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Modulation of Calmodulin mRNA and Protein Levels in Barley Aleurone[1]

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Changes in calmodulin (CaM) mRNA and protein were investigated in aleurone layers of barley (*Hordeum vulgare* L. cv Himalaya) incubated in the presence and absence of calcium, gibberellic acid (GA_3), and abscisic acid (ABA). CaM mRNA levels increased rapidly and transiently following incubation of aleurone layers in H_2O, $CaCl_2$, or GA_3. The increase in CaM mRNA was prevented by ABA. This increase in CaM mRNA was brought about by physical stimulation during removal of the starchy endosperm from the aleurone layer. CaM protein levels did not increase in response to physical stimulation. Only incubation in GA_3 plus $CaCl_2$ brought about a rapid increase in CaM protein levels in the aleurone cell. ABA reduced the level of CaM protein below that found at the beginning of the incubation period. The rise in CaM protein preceded increases in the synthesis and secretion of α-amylase. Immunocytochemistry with monoclonal antibodies to carrot and mung bean CaM was used to localize CaM in aleurone protoplasts. Monoclonal antibodies to tubulin and polyclonal antibodies to tonoplast intrinsic protein and malate synthase were used as controls. CaM was localized to the nucleus, the vacuolar membrane, and the cytosol, but was not associated with microtubules.

The GA_3-induced synthesis and secretion of hydrolytic enzymes by the cereal aleurone layer is dependent on Ca^{2+} (reviewed by Jones and Jacobsen, 1991; Bush, 1995). Isolated aleurone layers of barley (*Hordeum vulgare* L.), rice, wheat, and wild oat require externally supplied Ca^{2+} at millimolar concentrations to synthesize and secrete hydrolases such as α-amylase. In the presence of millimolar concentrations of Ca^{2+}, GA_3 brings about a sustained increase in $[Ca^{2+}]_i$ in aleurone cells of barley and wheat. Resting levels of $[Ca^{2+}]_i$ in barley and wheat aleurone cells are in the range of 50 to 100 nM, but following incubation in GA_3 plus Ca^{2+}, $[Ca^{2+}]_i$ increases to 200 to 500 nM and above (reviewed by Gilroy et al., 1993; Bush, 1995). In wheat aleurone the effects of GA_3 on $[Ca^{2+}]_i$ are evident within a few minutes of exposure of tissue to GA_3, and new steady-state levels of $[Ca^{2+}]_i$ are established within 30 to 90 min of hormone application (Bush, 1996).

Several lines of evidence indicate that increased $[Ca^{2+}]_i$ is required for GA_3-regulated hydrolase production by aleurone layers. Changes in $[Ca^{2+}]_i$ in response to GA_3 are rapid and precede the GA_3-induced increase in α-amylase mRNA and protein synthesis. On the other hand, the inhibitory effects of ABA on GA_3-induced enzyme synthesis are preceded by a lowering of $[Ca^{2+}]_i$ (Gilroy and Jones, 1992; Bush, 1995). Yet, the inactive GA, GA_8, does not bring about an increase in either α-amylase synthesis or $[Ca^{2+}]_i$ in wheat aleurone, and the GA_3-insensitive wheat mutant D6899, which does not respond to GA_3 by synthesizing α-amylase (Ho et al., 1981), shows no increase in $[Ca^{2+}]_i$ in response to GA_3 (Bush, 1996). Mannitol, which inhibits GA_3-induced α-amylase synthesis in barley (Jones and Armstrong, 1971) and wheat aleurone layers, also lowers $[Ca^{2+}]_i$ in GA_3-treated wheat aleurone cells (Bush, 1996). Taken together, these data indicate a strong coupling between the effects of GA_3 and ABA and $[Ca^{2+}]_i$ on hydrolytic enzyme synthesis and secretion in the cereal aleurone.

Altered $[Ca^{2+}]_i$ has many effects in the cereal aleurone cell. Since α-amylase is a Ca^{2+}-containing metalloprotein, Ca^{2+} is essential for the synthesis of active and stable α-amylase molecules (Bush et al., 1989). α-Amylase is synthesized on membranes of the ER, and Ca^{2+} binds to the enzyme in the lumen of the ER. This is facilitated by the presence of Ca^{2+}-binding proteins and high concentrations of Ca^{2+} in the ER (Bush et al., 1989; Jones and Bush, 1991). Ca^{2+} may also play important roles as a signaling molecule, either by interacting directly with target proteins, such as membrane transport proteins (Blatt and Thiel, 1993), or indirectly by binding to Ca^{2+}-binding regulatory proteins (reviewed by Clapham, 1995).

CaM is one of the best understood of the Ca^{2+}-binding regulatory proteins in eukaryotic cells and is considered to be the primary decoder of Ca^{2+} information in the cell (James et al., 1995). In animal cells, CaM regulates cyclic nucleotide metabolism, protein phosphorylation and dephosphorylation, and calcium transport and also interacts with components of the cytoskeleton. Although less is known about CaM in plant cells, CaM genes and proteins have been isolated from a variety of plant species, and details of its roles in regulating plant cell function are beginning to be known (see reviews by Roberts et al., 1986; Roberts and Harmon, 1992; Poovaiah and Reddy, 1993). Multiple CaM genes and cDNAs have been cloned from

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Abbreviations: ACaM-2, Arabidopsis CaM-2; $[Ca^{2+}]_i$, cytoplasmic calcium concentration; Ca-CaM, calcium-calmodulin; CaM, calmodulin; DAPI, 4,6-diamidino-2-phenylindole; PSV, protein storage vacuole; RT, room temperature; α-TIP, tonoplast intrinsic protein.

Arabidopsis (Braam and Davis, 1990; Perera and Zielinski, 1992a; Gawienowski et al., 1993), *Vigna radiata* (Botella and Arteca, 1994), potato (Takezawa et al., 1995), and pea (Smith et al., 1995), and several of these genes are differentially expressed during development. So far, only one CaM cDNA has been cloned from barley (Ling and Zielinski, 1989; Zielinski et al., 1990).

