

Barley Aleurone Cells Contain Two Types of Vacuoles: Characterization of Lytic Organelles by Use of Fluorescent Probes

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Light microscopy was used to study the structure and function of vacuoles in living protoplasts of barley (*Hordeum vulgare* cv Himalaya) aleurone. Light microscopy showed that aleurone protoplasts contain two distinct types of vacuole: the protein storage vacuole and a lysosome-like organelle, which we have called the secondary vacuole. Fluorescence microscopy using pH-sensitive fluorescent probes and a fluorogenic substrate for cysteine proteases showed that both protein storage vacuoles and secondary vacuoles are acidic, lytic organelles. Ratio imaging showed that the pH of secondary vacuoles was lower in aleurone protoplasts incubated in gibberellic acid than in those incubated in abscisic acid. Uptake of fluorescent probes into intact, isolated protein storage vacuoles and secondary vacuoles required ATP and occurred via at least two types of vanadate-sensitive, ATP-dependent tonoplast transporters. One transporter catalyzed the accumulation of glutathione-conjugated probes, and another transported probes not conjugated to glutathione.

INTRODUCTION

Vacuoles are present in all plant cells, but the form and function of this organelle vary with cell type (Boller and Wiemken, 1986; Chrispeels, 1991; Okita and Rogers, 1996). Meristematic cells contain many small vacuoles that fuse after cell enlargement to form the large central vacuole (Herman, 1994). Storage tissues of fruits and seeds contain specialized protein storage vacuoles (Higgins, 1984; Okita and Rogers, 1996). Although vacuoles among tissues and cell types are diverse in their morphology, ontogeny, and specialized functions, there is limited evidence that such diversity could occur within a single plant cell.

Recent studies, however, indicate that some plant cells contain more than one kind of vacuole (Hoh et al., 1995; Paris et al., 1996). In cells of developing pea cotyledons, two kinds of vacuoles appeared to be present: the degenerating vegetative vacuole and the newly formed protein storage vacuoles (Robinson et al., 1995). Evidence from electron microscopy suggests that protein storage vacuoles originated de novo from the rough endoplasmic reticulum and not from fragmentation of the vegetative vacuole (Robinson et al., 1995). In suspension-cultured tobacco BY-2 cells subjected to sucrose starvation in the presence of cysteine protease inhibitors, acidic organelles containing acid phosphatase activity accumulated in the cytosol (Moriyasu and Ohsumi,

1996). These organelles were seen in cells with well-formed central vacuoles, and the authors speculated that they might be lytic organelles that function in autophagy.

Using antibodies to specific tonoplast intrinsic proteins (TIPs; Maurel, 1997), Paris et al. (1996) identified two kinds of vacuoles in root tip cells of barley and pea. One of the organelles labeled with α -TIP antibodies and contained barley lectin, a protein normally stored in root tip cells (Lerner and Raikhel, 1989). The other organelle labeled with TIP-Ma27 antibodies and contained the cysteine protease aleurain (Paris et al., 1996). Because the anti- α -TIP antibody is known to label protein storage vacuoles in storage tissues (Johnson et al., 1989), it was argued that barley lectin is located in protein storage vacuoles, whereas the cysteine protease aleurain is contained in a different class of vacuole (Paris et al., 1996).

Localization of aleurain in barley aleurone cells also supports the idea that this enzyme is not present in protein storage vacuoles but rather is located in a separate organelle (Holwerda et al., 1990). Immunolocalization and observation using electron microscopy had shown that aleurain is absent from protein storage vacuoles but is found in a smaller organelle of unknown origin (Holwerda and Rogers, 1992). This observation is supported by biochemical evidence showing that although the barley aspartic protease HvAP (Törmäkangas et al., 1994) is present in barley aleurone protein storage vacuoles, aleurain is absent from this organelle (Bethke et al., 1996). These observations indicate that in

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barley aleurone, as in pea and barley roots, there may be two separate types of vacuoles: the protein storage vacuoles that contain HvAP, and storage proteins and another organelle that contain aleurain.

In previous work from our laboratory, we began our characterization of aleurone protein storage vacuoles. In common with many plant vacuoles (Sze, 1985; Rea and Poole, 1993; Maurel, 1997), the protein storage vacuole tonoplast contains vacuolar H⁺-ATPase (V-ATPase) and vacuolar H⁺-pyrophosphatase (V-PPase) activities (Swanson and Jones, 1996), slow vacuolar channels (Bethke and Jones, 1994), and the aquaporin α -TIP (Schuurink et al., 1996). The proton pumps in the protein storage vacuole tonoplast play an important role in gibberellic acid (GA)-induced acidification of the protein storage vacuole lumen (Swanson and Jones, 1996). Protein storage vacuoles from aleurone cells that have not been exposed to GA have a luminal pH near neutrality, whereas the lumen of protein storage vacuoles treated with GA is acidified to pH 5.5 or below (Swanson and Jones, 1996). Because GA does not increase the amount of V-ATPase or V-PPase in the protein storage vacuole tonoplast, a GA-stimulated increase in the activity of these pumps has been proposed (Swanson and Jones, 1996).

The lumen of barley aleurone protein storage vacuoles contains globulin storage proteins (Yupsanis et al., 1990), acid phosphatases (Gabard and Jones, 1986; Jones, 1987), aspartic proteases (including HvAP), and several cysteine proteases (Bethke et al., 1996). Although low rates of aspartic and cysteine protease activities were found in protein storage vacuoles before GA treatment, hormone stimulation resulted in a marked increase in cysteine protease activity (Bethke et al., 1996). The aspartic and cysteine proteases of aleurone protein storage vacuoles have pH optima near 4 (P.C. Bethke and R.L. Jones, unpublished data), and we have proposed that the pH of the protein storage vacuole lumen plays a key role in regulating lytic activity in this organelle (Swanson and Jones, 1996). GA may initiate storage protein breakdown by increasing the synthesis and transport of proteases to protein storage vacuoles and/or by lowering the luminal pH of these organelles so that proteolytic enzymes become active (Bethke et al., 1996; Swanson and Jones, 1996).

In this study, we report on the use of fluorescent probes and light microscopy to further address questions related to vacuole function and tonoplast transport in living barley aleurone cells and in isolated intact vacuoles. We show that in addition to protein storage vacuoles, aleurone cells contain a second type of lytic organelle, referred to as the secondary vacuole, that develops independently of GA or abscisic acid (ABA) treatment. The secondary vacuole has many features of plant vacuoles. It is an acidic compartment containing proteolytic activity, and fluorescence microscopy indicates that secondary vacuoles are dynamic organelles. Like protein storage vacuoles, secondary vacuoles accumulate organic solutes, including glutathione conjugates, by way of ATP-dependent tonoplast transporters. We show that these

directly energized tonoplast transporters are responsible for the accumulation of ion-sensitive fluorescent probes in aleurone protein storage vacuoles and secondary vacuoles.

RESULTS

Aleurone Protoplasts Contain Two Morphologically Distinct Types of Vacuole

Figure 1 shows that barley aleurone cells contain protein storage vacuoles and a distinctly different kind of organelle, which we have called secondary vacuoles. Differential interference contrast (DIC) microscopy showed that these two types of vacuole occur in both GA- and ABA-treated aleurone protoplasts (Figure 1). In Figure 1, the plane of focus was selected to most clearly reveal the characteristics of the secondary vacuoles. The location of some protein storage vacuoles is indicated in Figure 1, but with the exception of Figures 1B and 1F, they are out of the plane of focus. The characteristics of the barley aleurone protein storage vacuole have been described previously (Jacobsen et al., 1971; Fernandez and Staehelin, 1985; Bush et al., 1986). Protein storage vacuoles are the predominant organelle of barley aleurone cells and are present at grain maturity. They contain numerous inclusions of phytin and protein/carbohydrate (Figures 1A and 1E; Jacobsen et al., 1971) and are delineated by a tonoplast that has oleosomes embedded between the inner and outer leaflets of the membrane (Fernandez and Staehelin, 1985). The presence of oleosomes makes the protein storage vacuole tonoplast refractile and easily visible by light microscopy. Conversely, secondary vacuoles appear as clear patches in the cytoplasm and are typically <10 μ m in diameter (Figure 1, arrowheads). They are generally devoid of inclusions that are visible with the light microscope, and the secondary vacuole tonoplast is almost perfectly translucent, which is true especially in younger cells. This latter observation suggests that oleosomes are absent from the secondary vacuole tonoplast or are present at a much lower density than those in the protein storage vacuole tonoplast.

