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Gibberellic Acid Induces Vacuolar Acidification in Barley Aleurone

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The roles of gibberellic acid (GA_3) and abscisic acid (ABA) in the regulation of vacuolar pH (pH_v) in aleurone cells of barley were investigated using the pH-sensitive fluorescent dye 2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). BCECF accumulated in vacuoles of aleurone cells, but sequestration of the dye did not affect its sensitivity to pH. BCECF-loaded aleurone cells retained their ability to respond to both GA_3 and ABA. The pH_v of freshly isolated aleurone cells is 6.6, but after incubation in GA_3, the pH_v fell to 5.8. The pH_v of cells not incubated in hormones or in the presence of ABA showed little or no acidification. The aleurone tonoplast contains both vacuolar ATPase and vacuolar pyrophosphatase, but the levels of pump proteins were not affected by incubation in the presence or absence of hormones. We conclude that GA_3 affects the pH_v in aleurone cells by altering the activities of tonoplast H^+ pumps but not the amounts of pump proteins.

INTRODUCTION

The vacuole is the most prominent organelle in most plant cells and can occupy >90% of the volume of the cell. In addition to facilitating cell growth by maintaining turgor, the vacuole is the main subcellular compartment for toxin sequestration, nonphotosynthetic pigment storage, and phenolic and alkaloid accumulation (Taiz, 1992). Vacuoles also serve as lytic organelles, analogous to the mammalian lysosome, during senescence and programmed cell death (Boller and Wiemken, 1986). Although the function of the vacuole depends on developmental stage and cell type, most vacuoles are acidic and contribute to the regulation of cytoplasmic pH and ionic homeostasis via transport across the tonoplast (Taiz, 1992). The endosperm and cotyledons of fruits and seed contain vacuoles for long-term storage (Okita and Rogers, 1996). Stored proteins, carbohydrates, organic acids, and inorganic ions such as phosphate and calcium are remobilized during germination (Chrispeels, 1991). Vacuoles in the aleurone cells of cereal grains function in this respect and participate in the hormonal responses of aleurone tissue.

Aleurone cells from barley respond to gibberellic acid (GA_3) by upregulating metabolism, altering gene expression, modifying vacuolar morphology, and ultimately secreting hydrolases. The secreted hydrolases, including α-amylase, degrade stored reserves in the starchy endosperm, making them available for uptake by the scutellum and use by the growing embryo. Abscisic acid (ABA), an endogenous inhibitor of grain germination, antagonizes these effects of GA_3 and induces other responses in aleurone cells (Fincher, 1989; Jones and Jacobsen, 1991). Hydrolases secreted by the aleurone cell during the GA_3 response are newly synthesized from amino acids previously incorporated as storage protein in aleurone vacuoles (Filner and Varner, 1967). Thus, breakdown of vacuolar reserves and transport of amino acids and ions across the tonoplast are key aspects of the aleurone cell's response to GA_3. Vacuolar hydrolases, such as the proteases responsible for the mobilization of storage proteins and the acid phosphatases responsible for the mobilization of phyin, have acidic pH optima of 5 or below (Runeberg-Roos et al., 1991; Holwerda and Rogers, 1992; Bethke et al., 1996), suggesting a role for pH in the regulation of lytic metabolism in aleurone vacuoles.

The mammalian lysosome undergoes lumenal acidification by ATP-driven proton pumps to activate proteolytic digestion. As endosomes progress to biochemically distinct lysosomes, the internal pH drops from a mildly acidic pH 6 to a pH of ~5, the optimal pH for activity of lysosomal hydrolases (Bainton, 1981). Several observations suggest that the plant vacuolar pH (pH_v) is also regulated. In photosynthetic cells, the pH_v can fluctuate in the light (Yin et al., 1990a, 1990b). In plants with crassulacean acid metabolism, the pH_v can vary diurnally from a pH 3 at night to a pH 6 during the day (Lüttge, 1987). pH_v can also change during development; for example, during lemon fruit maturation, the pH_v can drop from 6.2 to as low as 2.2 (Sinclair, 1984). Stresses, such as anoxia (Menegus et al., 1991) and chilling (Yoshida, 1995), can also alter pH_v. Little is known, however, about the pH_v in plant cells that respond to hormones.

Similar to the mammalian lysosomal membrane, plant tonoplasts contain proton pumps able to alter the pH_v. These H^+ pumps establish the inside-positive electrochemical

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gradient for H^+, which drives secondary transport of solutes (Blumwald and Poole, 1985, 1986). Tonoplast pumps lower the pH of the vacuole lumen to values that are often two pH units or more below that of the cytosol (Sze, 1985; Boller and Wiemken, 1986). Plants possess two H^+-translocating phosphohydrolases in the tonoplast, the vacuolar ATPase (V-ATPase; EC 3.6.1.3) and the vacuolar pyrophosphatase (V-PPase; EC 3.6.1.1). Although the V-ATPase is found in endomembranes of all eukaryotes, the V-PPase is found only in plants (Nelson and Taiz, 1989; Rea and Poole, 1993).

In this article, we describe experiments using the fluorescent pH indicator BCECF (2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein) to make noninvasive measurements of pH_v in barley aleurone protoplasts. We show that protoplasts responding to GA_3 acidify the vacuole lumen in the course of a few hours from pH 6.6 to 5.8 or below. Vacuoles in protoplasts incubated in ABA or in the absence of hormones become acidified at a much slower rate or not at all. The tonoplast of aleurone vacuoles contains both V-ATPase and V-PPase, and our experiments show that these proton pumps are present in vacuoles of protoplasts treated with GA_3 or ABA as well as in untreated control protoplasts. Our data indicate that GA_3 stimulation of vacuole acidification in the aleurone cell is caused by increased activity of tonoplast proton pumps and not by increased amounts of pump proteins in the vacuolar membrane.