CaM has been implicated in only a limited number of signal transduction events in plants. Environmental stimuli such as touch cause a rapid, transient increase in $[Ca^{2+}]_i$ in tobacco (Knight et al., 1991) and an increase in CaM transcript levels in Arabidopsis (Braam, 1992), *Bryonia* (Galaud et al., 1993), and mung bean (Botella and Arteca, 1994). Transcript levels encoding other Ca^{2+}-binding proteins increase in Arabidopsis following physical stimulation (Sistrunk et al., 1994). There have been fewer studies of CaM protein than on CaM mRNA levels in plant cells. CaM levels rise during differentiation of *Zinnia* tracheary elements (Kobayashi and Fukuda, 1994), but during cell proliferation in cultured carrot cells CaM is rapidly turned over (Perera and Zielinski, 1992b). More direct evidence for a role of CaM in regulating plant cell development comes from experiments with the *aurea* mutant of tomato (Neuhaus et al., 1993; Bowler et al., 1994). In these experiments, microinjection of CaM into tomato cells that are defective in phytochrome synthesis induces developmental pathways that are normally dependent on phytochrome.

Several Ca-CaM target proteins have been identified in plants (Roberts and Harmon, 1992), and cDNAs encoding some of these CaM-binding proteins have been cloned. NAD kinase and Ca-ATPases are particularly well-studied CaM-stimulated proteins, and in the case of NAD kinase, enzyme activity is completely dependent on the presence of CaM (Muto, 1992; Roberts and Harmon, 1992; Evans, 1994). Glutamate carboxylase is the only CaM-binding protein whose function is known and whose cDNA has been cloned from plants (Baum et al., 1993). CaM increases the activity of this enzyme 2-fold in vitro. The enzyme is involved in the regulation of γ-aminobutyric acid synthesis. Two putative CaM-dependent protein kinases have recently been cloned from plants (Patil et al., 1995; Lu et al., 1996), but functions have not been established for these proteins.

We have identified two Ca-CaM-regulated proteins in barley aleurone: a Ca^{2+} ATPase on the ER that drives unidirectional Ca^{2+} uptake into the ER (Gilroy and Jones, 1993) and a cation channel on the membrane of the PSV (Bethke and Jones, 1994). More recently, we used CaM covalently coupled to horseradish peroxidase to isolate cDNAs encoding CaM-binding proteins from barley aleurone cDNA libraries. This approach resulted in the isolation of seven cDNAs encoding proteins that bind CaM.

Since there are many putative CaM-regulated proteins in the barley aleurone cell, we have begun a detailed study of the regulation of CaM synthesis in this cell. In this paper, we show that levels of CaM mRNA and protein are regulated by different stimuli. Whereas physical stimulation brings about an increase in CaM mRNA, only incubation in the presence of GA_3 and $CaCl_2$ results in a rapid increase in CaM protein. ABA decreases steady-state levels of both CaM mRNA and protein. We also use immunocytochemical methods to localize CaM in the aleurone cell. These data show that CaM is present in the nucleus, on the membrane of the PSV, and throughout the cytoplasm.

MATERIALS AND METHODS

Plant Material

Barley (*Hordeum vulgare* L. cv Himalaya, 1985 and 1991 harvests; Agronomy Department, Washington State University, Pullman) grains were de-embryonated, surface sterilized with 0.37% (v/v) sodium hypochlorite (20 min, RT), washed in 0.01 N HCl (10 min, RT), rinsed with sterile H_2O, and allowed to imbibe in sterile H_2O for 4 d in the dark at 25°C. Aleurone layers were isolated by gently scraping away the starchy endosperm with metal spatulas. Layers (25) were incubated in 25-mL Erlenmeyer flasks in 2 mL of H_2O with or without 10 mM $CaCl_2$, 5 μM GA_3, and 5 μM ABA with shaking at 135 rpm at RT for up to 24 h.

Enzyme Assays

α-Amylase was assayed as described by Bush et al. (1986). The activity of the purified recombinant Arabidopsis CaM-2 was determined by measuring the stimulation of phosphodiesterase (Sigma) activity. Spinach CaM (Sigma) was used as a control. Phosphate release was measured using the Fiske-Subbarow reagent (Sigma).

RNA Isolation and Northern Blotting

Aleurone layers (25) were ground to a fine powder in a mortar and pestle in liquid N_2, and total cellular RNA was isolated and purified according to the method of Slater (1984). The RNA was separated from small molecules on a Sephadex G-50 spin column. Northern blots were made by separating RNA on a glyoxal:DMSO agarose (1.4%) gel followed by blotting onto a nylon membrane (Hybond-N, Amersham) as suggested by the manufacturer. Membranes were hybridized at 65°C in 1% SDS, 1 M NaCl, 10% dextran sulfate (Pharmacia), and 0.1 mg/mL sonicated herring sperm DNA (Boehringer), or in 7% SDS, 1 M Na_2PO_4, pH 7.2, and 0.1 mg/mL sonicated herring sperm DNA. cDNA probes were labeled with [α-^{32}P]dCTP by random priming according to the manufacturer's instructions (Amersham). Plasmid pBCaM-3Δ1 containing a full-length barley CaM cDNA was kindly provided by Dr. V. Ling (Ling et al., 1991). The *Hin*dIII-*Eco*RI fragment containing the open reading frame of CaM, the 3′ untranslated region, and 21 bp of the 5′ untranslated region was used as a probe unless stated otherwise. For some hybridizations, only a 279-nucleotide fragment of the 3′ untranslated region of the CaM cDNA was used. This probe was generated from the pBCaM-3Δ1 plasmid DNA by PCR using specific primers. Subsequently, the nucleotide sequence of the PCR products was determined. Sequence reactions with *Taq* polymerase and fluorescent terminators were carried out according to the manufacturer's instructions (ABI, Columbia, MD) and analyzed on an ABI PRISM 377 sequencer. After hybrid-

ization, RNA blots were washed twice at 65°C in 2× SSC and 1% SDS. The amount of ^{32}P-labeled cDNA probe hybridized to specific mRNA was determined semiquantitatively by measuring the absorbance on autoradiographs with an Ultroscan KI densitometer (LKB, Bromma, Sweden). The blots were reprobed or probed simultaneously with a maize actin cDNA probe to standardize loading and normalize the CaM mRNA signals. The maximum signal on each autoradiogram was considered to be 100%.

