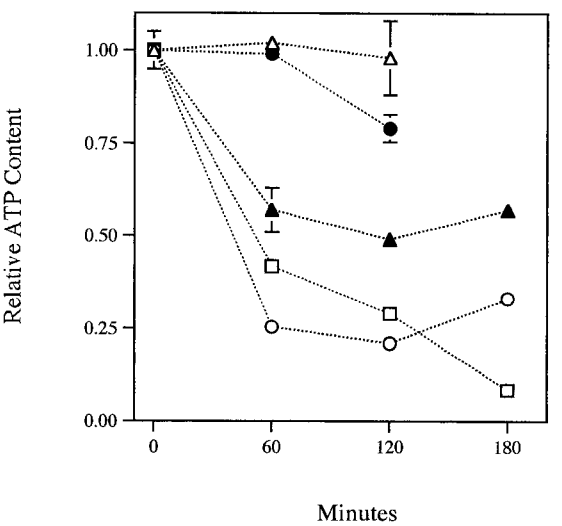

Figure 3. Effects of 2 μM BAF, 10 mM KCN, or 50 mM deoxy-Glc and anoxic treatment on total ATP in root segments comprising the root hair zone. Root segments comprising the root hair zone were incubated in the pH 7.0 perfusion buffer (△) or the buffer containing 2 μM BAF (●), 10 mM KCN (○), or 50 mM deoxy-Glc (□) for up to 180 min. The anoxic treatment (▲) was imposed by perfusing root segments with the buffer that had been vigorously purged with N_2. The data are the average of six determinations from three experiments. Bars representing the standard error are presented where the variation exceeds that of the data symbol.

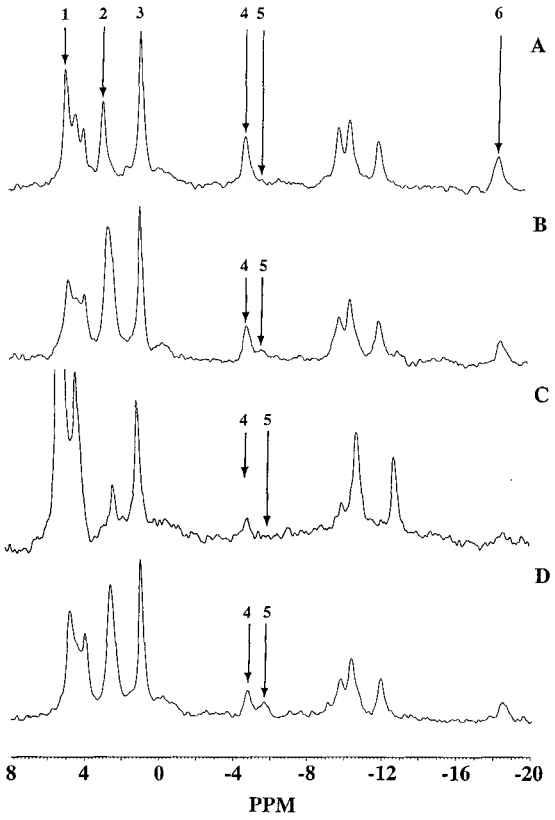

Figure 4. Effects of KCN, deoxy-Glc, or anoxic treatment on ^{31}P metabolites in corn root tissue. Root tissue was incubated with the pH 7.0 perfusion buffer (A), anoxia-treated perfusion buffer (B), the perfusion buffer containing 50 mM deoxy-Glc instead of Glc (C), and perfusion buffer containing 10 mM KCN (D). After 90 to 100 min of incubation, ^{31}P-NMR spectra were acquired over a 40- to 50-min period and presented as described in "Materials and Methods." Peaks designated by arrows are: Glc-6-phosphate (1); cytoplasmic Pi (2); vacuolar Pi (3); γ-P of ATP (4); β-P of ADP (5); and uridinediphosphate Glc (6).

treatment (Fig. 4B). There was no detectable quantity of ADP during the 2nd h of treatment with deoxy-Glc.