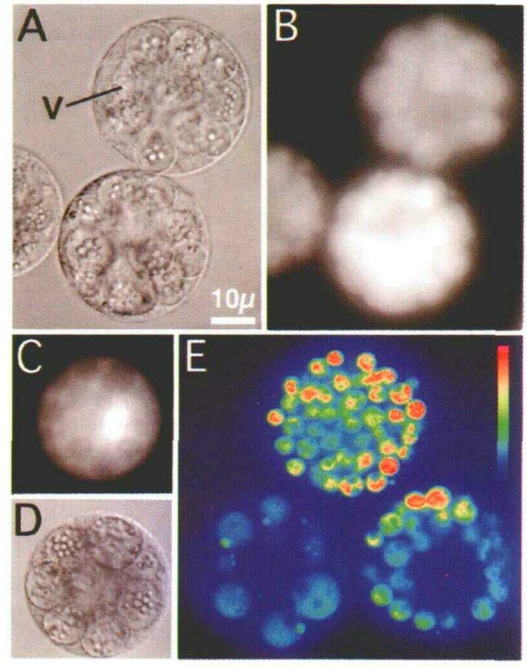

Figure 1. BCECF Loading in Barley Aleurone Protoplasts.

(A) Protoplast loaded with BCECF and observed using differential interference contrast (DIC) optics.

(B) Fluorescence emission (525 ± 25 nm) from 440-nm excitation of BCECF in the vacuoles 40 min after loading. Same protoplasts as are shown in **(A)**.

(C) Fluorescence emission (525 ± 25 nm) from 440-nm excitation of BCECF in the cytoplasm 10 min after loading.

(D) Unloaded (control) protoplast observed using DIC optics.

(E) Confocal image (488-nm excitation) of protoplasts containing vacuolar BCECF. Pseudocolor enhances visualization of dye distribution and indicates intensity of fluorescence, where blue is minimum and red is maximum intensity.

Bar in **(A)** = 10 μm for **(A)** to **(E)**. V, vacuole.

RESULTS

BCECF Accumulates in the Vacuoles of Aleurone Protoplasts

As seen in Figures 1A and 1B, barley aleurone protoplasts contain numerous vacuoles that accumulate the fluorescent pH indicator BCECF when the protoplasts are incubated with BCECF-AM, the membrane-permeant acetoxymethyl ester form of the dye. Each vacuole contains crystalline inclusions of phytin (a Ca^{2+}, K^+, and Mg^{2+} salt of inositol hexaphosphate), storage proteins, and carbohydrates, but total autofluorescence from these inclusions and the cytosol is <1% of the fluorescence signal from BCECF. BCECF fluorescence first appeared in the cytoplasm 6 to 10 min after BCECF-AM was added to the protoplast suspension, as shown in Figure 1C. We conclude that hydrolysis of the AM ester groups of the dye occurs in the cytoplasm. This conclusion is supported by our observation that no measurable BCECF fluorescence could be found in vacuoles during the first 10 min of incubation in BCECF-AM, making it unlikely that the dye is hydrolyzed in the vacuole. Cytoplasmic pH could be measured during the first 15 min or so of dye loading when BCECF had accumulated in the cytosol but was absent from the vacuole. Emission ratios indicate an average cytoplasmic pH of 7.35 ± 0.22 in freshly isolated protoplasts. With increasing time of incubation, BCECF disappeared from the cytosol and accumulated inside vacuoles. Figure 1A shows aleurone protoplasts with BCECF completely sequestered into the vacuoles. In Figure 1E, laser scanning confocal microscopy shows that after incubation for 40 min or more, all of the detectable BCECF fluorescence originates from the vacuoles.

BCECF Does Not Affect the Response of Aleurone Protoplasts to GA_3 or ABA

To establish that BCECF could be used to monitor pH continuously in living aleurone cells, we measured viability and hormone responsiveness of protoplasts after exposure to BCECF-AM. Protoplast viability was assessed using phenosafranin, a dye that enters dead but not living cells (Lin et al., 1996) before (91 ± 8% viability) and 40 min after (84 ±

11% viability) BCECF-AM loading. These data demonstrated that most protoplasts are viable after BCECF-AM uptake. When protoplasts were stained with both phenosafranin and BCECF, these dyes showed mutually exclusive distribution, indicating that BCECF-AM can function as a viability indicator in barley aleurone protoplasts (only living, intact protoplasts take up BCECF-AM and exclude phenosafranin). The concentration of BCECF in the vacuolar lumen ranged from ~50 to 400 μM, based on comparisons to in vitro imaging of vacuole-sized droplets containing a range of dye concentrations. Dye loading at these concentrations would not be expected to contribute significantly to the buffering capacity of aleurone vacuoles, which we determined to range from 25 to 85 mM H^+ pH^{-1}. Treatment of aleurone protoplasts with GA_3 or ABA had no effect on the uptake and accumulation of BCECF.

The morphology of barley aleurone cells is markedly influenced by GA_3. GA_3 treatment accelerates the coalescence of many hundreds of vacuoles into one large central vacuole in the barley aleurone cell (Bush et al., 1986). Aleurone protoplast morphology was unchanged by vacuolar accumulation of BCECF, as seen in Figures 1B (dye-loaded protoplasts) and 1D (unloaded protoplast). Vacuoles coalesced typically in response to GA_3 after dye loading.

Vacuolar loading of BCECF does not affect the hormone responsiveness of barley aleurone protoplasts. GA_3 responsiveness of aleurone cells was assessed by measuring α-amylase secreted from single protoplasts and from populations of protoplasts. GA_3-induced synthesis and secretion of α-amylase were unaffected by vacuolar BCECF when assessed in individual dye-loaded protoplasts embedded in a starch–agarose layer or when measured by the accumulation of α-amylase in the medium in flasks containing a population of dye-loaded protoplasts treated with GA_3 for 3 days (data not shown). In addition, BCECF had no effect on the ability of aleurone protoplasts to synthesize the amylase/subtilisin inhibitor (ASI) (Mundy et al., 1986) in response to added ABA. The presence of ASI protein in dye-loaded, ABA-treated protoplasts was confirmed by detection on a protein gel blot by using antibodies raised against ASI (data not shown).