CaM Purification

Arabidopsis CaM-2 cDNA inserted into the pET-5a vector (Novagen, Madison, WI) was kindly provided by Dr. R. Zielinski (University of Illinois, Urbana) and expressed in *Escherichia coli* BL21DE3. An overnight culture was diluted 1:100 in 1 L of fresh Luria broth medium and grown to an A_{550} of 0.6 before adding 1 mM isopropylthio-β-galactoside for an additional 2 h. Cells were collected at 5,000*g* for 10 min and washed in 50 mM Tris-HCl, pH 7.5. Subsequently, the cells were lysed in 50 mM Tris-HCl, pH 7.5, 2 mM NaEDTA, 1 mM DTT, and 200 μg/mL lysozyme. The viscosity of the solution was reduced by adding DNase I (Pharmacia) and incubating for 30 min on ice. The supernatant was collected after centrifuging at 27,000*g* for 30 min at 4°C. Saturated ammonium sulfate was added to a final concentration of 55% before centrifuging at 27,000*g* for 30 at 4°C. The pH of this supernatant was adjusted to 4.0 with 50% H_2SO_4, and the precipitated protein was collected by centrifugation at 27,000*g* for 30 min at 4°C. The pellet was resuspended in 5 mL of 50 mM Tris-HCl, pH 7.5, and 1 mM DTT and dialyzed against H_2O for 2 h and then overnight at 4°C against W7 buffer (10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 200 mM NaCl, and 2 mM $CaCl_2$). The proteins were warmed to RT and applied to a W7-agarose column (Sigma). CaM was eluted with W7 buffer in which 2 mM $CaCl_2$ was replaced by 5 mM EGTA. After dialysis against phenyl-Sepharose buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM $CaCl_2$, and 0.5 mM DTT), the fractions containing CaM were loaded onto a phenyl-Sepharose column (Sigma). CaM was eluted with 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, and 0.5 mM DTT and dialyzed against H_2O before being frozen in liquid N_2, lyophilized, and stored at −80°C. Since the extinction coefficient of ACaM-2 is not known, the amount of CaM was determined with Ellman's reagent (Sigma), which reacts with thiol groups to give an A_{412} with an extinction coefficient of 16,300. This quantitation is possible, since ACaM-2 has only one Cys residue (Ling et al., 1991).

Protein Isolation and Western Blotting

Aleurone layers (25) were extracted in 1 mL of extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT) with a Brinkmann Polytron homogenizer (model PT 10/35) at 4°C using the large probe at maximum speed. After centrifugation for 10 min at 10,000*g* at 4°C, the supernatant was retained and the pellet was reextracted in 1 mL of extraction buffer and recentrifuged. The two supernatants were combined, and aliquots were frozen in liquid N_2 and stored at −80°C. Polyacrylamide gels (15%) were prepared with (denaturing) or without (nondenaturing) SDS. Equal volumes of extracts representing equal amounts of aleurone layers were loaded on the gels. After electrophoresis, the proteins in nondenaturing gels were blotted onto PVDF (Millipore) membranes in 25 mM K_2HPO_4, pH 7.5. SDS-containing gels were blotted onto nitrocellulose in 50 mM Tris base, 40 mM Gly, 0.04% (w/v) SDS, and 20% (v/v) methanol. Membranes were incubated in TBSC (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 0.1 mM $CaCl_2$) and blocked with 2% BSA (Sigma) in TBSC. Subsequently, membranes were incubated with carrot CaM monoclonal antibodies diluted 1:250 (Fischer and Cyr, 1993) or with mung bean CaM monoclonal antibodies diluted 1:10 (Jablonsky et al., 1991) in TBSC-BSA for 2 h at RT or for 1 h at 37°C. Secondary antibodies (goat anti-mouse IgG) coupled to horseradish peroxidase (Sigma) were visualized with luminol (New England Nuclear), a chemiluminescent substrate. Membranes were exposed for 10 to 60 s to Kodak XAR-5 film. The amount of CaM protein was determined semiquantitatively by measuring the absorbance on fluorographs with a Molecular Dynamics (Sunnyvale, CA) densitometer. The maximum signal on each fluorograph was considered to be 100%.

Protoplast Isolation and Immunolocalization

Aleurone protoplasts were prepared using the method of Bush et al. (1986). Unless otherwise specified, protoplasts were incubated for 17.5 h in 10 mM $CaCl_2$ with either 5 μM GA_3 or 5 μM ABA. Protoplasts were fixed in the release medium containing one-fifth or one-sixth volume of 37% (v/v) formaldehyde for 0.5 h at RT. Protoplasts were carefully washed three times with release medium by allowing them to settle at 1*g* for 30 min and removing the supernatant with a Pasteur pipette. A small volume (2-3 μL) of protoplasts was pipetted onto poly-L-Lys-coated slides and allowed to dry briefly, but not to completion. Nonspecific binding sites were blocked with PBS containing 3% BSA. Antibodies were used in dilutions ranging from 1:25 to 1:100 and incubated overnight in a moist chamber at RT. Monoclonal antibodies to *Drosophila* β-tubulin were obtained from the National Institute of Child Health and Human Development (Bethesda, MD). Polyclonal antibodies to α-TIP were from Dr. K.D. Johnson (California State University, San Diego) and polyclonal antibodies to malate synthase were from Dr. R. Trelease (Arizona State University, Tempe). Fluorescein isothiocyanate-labeled secondary antibodies (Sigma) were diluted 1:50 and 1:100. Protoplasts were mounted in 0.1% *N*-propylgallate and 50% glycerol containing 20 μg/mL DAPI to stain DNA. Specimens were examined and photographed with a Zeiss Axiophot microscope equipped with a 100-W mercury epifluorescent illuminator and 40× and 100× plan-Neofluar objectives (Zeiss). Collages of photomicrographs were made using Photoshop software (Adobe Systems, Mountain View, CA) and printed on a Tektronix (Willsonville, OR) Phaser 440. Images were captured using a Zeiss ZVS-47 DEC integrating video camera.