In summary, the results from ^{31}P-NMR spectroscopy indicated that ADP accumulated during the anoxic treatment and in the presence of KCN. However, no detectable quantity of ADP was observed during treatment with deoxy-Glc. In addition, the relative effects of treatments on ATP levels as determined by in vivo ^{31}P-NMR spectrometry were similar to those found by the bioluminescence assay after extraction. These results are consistent with those published previously (Gerasimowicz et al., 1986; Pfeffer et al., 1987).

Effects of KCN, Deoxy-Glc, and Anoxia on Vacuolar pH

During the hour before the addition of either 10 mM KCN or 50 mM deoxy-Glc to the pH 7.0 perfusion, the fluorescence ratio of BCECF in the vacuoles of root hair cells averaged 4.3 ± 0.2 (Fig. 5), corresponding to a vacuolar pH of 5.2 ± 0.1. There was no significant change in the fluorescence ratio during the 120-min exposure to either metabolic inhibitor. In addition, there was no significant change in the fluorescence ratio of BCECF in the vacuoles of the root hair cells of root tissue subjected to the anoxic treatment. Therefore, the root hair cells were able to maintain vacuolar acidity despite greatly decreased levels of ATP.

To determine if H^+-ATPase was responsible for the maintenance of vacuolar acidity during treatment with deoxy-Glc, KCN, and anoxia, the effects of 2 μM BAF on the vacuolar pH of root hair cells was measured under these conditions (Fig. 6). In these experiments the perfusion solution was supplemented for 60 min before the addition of BAF with 10 mM KCN or 50 mM deoxy-Glc or purged with N_2 to ensure that the changes in metabolism had begun before assessing the effects of BAF. In the presence of 50 mM deoxy-Glc the addition of 2 μM BAF led to the alkalization of the vacuolar pH, as reflected by an increase in the fluorescence ratio (494/440) of BCECF. The fluorescence ratio in the presence of BAF and deoxy-Glc rose from 4.1 ± 0.1 to 7.9 ± 0.3, corresponding to a change in vacuolar pH from 5.2 ± 0.1 to 6.6 ± 0.1. The dissipation of vacuolar acidity in the presence of deoxy-Glc was similar to that found with normally respiring root hair cells (Fig. 2). When the data for the dissipation of vacuolar acidity were fitted to first-order kinetics as in Table I, the rate constant for dissipation was 0.020 min^{-1} and the length of the lag was 7 min. The correlation coefficient (r) and number of observations (n) in the regression analysis were 0.97 and 5,

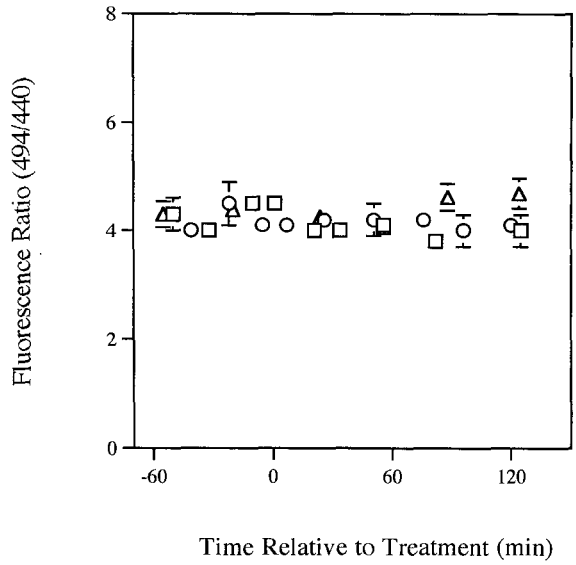

Figure 5. Effects of 10 mM KCN, 50 mM deoxy-Glc, or anoxic treatment on vacuolar pH in root hair cells. Vacuoles of root hair cells were loaded with BCECF, and the pH was followed by fluorescence microscopy as described in "Materials and Methods." Root hair cells were observed while being perfused with the pH 7.0 perfusion buffer up to 120 min after the addition of either KCN or deoxy-Glc. At time 0, the perfusion solution was adjusted to 10 mM KCN (○) or 50 mM deoxy-Glc (□). Root hair cells exposed to the anoxic treatment were perfused in the buffer that had been vigorously purged with N_2 (△). The data are the average of 24 cells from 4 different experiments. Bars representing the standard error are presented where the variation exceeds the size of the data symbol.