Treatment of aleurone protoplasts with GA caused the protein storage vacuoles to coalesce and form a large vacuole that nearly filled the cell (Jacobsen et al., 1985; Bush et al., 1986). As illustrated in Figures 1A to 1C, this occurred \sim 4 days after GA treatment. Fusion of protein storage vacuoles also occurred in ABA-treated protoplasts, but at a much slower rate (Figures 1E to 1H). Formation of a single large protein storage vacuole was rarely observed in ABA-treated cells. Secondary vacuoles, on the other hand, increased in number after incubation in the presence or absence of GA and ABA. When examined by light microscopy, few secondary vacuoles were visible in freshly isolated protoplasts (Figures 1A and 1E), but with increasing incubation times, the number of secondary vacuoles increased (Figures 1C, 1D,

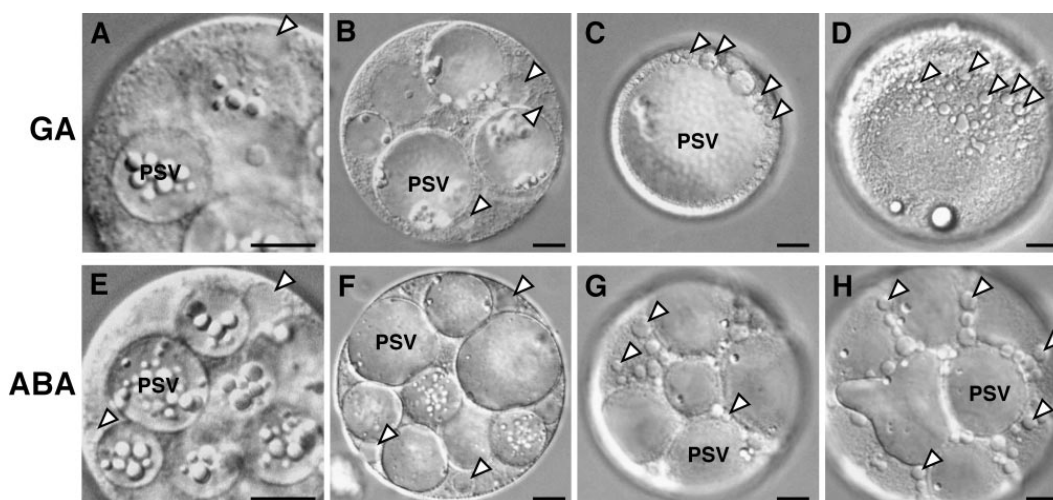


Figure 1. Barley Aleurone Protoplasts Contain Protein Storage Vacuoles and Secondary Vacuoles.

(A) to (D) Protoplasts treated with GA and photographed with DIC optics 1 day (A), 2 days (B), 4 days (C), or 6 days (D) after hormone treatment. The plane of focus is near medial in (A) and (B) and tangential in (C) and (D).

(E) to (H) Protoplasts treated with ABA and photographed with DIC optics 2 days (E), 4 days (F), 6 days (G), or 8 days (H) after hormone treatment. The plane of focus is near medial in (E) and (F) and tangential in (G) and (H).

Some of the secondary vacuoles within each cell are indicated by arrowheads. PSV, protein storage vacuole. Bars = 10 μm .

1G, and 1H). Protoplasts often accumulated ≥ 10 smaller secondary vacuoles (Figures 1D, 1G, and 1H).

We have classified protein storage vacuoles and secondary vacuoles as vacuoles based on several criteria, including the demonstration that the tonoplast of both organelles labeled with antibodies raised against α -TIP. As shown in Figure 2, antibodies raised against α -TIP labeled the tonoplast of protein storage vacuoles and the limiting membrane of secondary vacuoles in fixed aleurone protoplasts (Figures 2A and 2B and Schuurink et al., 1996).

Protein Storage Vacuoles and Secondary Vacuoles Are Acidic, Lytic Organelles

Figure 3 shows that in living barley aleurone protoplasts, the pH-sensitive fluorescent probe 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) accumulates in the lumen of protein storage vacuoles and secondary vacuoles in GA-treated (Figure 3A) and ABA-treated (Figure 3B) protoplasts. Because the probe accumulated in vacuoles, the signal from the cytosol did not contribute significantly to vacuolar pH measurements made by using ratio imaging analysis (Swanson and Jones, 1996). Secondary vacuoles were brighter than were protein storage vacuoles in protoplasts that were incubated in BCECF-acetoxymethyl ester (BCECF-AM) for 40 min. As shown in Figure 3, this difference in fluorescence intensity was an aid in distinguishing the two types of organelles. The luminal pH of protein storage vacuoles in

freshly isolated aleurone protoplasts was ~ 6.7 and dropped to ≤ 5.5 after incubation for 1 day in GA (Swanson and Jones, 1996). Ratio imaging of protoplasts incubated in GA for 4 days showed that the pH of protein storage vacuoles remained at ≤ 5.5 . This is shown in the pseudocolored protoplasts in Figure 3A. The pH of secondary vacuoles in these protoplasts was also acidic (Figure 3A). Secondary vacuoles in GA-treated protoplasts had a luminal pH similar to that of protein storage vacuoles, as indicated by the uniform blue color of the vacuoles in the pseudocolored images shown in Figure 3A.

Although a 20-hr incubation in ABA did not induce acidification of protein storage vacuoles in aleurone protoplasts (Swanson and Jones, 1996), these organelles were acidic after a 6-day incubation in ABA (Figure 3B). Ratio imaging showed that the pH of protein storage vacuoles in protoplasts incubated in ABA for 6 days was higher than that in protein storage vacuoles of protoplasts incubated in GA for 4 days (Figures 3B and 3A, respectively). Furthermore, the pH of secondary vacuoles was often higher than that of protein storage vacuoles in the same ABA-treated protoplast. This is shown in the pseudocolored protoplasts of Figure 3B by punctate areas of higher pH that correspond to secondary vacuoles. Additionally, we used Lysosensor yellow/blue, a pH-sensitive probe chemically unrelated to BCECF, to confirm that protein storage vacuoles and secondary vacuoles are acidic organelles. Lysosensor yellow/blue ($pK_a = 4.2$) emits yellow/green fluorescence at pH 3.5, blue/green fluorescence at pH 4.5, bright pale blue fluorescence at pH

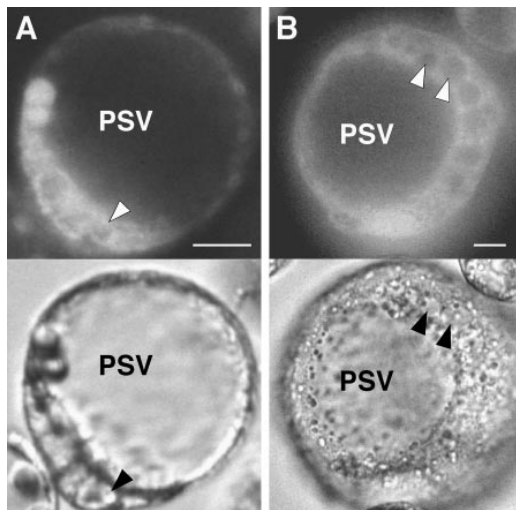


Figure 2. Antibodies Raised against the Tonoplast Protein α -TIP Labeled Protein Storage Vacuoles and Secondary Vacuoles in Barley Aleurone Protoplasts.