Vacuolar Contents Do Not Affect the pH Sensitivity of BCECF

The main advantage of BCECF over other fluorescent pH indicators is the ability to calculate a ratio of fluorescence emission from two excitation wavelengths and to calibrate this ratio to pH, allowing quantitation of pH as well as comparisons between different protoplasts that may vary in absolute fluorescence intensity. Figure 2A shows that the calibration of ratio to pH can be achieved in vitro by using calibration buffers containing free BCECF or in situ by using dye-loaded, isolated vacuoles. Aleurone vacuoles were isolated by lysis of BCECF-loaded protoplasts through a syringe needle into isolation buffer. Isolated vacuoles, shown in Figures 2B and 2C, were placed into a Sykes-Moore chamber for pH_v measurement. During isolation, aleurone vacuoles do not rupture and reseal; thus, after isolation, they contain BCECF as well as the inclusions present in vivo in the vacuolar lumen (Bethke et al., 1996). When incubated in a buffer series containing 50 mM ammonium acetate, the lumen of isolated aleurone vacuoles can be equilibrated over a range of pH values by weak acid/weak base partitioning across the tonoplast and imaged to produce the in situ calibration. Calibration in vitro was performed by imaging droplets of the same equilibration buffers containing 50 μM BCECF dispersed in immersion oil. The two calibrations shown in Figure 2 produced almost identical results, indicating that the contents of the aleurone vacuole do not affect the response of BCECF to pH. The data in Figure 2A also show that this dye is sensitive to pH over a range of 5.5 to 8.5.

GA_3 Regulates pH_v

The pH_v was measured in barley aleurone protoplasts before and after exposure to GA_3 and ABA for 20 hr. Significant pH_v changes were observed, as shown in Figure 3A, after 20 hr of incubation in 5 μM GA_3 compared with incubation without

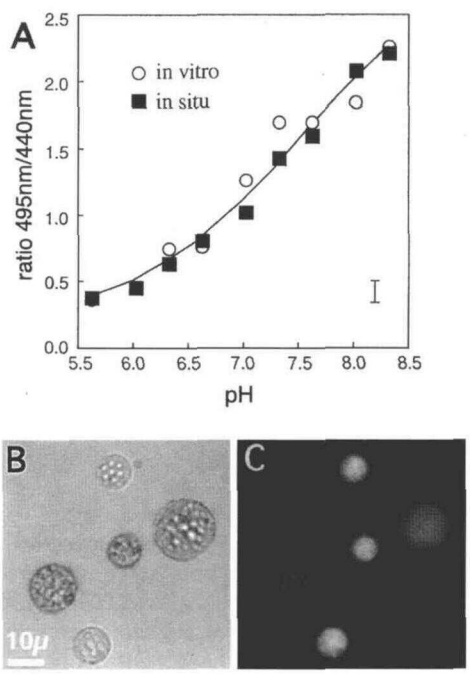

Figure 2. Calibration Curve of BCECF in Situ and in Vitro.
(A) Fluorescence ratio (495/440 nm) versus pH obtained in situ and in vitro under the microscope. The bar represents the maximum standard deviation from any point.
(B) and **(C)** Isolated vacuoles used for in situ calibration observed using differential interference contrast optics **(B)** and epifluorescence at 440-nm excitation **(C)**. Bar in **(B)** = 10 μm for **(B)** and **(C)**.

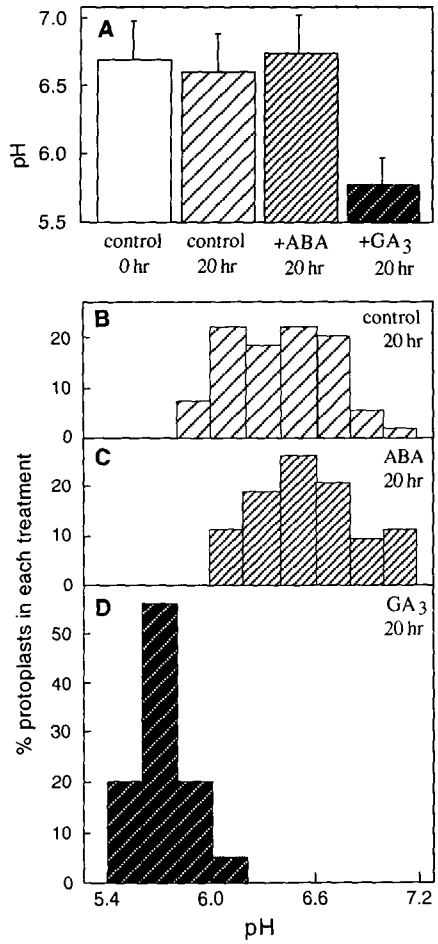

Figure 3. Effects of GA_3 and ABA on the pH_v of Barley Aleurone Protoplasts.

(A) pH_v measured before and 20 hr after hormone treatment. The data are a summary of pH_v in individual protoplasts from 20 different protoplast preparations and represent a mean ±SD.

(B) to **(D)** Histograms of the data in **(A)** showing the percentage of protoplasts at each measured vacuolar pH after 20 hr without hormone control **(B)**, with 5 μM ABA **(C)**, or with 5 μM GA_3 **(D)**.

hormone or with 5 μM ABA. Incubation of protoplasts in GA_3 for 20 hr caused the pH_v to fall from 6.63 ± 0.66 (n = 25) to 5.85 ± 0.31 (n = 21). Incubation in ABA for 20 hr did not cause a drop in pH_v (6.72 ± 0.64; n = 56) compared with control protoplasts incubated for 20 hr without hormone (pH_v = 6.58 ± 0.68; n = 53). Histograms showing the frequency distribution of pH_v in the 20-hr data sets from Figure 3A are shown in Figures 3B to 3D. These illustrate that the pH_v of any individual protoplast from each treatment could vary as much as 1 pH unit from other protoplasts in the same experiment.

