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Distribution of calmodulin in pea seedlings: Immunocytochemical localization in plumules and root apices

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Abstract. Immunofluorescence techniques have been used to study the distribution of calmodulin in several tissues in young etiolated pea (*Pisum sativum* L.) seedlings. A fairly uniform staining was seen in the nucleoplasm and background cytoplasm of most cell types. Cell walls and nucleoli were not stained. In addition, patterned staining reactions were seen in many cells. In cells of the plumule, punctate staining of the cytoplasm was common, and in part this stain appeared to be associated with the plastids. A very distinctive staining of amyloplasts was seen in the columella of the root cap. Staining associated with cytoskeletal elements could be shown in division stages. By metaphase, staining of the spindle region was quite evident. In epidermal cells of the stem and along the underside of the leaf there was an intense staining of the vacuolar contents. Guard cells lacked this vacuolar stain. Vacuolar staining was sometimes seen in cells of the stele, but the most distinctive pattern in the stele was associated with young conducting cells of the xylem. These staining patterns are consistent with the idea that the interactions of plastids and the cytoskeletal system may be one of the Ca^{2+}-mediated steps in the response of plants to environmental stimuli. Nuclear functions may also be controlled, at least in part, by Ca^{2+}.

Key words: Calmodulin – *Pisum* (calmodulin).

Introduction

The fine control of the levels of free Ca^{2+} within different intracellular compartments is now thought to be an essential feature for normal functioning in most eukaryotic cells. There is increasing evidence that modulation of intracellular $[Ca^{2+}]$ is an important step in initiating or coordinating the cellular activities involved in developmental processes or in the response of cells to external stimuli. Changes in $[Ca^{2+}]$ may occur within restricted regions of the cell, and such changes depend in part on the location and activation of calcium-binding proteins that regulate Ca^{2+} transport. Calmodulin is one of the most common intracellular receptors for Ca^{2+}. A highly conserved protein characteristic of eukaryotic organisms, it has been implicated as a key regulatory protein in animal cells (e.g. Cheung 1980; Means et al. 1984). Current evidence makes it highly probable that calmodulin plays and equally essential regulatory role in plants (for reviews and discussions, see Schleicher et al. 1982; Roux 1983). Chloroplast rotation in algae (Wagner et al. 1984; Serlin and Roux 1984), bud initiation in mosses (Saunders and Hepler 1981, 1983), polarization of growth in tip-growing cells (Weisenseel and Kicherer 1981), morphogenetic changes mediated by phytochrome (Roux 1983; Wayne and Hepler 1984), and gravitropic responses (Biro et al. 1982; Slocum and Roux 1983; Sun et al. 1984), have all been linked to fluxes in Ca^{2+} levels and have been shown to be sensitive to disruption of the activated calmodulin-Ca^{2+} balance within the cells. A number of cellular processes thought to be involved in bringing about these responses are affected or controlled by the binding of Ca^{2+} to calmodulin (Schleicher et al. 1982; Marmé and Dieter 1983; Hepler and Wayne 1985). These include the activities of the cytoskeletal components that affect general cytoplasmic motility, and special functions such as cell division and exocytosis, regulation of activity of a number of different enzymes, and the mediation of Ca^{2+} transport across membranes. Recent studies in our laboratory have indicated that calmo-

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dulin-Ca^{2+} mechanisms may also function in plant nuclei. Nuclei isolated from pea plumules were shown to exhibit a phytochrome-sensitive, Ca^{2+}-stimulated phosphorylation of several nuclear proteins, and this phosphorylation was blocked by calmodulin antagonists (Datta et al. 1985).

Because of the widespread occurrence and the apparent pleiotropic effects of calmodulin, it will be important to clarify its particular functions, especially in plants where much of the evidence is still circumstantial. In this laboratory, work has been done to test more critically the hypothesis that organelle-, cell- and tissue-specific fluxes of Ca^{2+} are a principal factor in the responses that follow photo- and gravistimulation (Roux et al. 1983), and to identify the sites and mechanisms by which Ca^{2+}-calmodulin might exert a directive force in bringing about these changes. As part of these investigations, immunochemical studies were undertaken to demonstrate the presence of calmodulin in isolated cell fractions (Biro et al. 1984) and in situ, and to assess its distribution on a subcellular and tissue level. The data presented here show the intracellular localization of calmodulin in different tissues in the plumule and root tip of pea seedlings, both of which show responses to light and gravity.

Material and methods

Plant material. Peas (*Pisum sativum* L., cv. Alaska; W. Atlee Burpee Co., Warminster, Penn., USA) were germinated and grown on vermiculite in the dark for 7 d at 25° C. For fixation, samples were taken from the plumule (including leaves and epicotyl from the hook region) and from root tips.