Protoplasts were fixed, probed with an antibody raised against α -TIP, washed, and reprobbed with fluorescently tagged secondary antibodies.

(A) Fluorescence and DIC images of a 5-day GA-treated aleurone protoplast.

(B) Fluorescence and DIC images of an 11-day ABA-treated aleurone protoplast.

Both protein storage vacuoles (PSV) and secondary vacuoles (arrowheads) are labeled by anti- α -TIP. Bars = 10 μ m.

5.5, and dim blue fluorescence near neutral pH. The fluorescence from Lysosensor yellow/blue in both protein storage vacuoles and secondary vacuoles of GA-treated protoplasts indicated that the luminal pH of these organelles was <6 (Figure 3C).

As shown in Figure 4, we used the probe 7-amino-4-chloromethylcoumarin benzyloxycarbonyl-L-phenylalanyl-L-arginine amide hydrochloride (ZFR-CMAC) to identify regions of proteolytic activity in living aleurone protoplasts. ZFR-CMAC is a nonfluorescent protease substrate that releases fluorescent CMAC when the peptide bond between the CMAC fluorophore and an adjacent arginine residue is cleaved. A diagram illustrating the transport and proteolysis of this substrate is presented in Figure 4A. In this diagram, transport of the substrate into vacuoles is facilitated by its conjugation to glutathione. Like the other fluorescent probes used in this study, ZFR-CMAC can be loaded into aleurone cells noninvasively, and the probe does not interfere with aleurone cell function (data not shown). Fluorescent CMAC accumulated in protein storage vacuoles and secondary vacuoles of barley aleurone cells, but fluorescence was initially more intense in secondary vacuoles (Figures 4B and 4C). Greater fluorescence from CMAC in the lumen of sec-

ondary vacuoles compared with protein storage vacuoles may reflect higher proteolytic activity in this compartment or a higher surface-to-volume ratio of secondary vacuoles relative to protein storage vacuoles (Figures 4B and 4C). ZFR-CMAC, like other fluorescent probes that label aleurone vacuoles, confirms that secondary vacuoles are present in aleurone protoplasts independent of hormone treatment (Figures 4B and 4C).

Because barley aleurone protein storage vacuoles contain cysteine and aspartic protease activities (Bethke et al., 1996), we hypothesized that proteolysis of ZFR-CMAC was occurring within both protein storage vacuoles and secondary vacuoles. We confirmed this by demonstrating that enzymes in intact isolated vacuoles can proteolyze ZFR-CMAC. As seen in Figure 5A, covalent addition of glutathione to the ZFR-CMAC molecule (ZFR-CMAC-GS) led to the accumulation of fluorescent CMAC in protein storage vacuoles and in secondary vacuoles. This most likely was a result of ATP-dependent uptake of the glutathione-conjugated probe ZFR-CMAC-GS and subsequent proteolysis. Uptake of ZFR-CMAC-GS by protein storage vacuoles or secondary vacuoles was not observed in the absence of added ATP (data not shown). When the protease substrate was not conjugated to glutathione, isolated vacuoles incubated with ZFR-CMAC did not accumulate fluorescent CMAC (data not shown).

Further evidence that accumulation of fluorescent CMAC in the lumen of aleurone vacuoles is a result of proteolytic activity inside of these organelles comes from experiments with protease inhibitors. We have shown previously that proteolytic activity in aleurone protein storage vacuoles could be inhibited by the cysteine protease inhibitor E-64 (Bethke et al., 1996). When isolated protein storage vacuoles and secondary vacuoles were preincubated for 30 min with E-64, leupeptin (another cysteine protease inhibitor), or a mixture of both inhibitors and then incubated in ZFR-CMAC-GS in the presence of ATP, CMAC fluorescence from both kinds of vacuole was reduced compared with that from vacuoles that were not preincubated with the inhibitors (Figures 5B to 5D).

Secondary Vacuoles of Aleurone Protoplasts Are Dynamic Organelles

Aleurone protoplasts were pulse labeled with fluorescent probes to investigate the dynamic nature of secondary vacuoles. As shown in Figure 6A, secondary vacuoles of aleurone protoplasts were more fluorescent than were protein storage vacuoles when observed after a 3-hr incubation in ZFR-CMAC. When protoplasts labeled in this manner were incubated for 2 days in a solution free of probe, protein storage vacuoles remained fluorescent and were of comparable intensity regardless of size (Figures 6B and 6C). Fluorescence from secondary vacuoles, however, was absent or much reduced relative to fluorescence from protein storage

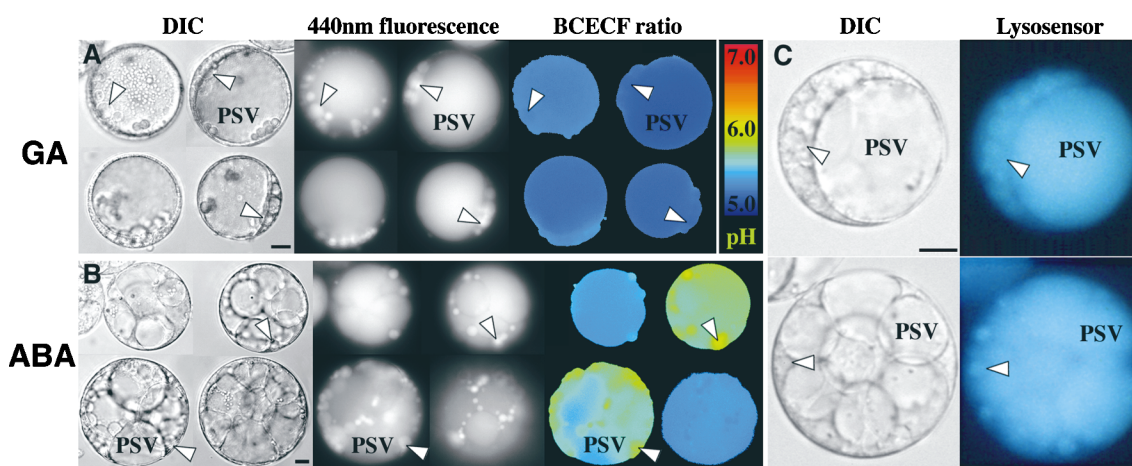


Figure 3. Luminal pH of Barley Aleurone Protein Storage Vacuoles and Secondary Vacuoles Is Acidic.

(A) Four representative 4-day GA-treated aleurone protoplasts showing bright-field images, BCECF fluorescence from 440-nm excitation, and the 495/440 ratio. Ratio images have been pseudocolored so that pH corresponds to the color scale shown.

(B) Four representative 6-day ABA-treated aleurone protoplasts showing bright-field images, BCECF fluorescence from 440-nm excitation, and the 495/440 ratio. Ratio images are pseudocolored as shown in (A).

(C) DIC and Lysosensor yellow/blue fluorescence from UV excitation showing vacuolar pH from a 4-day GA-treated protoplast (top) and a 6-day ABA-treated protoplast (bottom).

Arrowheads indicate some of the secondary vacuoles within each cell. PSV, protein storage vacuole. Bars = 10 μm.

vacuoles. For some secondary vacuoles, fluorescence intensity was similar to that from protein storage vacuoles (Figure 6B). For many secondary vacuoles, however, there was little or no detectable fluorescence (Figure 6C). An identical pattern of fluorescence was observed using fluorescein as a probe (data not shown). Because fluorescence from secondary vacuoles decreased relative to fluorescence from protein storage vacuoles, we conclude that turnover (i.e., degradation or export) of fluorescent CMAC is faster in secondary vacuoles than in protein storage vacuoles. Secondary vacuoles may also expand after the pulse labeling, resulting in lower luminal fluorescence intensity. The observation that all protein storage vacuoles remained fluorescent is additional support for our conclusion that secondary vacuoles are more dynamic organelles than are protein storage vacuoles.

