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Calsequestrinlike calcium-binding protein is expressed in calcium-accumulating cells of *Pistia stratiotes*

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ABSTRACT **To contend with high calcium (Ca) levels in the environment, many plant species contain crystal idioblasts, specialized cells which accumulate large amounts of Ca as oxalate crystals. The biochemical processes involved in the accumulation of Ca in crystal idioblasts are unknown, as these cells constitute only a minor proportion of the total plant tissue. To address how crystal idioblasts buffer cytosolic Ca during crystal formation, we purified these cells from water lettuce and assessed their biochemistry. We show here that crystal idioblast cells contain three Ca-binding proteins not detectable in mesophyll cells. One of the Ca-binding proteins shares antigenicity with rabbit calsequestrin, a high-capacity low-affinity Ca-binding protein, and is encoded by related nucleotide sequences. Immunocytochemical localization studies further demonstrate that a calsequestrinlike protein is present primarily in crystal idioblasts and is preferentially localized in the endoplasmic reticulum, an organelle enriched in Ca as evidenced by vital staining. We thus conclude that crystal idioblasts possess a buffering system involving calsequestrin-like proteins, a process that likely plays an essential role in the bulk control of Ca in plant cells.**

Calcium serves as an important effector molecule in the signal transduction pathways of many physiological and developmental processes in plants (1–4). To serve this role, intracellular Ca levels are maintained within strict limits. Most plant cells modulate the cytoplasmic levels of Ca by sequestration of this molecule within mitochondria, chloroplasts, and endoplasmic reticulum (ER) or by active pumping of Ca into the apoplast (5, 6). Many plant species, however, display very little discrimination of Ca in the root zone (7). This inability to restrict Ca uptake results in transport of Ca throughout the plant via the transpiration stream to levels directly proportional to those available in the environment. As most plants do not excrete appreciable amounts of Ca under normal growth conditions, Ca accumulates in the apoplast of those organs where evapotranspiration occurs. As up to a 10,000-fold difference in Ca concentration (total) may occur between the apoplast and cytosol (3), and the electrical potential difference is inside negative, there is a tremendous driving force for inward diffusion of Ca. Mechanisms involving sequestration of Ca, as discussed above, have limited capacity and become easily saturated at low concentrations, especially in those plants subjected to a continuous and relatively high uptake of Ca.

To cope with high Ca levels in the environment and, in turn, avoid intracellular Ca toxicity, many plants have evolved a mechanism to regulate bulk quantities of Ca by formation of Ca oxalate crystals (8–10). Ca oxalate formation often occurs in specialized cells called crystal idioblasts (11). It is thought that the primary function of these idioblasts is to serve as a Ca sink—i.e., accumulate Ca as oxalate crystals, thereby reducing the apoplastic concentration of this molecule around adjacent cells (12, 13). The importance of these specialized Ca sink cells is reflected by the fact that greater than 90% of the total Ca may be present in this physiological and osmotically inert compound (14, 15). Likewise, a value of 10% Ca oxalate by dry weight is not uncommon in plants (16).

There have been numerous developmental and structural studies on crystal idioblasts which have described the unique ultrastructural features presumably involved in Ca transport and sequestration into Ca oxalate crystals (reviewed in refs. 8 and 9). There has been, however, almost no biochemical information addressing the mechanisms involved in regulating Ca transport in these specialized cells. One major element that must be operating in crystal idioblasts is a Ca buffering system that will accommodate large fluxes of Ca while maintaining biochemical functions within the cytoplasm. In animal cells which are capable of large transitory fluxes of Ca (e.g., muscle cells), specialized Ca-binding proteins (CBP) have been found which appear to be involved in buffering Ca activity (17–20). The most extensively studied of these proteins is calsequestrin, a high-capacity (up to 50 Ca molecules per calsequestrin molecule) low-affinity (K_d = 1 mM) CBP (21–25). A calsequestrin-like protein has recently been found in spinach leaves and *Streptanthus tortuosus* cell cultures (26, 27); however, its tissue and subcellular locations have not been determined. A high-capacity CBP, such as calsequestrin, could play an important role in cells such as the crystal idioblasts which are specialized for Ca transport and compartmentation.