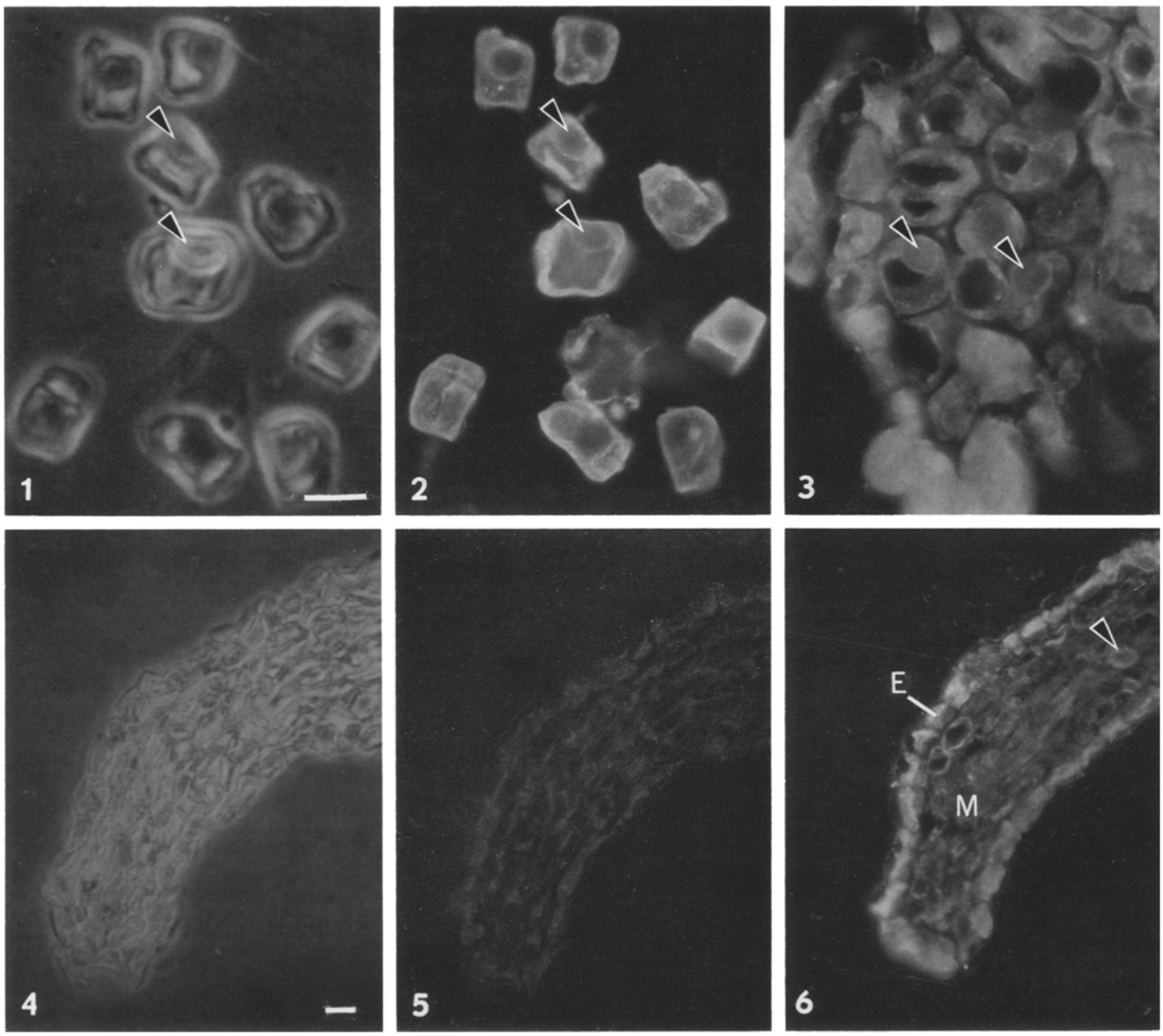
Sample processing. Tissue samples were fixed in 2% formaldehyde (prepared from *p*-formaldehyde) in 0.1 M Na-phosphate buffer, pH 7.4, for 3–6 h at room temperature. Samples were washed in buffer, and if necessary, stored overnight in buffer at 4° C. Isolated cells were prepared by teasing leaf samples in a droplet of dilute buffer or distilled water on slides treated with chrom-gelatin (0.5% gelatin, 0.05% chromium potassium sulfate) and allowing the sample to dry (usually overnight) on a slide warmer at 37° C. Additional tissue samples were dehydrated in ethanol and embedded in polyethyleneglycol (PEG) (Carbowax 1540; Carbide and Carbon Chemicals Co., New York, N.Y., USA, catalog No. CCC-1837 D). Infiltration was done under vacuum for 2–6 h at 60° C. Blocks were hardened at −20° C. Sections (4–6 μm) were cut on a microtome and adhered to chrom-gelatin- or polylysine- (Sigma Chemical Co., St. Louis, Mo., USA) treated slides.