Barley Aleurone Vacuoles Accumulate Many Different Fluorescent Probes

To further characterize the vacuoles in aleurone protoplasts, we have used a range of fluorescent probes as aids in visualizing secondary vacuoles and protein storage vacuoles and as reporters of specific vacuolar activities or properties. In protoplasts that contained secondary vacuoles, which were easily observed, it was clear that the fluorescence intensity from secondary vacuoles after incubation of proto-

plasts in various fluorescent dyes was initially greater (<1 hr) than that from protein storage vacuoles. Figure 7 shows that compounds that are nonfluorescent until conjugated to glutathione, such as monobromobimane (MBB; Kosower and Kosower, 1995; Figure 7A), Cell Tracker Green (CTG; Haugland, 1996; Figure 7B), and monochlorobimane (MCB; Kosower and Kosower, 1995; Coleman et al., 1997b; Figure 7C), accumulate in both types of aleurone vacuoles in vivo. Fluorescence from these compounds was first observed in the cytoplasm, indicating that conjugation to glutathione takes place there (data not shown). Vacuolar accumulation of glutathione-conjugated fluorescent probes in vivo could be prevented by probenecid, an inhibitor of organic anion transport (Cunningham et al., 1981). When protoplasts were incubated with MCB in the presence of probenecid, fluorescence was absent from vacuoles but accumulated in the cytosol (Figure 7D).

To establish that the loading of fluorescent dyes into vacuoles of aleurone protoplasts was not influenced by removal of cell walls during protoplast isolation, we loaded a range of fluorescent probes into barley aleurone layers. Figure 7E shows bright-field and fluorescence images of aleurone layers incubated in CTG. As was the case in protoplasts, fluorescence from CTG was more intense from secondary vacuoles compared with the larger protein storage vacuoles in the cells of the intact aleurone layer (Figure 7E).

Not all fluorescent probes tested were taken up by aleurone protoplasts and sequestered into vacuoles. Some

probes, such as BCECF free acid and Lucifer Yellow, were not taken up by aleurone protoplasts, although BCECF was taken up readily in the membrane-permeant AM form (Table 1). Other probes, such as the Ca^{2+} indicators indo-1 ff, indo-1 (Bush and Jones, 1987), and fluo-3 (Gilroy and Jones, 1992), remained in the cytosol of aleurone cells whether they were loaded by microinjection or by acid loading. Table 1 summarizes the subcellular localization of fluorescent probes in barley aleurone. Transport of BCECF and fluorescein from the cytoplasm into the vacuoles of intact protoplasts could be prevented by probenecid (data not shown; Wright and Oparka, 1994; Wright et al., 1996).

Fluorescent Probes Are Transported into Aleurone Vacuoles by ATP-Dependent Transporters

As shown in Figures 8 and 9, we have used isolated protein storage vacuoles to study the accumulation of fluorescent probes into these organelles. Our data show that there are at least two kinds of ATP-dependent transporters in the tonoplast of protein storage vacuoles that are responsible for fluorescent probe uptake. Probes such as ZFR-CMAC (Figures 4 and 5), MCB (Figures 8 and 9), MBB, and CTG (Figure 7) are transported across the tonoplast of isolated vacuoles after conjugation to glutathione. BCECF, fluorescein, and indo-1 ff, on the other hand, do not require glutathione conjugation (Figure 8; data not shown). Figures 8A and 8E show that autofluorescence from isolated protein storage vacuoles does not contribute to measurements of

probe fluorescence. Figures 8B and 8C show that uptake of BCECF into isolated vacuoles requires ATP. ATP is also needed for uptake of the glutathione conjugate of MCB, MCB-GS, into isolated aleurone protein storage vacuoles (Figures 8F and 8G). Transport of these two probes is not prevented by including ammonium acetate in the loading buffer (Figures 8D and 8H). We have shown that ammonium acetate equilibrates the internal pH of aleurone protein storage vacuoles with that of the external buffer (Swanson and Jones, 1996; see also Yoshida, 1994). Because uptake of BCECF and MCB-GS occurs in the absence of a proton gradient, we conclude that accumulation of these fluorescent probes into aleurone vacuoles is via an ATP-dependent transporter on the tonoplast rather than coupled to a proton-motive force.

Figure 9 shows that ATP-dependent transport of free probes, such as BCECF (Figure 9A), and glutathione conjugates, such as MCB-GS (Figure 9B), is strongly (>90%) inhibited by vanadate. Vanadate does not inhibit the proton translocating vacuolar ATPase but does inhibit glutathione conjugate pumps (Martinoia et al., 1993; Lu et al., 1997). Substrates used by others to characterize ATP-dependent glutathione conjugate transporters and ATP-dependent organic anion transporters (Martinoia et al., 1993; Blake-Kalff and Coleman, 1996) also inhibited fluorescent probe uptake into isolated aleurone vacuoles (Figure 9). Glutathione-conjugated *N*-ethylmaleimide glutathione (NEM-GS; 25 μM) inhibited MCB-GS (25 μM) uptake by 44% and BCECF (25 μM) uptake by 33%. The anion taurocholate inhibited BCECF accumulation by 71% and MCB-GS uptake by 17%

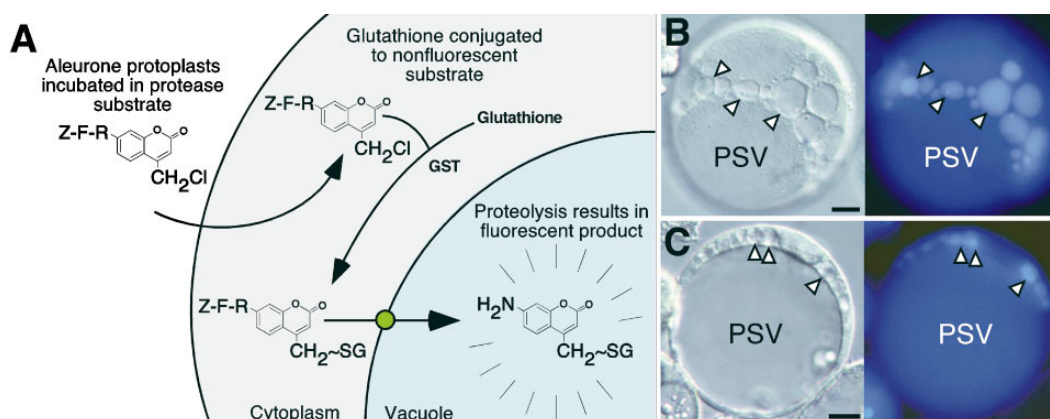


Figure 4. Barley Aleurone Vacuoles Accumulate the Proteolysis Product of the Protease Substrate ZFR-CMAC.

(A) A diagram showing the transport and proteolysis of the substrate ZFR-CMAC. Upon entering the cytoplasm, the substrate is conjugated to glutathione. This conjugation reaction may be mediated by a cytosolic glutathione *S*-transferase (GST). The conjugated substrate is then transported via a tonoplast transporter into the vacuole, where proteases release the fluorescent product CMAC.

(B) and **(C)** DIC and fluorescence images of aleurone protoplasts that have been incubated in ZFR-CMAC for 3 hr. The protoplasts were treated with ABA **(B)** or GA **(C)**. Arrowheads point to some of the secondary vacuoles within each cell. Regardless of hormone treatment, both secondary vacuoles and protein storage vacuoles (PSV) accumulate the proteolysis product of ZFR-CMAC (blue fluorescence). Bars = 10 μm .