To study the biochemical mechanisms involved in the accumulation and sequestration of Ca in crystal idioblasts, we have developed an efficient procedure for the isolation of protoplasts of these specialized cells as well as those from the bulk mesophyll tissue from *Pistia stratiotes*. Using biochemical, immunochemical, and molecular techniques, we demonstrate that calsequestrin-like proteins are present in crystal idioblasts but not in surrounding mesophyll cells of leaves of this species. The data indicate that crystal idioblasts possess a Ca buffering system which is critical to this mechanism of high-capacity Ca regulation in plants.

METHODS

Isolation of Idioblast and Mesophyll Protoplasts. The adaxial surface of young leaves of *Pistia stratiotes* L. (water lettuce) was gently abraded with emery cloth to remove trichomes. The leaves were cut into 1- to 2-cm^2 pieces, placed adaxial surface down onto digestion medium [wt/vol: 2% cellulase (Worthington CELF), 1% Macerase (Calbiochem), 1% pectolyase Y-23 (Seishin Pharmaceutical), and 1% bovine

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Abbreviations: ER, endoplasmic reticulum; CBP, calcium-binding protein.

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serum albumin in 5 mM $CaCl_2$/0.4 M mannitol/10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.5] and vacuum infiltrated for 30 sec. After 1 hr at 34°C in a shaker bath, the digestion was essentially complete. The digest was filtered through 120 μm pore nylon mesh and the protoplasts were pelleted at 150 × *g* for 1 min, suspended in cold sucrose buffer [0.4 M sucrose/2 mM $CaCl_2$/5 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes), pH 7.0] and placed on ice. After 5 min most of the crystal idioblasts settled out, while mesophyll protoplasts remained suspended. The idioblast protoplasts were suspended in mannitol buffer (0.4 M mannitol/2 mM $CaCl_2$/5 mM Hepes, pH 7.0) and placed on a step gradient consisting of 2 ml of Percoll (Sigma), 2 ml of sucrose buffer plus 9.1% dextran T35-50 (United States Biochemical), and 2 ml of sucrose buffer. The gradient was centrifuged at 150 × *g* for 2 min. Raphide idioblast protoplasts banded at the top of the Percoll layer, while druse idioblast protoplasts pelleted at the bottom of the Percoll layer. The mesophyll protoplast fraction was placed on a similar gradient and after centrifugation was collected at the top of the sucrose buffer plus 9.1% dextran step.

Vital Stain for Ca Compartmentation. Protoplasts were incubated with vital stains to determine if organelle compartmentation of Ca could be identified at the light microscope level. The protoplasts were incubated for 15 min with the following fluorescent probes dissolved in isolation medium: chlortetracycline (10^{-4} M; Calbiochem) for bound Ca, $DiOC_6$ (5 μg/ml; Molecular Probes) for ER, and rhodamine 123 (5 μg/ml; Sigma) for mitochondria. After a brief wash with isolation medium, the protoplasts were viewed by using a Leitz Aristoplan microscope set up for epifluorescence.

$^{45}Ca^{2+}$ Overlay Assay. The protoplast samples were freeze-thawed several times and then mixed with an equal volume of a solution containing 50 mM Tris·HCl at pH 7.3, 10% (wt/vol) 2-mercaptoethanol, 2% (wt/vol) sodium dodecyl sulfate (SDS), and 10% (vol/vol) glycerol. The samples were heated at 95°C for 5 min and centrifuged at 16,000 × *g* for 2 min, and the supernatant was analyzed by SDS/polyacrylamide gel electrophoresis. After electrophoresis the proteins were transferred to a nitrocellulose membrane which had been incubated for 10 min in the binding buffer (60 mM KCl/5 mM $MgCl_2$/10 mM imidazole·HCl, pH 7.4) (28). The membranes were then transferred to 10 ml of binding buffer containing 6.25 μM $^{45}CaCl_2$ (25 μCi; 1 Ci = 37 GBq). After an incubation of 10 min, the membranes were washed with distilled water, dried, and then exposed to x-ray film.

Immunolocalization. Immunolocalization was conducted according to Li and Franceschi (29). Sections were incubated with the primary antibody (1:100 dilution) for 2 hr at room temperature. After washing, the sections were incubated with staphylococcal protein A-gold (Amersham) for 1.5 hr at 1:100 dilution, followed by washing. For transmission electron microscopy examination, the ultrathin sections from the same tissue blocks were poststained as described (29) and examined with a Hitachi 600 electron microscope. For light microscopy examination, the gold particles bound to the semithin sections were silver enhanced by using a kit (Amersham), examined, and photographed on a Leitz Aristoplan microscope. Images of the labeled cells were recorded by making double exposures, with one exposure using epiillumination and a polarizing filter cube to make the label show as bright spots, and the second exposure using phase-contrast optics to make the cellular details show up.