Staining procedure. The isolated-cell preparations were immersed in prechilled absolute methanol for 6–15 min and then rehydrated. Sections were likewise treated with methanol or simply hydrated in two changes of water. All samples were stained by the same procedure. Samples were treated with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.0–7.2, or with 2% BSA in borate buffer (0.1 M boric acid, 25 mM sodium tetraborate, 75 mM NaCl), pH 8.3–8.4, with or without 1 mM ethyleneglycol-bis-(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), for approx. 1 h at room temperature in a moist chamber. Prior to application of the antibody the samples were rinsed briefly in borate buffer. Monospecific antibody to calmodulin (obtained as a generous gift from J.G. Chafouleas, Baylor College of Medicine, Houston, Tex., USA) was prepared in sheep against native rat testis protein and was purified on a calmodulin affinity column (Chafouleas et al. 1979, 1983). The antibody was lyophilized with an equal amount of BSA and stored frozen. Samples were reconstituted in EGTA-borate with an antibody concentration of 200–1 000 $\mu g \cdot ml^{-1}$. For staining, the stock solution was diluted 1:10 in borate buffer (with or without EGTA) and applied to the small droplet of buffer covering the samples. Staining was for 1–3 h at room temperature. The samples were washed up to 1 h with two changes of borate buffer, washed with PBS, and stained with rhodamine-conjugated affinity-purified rabbit anti-sheep immunoglobulin G (IgG; Pel-Freez Biologicals, Rogers, Ark., USA) diluted 1:20 in PBS and applied as a few drops to the PBS-covered samples. Staining was for 45 min to 1 h at room temperature in a moist chamber in the dark. Control samples were mounted on the same slides as those treated with antibody and were processed in parallel with the antibody-treated samples. Three control procedures were tested: (1) the first antibody was omitted, (2) samples were treated with preimmune serum instead of the first antibody, and (3) anti-calmodulin was absorbed with a fivefold excess of calmodulin purified from oats, and the resultant solution was used. Material was nonspecifically stained for 30 s to 1 min with methylene blue (Loefflers; Fisher Scientific Co., Fair Haven, N.J., USA) and rinsed once with H_2O.

Microscopy. Stained samples were mounted in Aqua-Mount (Lerner Laboratories, New Haven, Conn., USA) and analyzed on Zeiss (Carl Zeiss, Oberkochen, FRG) microscopes equipped for epi-fluorescence, with the filter system appropriate for rhodamine fluorescence. Kodak Tri-X film (Eastman-Kodak, Rochester, N.Y., USA) was used for photography.

Results

Staining of isolated cells. Even without cellulase treatment, cells from the young leaves could be isolated quite readily when the tissue was gently teased. The mesophyll cells in these leaves were not highly differentiated; most were relatively small, cuboidal cells with fairly dense cytoplasm around a central vacuole. The isolated cells appeared well-preserved when stained with methylene blue or viewed with phase-contrast microscopy (Fig. 1). These preparations yielded good, consistent calmodulin staining (Fig. 2). The cytoplasm of most cells showed relatively uniform fluorescence although some cells had a slightly heavier staining in regions around the nuclei and along the vacuolar membrane. A few cells were very brightly stained. Because whole cells were being viewed, the degree of staining in cellular compartments was difficult to discern. The fluorescence patterns of mesophyll cells in sectioned samples



Figs. 1, 2. Phase-contrast and fluorescence micrographs of whole isolated cells from pea leaves demonstrating the distribution of calmodulin-associated fluorescence. Nuclei are indicated at the *arrowheads*. ×925; bar = 10 μm

Fig. 3. Comparable fluorescence staining is seen in similar cells as in Figs. 1 and 2 from a section of PEG-embedded material. The nuclei (*arrowheads*) are stained; some patterning of the cytoplasmic staining is evident. Region from a young leaf. ×925

Figs. 4, 5. Phase-contrast and fluorescence micrographs of a section from a leaf processed without application of the first antibody (anti-calmodulin). Staining of all cell types is negligible. ×375; bar = 10 μm

Fig. 6. Fluorescence staining of an adjacent section showing the typical distribution of calmodulin-associated fluorescence. Epidermal cells (*E*) are most highly stained, and mesophyll (*M*) cells are well stained. A tracheary element is indicated at the *arrowhead*. ×375

(Fig. 3) were comparable to those seen in the isolated cells. With sectioned material fluorescence was observed in the nucleus as well as the cytoplasm. Again there was often a greater fluorescence adjacent to the nuclei and along vacuolar membranes. Some punctate staining was seen in the cytoplasm.

Staining of control samples. Overall, the control samples had very low levels of background fluorescence with no evidence of tissue specificity. Figures 4 and 5 show a phase-contrast and fluorescence image of a sectioned leaf that had no first-antibody treatment; Fig. 6 is of an adjacent section treated with anti-calmodulin and typifies the stain-

ing patterns. With this control procedure the fluorescence in all samples (isolated cells and sections from all regions of the plant) was negligible. A very similar level of staining was seen in samples treated with pre-immune serum. When antibody that had been preabsorbed with purified oat calmodulin was used, there was a slightly higher level of nonspecific fluorescence. Also with this preabsorbed antibody, one staining pattern similar to that in anti-calmodulin-treated tissue was seen in the tracheary elements of the plumule. This was the only case of selective staining observed in the control samples (see *Discussion*).