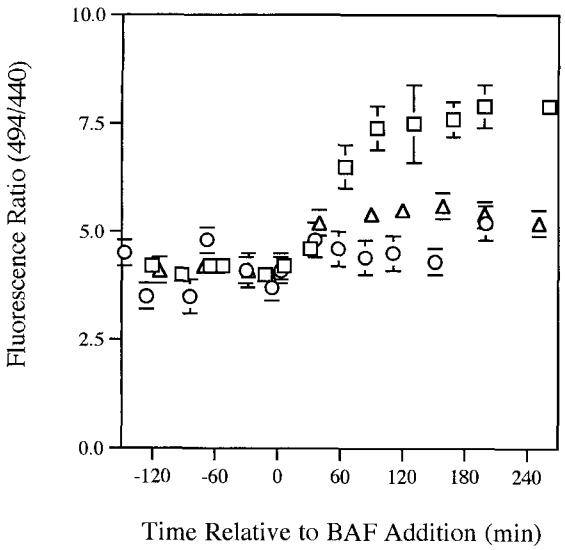

Figure 6. Effects of 2 μM BAF on the vacuolar pH of root hair cells treated with deoxy-Glc, KCN, or anoxia. Vacuoles of root hair cells were loaded with BCECF, and the pH was followed by fluorescence microscopy as described in "Materials and Methods." Root hair cells were observed while being perfused with the pH 7.0 perfusion buffer up to 240 min after the addition of 2 μM BAF. At time −60 min, the perfusion solution was adjusted to either 10 mM KCN (○) or deoxy-Glc (□), or purged vigorously with N_2 (△). At time 0, the perfusion solution was adjusted to 2 μM BAF. The data are the average of 24 cells from 4 different experiments. Bars representing the standard error are presented where the variation exceeds the size of the data symbol.

respectively. These values were not significantly different from those obtained with 2 μM BAF in Table I. Therefore, the presence of deoxy-Glc did not affect the sensitivity of vacuolar acidity to BAF, indicating that the majority of the vacuolar acidity was being maintained by the tonoplast H^+-ATPase, despite a significant reduction in ATP content.

However, the addition of 10 mM KCN to the perfusion solution had a profound effect on the sensitivity of vacuolar acidity to 2 μM BAF (Fig. 6). In the presence of KCN, there was no change in the fluorescence ratio (494/440) of BCECF in the vacuoles of root hair cells for at least 150 min after the addition of BAF. There was a slight increase in the fluorescence ratio (494/440) to 5.2 ± 0.4 after 200 min of exposure to BAF in the presence of KCN. These results indicated that the sensitivity of vacuolar acidity to BAF was much less in the presence of KCN as compared with that in control and deoxy-Glc-treated root hair cells.

The effects of the anoxic treatment on the ability of BAF to dissipate vacuolar acidity more closely resembled those found in the presence of KCN than in either the control (Fig. 2, 2 μM BAF) or presence of deoxy-Glc (Fig. 6). During the first 120 min of treatment to BAF under the anoxic treatment, there was a tendency for the fluorescence ratio of vacuolar BCECF to increase slightly, indicative of a small increase in pH. However, the rate of increase in the fluorescence ratio and extent of change between 120 and 240 min of exposure to BAF was significantly less than that observed in the presence of deoxy-Glc (Fig. 6) or under control circumstances (Fig. 2).

Effects of KCN and ADP on Proton Transport in Vitro

The concentration dependence of BAF inhibition of the maize root V-type H^+-ATPase was assessed in highly purified tonoplast vesicles to explore the possibility that either KCN or ADP has an intrinsic effect on the sensitivity of the vacuolar H^+-ATPase to BAF (Fig. 7). In the absence and presence of 10 mM KCN, proton transport was completely inhibited by less than 2 nM BAF. The BAF concentration causing 50% inhibition in the initial rate of proton transport was about 0.3 nM, independent of the presence of KCN. Therefore, the presence of KCN had no effect on the sensitivity of the tonoplast H^+-ATPase to BAF in vitro. Also, the addition of 0.05 mM ADP had no significant effect on the apparent K_I for BAF (Fig. 7). An ADP concentration of 0.05 mM was chosen because it approximates the apparent K_I (Brauer and Tu, 1994). In agreement with earlier findings, the addition of 0.05 mM ADP reduced the maximum initial rate of proton transport from 1.12 ± 0.08 A min^{-1} mg^{-1} protein in controls to 0.60 ± 0.05 A min^{-1} mg^{-1} protein (see legend of Fig. 7).