RESULTS

CaM mRNA Levels in Barley Aleurone

To determine whether CaM mRNA levels in barley aleurone layers were regulated by GA and ABA, northern blot analyses were performed. Northern blots of RNA isolated from barley aleurone layers incubated in H_2O for up to 8 h and probed with the full-length barley CaM cDNA (pBCaM-3Δ1) and the 3′ untranslated region of barley CaM (CaM-3′UT) are shown in Figure 1, A and B. A single band was detected on autoradiograms corresponding to the predicted molecular mass of CaM mRNA of about 850 nucleotides. Although the strength of the signal was greater when the full-length cDNA probe was used, the qualitative nature of the hybridization of the two probes was identical. The qualitative nature of the hybridization of the two probes to RNA isolated from aleurone layers incubated in GA_3 plus $CaCl_2$ and ABA plus $CaCl_2$ for up to 8 h was also identical (data not shown). Since the amounts of RNA hybridizing with both probes were qualitatively similar (Fig. 1, A and B), all subsequent RNA blots were probed with the full-length CaM probe, pBCaM-3Δ1. To ensure equal loading of RNA onto gels, northern blots were also probed, either simultaneously or following pBCaM-3Δ1, with a maize actin probe (Fig. 1C). We have shown that actin mRNA levels do not change relative to rRNA levels in the barley aleurone layer under our experimental conditions (data not shown). Previous work from our laboratory (Deikman and Jones, 1985) showed that rRNA levels in barley aleurone layers did not change over a 24-h incubation period in H_2O, $CaCl_2$, or GA_3 plus $CaCl_2$.

Detailed time courses of steady-state CaM mRNA levels are shown in Figures 2 and 3. Total RNA was isolated from aleurone layers incubated in H_2O or in 10 mM $CaCl_2$ containing GA_3 or ABA for different lengths of time. CaM mRNA levels increased rapidly and reached a level almost 4 times above initial levels within 2 h of incubation in H_2O or GA_3 plus $CaCl_2$, but thereafter they declined to a level about twice that at the start of incubation (Fig. 2A). The response of aleurone layers incubated in $CaCl_2$ was similar to that of layers incubated in H_2O (data not shown). A more detailed dissection of the kinetics of CaM mRNA levels showed that the levels of RNA increased more than three times during the first 60 min of incubation in GA_3 plus $CaCl_2$ (Fig. 3). The increase in CaM RNA levels was much less pronounced in aleurone layers, incubated in ABA plus $CaCl_2$ (Fig. 2A). In ABA-plus-$CaCl_2$-treated layers, CaM mRNA increased to twice the initial level during the 1st h of incubation and then declined to levels slightly lower than those found at the beginning of the incubation period (Fig. 2A).

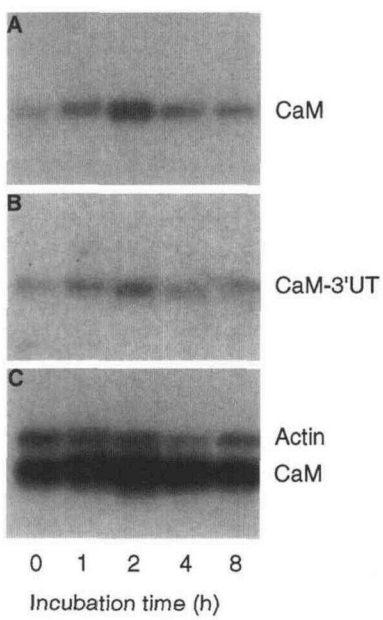

Figure 1. Steady-state CaM mRNA levels in barley aleurone layers incubated in H_20 for up to 8 h. Northern blots were probed with the full-length barley CaM cDNA (A), with the 3′ untranslated region of barley CaM (B), and simultaneously with the full-length barley CaM cDNA and maize actin (C).

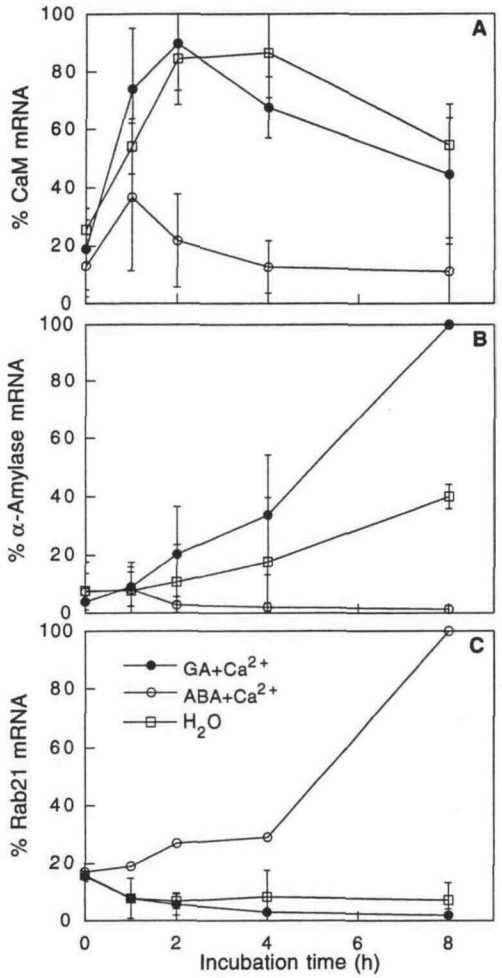

Figure 2. Time course of the effect of incubation in GA_3 and ABA on CaM (A), high-pI α-amylase (B), and Rab21 (C) steady-state mRNA levels in barley aleurone layers. Autoradiograms of northern blots were scanned with a densitometer using actin signals to compensate for unequal loading. The data represent the mean and SD of six GA_3-plus-Ca^{2+}, three H_2O, and two ABA-plus-Ca^{2+} experiments.

To establish that aleurone layers were responding to GA_3 and ABA, the RNA blots shown in Figure 2A were rehybridized with probes to well-characterized GA_3- and ABA-inducible genes. GA_3 responsiveness was determined by probing with the high-pI α-amylase cDNA, 1-28 (Fig. 2B; Deikman and Jones, 1985), and ABA responsiveness was determined with the Rab21 cDNA (Fig. 2C and Mundy and Chua, 1988). GA_3 brought about a 20-fold increase in high-pI α-amylase mRNA levels during 8 h of incubation, whereas ABA reduced the amount of this RNA to almost undetectable levels (Fig. 2B). Conversely, ABA brought about more than a 5-fold increase in the level of the Rab21 RNA during the first 8 h of incubation (Fig. 2C). When ABA was omitted from the incubation medium and layers were incubated in either H_2O or GA_3 plus $CaCl_2$, the levels of Rab21 RNA declined to levels less than one-half the initial value (Fig. 2C).