To determine the kinetics of vacuolar acidification, we monitored pH_v in individual protoplasts before and after hormone treatment. BCECF-loaded protoplasts were immobilized on a poly-L-lysine–coated cover slip within a Sykes-Moore chamber and imaged every 2 hr after the release buffer in the chamber was replaced with release buffer containing either 5 μM GA_3 or 5 μM ABA. Protoplasts observed after ABA treatment showed little vacuolar acidification when observed over 10 hr (n = 6). GA_3-treated protoplasts, in contrast, exhibited a much greater drop in a shorter time period (n = 6). Representative GA_3- and ABA-treated protoplasts are shown in pseudocolor in Figure 4A to visualize pH_v differences. The ratioed images show that all of the protein storage vacuoles in an individual protoplast appear to have the same pH. Data from these two cells imaged over a 10-hr incubation period are shown in Figure 4B. The ABA-treated protoplast showed a pH_v drop of 0.1 unit over 10 hr, whereas the GA_3-treated protoplast showed a drop of 1.1 unit over the same time period. We conclude that GA_3 brings about an increase in the rate of vacuolar acidification.

V-ATPase and V-PPase Are Present in Aleurone Tonoplasts

We identified the H^+ pumps that could contribute to vacuolar acidification by monitoring fluorescence from intact, isolated vacuoles loaded in vivo with BCECF and incubated in a Sykes-Moore chamber on poly-L-lysine–coated glass. The lumen of BCECF-loaded, isolated vacuoles could be acidified by adding either ATP or PPi to the incubation medium. As shown in Figure 5A, the addition of 1 mM ATP to isolated vacuoles caused an immediate acidification of the vacuole lumen, and ATP-induced acidification could be prevented by 10 mM NO_3^-, an inhibitor of V-ATPase activity. ATP brought about a rapid pH_v drop in vacuoles isolated from protoplasts incubated for 20 hr in the presence or absence of GA_3 or ABA. The addition of 1 mM PPi also caused an immediate acidification of the lumen of isolated vacuoles, as shown in Figure 5B, observable in vacuoles from protoplasts incubated for 20 hr in the presence or absence of GA_3 or ABA. This PPi-induced acidification could be prevented by the presence of 100 μM $CaCl_2$. Results of the proton pump inhibitor experiments in Figures 5A and 5B show a slight decline at every time point. With repeated exposure to excitation energy, BCECF undergoes a small amount of photobleaching, which affects the 495/440 nm ratio independent of pH.

To establish whether proton pumping activities of V-PPase and V-ATPase were colocalized in the tonoplast of the same vacuole, we added PPi and ATP sequentially to isolated, BCECF-loaded vacuoles. In the results shown in Figure 5C, the pH_v was monitored as the composition of the medium was changed by adding ATP or PPi. The pH_v was first reset to ~7.2 with equilibration buffer containing ammonium acetate. The equilibration buffer was replaced with isolation buffer, and the pH_v was monitored before and after the addition of PPi. Next, the chamber was filled with isolation buffer including ATP and finally with equilibration buffer. PPi brought about a decrease

in the pH_v to ~6.3, and the addition of ATP lowered the pH_v to ~5.5. These data indicate that both H^+ pumps coexist and can function on the same vacuolar membrane.

Antibodies raised against V-ATPase and V-PPase were used to identify V-ATPase and V-PPase by immunocytochemistry and protein gel blotting. Immunolocalization of V-PPase, shown in Figure 6, was performed on formaldehyde-fixed aleurone protoplasts that had been incubated with or without GA_3 and ABA for 20 hr. Tonoplasts of GA_3- and ABA-treated cells (Figures 6A and 6B), as well as protoplasts not exposed to hormone (Figure 6C), were prominently labeled with antibodies raised against mung bean V-PPase. Fixed protoplasts with only secondary antibodies applied, shown in Figure 6D, displayed negligible signal. Aleurone protoplasts labeled with antibodies raised against the 57-kD subunit of V-ATPase indicate that the tonoplast as well as other endomembranes contain this protein (data not shown).

The V-PPase and V-ATPase antibodies were used to quantify the corresponding antigens on protein gel blots of aleurone protoplasts proteins separated by SDS-PAGE. Proteins were analyzed from protoplasts incubated for 20 hr in the presence or absence of GA_3 or ABA. Detection of the V-PPase on a protein gel blot is shown in Figure 7A. Although there is less V-PPase protein in control compared with hormone-treated protoplasts, the V-PPase protein appears in approximately equal amounts in ABA- and GA_3-treated protoplasts. GA_3 treatment causes the appearance of a degradation band at a lower molecular mass detected by the V-PPase antibody. As shown in Figure 7B, neither GA_3 nor ABA affected the amount of the V-ATPase 57-kD subunit protein in barley aleurone protoplasts.

DISCUSSION

The data presented in this study show that BCECF, a pH-sensitive fluorescent dye, can be introduced into barley aleurone protoplasts noninvasively and that accumulation of the dye in vacuoles does not affect the response of the protoplast to GA_3 or ABA (Figure 1). We can monitor vacuolar pH continuously in intact protoplasts or individual isolated vacuoles and can measure cytoplasmic pH briefly during dye loading. Our experiments with BCECF show that the pH_v in aleurone cells is maintained at ~6.6 in the absence of GA_3, but exposure to GA_3 induced a drop in pH_v of >1 pH unit (Figures 3 and 4). This change in pH_v could be detected as early as 2 hr after exposure to GA_3. The addition of ABA did not induce vacuolar acidification. Results from experiments with isolated vacuoles from both GA_3- and ABA-treated protoplasts indicate the presence of the V-ATPase and V-PPase, the tonoplast proton pumps that may regulate hormone-induced changes in pH_v (Figure 5). Our data support the hypothesis that vacuolar pH plays a key role in regulating the mobilization of reserves from the aleurone vacuole during the GA_3 response.

Vacuolar loading of BCECF did not affect the synthesis and secretion of α-amylase by aleurone cells in response to GA_3 and did not impact ABA-induced protein synthesis. Protoplast morphology was unaltered, and coalescence of vacuoles in response to GA_3 progressed typically in the presence of vacuolar BCECF. In addition, because the buffering capacity of the aleurone vacuole was at least an order of magnitude greater than that of lumenal BCECF concentrations, any pH buffering effects as a result of the presence of dye should have been negligible. Because the in situ and in vitro calibration curves are comparable, we are confident that BCECF is not adversely modified after sequestration into the vacuole and that the vacuolar lumenal milieu does not affect the pH sensitivity of BCECF. These observations confirm that BCECF is a suitable dye for in vivo measurement of pH_v in barley aleurone protoplasts.