Gel Electrophoresis and Immunoblotting. SDS/PAGE was performed according to Laemmli (30) and gels were stained with silver as described by Ohsawa and Ebata (31). For immunoblot analysis, the proteins in the gel were transferred to a nitrocellulose membrane and probed with primary antiserum (1:500 dilution). After washing the membrane was probed with a secondary antibody (sheep anti-goat IgG) tagged with horseradish peroxidase and detected as described by the supplier (Sigma).

RNA Gel Blot Analysis. Poly(A)$^+$ RNA was isolated, fractionated on an agarose gel, and transferred to a nitrocellulose membrane as described by Sambrook *et al.* (32). The 5′ coding region (about 800 bp) of the cDNA encoding rabbit calsequestrin (33) was inserted into Bluescript plasmid (Stratagene). The plasmid was used to prepare antisense RNA probe by *in vitro* transcription using T3 RNA polymerase as described by the supplier (Promega). Hybridization was performed at 42°C overnight in a solution containing 50% (vol/vol) formamide, 5× SSPE (32), 3× Denhardt's solution (32), 2% SDS, salmon sperm single-stranded DNA at 75 μg/ml, poly(A)$^+$ RNA at 5 μg/ml, and 2 μg of radiolabeled antisense or sense strand RNA probe. After hybridization, the membrane was washed sequentially for 15 min each at 42°C, with 2× SSPE/0.1% SDS, 1× SSPE/0.1% SDS, and 0.5× SSPE/0.1% SDS. The membrane was air dried and exposed to x-ray film.

RESULTS

Purification of Idioblast and Mesophyll Protoplasts. Crystal idioblasts comprise less than 1% of the total leaf cells of *Pistia*, and so biochemical dissection of the mechanisms involved in Ca oxalate formation necessitated the isolation of this cell type. As is demonstrated in Fig. 1, our protoplast isolation procedure (see *Materials and Methods*) results in highly purified preparations of intact idioblast and mesophyll protoplasts.

Vital Staining Indicates Ca Is Sequestered in the Idioblast ER. Chlortetracycline, which labels bound Ca, gave a general greenish-yellow fluorescence of idioblasts, and a distinctly brighter reticulate pattern could be seen within the fainter cytoplasmic fluorescence (Fig. 2). A similar reticulate pattern of fluorescence was seen when $DiOC_6$ was used to visualize the ER in the idioblast cells (Fig. 3). The reticulate pattern of fluorescence could not be detected in mesophyll protoplasts (see Fig. 3). This is consistent with less ER in mesophyll cells as determined by electron microscopy examination (data not shown), although it is also likely that ER fluorescence in mesophyll cells was obscured by the bright red autofluorescence of chlorophyll (see Fig. 3). Rhodamine 123 gave a punctate pattern of fluorescence within the idioblasts (data not shown), which is consistent with staining of mitochondria. These results indicate that Ca is accumulated in the ER of developing idioblast cells.

Identification of Idioblast-Specific CBPs. The large Ca influx into the crystal idioblasts during crystal deposition suggests that a significant increase in the cytoplasmic free Ca concentration in the idioblasts could occur. The excess Ca in the idioblast cytosol must be buffered, perhaps through a

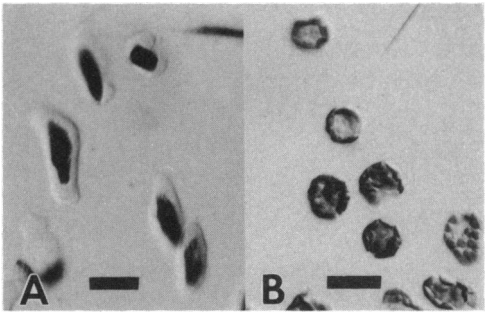

FIG. 1. Bright-field microscopy of protoplasts isolated from leaves of *Pistia stratiotes*. (*A*) Purified raphide crystal idioblast protoplasts. (Bar = 50 μm.) (*B*) Mesophyll protoplasts from the same digest. (Bar = 25 μm.)