Since CaM mRNA levels in Arabidopsis increase rapidly following physical stimulation (Braam and Davis, 1990), we carried out experiments to determine whether the increase in CaM mRNA levels in aleurone layers that occurred rapidly (Fig. 3) and independently of the presence of GA_3 (Fig. 2A) also was due to physical perturbation of the tissue. The isolation of barley aleurone layers from half grains that had imbibed and their subsequent incubation involves physical manipulation of the tissue. After scraping the starchy endosperm from the aleurone layer with spatulas, layers were picked up with forceps and dropped into 2 mL of medium in 25-mL Erlenmeyer flasks and incubated with shaking at approximately 135 rpm. Two types of experiments were performed to test the hypothesis that the rapid rise in CaM mRNA was due to one or the other of these manipulations. First, embryoless half grains of barley that had imbibed, from which the aleurone layer was not removed, were incubated with shaking at 135 rpm. The initial levels of CaM mRNA were much lower in half grains than in isolated aleurone layers (compare Fig. 4, A and B). Following incubation in GA_3 plus $CaCl_2$, there was almost twice as much CaM mRNA as initially, but in ABA-plus-$CaCl_2$-incubated half grains CaM, mRNA decreased over the 8-h incubation period (Fig. 4A). A parallel experiment with isolated aleurone layers (Fig. 4B) showed that the amount of CaM mRNA accumulated in half grains

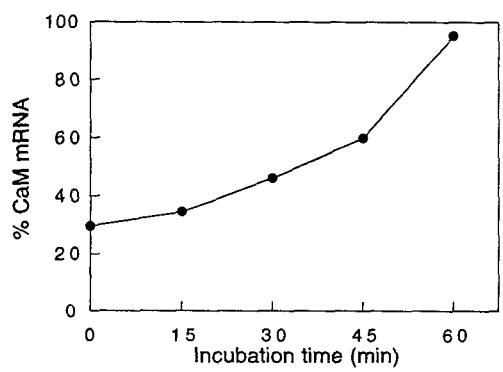

Figure 3. Time course of the effect of incubation in GA_3 on steady-state CaM mRNA levels in barley aleurone layers. The data represent the mean of three experiments.

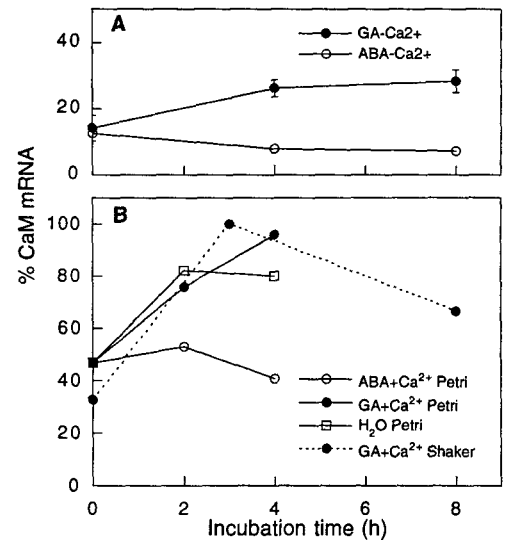

Figure 4. The effect of physical stimuli on steady-state CaM mRNA levels in half grains (A) and aleurone layers (B). A, Half grains were incubated in GA_3 plus Ca^{2+}, H_2O, or ABA plus Ca^{2+} with shaking; the data represent the mean and SD of three experiments for GA_3 plus Ca^{2+} and one experiment for ABA plus Ca^{2+}. B, Aleurone layers were incubated either on moist filter paper in Petri dishes with GA_3 plus Ca^{2+}, ABA plus Ca^{2+}, H_2O, or GA_3 plus Ca^{2+} with shaking. Data in both A and B are presented as percent highest value in B.

over 8 h of incubation as well as the initial levels of this RNA were much smaller in half grains than in isolated aleurone layers (compare Fig. 4, A and B). These results suggest that the differences in CaM mRNA levels result in part from the aleurone layer isolation process and in part from the handling of the isolated layers. These experiments also establish that shaking at 135 rpm does not induce the large increase in the amount of CaM mRNA.

Our second experiment also indicates that shaking of isolated aleurone layers does not bring about a further increase in CaM mRNA. Isolated aleurone layers were incubated on moistened filter paper without shaking or under standard conditions with shaking at 135 rpm (Fig. 4B). There was a large and transient increase in CaM mRNA levels in both shaken and nonshaken aleurone layers, indicating that it was the procedure of aleurone layer preparation and not the shaking that caused the increase in CaM mRNA over the course of the experiment (Fig. 4B). An ABA treatment included in this experiment confirmed that ABA prevents the rise in CaM mRNA brought about by physical stimulation (Fig. 4B).

CaM Protein Levels Increase in GA_3-plus-$CaCl_2$-Treated Barley Aleurone Layers

It was of interest to determine if the changes in CaM mRNA were reflected in changes in CaM protein levels. Therefore, several antibodies to CaM were screened for one that cross-reacted strongly with CaM but not with other aleurone proteins on western blots. Two monoclonal antibodies, raised against carrot CaM (Fisher and Cyr, 1993) and mung bean CaM (Jablonsky et al., 1991), were superior

in this regard to others tested. Since identical results were obtained with both antibodies, all protein blotting experiments were done with the monoclonal antibodies against carrot CaM. This antibody recognized two bands corresponding to CaM and Ca-CaM on a protein blot after SDS-PAGE (Fig. 5A) and a single band on a protein blot following nondenaturing PAGE (Fig. 5B). Cell-free homogenates representing equal numbers of layers that were purified by centrifugation, separated by nondenaturing PAGE, and transferred to PVDF membranes in phosphate buffer allowed reproducible quantitation of CaM (Fig. 5). This protocol permitted the detection of 5 ng of CaM using recombinant Arabidopsis CaM-2 as a standard (Fig. 5B). Arabidopsis CaM-2 differs in its mobility from barley CaM when electrophoresed under nondenaturing conditions (Fig. 5B), but on SDS-PAGE Arabidopsis and barley CaM are almost indistinguishable (Fig. 5A).