Vacuolar pH in plants shows considerable variation, and previous work indicates that the pH_v can be regulated developmentally (Sinclair, 1984) and by light (Lüttge, 1987; Yin

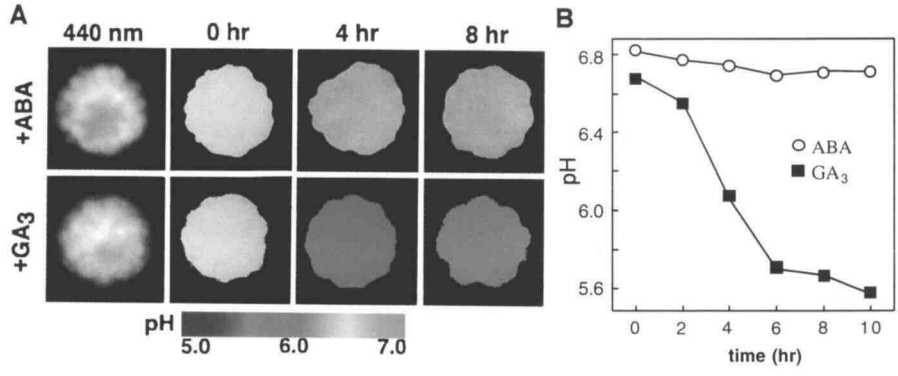

Figure 4. pH_v in Individual Barley Aleurone Protoplasts after the Addition of GA_3 or ABA.

(A) Protoplasts containing vacuolar BCECF observed 0, 4, and 8 hr after treatment with 5 μM GA or 5 μM ABA. Fluorescence emission (525 ± 25 nm) is from 440-nm excitation of BCECF. Ratio images have been pseudocolored to show pH.
(B) pH_v of the protoplasts shown in **(A)** monitored at 2-hr intervals.

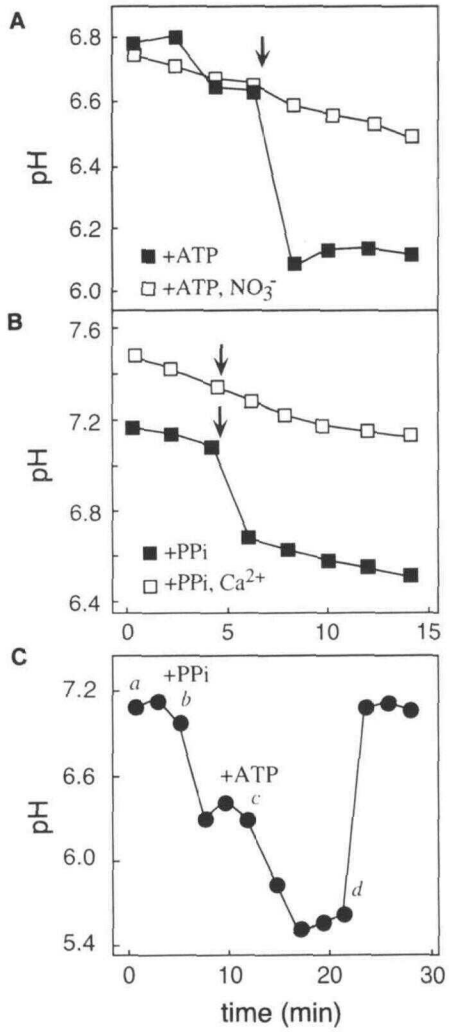

Figure 5. Proton Pumping Activity in Individual Isolated Vacuoles and Effects of Inhibitors.

(A) ATP-dependant proton pumping and inhibitory effect of NO_3^-. The arrow indicates the addition of 1 mM ATP or 1 mM ATP plus 10 mM NO_3^-. The vacuoles were isolated from ABA-treated protoplasts.
(B) PPi-dependent proton pumping and inhibitory effect of Ca^{2+} in isolated vacuoles from barley aleurone protoplasts. The arrows indicate addition of 1 mM PPi or 1 mM PPi plus 100 μM Ca^{2+}. The vacuoles were isolated from ABA-treated protoplasts.
(C) V-PPase and V-ATPase activities on the tonoplast of the same vacuole. The lumenal pH over time is indicated. The pH_v was reset to 7.2 using equilibration buffer; ATP and PPi were then added sequentially. Test solutions were added where indicated: isolation buffer, calcium free (*a*); PPi in vacuole isolation buffer, calcium free (*b*); ATP in isolation buffer (*c*); pH 7.2 equilibration buffer (*d*). The vacuoles were isolated from GA_3-treated protoplasts.

et al., 1990a, 1990b). Stresses such as anoxia (Menegus et al., 1991), osmotic shock (Okazaki et al., 1996), and temperature shock (Yoshida, 1995) can affect the pH_v. pH_v also varies according to cell function. In specialized cells, such as the lemon fruit juice sac, pH_v values are as low as 2.2, whereas in epicotyl cells of lemon, the pH of vacuoles is ~5.5 (Muller et al., 1996). The pH_v in cells undergoing crassulacean acid metabolism can range from as low as 3.5 at night when organic acids are accumulated to pH 6 during the day when organic acids are metabolized (Lüttge, 1987). In barley aleurone, pH depends on the physiological state of the cell (Figures 3 and 4). Cells that are stimulated to synthesize and secrete hydrolytic enzymes by GA_3 acidify the vacuole lumen rapidly, whereas those cells that are not GA_3 stimulated or are incubated in ABA acidify their vacuoles very slowly or not at all. After as little as a 6-hr incubation in GA_3, the pH_v of barley aleurone cells fell from 6.7 to 5.7 (Figure 4). Because the pK_a of BCECF in barley aleurone vacuoles is near neutral, the dye