Staining of plumule sections. The characteristic staining pattern seen in the leaves is shown in Fig. 6. There was a general staining of the mesophyll cells. A slightly brighter fluorescence in the vascular tissue appeared to reflect, for the most part, a higher cytoplasm-to-vacuole ratio in these tissues. In addition, a strong staining was seen in some of the conducting cells of the xylem. The most intensely stained cells were in the epidermis. This staining was asymmetric: in these etiolated plants the epidermal layer exposed to the environment, i.e., the abaxial side, was highly fluorescent whereas the upper leaf surface was stained much less or equivalent to that of the mesophyll. At higher magnification (Fig. 7) it is seen that this enhancement was primarily a consequence of staining of the vacuolar contents in addition to the nucleus and cytoplasm. This vacuolar stain was usually nonhomogeneous, with most of it distributed near the periphery. In all samples, cell walls were usually devoid of anti-calmodulin fluorescence.

The basic staining pattern in the stem was quite similar to that in the leaf (Fig. 8). Cells in the vascular tissues were slightly more fluorescent than the cortical cells, and in some of the vascular cells there was a light stain in the vacuoles. Conducting cells in the xylem were often well stained. Again the most intensely labeled cells were in the epidermis, because of a strong reaction of materials in the vacuoles (Fig. 9). Guard cells in the leaf and stem showed fluorescence in the cytoplasm but not in vacuoles (Fig.10).

Excluding the tissue-specific staining of vacuolar material, the staining pattern of structures at the cellular level was consistent throughout the plumule. In these rather thick sections the intracellular patterns were difficult to demonstrate in the small cells of the leaf (see Fig. 3), although evidence of specificity could be seen. In the stem, details of the staining of both nuclei and cytoplasmic regions were evident. In the nuclei (Fig. 11), fluorescence was mainly in the nucleoplasm; nucleoli did not stain. In the cytoplasm, some generally higher staining along the vacuolar membrane and close to nuclei was seen, similar to that noted in leaf cells (Figs. 2, 3). In addition, evidence for plastid staining was routinely observed (Figs. 11, 12). Further patterning of fluorescence in cytoplasmic regions was visible particularly where thin regions of cytoplasm were viewed en face (Fig. 12). Both punctate and possibly fibrillar staining were observed, but the organelles which were labeled were not discernable at this level of resolution. A distinctive distribution of stain was seen in division figures. By metaphase (Fig. 13) strong fluorescence was seen in the spindle, and, in general, the staining of subsequent stages paralleled the location of spindle tubules. Chromosomes were not stained.

A striking localization of stain was seen in tracheary elements in both leaves and stems (Fig. 14). Some, but not all elements showed this pattern. When viewed with the filter system for fluorescein, wall thickenings produced a strong inherent fluorescence. Comparison of this pattern of fluorescence with that observed by rhodamine staining confirmed that the rhodamine staining was not coincident with the thickenings; rather this labeling occurred between the thickenings in either the cytoplasm or some cytoplasmic remnant still present prior to complete maturation of these elements. As noted previously, this pattern was seen in samples treated with antibody preabsorbed with calmodulin. It was not, however, seen with either of the other control procedures.

Staining of root-tip sections. There was more anti-calmodulin fluorescence in the cortical cells of the root than in epidermal cells or cells of the stele (Fig. 15). In contrast to fluorescence patterns in the plumule, the relative amount of staining of the cortex and stele in this region inversely reflects the cytoplasmic density of these cells. Within the stele, cells of developing vascular elements were more highly stained than stelar parenchyma cells. Labeling in the nuclei was somewhat less evident in the meristematic region than it was basipetally or in the rootcap.

The rootcap showed a very distinctive staining pattern. Cells in the columella were intensely stained whereas adjacent cells in the secretory region had little stain (Figs. 16, 17). Observations with phase-contrast microscopy (Fig. 16) and of methylene-blue-stained sections indicated that this distribution was not simply a result of a change in cytoplasmic density. Higher magnification indicated a strong staining associated with the amylo-