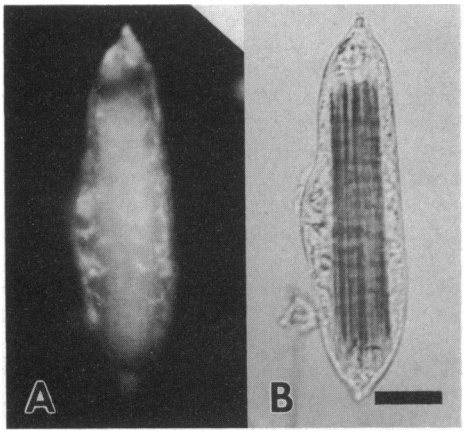

FIG. 2. Localization of bound Ca in crystal idioblasts as shown with chlortetracycline. (Bar = 20 μm.) (*A*) Fluorescence image of an idioblast protoplast gives a reticulate pattern of fluorescence after treatment with chlortetracycline. The crystal bundle also fluoresces. (*B*) Bright-field image of the same protoplast.

mechanism similar to that in animals, where Ca is normally associated with low-affinity high-capacity CBPs (19). Fig. 4 is a ^{45}Ca blot for detection of CBP and shows that three CBPs are evident in the idioblasts, corresponding to molecular masses of about 70, 42, and 24 kDa, respectively. In contrast, only the 24-kDa protein was detectable in mesophyll cells when equal protein loading was used. These results indicate the presence of CBPs in idioblasts which, due to their cell-specific accumulation, may play a role in buffering cytosolic Ca in this cell type.

A CBP Antigenically Similar to Calsequestrin Is Present in Crystal Idioblasts. To test whether any of the proteins identified by ^{45}Ca overlays were similar in structure to CBPs of animal systems, we performed immunoblotting, using a variety of antibodies to animal CBPs, including calsequestrin, calreticulin, and gelsolin. Only the antibodies to rabbit muscle calsequestrin reacted with a protein from *Pistia* extracts. As shown in Fig. 5, this protein was detected by immunoblot analysis of extracts from idioblast protoplasts but not from mesophyll protoplasts when equal protein loading was used. The protein recognized by the antibody had a molecular mass of about 42 kDa, slightly smaller than that of rabbit calsequestrin but similar to that of an idioblastic CBP identified by ^{45}Ca overlay (Fig. 4). Probing the blot with ^{45}Ca showed that the band detected with the antibody also could bind Ca (Fig. 5). On the basis of these results we conclude that the 42-kDa CBP is accumulated in idioblasts relative to mesophyll cells and shares immunological epitopes with rabbit calsequestrin, and we will refer to it as a calsequestrinlike protein.

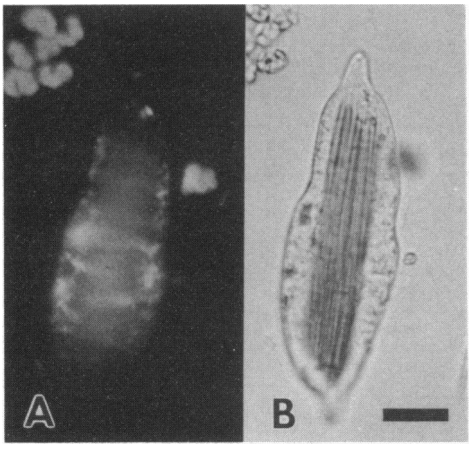

FIG. 3. Visualization of the ER in crystal idioblasts by using the ER-selective fluorescent probe $DiOC_6$. (Bar = 20 μm.) (*A*) A reticulate pattern of fluorescence similar to that seen with chlortetracycline is evident, but the crystal bundle does not fluoresce. The faint round bodies in the upper left are mesophyll protoplasts that are fluorescing red due to their chlorophyll content. The idioblasts have little chlorophyll. (*B*) Bright-field image of the same protoplast.

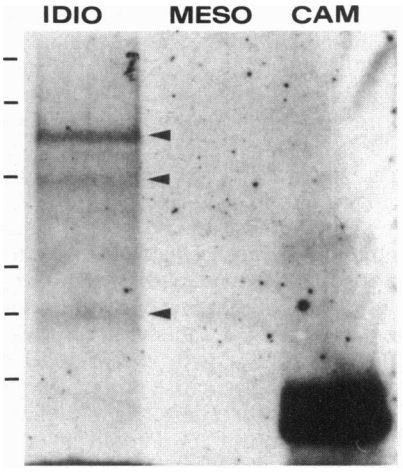

FIG. 4. ^{45}Ca overlay of proteins (≈30 μg per lane) from crystal idioblast (IDIO) and mesophyll (MESO) protoplasts. Three proteins with an affinity for Ca are seen in the idioblast sample (arrowheads). Purified calmodulin (CAM) was used as a control for Ca binding. Bars are approximate position of prestained standards of 110, 84, 47, 33, 24, and 16 kDa.