There was twice as much CaM protein in GA_3-incubated aleurone layers in the presence of $CaCl_2$ (Fig. 6A) as at the start of the incubation. The increase in CaM protein preceded the onset of α-amylase secretion, which does not begin until after 4 h of incubation in GA_3 (compare Figs. 6A and 7 with Fig. 6B). CaM protein levels decreased rapidly in layers incubated in H_2O, even though CaM mRNA levels were as high as those in GA_3-treated layers (compare Figs. 6A and 2A). Incubation of layers in ABA plus $CaCl_2$ also brought about a decline in CaM protein (Fig. 6A). CaM protein levels in barley aleurone correlated well with the levels of α-amylase synthesized and secreted under the various conditions of incubation; thus, only GA_3-plus-$CaCl_2$ treatments brought about an increase in CaM and α-amylase protein levels (Fig. 6B).

Since one of the most rapid effects of GA_3 in the cereal aleurone is to increase $[Ca^{2+}]_i$ as a result of Ca^{2+} transport from outside the cell (Gilroy and Jones, 1992; Bush, 1995, 1996), we designed experiments to determine whether the

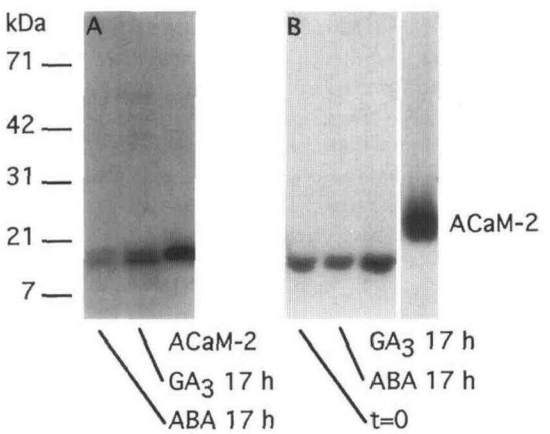

Figure 5. Western blot of CaM protein in barley aleurone layers. Cell-free protein extracts from equal numbers of aleurone layers that had been incubated in GA_3 plus Ca^{2+} (GA_3) or ABA plus Ca^{2+} (ABA) for 17 h or not incubated at all (time = 0) were separated by SDS-PAGE (A) or native-PAGE (B). CaM protein was detected with monoclonal antibodies against carrot CaM. Purified recombinant ACaM-2 was used as a control. Molecular mass markers for the SDS polyacrylamide gel (A) are shown along the left margin.

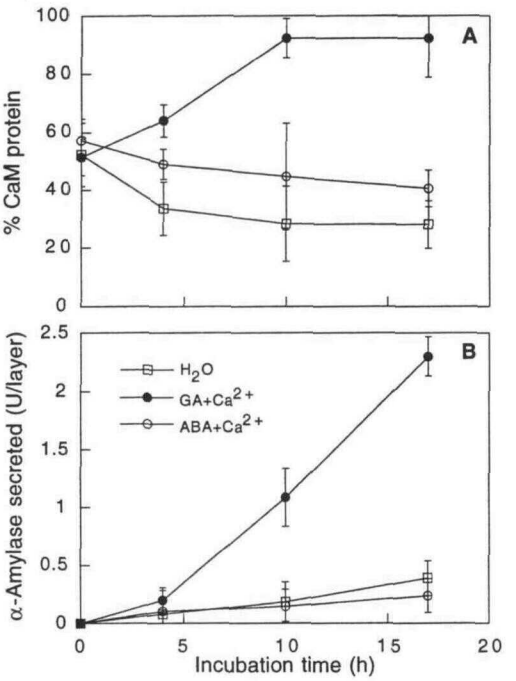

Figure 6. Time course of the effects of incubation in GA_3 plus Ca^{2+}, ABA plus Ca^{2+}, and H_2O on CaM protein levels (A) and α-amylase secretion (B) in barley aleurone layers. Fluorographs were scanned with a densitometer. The mean and SD of three experiments are presented.

GA_3-stimulated rise in CaM levels in the aleurone layer was dependent on the presence of external $CaCl_2$. CaM protein levels were determined in aleurone layers incubated in GA_3 in the presence or absence of added $CaCl_2$. As in our previous experiments, incubation in GA_3 plus $CaCl_2$ brought about a 2-fold increase in CaM protein levels, and an increase in CaM protein was detected as early as 2 h after the start of incubation. CaM levels in layers incubated in GA_3 in the absence of added Ca^{2+} did not change during the first 4 h of incubation and were the same as those found in layers incubated in $CaCl_2$ alone (Fig. 7). After 8 h of incubation in GA_3 in the absence of $CaCl_2$, however, CaM

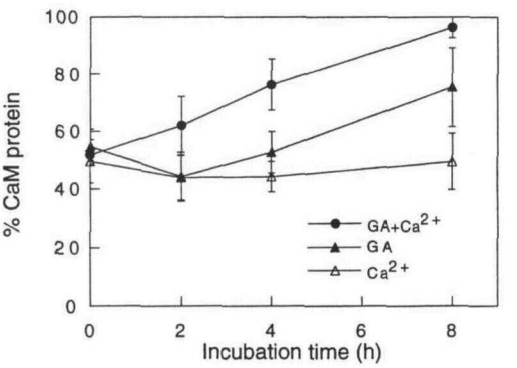

Figure 7. Time course of the effect of incubation in Ca^{2+} and GA_3 on CaM protein levels in barley aleurone layers. The data represent the mean and SE of three experiments.

levels increased, but they were not as high as those in GA_3-plus-$CaCl_2$-treated layers (Fig. 7).

Using the Arabidopsis CaM-2 protein as a standard, we quantified the amount of CaM protein present in the barley aleurone layer. Assuming that more than 95% of the CaM was extracted with our procedure (Walker et al., 1993), we estimated that about 50 ng of CaM protein was present in a freshly isolated aleurone layer. This amount increased to about 100 ng in GA_3-plus-$CaCl_2$-treated layers after 17 h. This is equivalent to a cellular CaM concentration of approximately 1.3 μM, assuming that 50% of the fresh weight of an aleurone layer is dry matter.