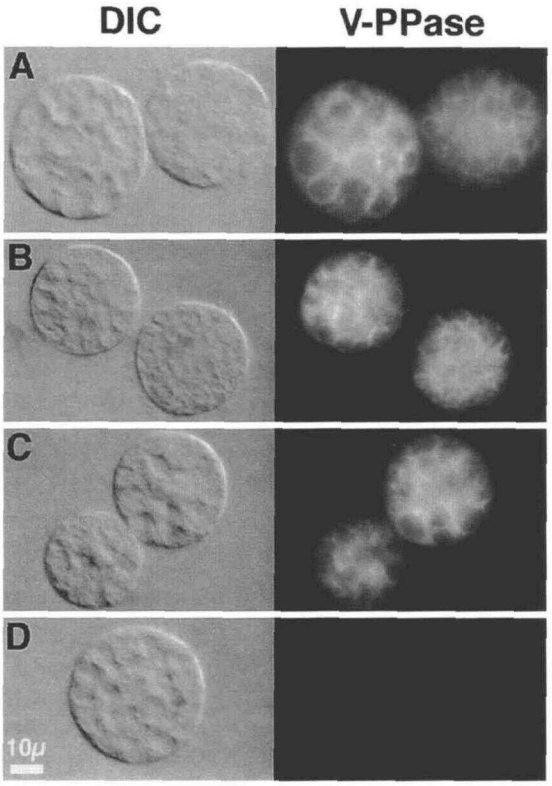

Figure 6. Indirect Immunolocalization of V-PPase by Using Formaldehyde-Fixed Barley Aleurone Protoplasts.

(A) to **(C)** Protoplasts treated for 20 hr with GA_3 **(A)** or ABA **(B)** or untreated protoplasts **(C)** were observed with differential interference contrast (DIC) optics or 440-nm excitation to visualize the V-PPase via the fluorescein-conjugated secondary antibody.
(D) Protoplasts were labeled with only the secondary antibody.
Bar in **(D)** = 10 μm for **(A)** to **(D)**.

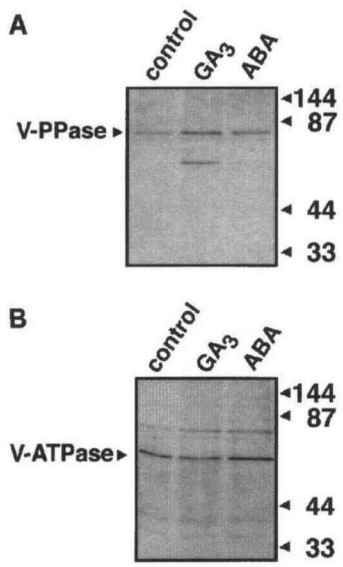

Figure 7. Proton Pump Protein Detection on Gel Blots of Proteins from Aleurone Protoplasts.

(A) Detection of the 78-kD V-PPase.
(B) Detection of the 57-kD V-ATPase.
Lanes include proteins from control protoplasts not treated with hormone and proteins from protoplasts treated for 20 hr with GA_3 or 20 hr with ABA. Molecular mass markers are shown at right in kilodaltons.

is less pH sensitive at lower pH values (Figure 2). It is therefore likely that the pH of GA_3-treated aleurone vacuoles is lower than is the average of pH 5.8 measured in our experiments.

The pH of the vacuole in wild oat aleurone protoplasts is also developmentally regulated (Davies et al., 1996). In these experiments, the pH_v in wild oat protoplasts was measured using carboxyfluorescein diacetate and declined slowly from pH 7.0 to 5.6 during 6 days of incubation. This drop in pH_v was correlated with the development of the aleurone vacuole, not hormone treatment (Davies et al., 1996). Interestingly, the pH values of the vacuoles of freshly isolated barley (pH 6.6) and wild oat (pH 7.0) aleurone protoplasts are among the most basic that have been reported. The pH values of vacuoles in most plant tissues lie in the range of 4.0 to 6.0 (Kurkdjian and Guern, 1989). We speculate that maintenance of a near-neutral pH in aleurone vacuoles is related to regulation of lytic activity in this compartment. Thus, at a pH near neutrality, acidic hydrolases in the vacuole lumen are less active.

The vacuole of the barley aleurone cell is a unique organelle. Not only are stored reserves found within this compartment but also enzymes that are likely to be involved in their mobilization (Bethke et al., 1996). Hydrolytic enzymes found within the vacuole of barley aleurone have sharp pH optima (Gabard and Jones, 1986; Sarkkinen et al., 1992), indicating that pH_v may play a crucial role in regulating the activity of vacuolar hydrolases. The barley grain aspartic protease HvAP, an enzyme with a pH optimum between 3.5 and 3.9 (Sarkkinen et al., 1992), provides an excellent example of a protein whose activity is likely to be regulated by pH. HvAP is present in the lumen of the vacuole in aleurone from dry barley grain, and the protein level of HvAP does not change after imbibition of the grain or after incubation of the aleurone layer in media with or without GA_3 or ABA (Bethke et al., 1996). Although it is unlikely that HvAP participates directly in the hydrolysis of storage proteins, we speculate that it may play an important role in proteolytic processing of other acid hydrolases.

In addition to the activation of enzymes in aleurone vacuoles, a low pH_v would increase the electrochemical H^+ gradient across the tonoplast of a GA_3-stimulated cell, providing energy for secondary transport processes. The increased pH differential between the vacuolar compartment and cytosol may therefore be important for the transport of vacuolar reserves to the cytosol. Amino acids released from storage proteins in the vacuole are used for the synthesis of secreted hydrolases (Filner and Varner, 1967), and the inorganic ions liberated during the mobilization of vacuolar phytin are released out of the aleurone and into the endosperm (Jones, 1973).