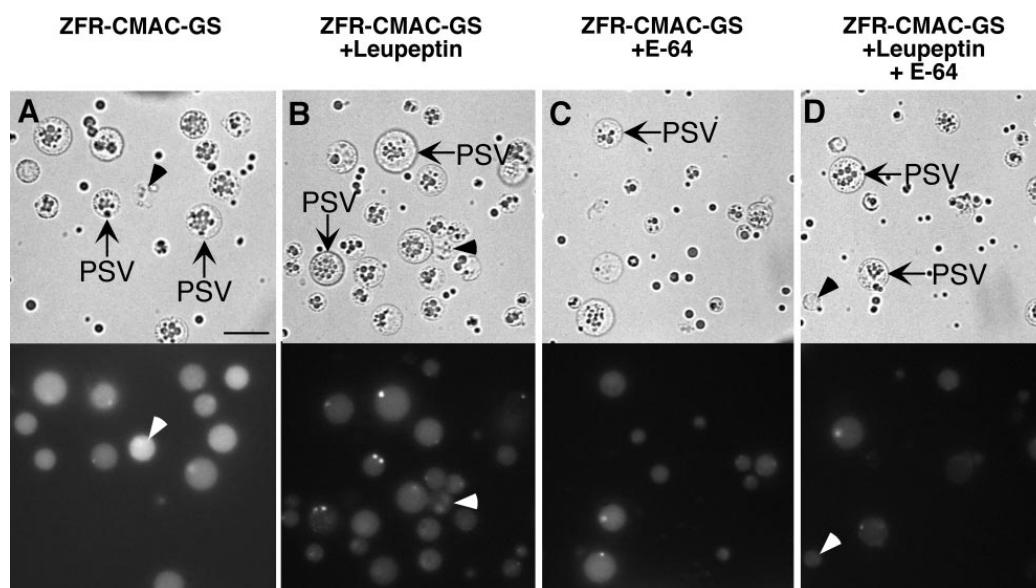


Figure 5. Proteolysis of the Protease Substrate ZFR-CMAC-GS by Isolated Barley Aleurone Protein Storage Vacuoles and Secondary Vacuoles Is Inhibited by the Cysteine Protease Inhibitors Leupeptin and E-64.

Isolated vacuoles were incubated in the presence or absence of protease inhibitors and subsequently incubated with added ZFR-CMAC-GS.

(A) DIC and fluorescence images of protein storage vacuoles (PSV) and secondary vacuoles (arrowheads) as well as free vacuolar inclusions. The larger organelles containing numerous inclusions are protein storage vacuoles. The fluorescent proteolysis product of ZFR-CMAC-GS accumulates in both protein storage vacuoles and secondary vacuoles.

(B) to (D) The intensity of vacuolar fluorescence is reduced by the protease inhibitors leupeptin, as shown in (B) and (D), and E-64, as shown in (C) and (D).

Punctate autofluorescence from some protein storage vacuole inclusions can be seen in the fluorescence images. Bar in (A) = 20 μm for (A) to (D).

(Figure 9). Similar to the *in vivo* result, probenecid inhibited uptake of BCECF into aleurone vacuoles by 61% (Figure 9A). In contrast, probenecid stimulated uptake of MCB-GS into isolated vacuoles by $\sim 20\%$ (Figure 9B), even though it prevented vacuolar accumulation of this probe *in vivo* (Figure 7D).

DISCUSSION

Our results show that barley aleurone cells contain two different types of vacuoles. In this study, we describe an organelle, referred to as the secondary vacuole, that is distinct from protein storage vacuoles (Figure 1). We demonstrate directly in living cells that the secondary vacuole is an acidic (Figure 3), lytic (Figure 4) organelle. The secondary vacuole has other properties in common with plant vacuoles, including a tonoplast that labels with antibodies to the aquaporin α -TIP (Figure 2) and contains ATP-dependent organic anion and glutathione conjugate transporter activity (Figures 7 to 9). We show that secondary vacuoles are dynamic organelles that increase in number with duration of incubation of aleurone protoplasts in GA or ABA (Figure 1). We also show that fluorescent probes are more rapidly turned over in

secondary vacuoles than in protein storage vacuoles (Figure 6). We suggest that the secondary vacuoles in aleurone are lysosome-like organelles.

Barley Aleurone Cells Contain Two Types of Lytic Organelles

We have used light microscopy to study secondary vacuoles and protein storage vacuoles *in vivo* and *in vitro*. By focusing our observations on living cells and intact organelles, we have avoided many of the limitations inherent in histochemical or immunological approaches that rely on fixed and permeabilized tissue. The fluorescent probes that we have used are plasma membrane permeable, are not cytotoxic, and do not affect the response of the aleurone cell to GA or ABA (Swanson and Jones, 1996; data not shown). The pH-sensitive probe BCECF and the protease substrate ZFR-CMAC preferentially accumulate in vacuoles, and this has allowed us to monitor pH and proteolytic activity in the secondary vacuoles and protein storage vacuoles of living aleurone protoplasts. Our data allow us to conclude that barley aleurone protoplasts have at least two types of vacuoles, of which one appears to have features and functions in common with lysosomes.

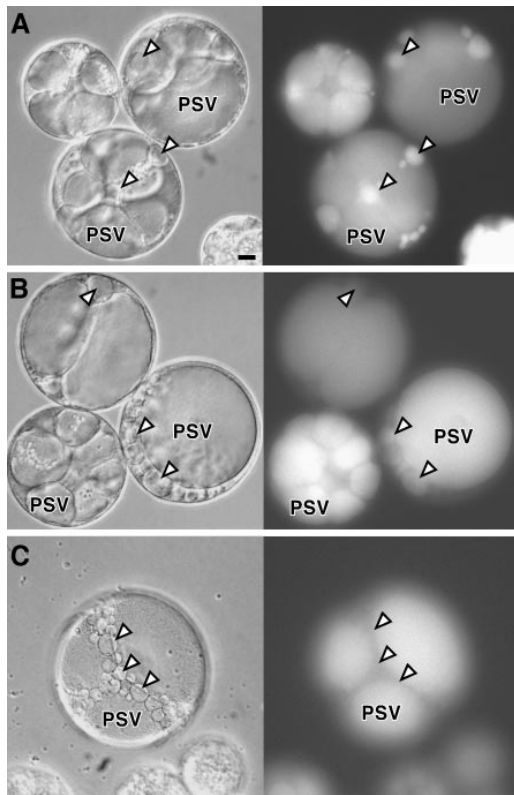


Figure 6. Pulse-Chase Labeling of Barley Aleurone Vacuoles Demonstrates That Secondary Vacuoles Are Dynamic Organelles.

(A) DIC and fluorescence images of barley aleurone protoplasts incubated for 3 hr with the protease substrate ZFR-CMAC. Note that the intensity of fluorescence from secondary vacuoles (arrowheads) is typically greater than that from protein storage vacuoles (PSV).

(B) and **(C)** Protoplasts fluorescently labeled as shown in **(A)** were washed free of unincorporated substrate and incubated in fresh media for 2 days. Protoplasts were then photographed using DIC and fluorescence microscopy. Note that secondary vacuoles are either no brighter than protein storage vacuoles **(B)** or are nonfluorescent **(C)**.

Bar in **(A)** = 10 μm for **(A)** to **(C)**.