Immunolocalization of CaM in Aleurone Protoplasts

To determine where CaM was localized in the barley aleurone cell and whether CaM was differentially localized in GA_3- and ABA-treated aleurone cells, we used the monoclonal antibodies to carrot and mung bean CaM to localize CaM in barley aleurone protoplasts by immunofluorescence microscopy. Monoclonal antibodies raised against *Drosophila* tubulin and polyclonal antibodies to α-TIP, a tonoplast marker enzyme, and to the glyoxysomal enzyme malate synthase were used as controls (Fig. 8). The nucleus was localized using DAPI fluorescence staining. CaM was localized primarily to the nucleus, the tonoplast membrane of the PSV, and the cytoplasm of aleurone protoplasts treated with GA_3 for 17.5 h (Fig. 8, A–H). CaM was noticeably absent from the microtubules (Fig. 8L). CaM distribution in the aleurone protoplasts was not altered by incubation in GA_3 for up to 17.5 h, nor was its distribution different in the absence of GA_3 or in the presence of ABA (data not shown). Protoplasts incubated with fluorescein isothiocyanate-labeled secondary antibodies alone showed little fluorescence (Fig. 8I). Antibodies to α-TIP, tubulin, and malate synthase localized to the vacuolar membrane (Fig. 8J), microtubules (Fig. 8L), and punctate regions in the cytosol corresponding to the size of glyoxysomes (Fig. 8K), respectively. Only carrot and mung bean anti-CaM labeled the nucleus of the aleurone protoplast in these experiments, indicating that the labeling of the nucleus with the CaM antibody under these conditions of tissue fixation was specific.

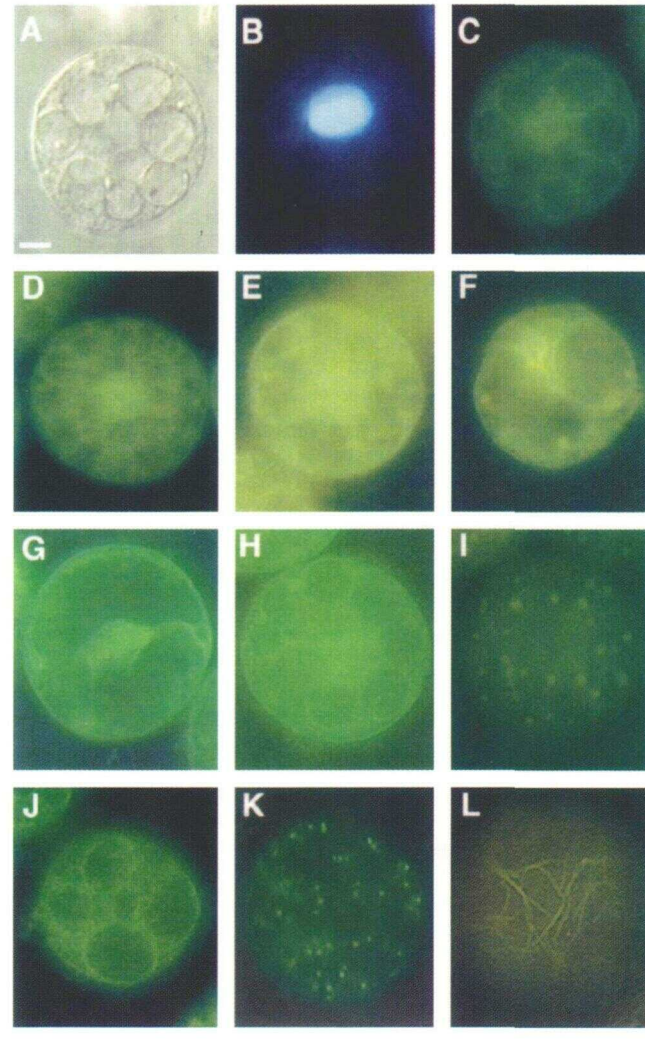

Figure 8. Immunolocalization of CaM protein in barley aleurone protoplasts using monoclonal antibodies to carrot (C–F) and mung bean (G and H) CaM. Differential interference contrast image (A), DAPI staining (B), and CaM labeling (C) of the same protoplast. I, Protoplast incubated with fluorescein isothiocyanate-labeled secondary antibodies alone. J, K, and L, Protoplasts labeled for α-TIP, malate synthase, and β-tubulin, respectively. Magnification bar = 10 μm.

DISCUSSION

Our results show that the levels of CaM mRNA and protein in barley aleurone layers are regulated by a complex set of stimuli. CaM mRNA levels increase rapidly and transiently following physical stimulation, and ABA prevents this increase. The touch-induced increase in CaM mRNA does not result in a commensurate increase in CaM protein. Only when aleurone layers are incubated in GA_3 plus $CaCl_2$ do levels of CaM protein increase within 4 h. Incubation of aleurone layers in GA_3 plus $CaCl_2$ brings about a sustained increase in CaM protein levels at twice the initial level. Immunocytochemical localization shows that CaM protein is prominent in the nucleus and on the vacuolar membrane as well as in the cytosol of the aleurone cell.

Experiments with barley half grains and aleurone layers establish that physical stimuli play an important role in regulating levels of CaM mRNA in barley aleurone layers, but physical stimulation does not bring about an increase in CaM protein levels. The rapid rise in CaM mRNA in barley aleurone layers results from the physical effects of tissue manipulation and is analogous to the response of *Arabidopsis thaliana* to physical stimuli described by Braam and Davis (1990). CaM mRNA levels in freshly isolated aleurone layers are higher than in half grains allowed to imbibe and increase further on subsequent incubation (Figs. 3 and 4). These effects of physical stimulation are transient, however, since after about 4 h of incubation, CaM mRNA levels decline (Figs. 1 and 2A). Physical stimulation does not bring about an increase in the levels of all

mRNAs in the aleurone layer. The levels of α-amylase and Rab21 mRNAs do not change in response to touch during the first 4 h of incubation, although the levels of these mRNAs increase in response to either GA_3 (α-amylase mRNA) or ABA (Rab21 mRNA), as has been reported previously (Deikman and Jones, 1985; Mundy and Chua, 1988).

The stimulatory effects of touch on CaM mRNA levels are found under all conditions of tissue incubation except when ABA is present in the incubation medium (Figs. 2A and 4B). ABA prevents most of the touch-induced increase in CaM mRNA, and following prolonged incubation in ABA, CaM mRNA levels are reduced slightly below those found in unincubated layers (Fig. 2A). In this respect, the effects of ABA on CaM mRNA are similar to the effects of ABA on the levels of α-amylase (Fig. 2B) and other mRNAs in the cereal aleurone (Jones and Jacobsen, 1991).

To our knowledge, our experiments with ABA are the first to show that the rise in CaM mRNA levels in response to physical stimuli can be prevented by hormone treatments. The ability of ABA to prevent the touch-induced rise in CaM mRNA in the aleurone cell is consistent with the overall inhibitory effect of this hormone in the cereal aleurone (Jones and Jacobsen, 1991) as well as in other tissues (Giraudet et al., 1994). It will be of interest to establish how ABA down-regulates CaM mRNA in the aleurone.