A Transcript From *Pistia* Is Recognized by a cDNA for Rabbit Muscle Calsequestrin. To further test the homology between the idioblast CBP and calsequestrin, Northern analysis using a cDNA for rabbit muscle calsequestrin was conducted. Poly(A)$^+$ RNA was isolated from developing *Pistia* leaves, where crystal idioblasts were undergoing most rapid development. As shown in Fig. 6, the antisense probe, but not the sense-strand probe (not shown), hybridized to an mRNA of about 1.5 kb, which is of sufficient size to encode the calsequestrin-like 42-kDa protein. Thus a *Pistia* mRNA, possibly encoding the calsequestrinlike protein, shows enough homology to mRNA for rabbit calsequestrin to give a signal by Northern analysis.

The Calsequestrinlike Protein Is Localized to the Idioblast Cytoplasm. To determine the subcellular location of the

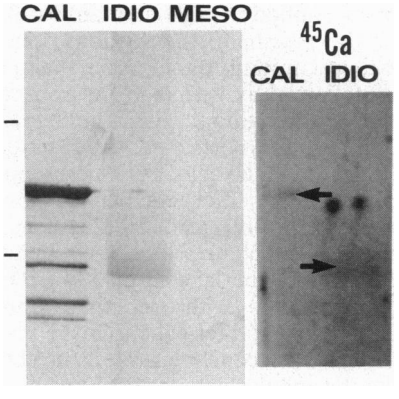

FIG. 5. Western immunoblot of idioblast (IDIO) and mesophyll (MESO) protoplast proteins (≈22 μg per lane) using antibody to rabbit muscle calsequestrin (W. Trumble, University of Idaho). Only the idioblasts have a reactive protein band. The first lane (CAL) is purified calsequestrin (63 kDa), which shows breakdown products due to freezing and thawing. Bars indicate 84 and 47 kDa. The last two lanes show the first two lanes after probing with ^{45}Ca.

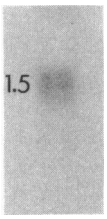

FIG. 6. Northern analysis of *Pistia* leaf mRNA probed with a cDNA to rabbit muscle calsequestrin. A positive transcript of about 1.5 kb is seen with the antisense probe.

calsequestrinlike protein, we performed immunolocalization using antibodies to rabbit calsequestrin. Fig. 7*A* shows that the protein is concentrated in the crystal idioblasts relative to the surrounding mesophyll cells. At the resolution of light microscopy, the calsequestrinlike protein appeared to be localized in the bulk cytosol, which is consistent with its proposed function. Labeling of the crystals also occurred, but it is probably an interaction of the silver reagent with Ca oxalate, since the preimmune serum (and also other antibodies to various proteins) also gave crystal labeling (Fig. 7*B*).

Transmission electron microscopy localization studies of very young idioblasts gave light but consistent labeling of the ER (Fig. 8). Preimmune serum gives no labeling in the thin sections (not shown).

DISCUSSION

It has been demonstrated that Ca oxalate precipitation can occur very rapidly (13), which points to the specialized nature of the crystal idioblasts with respect to Ca transport and sequestration. The large Ca influx into crystal idioblasts during crystal deposition would necessitate a mechanism for controlling cytoplasmic Ca activity. The Ca regulation mechanisms operating in the general plant cell would likely be overcome by the magnitude of the Ca fluxes necessary to