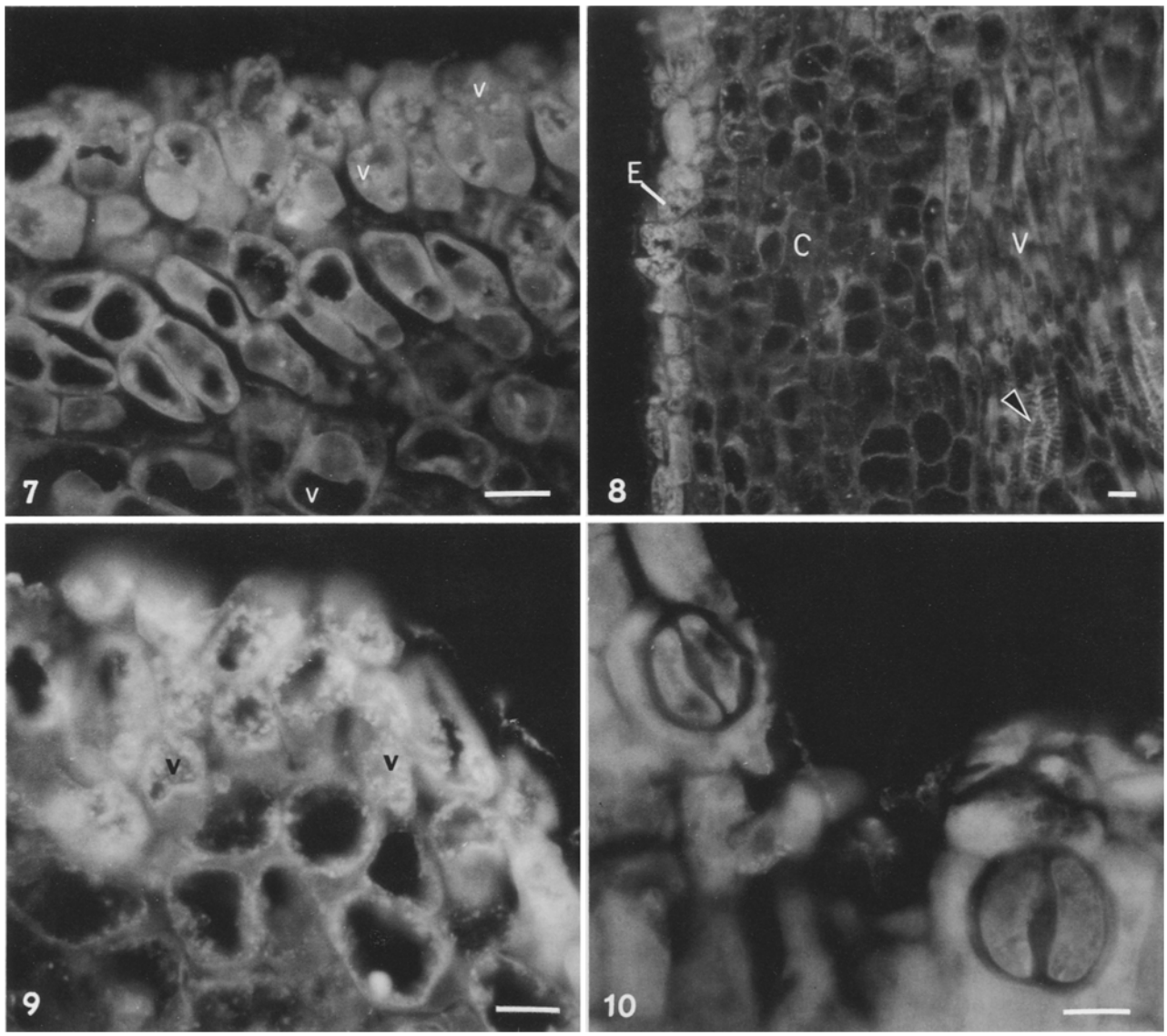


Fig. 7. Higher-magnification fluorescence micrograph from a pea leaf. In the epidermal cells, materials within the vacuole (*v*) are heavily stained whereas little staining is seen in the vacuoles of mesophyll cells. ×950; bar = 10 μm

Fig. 8. Fluorescence migrograph taken from the stem of a pea seedling. Epidermal cells (*E*) are most intensely stained. Cells in the vascular tissue (*V*) are usually more highly stained than are cortical cells (*C*). Tracheary elements are indicated at the *arrowhead*. ×400; bar = 10 μm

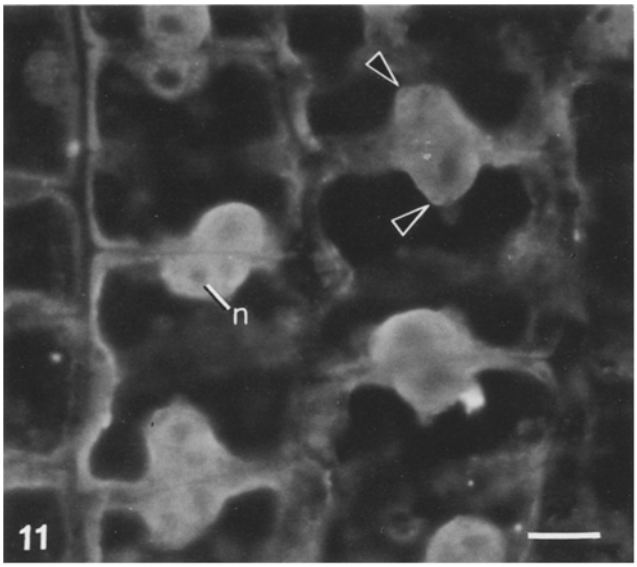
Fig. 9. Higher-magnification fluorescence micrograph demonstrating the epidermal staining in a pea stem. The section is slightly tangential. The intensely stained granular material is within the vacuoles (*V*) of the epidermal cells. ×975; bar = 10 μm

Fig. 10. Fluorescence micrograph demonstrating the staining of guard cells. ×975; bar = 10 μm

plasts in the columella cells (Figs. 18, 19), whereas in more peripheral cells, amyloplasts and other structures were only slightly fluorescent.