DISCUSSION

Root hair vacuoles of a root segment undergoing normal respiration were not able to maintain their acidity in the presence of relatively low concentrations of BAF (Fig. 2), a specific inhibitor of V-type H^+-ATPase (Bowman et al.,

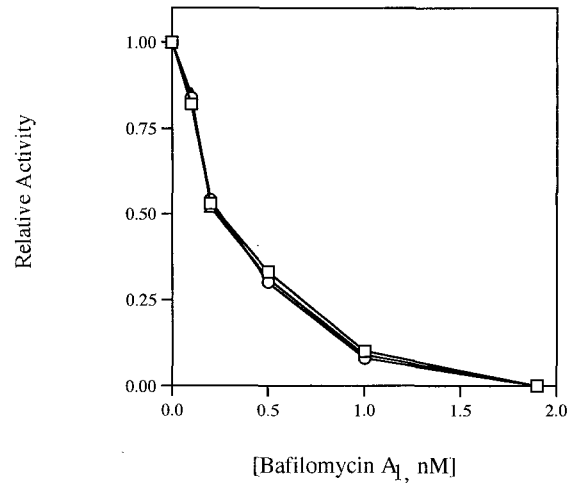

Figure 7. Effects of 10 mM KCN or 0.05 mM ADP on the sensitivity of proton transport by the V-type H^+-ATPase to BAF in isolated tonoplast vesicles. Tonoplast vesicles were isolated from maize roots and proton transport assayed as described in "Materials and Methods." The initial rate of ATP-dependent proton transport was assayed in the absence (□) and presence of either 10 mM KCN (○) or 0.05 mM ADP (△) after the addition of 0 to 2 nM BAF. The data are the average of two experiments, each with three replicates. Data obtained in the absence and presence of KCN are plotted relative to the activity in the absence of BAF and either KCN or ADP, which had an average activity of 1.12 ± 0.08 A min^{-1} mg^{-1} protein. The data symbols exceeded the range of the standard errors for all observations. Data collected in the presence of ADP are plotted relative to the initial rate observed in the presence of 0.05 mM ADP and 0 nM BAF, which averaged 0.60 ± 0.05 A min^{-1} mg^{-1} protein.

1988; Yoshimori et al., 1991). The sensitivity of vacuolar pH to BAF under these conditions suggested that a major portion of the proton transport across the tonoplast membrane was driven by the V-type H^+-ATPase. However, BAF was not able to dissipate all of the acidity in the vacuoles of maize root hair cells. In contrast, the addition of NH_4Cl or protonophores dissipated all of the acidity in vacuoles of root hair cells, as evident from the equilibration of pH in the vacuoles with that of the external buffer (Brauer et al., 1997). The vacuoles of root hair cells attained a maximum pH of about 6.7 in the presence of BAF at an external pH 7.0, as compared with a vacuolar pH of 7.0 after the addition of NH_4Cl. The significance of this observation is not clear. It is possible that proton transport by the H^+-PPase was able to maintain this small pH gradient in maize root hair cells performing aerobic metabolism.

Vacuoles of root hair cells were able to maintain vacuolar acidity under conditions in which total ATP was substantially reduced by the addition of deoxy-Glc (Figs. 3 and 5). The concentration of cytoplasmic ATP in corn root has been estimated to be about 2 mM (Roberts et al., 1985; Pfeffer et al., 1987). Such a level is consistent with the intensity for γ-P of ATP in the NMR spectra of control roots (Fig. 4) and a total tissue content of 57 μg ATP g^{-1} fresh weight (Fig. 3), assuming that the cytoplasm constituted about 5% of the total tissue volume. Therefore, a reduction in total ATP by 70% after 2 h of dexoy-Glc treatment corresponded to a cytoplasmic ATP concentration of about 0.6 mM. The K_m for ATP for the tonoplast H^+-ATPase from maize roots is about 0.2 mM based on in vitro characterization (Brauer and Tu, 1994; Tu et al., 1987). Therefore, the reduction in ATP by a 2-h treatment with deoxy-Glc probably was not sufficient to substantially reduce the activity of tonoplast H^+-ATPase in vivo, thereby accounting for the maintenance of vacuolar acidity. The involvement of the H^+-ATPase in maintaining vacuolar acidity in the presence of deoxy-Glc was supported by the observation that most of the vacuolar acidity was dissipated by 2 μM BAF (Fig. 6).