The existence of lysosomes in plants has long been debated (see, e.g., Moriyasu and Ohsumi, 1996). Matile (1975) recognized that catabolic enzymes were essential for sustained biological activity and that these enzymes must be compartmentalized to prevent their indiscriminate hydrolysis of biopolymers. He proposed that plant proteases, nucleases, phosphatases, and other degradative enzymes were constituents of a "lytic compartment," a compartment that included the extracellular space, vacuoles, and other organelles containing lytic enzymes. With improved techniques for vacuole isolation, it became clear that many plant vacuoles contain enzymes found in animal lysosomes (Matile, 1978;

Wink, 1993). Plant vacuoles were therefore seen as fulfilling the role of the animal lysosomal system (Boller and Wiemken, 1986). That the lysosomal system in plants is not unique but is analogous to that in animals was recognized by Marty et al. (1980), who considered vacuoles to be "a differentiated elaboration of lysosomal structures found in all eukaryotes." Marty et al. adopted de Duve's view that the term lysosome was an operational term based on function and that "only when considered as part of a system involved directly or indirectly in intracellular digestion does the term lysosome describe a physiological unit" (de Duve, 1969, quoted in Marty et al. 1980). It is in this context that Marty et al. suggested that acid hydrolase-containing provacuoles are primary lysosomes (Marty et al. 1980). Lysosomes are defined as acidic organelles within the lysosomal system that contain an array of hydrolytic enzymes and that function to degrade macromolecules to simple sugars, amino acids, and nucleic acids (Mellman, 1996). Although plant vacuoles have sometimes been called lysosomes (see, e.g., Holtzman, 1989), we propose that the term lysosome be used to designate an acidic organelle within the plant lysosomal system that contains a battery of hydrolytic enzymes and whose overriding function is intracellular digestion. By this definition, the multifunctional central vacuole of most plant cells and the protein storage vacuoles of seeds and grain are not plant lysosomes because they have significant storage functions aside from their roles in hydrolytic breakdown.

Lysosome-like organelles have recently been described in other plant cells. In sucrose-starved tobacco BY-2 cells treated with cysteine protease inhibitors, a specialized acidic vacuole 1 to 6 μm in diameter arose de novo in the cytosol. Although this compartment was not shown to contain proteolytic activities, it appeared coincident with an increase in cellular proteases and with increased protein degradation, and it contained acid phosphatase activity (Moriyasu and Ohsumi, 1996). Acid phosphatase is an enzyme closely associated with lysosomes in animal cells. Moreover, accumulation of these organelles required inhibition of cysteine protease activity. These data strongly indicate that these vacuoles of sucrose-starved BY-2 cells function as lysosomes. The evidence that the secondary vacuole of barley aleurone cells is a lysosome is equally compelling. Secondary vacuoles are membrane-bound acidic organelles containing cysteine protease activity. Moreover, the appearance of this organelle foreshadows the death of the aleurone cell (P.C. Bethke, J.E. Lonsdale, A. Fath, and R.L. Jones, unpublished data). Our working hypothesis is that the secondary vacuole is involved in autophagy and may be part of the process leading to programmed cell death of the aleurone cell (Wang et al., 1996).

Barley aleurone cells were predicted to have at least two vacuole types based on the localization of the cysteine protease aleurain and the aspartic protease HvAP (Holwerda and Rogers, 1992). HvAP is located in the protein storage vacuole of the mature aleurone cell (Bethke et al., 1996), whereas aleurain is located in a separate vacuole 1 to 2 μm

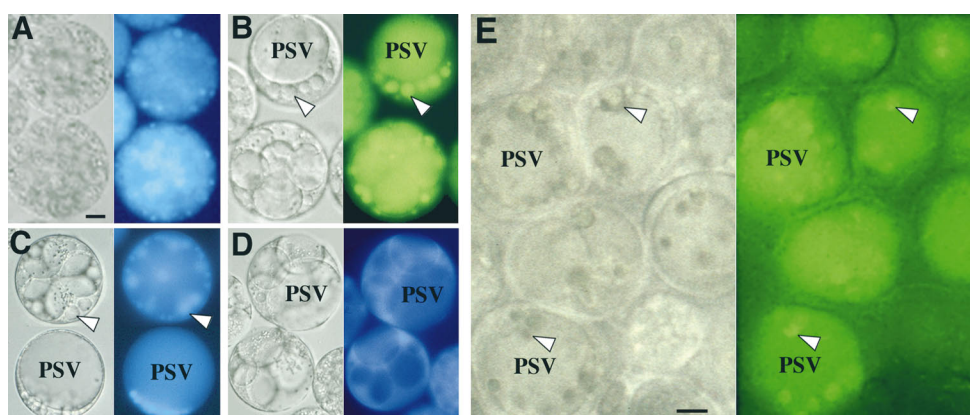


Figure 7. The Nonfluorescent Probes MBB, MCB, and CTG Are Conjugated to Cytosolic Glutathione in Barley Aleurone Cells, and the Fluorescent Conjugates Accumulate in Protein Storage Vacuoles and Secondary Vacuoles.

(A) to (D) DIC and fluorescence images of barley aleurone protoplasts labeled with MBB (A), CTG (B), or MCB without (C) and with probenecid (D). Note that both protein storage vacuoles (PSV) and secondary vacuoles (arrowheads) accumulate fluorescent conjugates [(A) to (C)] and that accumulation is inhibited by probenecid (D).

(E) DIC and fluorescence images of a barley aleurone layer labeled with CTG.

Bar in (A) = 10 μm for (A) to (D); bar in (E) = 10 μm for (E).

in diameter (Holwerda and Rogers, 1992). Our data do not allow us to conclude that the aleurain-containing vacuole and secondary vacuoles are developmentally related organelles, but this remains an attractive hypothesis. Immunolabeling experiments are under way to test this hypothesis.

Two Kinds of ATP-Dependent Transporters Load Fluorescent Probes into Aleurone Vacuoles

In addition to BCECF and ZFR-CMAC, the use of several other fluorescent probes has allowed us to distinguish secondary vacuoles from protein storage vacuoles in the aleurone cell. By following the uptake of these probes into intact isolated vacuoles, we have been able to identify transporters that result in the accumulation of fluorescent probes within protein storage vacuoles and secondary vacuoles (Figures 5, 8, and 9). Our data show the existence of at least two types of ATP-dependent transporters on aleurone tonoplasts, one that transports glutathione conjugates (Kreuz et al., 1996; Coleman et al., 1997a) and another that transports organic anions (Hortensteiner et al., 1993; Blake-Kalff and Coleman, 1996). To date in plant cells, these transporters have been found only on the tonoplast (Tommasini et al., 1993; Coleman et al., 1997a, 1997b), and their presence on both the protein storage vacuole and secondary vacuole provides further confirmation that these organelles have many properties in common with other plant vacuoles.

Using isolated intact protein storage vacuoles, we have studied the uptake and accumulation of fluorescent probes in detail. Transport of MCB (Figures 8 and 9), MBB, and ZFR-CMAC (Figure 5) into intact isolated vacuoles required ATP and attachment of glutathione to the probe. Uptake of

Table 1. Subcellular Distribution of Fluorescent Probes in Barley Aleurone Protoplasts

Probe	Location of Fluorescence
Glutathione/sulfhydryls	
Monochlorobimane	Cytoplasm then vacuole
Monobromobimane	Cytoplasm then vacuole
Cell Tracker Green	Cytoplasm then vacuole
pH	
BCECF-AM	Cytoplasm then vacuole
BCECF	No uptake
Lysosensor yellow/blue	Vacuole
Calcium	
Indo-1 ff	Cytoplasm
Indo-1 (Bush and Jones, 1987)	Cytoplasm
Fluo-3 (Gilroy and Jones, 1992)	Cytoplasm
Protease substrate	
ZFR-CMAC	Vacuole
Other fluorophores	
Lucifer yellow	No uptake
Oregon green	Cytoplasm then vacuole
Fluorescein	Cytoplasm then vacuole

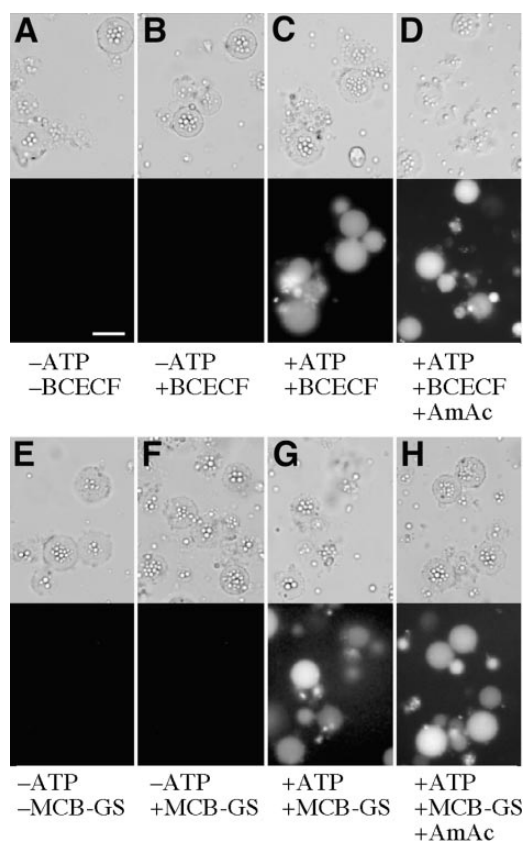


Figure 8. Fluorescent Probe Uptake into Isolated Aleurone Vacuoles Requires ATP but Not a Proton Gradient.