Our results showing regulation of pH_v in the aleurone cell by GA_3 raise interesting questions about the way GA_3 affects this process. Protoplasts incubated without hormone or in the presence of ABA and showing little or no vacuole acidification (Figures 3 and 4) contain functional V-ATPase and V-PPase in the tonoplast (Figure 5). In this context, it is interesting that the activity of another tonoplast transporter in the aleurone cell, the slow vacuolar channel, is not markedly affected by GA_3 or ABA (Bethke and Jones, 1994). The addition of ATP or PPi to vacuoles isolated from ABA-treated aleurone cells brings about immediate acidification of the vacuole lumen (Figure 5), indicating that the lack of acidification of vacuoles from ABA-treated protoplasts does not occur because proton pumps are absent. Immunocytochemistry shows that V-PPase is present on the tonoplasts from both ABA- and GA_3-treated protoplasts (Figure 6), and immunoblot analyses of V-ATPase and V-PPase proteins indicate that ABA- and GA_3-treated protoplasts contain approximately equal amounts (Figure 7). We conclude that GA_3 does not act to stimulate vacuolar acidification by influencing the synthesis of the V-ATPase or the V-PPase.

Because hormone treatment does not influence the presence of the proton pumps in barley aleurone, proton transport across the tonoplast must be regulated by signal transduction intermediates. Little is known about the mechanisms that regulate V-ATPase and V-PPase activities in plants. It has been postulated that phosphorylation of the B subunit of the V1 domain of the V-ATPase plays a role in regulating the activity of the enzyme (Martiny-Baron et al., 1992). Other mechanisms proposed to regulate the V-ATPase are cytosolic pH (Davies et al., 1994) and the redox state of the cytosol (Feng and Forgac, 1992; Muller et al., 1996).

The V-PPase is inhibited by low micromolar concentrations of Ca^{2+}, and the sensitivity of the V-PPase to Ca^{2+} suggests that this ion could play a role in regulating the pH of the vacuole

in plant cells (Maeshima, 1991; Rea et al., 1992). The barley aleurone V-PPase is also inhibited by Ca^{2+} (Figure 5). Because GA_3 increases cytosolic Ca^{2+} in cereal aleurone cells from ~50 to 200 nM to ~300 to 600 nM (Bush and Jones, 1988; Gilroy and Jones, 1992; Bush, 1996), it seems unlikely that V-PPase proton pumping would contribute to GA_3-stimulated vacuole acidification. The inhibitory effects of Ca^{2+} on V-PPase activity are markedly reduced by Mg^{2+} concentrations >1 mM (Rea et al., 1992). Because aleurone vacuoles contain substantial Mg^{2+} reserves (Stewart et al., 1988) that are released to the cell exterior via the cytosol (Jones, 1973), regulation of the V-PPase by cations in the aleurone cell is likely to be complex and will require information on the dynamics of Mg^{2+} concentration in the cytosol during response to GA_3. Whereas tonoplast proton pumps are responsible for the primary proton influx, pH_v is also dependent on protons accompanying the movement of anions and cations across the tonoplast and the buffering capacity of the lumen. Further work investigating barley aleurone pH_v will address these possibilities.

METHODS

Isolation and Incubation of Aleurone Protoplasts

Protoplasts were prepared from aleurone layers isolated from deembryonated barley grains (*Hordeum vulgare* cv Himalaya; 1991 harvest, Washington State University, Pullman), as described previously (Jacobsen et al., 1985; Bush et al., 1986). Protoplasts were incubated in Gamborg's B-5 medium (release medium; Bush and Jones, 1988) modified by the addition of 10 mM $CaCl_2$. Where indicated, 5 μM gibberellic acid (GA_3) or 5 μM abscisic acid (ABA) was included in the incubation medium. Secretion of α-amylase from populations of protoplasts was assayed according to Jones and Varner (1967). Secretion of α-amylase from individual protoplasts was monitored using the thin-layer agarose method of Hillmer et al. (1993). Protoplast viability was estimated by staining with 10 mg of phenosafranin per mL of protoplast suspension (Lin et al., 1996), and cell counts were determined using a hemocytometer.

Immunoblotting

Aleurone protoplasts were purified on a Nycodenz gradient (Bush and Jones, 1988) after 20 hr of incubation in release medium with or without 5 μM GA_3 or 5 μM ABA and 10 μM 2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM). Protoplasts were collected from the gradient and stored at −80°C. Protein concentration in each sample was determined by using a Bio-Rad protein assay, and 20 μg protein was loaded in each lane of the polyacrylamide gels (20% acrylamide for amylase/subtilisin inhibitor [ASI] and 12% for the vacuolar pyrophosphatase [V-PPase] and vacuolar ATPase [V-ATPase]). One-dimensional SDS-PAGE was performed, and the proteins from the gels were electrotransferred to polyvinylidene fluoride (Millipore, Bedford, MA) membranes in 50 mM Tris base, 40 mM glycine, 0.04% (w/v) SDS, and 20% (v/v) methanol. Membranes were blocked with 5% milk in PBS (20 mM $NaHPO_4$, 0.6 mM KH_2PO_4, 100 mM NaCl, pH 7.2), and then primary antibodies were added at a 1:250 dilution and incubated overnight. Secondary antibodies (goat anti-rabbit IgG) coupled to horseradish peroxidase (Sigma) were visualized chromogenically with 4CN Plus (Du Pont–New England Nuclear, Boston, MA). Antibodies raised against the V-PPase and V-ATPase were provided by M. Maeshima (University of Nagoya, Nagoya, Japan), and antibodies raised against ASI were provided by J. Mundy (University of Copenhagen, Denmark).

Loading of Protoplasts with BCECF-AM

The pH-sensitive fluorescent dye BCECF-AM was purchased from Molecular Probes (Eugene, OR). Aleurone protoplasts were purified on a Nycodenz gradient and then incubated in release medium containing 10 μM BCECF-AM diluted from a stock solution of 1 mM BCECF-AM in DMSO.

Laser Scanning Confocal Microscopy

Confirmation of the subcellular localization of BCECF was performed using a Molecular Dynamics (Sunnyvale, CA) laser scanning confocal microscope and Nikon Optiphot microscope (Tokyo, Japan). Excitation energy was provided by the 488-nm emission line of the argon ion laser. BCECF fluorescence passed through a 500-nm dichroic mirror and 530 ± 30-nm band pass filter before confocal detection. Images were collected as an average of three scans with a Nikon (Melville, NY) PlanApo Fluor ×60 oil objective having a numerical aperture of 1.4.