We decided to follow ADP accumulation in response to the treatments by in vivo ^{31}P NMR for several reasons. First, analysis of extractable ADP tends to overestimate the amount that is present because of hydrolysis of ATP to ADP during the procedures (Roberts, 1986). Second, Hooks et al. (1994) demonstrated that much of the ADP in extracts of corn roots undergoing anaerobic metabolism was bound tightly to protein. Therefore, it could be quite difficult to estimate the levels of free ADP and hence the degree of inhibition to the tonoplast H^+-ATPase from extract data without substantial information on the binding characteristics of many of the cytoplasmic proteins from corn roots. However, in vivo ^{31}P NMR provides an estimate of the free-cytoplasmic concentrations of ADP (Hooks et al., 1989), which can be compared directly with the inhibitory constants generated for ADP in vitro.

Either the anoxic treatment or the addition of KCN resulted in the accumulation of free ADP as detected by ^{31}P NMR spectroscopy (Fig. 4). The detection limit of ADP for in vivo ^{31}P NMR spectroscopy under these conditions is about 50 μM (Roberts et al., 1985; Roberts, 1986; Pfeffer et al., 1987). The in vitro activity of the maize tonoplast H^+-ATPase was substantially reduced in the presence of 0.5 to 1.0 mM ATP when the ADP concentration was either 60 or 100 μM (Brauer and Tu, 1994). Therefore, the activity of the tonoplast H^+-ATPase in vivo should have been substantially reduced by the anoxic and KCN treatments, based on estimated cytoplasmic concentrations of 0.5 to 1.0 mM ATP and 0.1 to 0.2 mM ADP (Figs. 3 and 4). However, no dissipation in vacuolar acidity was observed during the first 2 h of these two treatments (Fig. 5). There are at least two possible hypotheses to account for the lack of an effect of either anoxia or KCN on the ability of maize root hair cells to maintain vacuolar acidity. First, the in vivo characteristics of the tonoplast H^+-ATPase are different from those derived from in vitro characterization. Second, vacuolar acidity in root hair cells treated with anoxia or KCN was maintained by a process other than the tonoplast H^+-ATPase.

The following data are consistent with the second hypothesis. Vacuolar acidity in maize root hair cells treated with anoxia or KCN was not dissipated by 2 μM BAF (Fig. 6). This might suggest a direct effect of either ADP or KCN on the sensitivity of the tonoplast H^+-ATPase to BAF. However, in vitro, the presence of either KCN or ADP had no effect on the K_I for BAF to inhibit proton transport by the H^+-ATPase (Fig. 7), indicating that KCN or ADP did not alter the sensitivity of the H^+-ATPase to BAF. In addition, the inhibition mechanism of BAF on the V-type H^+-ATPase affects proton transport directly without an increase in proton leakage from the vesicles (Crider et al., 1994). Taking the above observations into consideration, the inability of BAF to dissipate vacuolar acidity during treatment with anoxia or KCN (Fig. 6) seems to indicate that proton transport by H^+-ATPase was not contributing to vacuolar acidity under these conditions, but rather some other pump, possibly the H^+-PPase, was responsible.

Therefore, the data in Figure 6 are consistent with the previously proposed hypothesis (Brauer et al., 1992; Rea and Poole, 1993; Brauer and Tu, 1994; Brauer et al., 1995b) that the V-type H^+-ATPase maintains vacuolar acidity in normally respiring root cells, whereas the H^+-PPase maintains vacuolar acidity under cellular conditions that favor the accumulation of ADP. Additional experiments indicated that changes in the sensitivity of vacuolar pH to BAF induced by the anoxic treatment were not associated with a change in the activities of the H^+-ATPase and H^+-PPase in isolated membranes. Microsomes isolated from roots after a 3-h incubation in perfusion buffer purged with N_2 had similar levels of H^+-PPase and H^+-ATPase as microsomes isolated from control roots. The initial rate of proton transport by the H^+-ATPase and H^+-PPase in microsomes from control roots averaged 1.32 ± 0.06 and 0.49 ± 0.02 A min^{-1} mg^{-1} protein, respectively, as compared with 1.30 ± 0.05 and 0.46 ± 0.02 A min^{-1} mg^{-1} protein, respectively, for microsomes isolated from roots purged with N_2 for 3 h ($n = 3$). Similar results were found with corn roots by Carystinos et al. (1995). In contrast, the activity, protein, and mRNA levels of the vacuolar H^+-PPase in rice increased by as much as