DIC and fluorescence images of isolated barley aleurone vacuoles are shown. Additions (+) or omissions (-) in each experiment are indicated under (A) to (H).

(A) to (D) Vacuoles were incubated without (A) or with ([B] to [D]) BCECF in the absence ([A] and [B]) or presence ([C] and [D]) of ATP and in the absence ([A] and [C]) or presence (D) of ammonium acetate (AmAc).

(E) to (H) Vacuoles were incubated without (E) or with ([F] to [H]) MCB-GS in the absence ([E] and [F]) or presence ([G] and [H]) of ATP and in the absence ([E] to [G]) or presence (H) of ammonium acetate.

Accumulation of the fluorescent probe requires ATP and is relatively insensitive to ammonium acetate, which collapses the proton gradient across the tonoplast. Bar in (A) = 10 μm for (A) to (H).

glutathione-conjugated probes into isolated vacuoles was not dependent on a proton gradient across the tonoplast because dissipation of the proton gradient with ammonium acetate did not inhibit ATP-dependent probe accumulation. Instead, transport of glutathione-conjugated fluorescent probes was most likely mediated by a transporter of glutathione-conjugated xenobiotics, as suggested by others, including those working with vacuoles isolated from barley leaves

(Tommasini et al., 1993; Blake-Kalff and Coleman, 1996; Kreuz et al., 1996; Coleman et al., 1997a). This idea is supported by our results showing that other glutathione conjugates, such as NEM-GS, inhibited accumulation of fluorescent MCB-GS in isolated aleurone vacuoles and by our data showing that uptake of MCB-GS is vanadate sensitive (Figure 9).

The uptake of BCECF and other fluorescent probes not conjugated to glutathione was likely by an ATP-dependent organic anion transporter (Hortensteiner et al., 1993). Our data show that BCECF transport into isolated aleurone vacuoles was reduced by taurocholate, a bile acid shown to accumulate in barley mesophyll vacuoles in an ATP-dependent manner (Blake-Kalff and Coleman, 1996). Taurocholate may

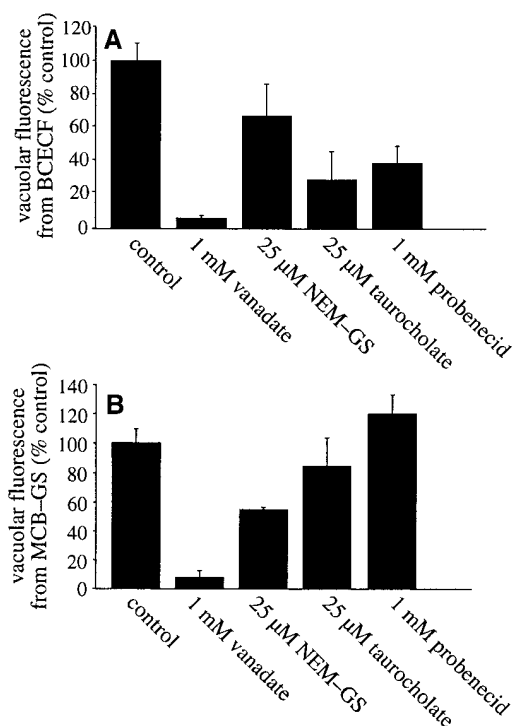


Figure 9. Uptake of MCB-GS by Isolated Barley Aleurone Vacuoles Is Mediated by Different ATP-Dependent Transporters Than Those Used to Transport BCECF.

(A) Effect of compounds on the uptake of BCECF by isolated aleurone vacuoles. The control is the fluorescence intensity of isolated vacuoles after ATP-dependent BCECF accumulation.

(B) Effect of compounds on the uptake of MCB-GS by isolated aleurone vacuoles. The control is the fluorescence intensity of isolated vacuoles after ATP-dependent MCB-GS accumulation.

For (A) and (B), vanadate, NEM-GS, taurocholate, or probenecid were added to the control reaction mixture, and vacuolar fluorescence after 30 min was expressed as the percentage of the control. Error bars indicate standard deviation.

act as a competitive inhibitor to BCECF in these experiments. Vanadate also inhibited the directly energized transporter that pumps these organic anions into barley aleurone vacuoles in a manner similar to that found for these transporters in other types of plant cells (Martinoia et al., 1993; Li et al., 1995b; Coleman et al., 1997a). Consistent with its ability to inhibit anion transport, probenecid decreased the ATP-dependent uptake of BCECF into isolated vacuoles. However, probenecid slightly stimulated uptake of MCB-GS (Figure 9). Surprisingly, when aleurone protoplasts were incubated in probenecid, vacuolar accumulation of MCB-GS was much reduced (Figure 7). Currently, we are unable to explain the differing effects of probenecid on in vivo accumulation compared with in vitro accumulation of MCB-GS in aleurone vacuoles.

Because probenecid stimulated MCB-GS uptake into isolated vacuoles and taurocholate had little effect, and because both of these compounds inhibited BCECF uptake by ~60%, we conclude that at least two kinds of ATP-dependent transporters are present in protein storage vacuoles. One of these is an organic anion transporter that can be inhibited by probenecid and transports BCECF. The other is a glutathione conjugate transporter that is not inhibited by probenecid and transports MCB-GS. Both transporters may belong to the superfamily of ABC transporters (reviewed in Rea et al., 1998). We suggest that BCECF, MCB-GS, and probenecid may help to discriminate between the activity of these two types of ATP-dependent transporters in tonoplasts from other plant cell types.

Conclusions

By using nontoxic fluorescent probes that can be loaded into barley aleurone protoplasts noninvasively, we have demonstrated the presence of a second type of vacuole that has many features of a lysosome. This organelle is present in the aleurone cell during incubation in both GA and ABA, and imaging with pH-sensitive probes indicates that the organelle is more acidic in GA-treated cells than in ABA-treated cells. This observation may have far-reaching implications, because GA brings about programmed cell death in aleurone and ABA prevents it (Kuo et al., 1996; Wang et al., 1996). Lower pH within the secondary vacuoles of GA-treated cells may be associated with increased rates of hydrolysis within this organelle and a commensurate increase in the rate of autophagy. Our experiments showing that the loading of fluorescent dyes into aleurone vacuoles is driven by at least two types of ATP-dependent transporters allow for the rational design of compounds that can be targeted to the plant vacuole. For example, cell-permeable electrophiles that readily form glutathione conjugates are likely to be taken up into the vacuole both in vivo and in vitro. In vitro conjugation of these same compounds to glutathione provides a means for their introduction into isolated vacuoles. The ability of probenecid to prevent the uptake of fluores-

cent probes into vacuoles in vivo provides a way to alter the partitioning of probes between vacuoles and cytoplasm.