It is interesting that embryoless half grains of barley do not display the large transient increase in CaM mRNA levels. It should be emphasized that in the barley grain the outer surface of the aleurone layer is protected by several layers of dead cells that are derived from the testa and pericarp, and the inner surface of the aleurone is adjacent to the nonliving starchy endosperm. In isolated aleurone layers, on the other hand, aleurone cells become exposed after starch removal and are accessible to direct physical stimulation. We believe that this explains the sensitivity of the aleurone layer, but not the sensitivity of the half grain, to physical stimulation. However, we cannot exclude the possibility that removal of the starchy endosperm itself brings about an increase in CaM mRNA levels.

Whereas physical stimulation of barley aleurone layers increases CaM mRNA levels, it does not increase levels of CaM protein. Only when aleurone layers are incubated in GA_3 is there an increase in CaM protein levels (Figs. 6 and 7). None of the other conditions of incubation that were tested brought about a rise in CaM protein levels. $CaCl_2$ must be present at millimolar concentrations in the incubation medium for GA_3 to bring about an increase in CaM protein. When aleurone layers are incubated in GA_3 in the absence of added $CaCl_2$, there is no increase in CaM protein during the first 4 h of incubation, although CaM levels rise between 4 and 8 h of incubation in GA_3 in the absence of added $CaCl_2$ (Fig. 7). The rise in CaM protein during extended incubation in GA_3 may result from the accumulation of Ca^{2+} in the incubation medium during long incubation periods in GA_3 (D.S. Bush and R.L. Jones, unpublished data). The presence of $CaCl_2$ in the incubation medium is also required to prevent a decline in CaM protein levels (Fig. 6A). CaM protein levels remain constant in aleurone layers incubated in $CaCl_2$, but in layers incubated in H_2O CaM protein declines by nearly 50% over a 17-h incubation (Figs. 6A and 7).

These experiments indicate a key role for external Ca^{2+} in the regulation of CaM stability and synthesis in the aleurone cell, and we hypothesize that $[Ca^{2+}]_i$ regulates steady-state levels of CaM protein in the aleurone cell. Braam and Davis (1990) and Braam (1992) also proposed that Ca^{2+} was important in regulating steady-state CaM mRNA levels in Arabidopsis, based on observations that physical stimuli such as touch and temperature brought about a change in $[Ca^{2+}]_i$ in plant cells (Knight et al., 1991). The link between altered $[Ca^{2+}]_i$ and CaM protein levels in cereal aleurone cells is more direct. GA_3 brings about a rise in $[Ca^{2+}]_i$ in the aleurone cell, and this rise is dependent on an external supply of Ca^{2+} (Bush and Jones, 1988; Gilroy and Jones, 1993; Bush, 1995, 1996). CaM protein levels also correlate well with $[Ca^{2+}]_i$ in layers incubated in $CaCl_2$ or H_2O. Incubation of aleurone layers in Ca^{2+} in the absence of GA_3 brings about transient but not sustained increases in $[Ca^{2+}]_i$ (Bush, 1996), but incubation in H_2O results in a lowering of $[Ca^{2+}]_i$ levels in the aleurone cell (D.S. Bush and R.L. Jones, unpublished data).

Changes in CaM mRNA do not correlate with the levels of CaM protein in the aleurone cell. There are several explanations that may account for the apparent discrepancy between the levels of the transcript and its translation product. First, there may be several CaM genes in barley, and because of the highly conserved nature of the CaM sequence (Takezawa et al., 1995), different CaM mRNAs may not be distinguished with the full-length cDNA probe used in this work. Our northern blotting data using a cDNA probe encoding the 3′ untranslated region of pBCaM-3Δ1 show that the pattern of CaM mRNA accumulation is similar to that observed when the full-length pBCaM-3Δ1 probe is used (Fig. 1; data not shown), indicating that differential expression of different CaM genes does not explain the lack of correlation between CaM RNA and protein levels. Second, posttranscriptional and posttranslational control mechanisms could also account for the discrepancy between CaM mRNA and protein levels. Perera and Zielinski (1992b) argued that the discrepancy between CaM mRNA and protein levels in suspension-cultured cells of carrot resulted from posttranslational modification of CaM that increased the protein's stability. Changes in posttranslational modification of CaM in the carrot cell-suspension system that correlated with the apparent turnover of CaM inferred by Perera and Zielinski (1992b) were demonstrated directly by Oh et al. (1992).

We favor the proposal that two different signal transduction pathways affect the accumulation of CaM mRNA and CaM protein in the barley aleurone cell. One of these pathways couples physical stimuli to the expression of the CaM gene, and the other involves hormonal regulation of CaM mRNA and protein accumulation. Regulation via GA_3 leads to an increase in CaM protein levels along a pathway involving a sustained increase in $[Ca^{2+}]_i$, as has been shown to occur in the aleurone cell (reviewed by Bush,

1995). Regulation by touch, on the other hand, may involve a different Ca^{2+} signaling pathway and results only in the accumulation of CaM mRNA, and not CaM protein, in isolated aleurone layers. We speculate that the response to touch in the aleurone cell involves large but not sustained increases in $[Ca^{2+}]_i$, as has been described to occur in response to physical stimuli in other plant cells (Knight et al., 1991). Since touch increases CaM mRNA in aleurone cells in the absence of external Ca^{2+} (Fig. 3B), we also speculate that increased $[Ca^{2+}]_i$ arises from internal pools (Bush, 1995).

The localization of CaM in the nucleus and cytosol as well as its association with endomembranes of the aleurone cell is consistent with our knowledge of the roles of CaM in the aleurone cell. We have previously identified CaM-regulated proteins at the ER and tonoplast of the aleurone cell (reviewed by Bush, 1995), and the cloning of a putative DNA-binding protein that also binds CaM is consistent with the localization of CaM in the nucleus (W. Lin and R. Jones, unpublished observation). There is at present great interest in the roles of CaM in the nuclei of eukaryotic cells (see reviews by Bachs et al., 1994; James et al., 1995), and barley aleurone cells may provide an excellent model system for investigating the role of CaM in regulating events in the nuclei of plant cells.

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