Discussion

A variety of immunocytochemical treatments were tested both with isolated whole cells and sectioned material from etiolated pea seedlings to assess their relative suitability. The techniques that proved most reliable and effective were then used to establish the distribution of calmodulin in pea plumules and roots. In general, the techniques used were modifications of those described by others (see Wick et al. 1981; Harper and Steiner 1983; Welsh 1983). Formaldehyde, or formaldehyde with low amounts of glutaraldehyde, has been shown to give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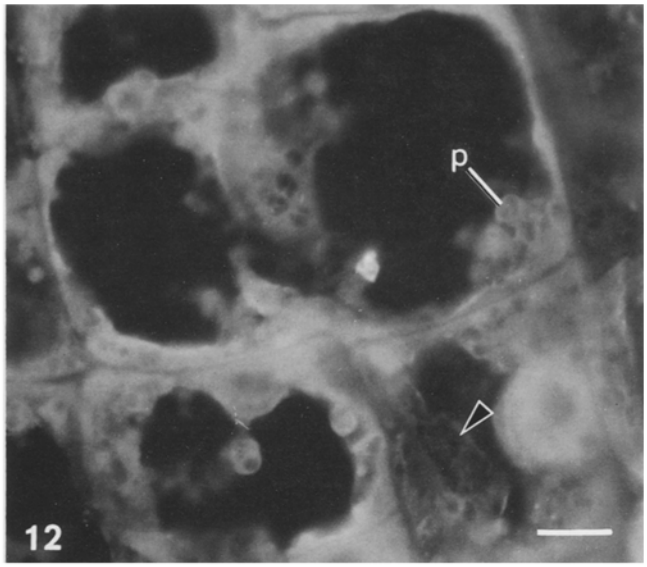
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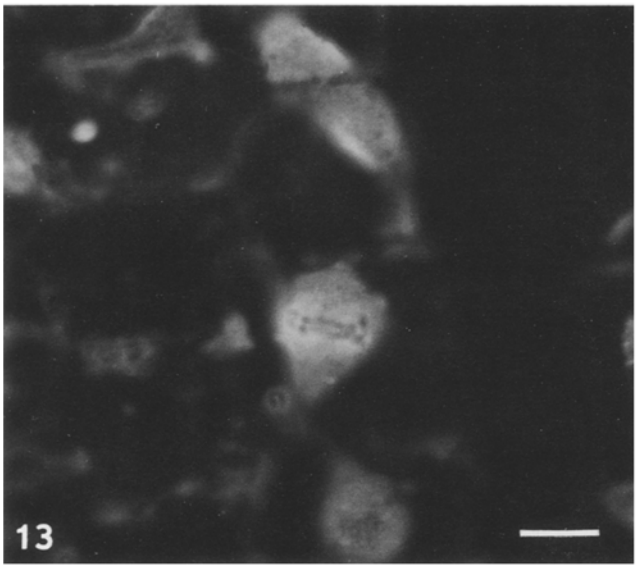
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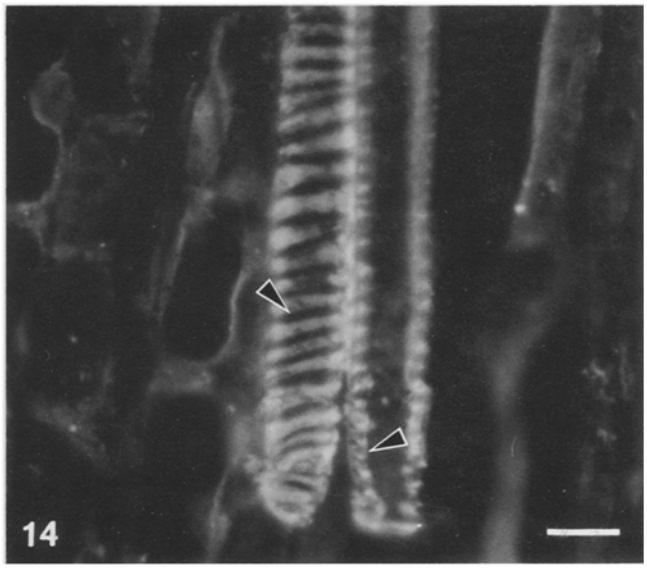
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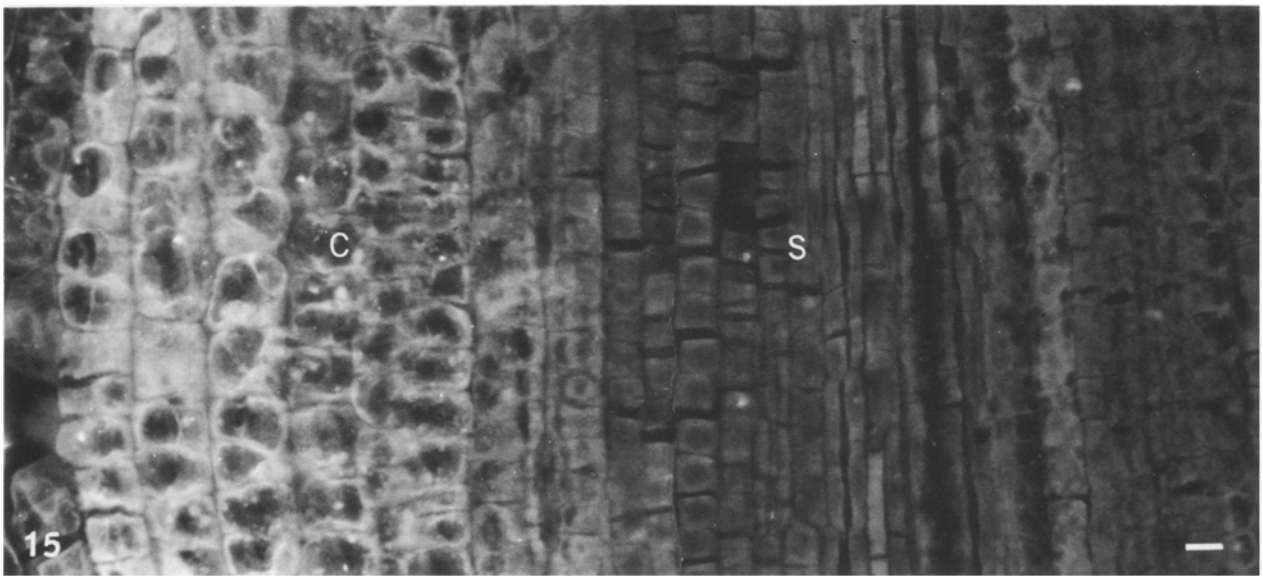
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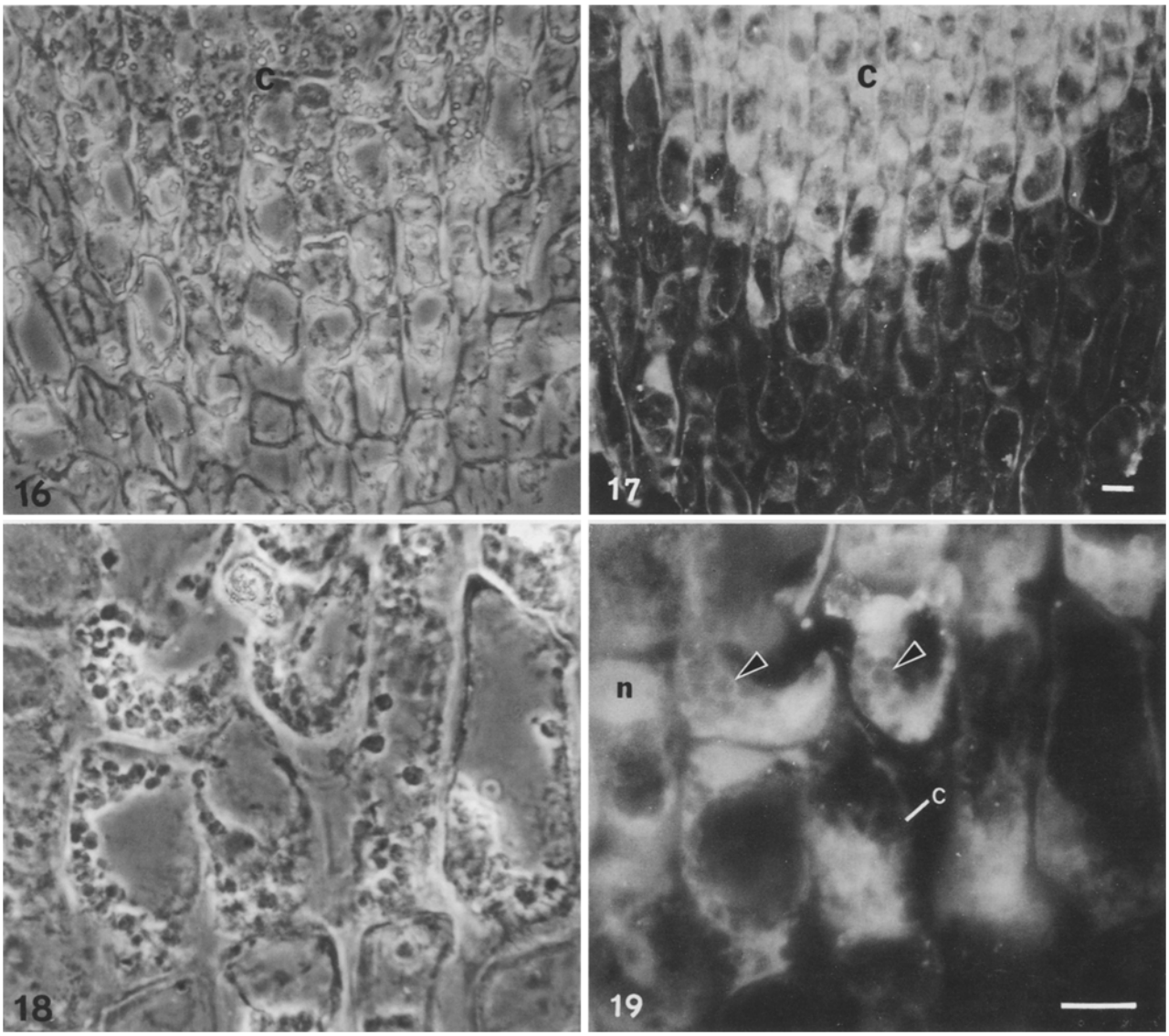