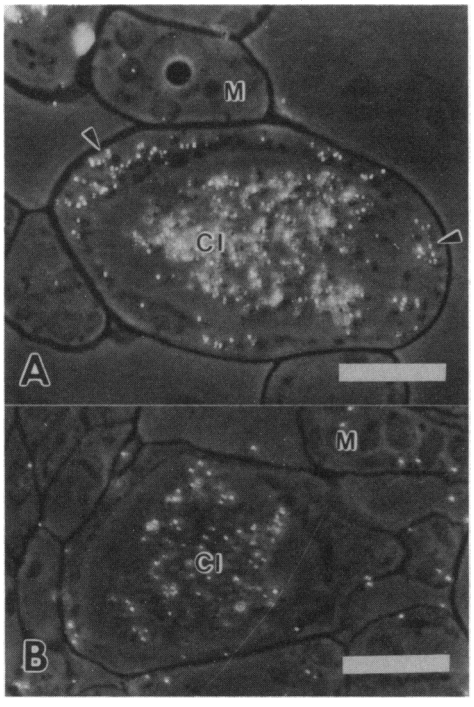

FIG. 7. Light microscope immunocytochemistry of young *Pistia* leaf sections probed with antibody to rabbit muscle calsequestrin. Labeling is indicated by bright spots. (Bars = 10 μm.) (*A*) The immune serum labels the cytoplasm (arrowheads) of crystal idioblasts (CI) but not the surrounding mesophyll cells (M). (*B*) The nonimmune serum does not label the idioblast cytoplasm, but labeling is seen on the crystals. This is interpreted as a nonspecific interaction of the silver enhancement reagent with Ca oxalate.

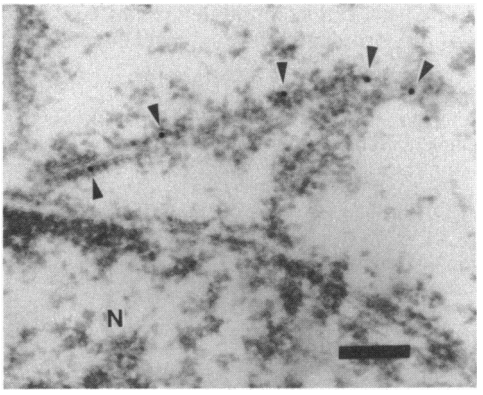

FIG. 8. Transmission electron microscope immunocytochemistry of a *Pistia* crystal idioblast probed with antibody to calsequestrin. A region of cytoplasm containing ER and part of the nucleus (N) is shown. The ER is specifically labeled as indicated by the gold particle (arrowheads) distribution. (Bar = 0.2 μm.)

generate the crystal growth rates observed (13), hence a need for an additional mechanism in the idioblasts. Our vital stain results demonstrate that the idioblasts are capable of compartmentalizing relatively large amounts of Ca in the ER, and we have performed various biochemical analyses to determine the nature of the mechanism responsible for this enhanced capacity. The Ca must eventually be transported to the vacuole, but we have little information on the mechanism of this transport. The idioblasts show considerable Golgi activity and it is possible that this organelle is involved in Ca transport, but this remains speculation at this time.

The data presented here indicate that the crystal idioblasts possess CBPs not detectable in the leaf mesophyll cells. This is similar to the situation in animal cell systems involved in transient fluxes of Ca, such as muscle cells. One of the major muscle proteins that is involved in buffering Ca is calsequestrin, a high-capacity, low-affinity CBP. A similar protein has been identified in plant extracts; however, its tissue and cellular localization as well as its functional significance in plants have not been determined (26, 27). Our results show that a similar protein, as determined by immunological recognition and by Northern blot analysis using probes to rabbit muscle calsequestrin, is present in *Pistia stratiotes*. Furthermore, we have shown that this protein has Ca-binding capacity, and so refer to it as a calsequestrinlike protein. We have demonstrated the potential functional significance of such a protein in plants. We have shown that this protein is found primarily in Ca oxalate crystal idioblasts, a cell type in plants that is involved in bulk Ca regulation.

We hypothesize that the calsequestrinlike protein is involved in buffering cytosolic free Ca in the crystal idioblasts. This is supported by the immunocytochemical studies showing that it is in the cytosolic compartment of this cell type. Transmission electron microscopy immunolocalization on thin sections from the same blocks used for light microscopy suggests that the ER is the primary site for compartmentation of this protein in young developing idioblasts. The results of our vital staining experiments also indicate that the ER of crystal idioblasts is involved in Ca accumulation, and they further support the idea that the calsequestrinlike protein is involved in buffering Ca activity. It is worth noting that crystal idioblasts are often enriched in ER relative to surrounding cells (8) and so would have an enhanced capacity for Ca sequestration by means of the activity of this organelle.

The calsequestrinlike protein could be important in other plant cell types which utilize transients in Ca concentrations for regulating specific functions, such as guard cells (34) or dividing cells (35). It may also be present in lower amounts in most other cells, where Ca regulation is important but large

amounts of Ca are not accumulated or stored. The next step is to isolate the protein for further biochemical characterization.

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