METHODS

Isolation of Aleurone Protoplasts and Layers

Aleurone layers were prepared as described previously (Jones and Jacobsen, 1982). Layers were incubated in water containing 20 mM CaCl₂ and either 5 μM abscisic acid (ABA) or 5 μM gibberellic acid (GA). Protoplasts were prepared from aleurone layers isolated from deembryonated barley grains (*Hordeum vulgare* cv Himalaya, 1991 harvest, Washington State University, Pullman), as described previously (Bush et al., 1986; Bethke et al., 1996), except that 5 μM ABA was added to all solutions used in the preparation of protoplasts. Protoplasts were incubated at 25°C in Gamborg's B-5 medium (release medium; Sigma) with 20 mM CaCl₂ and 5 μM ABA. Where indicated, 25 μM GA was also included in the release medium. Protoplasts were purified on a discontinuous Nycodenz density gradient, as described by Bethke et al. (1996).

Loading of Protoplasts and Layers with Fluorescent Probes

Monochlorobimane (MCB) and monobromobimane (MBB) were purchased from CalBiochem (La Jolla, CA). Indo-1 ff was purchased from Teflabs (Austin, TX). All other fluorescent probes were purchased from Molecular Probes (Eugene, OR). A small volume of stock probe solution was added to the medium in which protoplasts were cultured. Because the pH of the incubation medium is acidic (pH 5.6 to ~3, depending on hormone treatment and protoplast age), probe uptake across the plasma membrane is thought to occur by acid loading (Bush and Jones, 1987). 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was loaded into aleurone cells by using the membrane-permeant acetoxymethyl (AM) form. For experiments using Lysosensor yellow/blue, we briefly incubated protoplasts in medium buffered to pH 8.0 with Hepes-KOH. This allowed the probe to diffuse through the plasma membrane in its neutral form. The solvent, stock concentration, working concentration, and except when noted, the loading time for each of the fluorescent probes used are as follows: BCECF-AM (DMSO, 1 mM and 10 μM; 40 min), Lysosensor yellow/blue (DMSO, 1 mM and 10 μM; 1 hr), fluorescein (water, 1 mM and 10 μM; 20 min), indo-1 ff (water, 5 mM and 50 μM; 1 hr), MCB (DMSO, 5 mM and 50 μM; 1 hr), MBB (DMSO, 5 mM and 50 μM; 1 hr), Cell Tracker Green ([CTG] DMSO, 5 mM and 50 μM; 1 hr), 7-amino-4-chloromethylcoumarin benzyloxycarbonyl-L-phenylalanyl-L-arginine amide hydrochloride ([ZFR-CMAC] DMSO or ethanol, 20 mM and 20 μM; 3 hr).

For long-term localization of fluorescent probes, protoplasts were incubated with either ZFR-CMAC (20 μM) or fluorescein (20 μM) for 3 hr, settled at 1 g, and floated on a Nycodenz (Sigma) gradient to remove unincorporated probe. Cells were transferred to fresh release medium. After 2 days, the location and intensity of fluorescence were observed using standard UV (CMAC) or fluorescein filter sets.

Aleurone layers were loaded with fluorescent probes in the same way as were protoplasts, except that layers were incubated in water containing the probe at working concentration on a rotating shaker (75 rpm) for the indicated time.

Light and Epifluorescence Microscopy

Living protoplasts were examined on Teflon-coated 10-well microscope slides (Cel-line, Newfield, NJ) or on glass slides by using a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY) equipped with a Plan-Neofluor $\times 40$ dry objective, 0.75 numerical aperture objective, differential interference contrast (DIC) optics, and a 100-W mercury arc light source. Fluorescence was filtered using standard UV or fluorescein filter sets purchased from Chroma Corp. (Brattleboro, VT). Vacuolar pH measurements were made as described previously (Swanson and Jones, 1996).

Images were acquired using Kodak Ektachrome 160T film or a cooled charge-coupled device camera (Princeton Instruments, Trenton, NJ). For quantitative fluorescence measurements, a 400-msec exposure was used. The camera was controlled with IPLab software (Signal Analytics Corp., Vienna, VA) running on a Macintosh computer (Cupertino, CA).

Immunolocalization

For immunolocalization of α -TIP, protoplasts were purified on a Nyco-denz gradient and diluted 1:10 in fixative. Fixatives contained 4.5% (w/v) paraformaldehyde and 0.3% glutaraldehyde and were prepared in either Gamborg's B-5 medium or $2.3 \times$ PBS ($1 \times$ PBS is 5.0 mM Na_2HPO_4 , 0.59 mM KH_2PO_4 , and 100 mM NaCl). In both cases, mannitol was added when needed to make the fixative isotonic with the protoplast release medium. Cells were fixed for at least 1 hr and then washed three times for 20 min with isotonic wash buffer. The wash buffer contained either release medium plus KOH to pH 7.2 or $2.3 \times$ PBS plus mannitol. To permeabilize the cells, the first wash contained 0.2% (w/v) Triton X-100 (Sigma). Permeabilized cells were incubated for 2 hr with an anti- α -TIP antibody diluted 1:75 to 1:400 in wash buffer containing 3% BSA. Cells were washed three times for 20 min with wash buffer and then incubated for 2 hr with fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibodies (Sigma) diluted 1:100 to 1:200 in wash buffer containing 3% BSA. Cells were washed three times for 20 min with wash buffer and then photographed as described above. Polyclonal antibodies raised against α -TIP were provided by K.D. Johnson (California State University, San Diego).

Measurement of Transport into Intact Vacuoles

Intact vacuoles were isolated from aleurone protoplasts, as described by Bethke and Jones (1994). The method for loading compounds into isolated vacuoles is a modification of the approach described by Li et al. (1995a). Uptake was measured at room temperature in 50 μL of reaction mixtures containing 3 mM ATP, 3 mM MgSO_4 , 10 mM creatine phosphate, 16 units per mL creatine phosphokinase, buffer solution (50 mM KCl, 0.1% [w/v] BSA, 25 mM Tris-Mes, pH 8, and mannitol added to 1100 mmol/kg), and test compounds, as indicated in the text. Test compounds were as follows: 25 μM BCECF, 25 μM taurocholate, 25 μM *N*-ethylmaleimide-glutathione (NEM-GS), 25 μM MCB-glutathione (MCB-GS), 1 mM vanadate, 1 mM probenecid, and/or 50 mM ammonium acetate. Substrates requiring conjugation to glutathione (NEM and MCB) before uptake were prepared as described by O'Connell et al. (1988), using DMSO as the solvent for the compound. Uptake into isolated vacuoles was initiated by the addition of 8 μL of isolated vacuoles and allowed to proceed for 30 min. Uptake was terminated by allow-

ing the vacuoles to settle at 1g and then replacing the supernatant with buffer solution. After repeating the wash step, vacuoles were viewed on Teflon-coated 10-well microscope slides and fluorescence quantitated using the charge-coupled device camera. The background fluorescence was subtracted, and the signal from individual vacuoles was averaged. For each treatment, 40 to 60 vacuoles of similar diameter were observed.

For measurements of ZFR-CMAC proteolysis, protoplasts were passed through a fine syringe to isolate intact vacuoles. A sample of this lysate (2 μL) was added to 500 μL of incubation mixture containing 100 mM KCl, 2 mM MgCl_2 , 1 mM CaCl_2 , 5 mM Hepes, pH 7.2, and D-sorbitol to 1050 mmol/kg. The following reagents were added as indicated in the text: ATP, 2 mM; E-64, 50 μg ; and leupeptin, 50 μg . After 30 min, the glutathione conjugate of ZFR-CMAC was added to a final concentration of 5 μM . After 2 hr, vacuolar fluorescence was visualized on a Nikon Diaphot microscope (Tokyo, Japan) by using a Nikon Nicor $\times 40$ oil immersion lens with a numerical aperture of 1.3. Light from a 100-W mercury lamp was filtered through a 380-nm band pass excitation filter, a 400-nm dichroic mirror, and a 420-nm long pass emission filter. Images were captured on Ektachrome 160T film by using a 5-sec exposure. The glutathione conjugate of ZFR-CMAC was prepared as described above (O'Connell et al., 1988), using ethanol as a solvent for ZFR-CMAC.

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