75-fold over a 6-d anoxia treatment (Carystinos et al., 1995). Therefore, if changes in proton pumping are occurring in corn roots under conditions in which ADP content is high, it does not appear to result in changes in the activities of the pumps in isolated membranes.

Although these results indicated that metabolic conditions favoring ADP accumulation resulted in a change in the sensitivity of vacuolar acidity to BAF, the role of ADP may be to reduce the activity of the H^+-ATPase, rather than to activate some other H^+ transport process. One benefit from complete inhibition of the H^+-ATPase could be a decrease in the permeability of the tonoplast membrane to protons. The permeability of maize root tonoplast was substantially less when proton transport was catalyzed by the H^+-PPase alone (Brauer et al., 1992). Apart from the accumulation of ADP in root cells, the inhibition of aerobic respiration would be accompanied by other changes in the concentrations of metabolites and metal ions. For example, increases in cytosolic Mg^{2+} occur concomitantly with decreases in the ATP/ADP ratio (Yakasaki et al., 1988), as observed by changes in the chemical shift of α and β phosphate of ATP by ^{31}P-NMR. In our experiments very little change in the relative position of these two peaks was observed. However, our experimental conditions were not optimized to observe signals from the phosphates of ATP. The significance of changes in cytosolic Mg^{2+} may be of interest, because certain experiments have suggested that free Mg^{2+} is a positive allosteric modulator of the H^+-PPase (White et al., 1990). It seems apparent that further investigations are necessary to understand the factor(s) that result in the shift in the sensitivity of vacuolar pH to BAF under those conditions that promote ADP accumulation. Experiments to demonstrate the involvement of H^+-PPase in the maintenance of vacuolar acidity of maize root hair cells are not possible yet because a specific, cell-permeable inhibitor of the H^+-PPase is not available.

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LITERATURE CITED

Bensadoun A, Weinstein D (1976) Assay of proteins in the presence of interfering materials. Anal Biochem **70:** 241–250

Bowman EM, Siebers A, Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells and plant cells. Proc Natl Acad Sci **85:** 7972–7976

Brauer D, Conner D, Tu S (1992) Effects of pH on proton transport by vacuolar pumps from maize roots. Physiol Plant **86:** 63–70

Brauer D, Otto J, Tu S (1995a) Selective accumulation of the fluorescent pH indicator, BCECF, in vacuoles of maize roothair cells. J Plant Physiol **145:** 57–61

Brauer D, Otto J, Tu S-I (1995b) Nucleotide binding is insufficient to induce cold inactivation of the vacuolar-type ATPase from maize roots. Plant Physiol Biochem **33:** 555–559

Brauer D, Tu S (1994) Effect of ATP analogs on proton pumping by the vacuolar H^+-ATPase from maize roots. Physiol Plant **91:** 442–448

Brauer D, Uknalis J, Triana R, Tu S (1997) Effects of external pH and NH_4^+ on vacuolar pH in maize roothair cells. Plant Physiol Biochem (in press)

Carystinos GD, MacDonald HR, Monroy AF, Dhindsa, Poole RJ (1995) Vacuolar H^+-translocating pyrophosphatase is induced by anoxia or chilling in seedlings of rice. Plant Physiol **108:** 641–649

Crider BP, Xie XS, Stone DK (1994) Bafilomycin inhibits proton flow through the H^+ channel of vacuolar proton pumps. J Biol Chem **269:** 17379–17381

Ellebracht A, Heber V, Sonnewald V (1994) Light stimulated proton transport into vacuoles of leaf mesophyll cells does not require energization by tonoplast pyrophosphatase. Planta **193:** 203–207