Epifluorescence Microscopy and Image Acquisition

BCECF-loaded protoplasts were observed in release medium on a microscope slide or immobilized on a poly-L-lysine–coated cover slip in a Sykes-Moore chamber (Bellco Glass Inc., Vineland, NJ) for time course experiments and when solution changes were necessary. Poly-L-lysine–coated cover slips were prepared by immersion of cover slips in a 100 μg mL^{-1} solution of poly-L-lysine (molecular weight of 70,000) for 1 hr. Coated cover slips were dried and stored in a desiccator. Protoplasts were viewed using a Plan-Neofluor ×40 dry objective, 0.75 numerical aperture, on a Zeiss Axiophot microscope (Thornwood, NY) equipped with a 100-W mercury arc light source and a filter wheel (Sutter Instruments Co., Novato, CA) alternating between 440 ± 10-nm and 495 ± 10-nm excitation wavelengths. BCECF fluorescence passed through a 515-nm dichroic mirror and a 535 ± 25-nm band pass filter. A 50% transmittance neutral density filter was used between the light source and the filter wheel to decrease excitation energy and minimize photobleaching. Filters were purchased from Chroma Corporation (Brattleboro, VT).

Images of the fluorescence emission from vacuoles were acquired using a cooled charge-coupled device (CCD) camera (Princeton Instruments, Trenton, NJ) with a dynamic range of 0 to 4095 pixel intensity units. The camera shutter was set at a 400-msec exposure. Total time to collect 440- and 495-nm dual excitation fluorescence emission images was <1 sec. The CCD camera was also used for bright-field or Nomarski differential interference contrast image collection. The camera and filter wheel were controlled using IPLab software (Signal Analytics Corp., Vienna, VA) running on a Macintosh computer (Cupertino, CA).

Ratio Imaging Analysis

IPLab software was used for ratio analysis of raw data image pairs. Camera dark level (40 intensity units) was subtracted from every pixel intensity in each image. Background fluorescence intensity was measured based on average signal from an area next to the sample. Average background signal ranged from five to 50 intensity units and was calculated and subtracted from each individual image. After background and dark-level subtraction, the average emission signal from BCECF in aleurone vacuoles ranged from 200 to 1000 intensity units. Autofluorescence represented <1% of the total signal from dye-loaded protoplasts and did not change with time or experimental treatment. The image obtained from 495-nm excitation was divided by the image obtained from 440-nm excitation to produce the ratioed image. An average ratio was calculated and converted to a pH value, using the calibration described below.

Estimation of Vacuolar BCECF Concentration

Vacuolar dye concentrations were estimated by comparing absolute intensity from BCECF fluorescence in protoplasts to the fluorescence intensity of protoplast-sized spherical droplets (40 to 50 μm in diameter) of buffer containing free BCECF dispersed in oil and sandwiched under a cover slip bridge. The buffer consisted of pH 6.3 calibration buffer described below containing a range of BCECF concentrations (10 to 500 μM). Measurement of vacuolar buffering capacity in isolated vacuoles was made using the method of Boyarsky et al. (1988).

Vacuole Isolation

Aleurone vacuoles were isolated according to Bethke and Jones (1994). Isolation buffer contained 100 mM KCl, 2 mM $MgCl_2$, 5 mM Hepes-KOH, pH 7.2, 100 μM $CaCl_2$, and mannitol to 1100 mosmol kg^{-1}. Protoplasts were loaded with BCECF before vacuole isolation. During isolation, aleurone vacuoles remained intact; thus, after isolation, they contained the fluorescent dye as well as the inclusions present in the vacuole lumen in vivo.

Calibration of Fluorescence versus pH

For pH ratio calibration, vacuoles loaded with BCECF in vivo were isolated from aleurone protoplasts and immobilized on a poly-L-lysine–coated cover slip in a Sykes-Moore chamber. Vacuolar pH (pH_v) was equilibrated to that of the external solution by the partitioning effect of weak acid and weak base exchange. Equilibration solutions used for calibration were based on Yoshida (1994) and contained 50 mM Hepes–bis-tris-propane, pH 6.6 to 7.8, or Mes–bis-tris-propane, pH 5.5 to 6.6, 50 mM ammonium acetate, and mannitol to 1100 mosmol kg^{-1}. Immobilized vacuoles were stepped through pH values by exchanging solution in the chamber with equilibration buffer at a new known pH. pH_v ratios were determined after a new and stable value was reached (~5 min after solution exchange), and average ratios of five vacuoles were plotted against pH to produce the in situ calibration curve. For pH ratio calibration in vitro, droplets of the pH equilibration buffer containing 50 μM free BCECF were dispersed in oil and sandwiched under a cover slip bridge. Average ratios of five drops at each pH were used to generate the calibration curve.

Measurement of V-ATPase and V-PPase Proton Translocation in Intact, Isolated Vacuoles

Proton-translocating V-ATPase and V-PPase activities were assessed using intact vacuoles isolated from protoplasts loaded with BCECF. Vacuoles were immobilized on poly-L-lysine–coated cover slips in a Sykes-Moore chamber to facilitate solution changes. For some experiments, pH_v was reset to a higher value before measurement of proton pump acitivity by incubation of isolated vacuoles in an equilibration buffer (pH 7.2) for a few minutes, then followed by replacement with an isolation buffer. To initiate ATP-dependent inward proton pumping, we replaced the isolation buffer in the chamber with isolation buffer containing 1 mM ATP (diluted from a 100 mM stock solution, pH adjusted to 7.2). For some experiments, 10 mM KNO_3 was added to the ATP test solution. PPi-dependent acidification was initiated by replacing the isolation buffer in the chamber with a 1 mM PPi solution (diluted from a 100 mM sodium pyrophosphate stock solution) into an isolation buffer without $CaCl_2$ and including 5 mM EGTA, or with 100 μM $CaCl_2$ but no EGTA.

Immunolocalization

Immunolocalization of the V-ATPase and V-PPase in aleurone protoplasts was performed as described by Schuurink et al. (1996), except that fluorescence images were acquired using the CCD camera and IPLab software.

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