Gerasimowicz W, Tu S, Pfeffer P (1986) Energy facilitated Na^+ uptake in excised corn roots via ^{31}P and ^{23}Na NMR. Plant Physiol **81:** 925–928

Gogarten JP, Fichmann J, Braun Y, Morgan L, Styles P, Taiz SL, DeLapp K, Taiz L (1992) The use of antisense mRNA to inhibit the tonoplast H^+-ATPase in carrot. Plant Cell **4:** 851–864

Hooks MA, Clark RA, Nieman RH, Roberts JKM (1989) Compartmentation of nucleotides in corn root tips studied by ^{31}P-NMR and HPLC. Plant Physiol **89:** 963–969

Hooks MA, Shearer GC, Roberts JKM (1994) Nucleotide availability in maize (*Zea mays* L.) root tips: estimation of free and protein-bound nucleotides using ^{31}P-nuclear magnetic resonance and a novel protein-ligand-binding assay. Plant Physiol **104:** 581–589

Kurkdjian A, Guern J (1989) Intracellular pH: measurement and importance in cell activity. Annu Rev Plant Physiol **40:** 271–303

Maeshima M (1991) H^+-translocating inorganic pyrophosphatase of plant vacuoles: inhibition by Ca^{2+}, stabilization by Mg^{2+} and inorganic pyrophosphatases. Eur J Biochem **196:** 11–17

Nagahashi G, Baker AF (1984) β-glucosidase activity in corn root homogenates: problems in subcellular fractionation. Plant Physiol **72:** 837–846

Nelson N, Taiz L (1989) The evolution of H^+-ATPase. Trends Biochem Sci **14:** 113–116

Pfeffer P, Tu S, Gerasimozica W, Boswell R (1987) Role of the vacuole in metal ion trapping as studied by in vivo ^{31}P-NMR spectroscopy. In B Martin, ed, Plant Vacuoles. Plenum Publishing Corp., New York, pp 349–359

Rea PA, Poole RJ (1993) Vacuolar H^+-translocating pyrophosphatase. Annu Rev Plant Physiol **44:** 157–80

Roberts JKM (1986) Determination of the energy status of plant cells by ^{31}P-nuclear magnetic resonance spectroscopy. *In* HF Linskins, JF Jackson, eds, Modern Methods of Plant Analysis, Vol 2. Springer-Verlag, New York, pp 43–59

Roberts JKM, Lane AN, Clark KA, Nieman RH (1985) Relationships between the rate of synthesis of ATP and concentrations of reactants and products of ATP hydrolysis in maize root tips, determined by ^{31}P nuclear magnetic resonance. Arch Biochem Biophys **240:** 712–722

Saglio PH, Pradet A (1980) Soluble sugars, respiration and energy charge during aging of excised maize root tips. Plant Physiol **66:** 516–519

Shimmen T, MacRobbie EAL (1987a) Demonstration of two proton translocating systems in tonoplast of permeablized Nitella cells. Protoplasma **136:** 205–207

Shimmen T, MacRobbie EAL (1987b) Characterization of two proton transport systems in the tonoplast of plasmalemma permeablized Nitella cells. Plant Cell Physiol **28:** 1023–1031

Sze H (1985) H^+-translocating ATPases: advances using isolated vesicles. Annu Rev Plant Physiol **36:** 175–208

Tu S-I, Nagahashi G, Brouillette JN (1987) Proton transport and origin of nitrate inhibition of tonoplast-type H^+-ATPase. Arch Biochem Biophys **266:** 289–297

White PJ, Marshall J, Smith JAC (1990) Substrate kinetics of the tonoplast H^+-translocating inorganic pyrophosphatase and its activation by free Mg^{2+}. Plant Physiol **93:** 1063–1070

Yakasaki Y, Asukagawa N, Ishikawa Y, Ohta E, Sakata M (1988) Estimation of cytoplasmic free Mg^{2+} levels and phosphorylation potential in mung bean root tips by in vivo ^{31}P NMR spectroscopy. Plant Cell Physiol **29:** 919–924

Yoshimori T, Yamamoto A, Yoshinori Y, Futai M and Tashiro Y (1991) Bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPase inhibits acidification and protein degradation in lysosomes of cultured cells. J Biol Chem **266:** 17707–17712