Fig. 11. Fluorescence micrograph demonstrating the pattern of staining in nuclei. Stain is seen in the region containing chromatin; nucleoli (*n*) are not stained. Structures at the *arrowheads* are plastids appressed to the nuclei in these highly vacuolate cells. ×1000; bar = 10 μm

Fig. 12. Fluorescence micrograph demonstrating the pattern of staining of different structures in the cytoplasm of pea stem cells. Staining of the plastids (*p*) can be seen in several areas. Possibly fibrillar staining is seen in the very thin cytoplasmic region indicated at the *arrowhead*. ×1000; bar = 10 μm

Fig. 13. Fluorescence micrograph demonstrating the pattern of staining in a division figure at metaphase in a pea stem cell. ×1100; bar = 10 μm

Fig. 14. Fluorescence micrograph demonstrating the pattern of staining in the tracheary elements of a pea stem. The secondary wall thickenings (*arrowheads*) are not stained. ×1000; bar = 10 μm

Fig. 15. Montage showing distribution of stain across the cortex (*C*) and stele (*S*) in the meristematic region of the root apex of a pea seedling. Cortical cells show a characteristic intense staining. ×475; bar = 10 μm

Figs. 16, 17. Phase-contrast and fluorescence micrographs demonstrating the difference in staining in the columella (*C*) and in the more peripheral cells of the root cap of a pea seedling. ×425; bar = 10 μm

Figs. 18, 19. Higher-magnification phase-contrast and fluorescence micrographs close to the region showing the change in staining in a pea root cap. Amyloplast staining (*arrowheads*) is evident in the cells of the columella of the root cap; nuclei (*n*) and cytoplasmic (*c*) regions are also stained. In the adjacent cells toward the root-cap periphery, these cellular structures are visible in the phase-contrast micrograph but are not well stained. ×1000; bar = 10 μm

good immunostaining of calmodulin (Willingham et al. 1983). The polyethyleneglycol technique offers a rapid, relatively gentle embedding procedure that can be used for tissue-level investigations and for ultrastructural localizations (Wolosewick 1980; Wolosewick and De Mey 1982). The comparable staining patterns obtained between whole cells and the embedded tissues indicates good retention of calmodulin antigenicity with this processing.

The antibody used is against rat calmodulin; evidence from several laboratories indicates that such antibodies bind efficiently to plant calmodulin (see Marmé and Dieter 1983). In our material, the division figures offer the best opportunity for comparison of results with the work of others. The staining patterns obtained compare well with those reported by Wick et al. (1985) for division figures stained by antibodies prepared against plant calmodulin. The specificity of the staining reaction is further supported by the lack of staining in all but one of the control preparations. The selective staining of tracheary elements with antibodies that had been preabsorbed with oat calmodulin will be discussed below.

A common finding is an association between calmodulin and elements of the cytoskeletal system. The localization of calmodulin with respect to microtubules and division figures has been extensively studied in both plants and animals (Deery et al. 1984; Wick et al. 1985; Clayton and Lloyd 1985; Vantard et al. 1985). The staining patterns obtained here were similar to those reported by Wick et al. (1985). There was no apparent staining of preprophase microtubules, and the most intense fluorescence corresponded with the spindle at metaphase with additional staining of the plate region during cytokinesis. Wick et al. (1985) showed by use of double-labeling techniques that whereas there was a fairly close correspondence of calmodulin and microtubules until late cytokinesis, in the latter stages only part of the staining patterns of calmodulin and tubulin coincided. In the interphase cells in our study and in those studied by Wick et al. (1985) there did not appear to be an association of calmodulin with microtubules. These findings are in contrast to those reported in animal systems. Using double immunofluorescence, Deery et al. (1984) were able to show staining of calmodulin associated with microtubules in interphase mammalian cells treated with detergent and EGTA. They proposed a Ca^{2+}-independent association of calmodulin with microtubules that would affect the polymerization of microtubules only in the presence of micromolar Ca^{2+}. Whether a similar process is important in changing microtubular arrays in plants remains to be determined.

Calmodulin has also been shown to be associated with other elements of the cytoskeletal system such as microfilaments and intermediate filaments. In our system, staining at the periphery of the nucleus was often observed, and some fibrillar patterns were seen in thin cytoplasmic regions. Immunofluorescent studies of the localization of intermediate filaments in animal (Deery et al. 1984) and plant (Dawson et al. 1985) cells show some perinuclear localization. In plants, actin filaments often occur close to the nucleus and can appear to surround it (Clayton and Lloyd 1985). Thus the calmodulin staining pattern reported here shares features with those of the three cytoskeletal elements. It is possible that calmodulin is associating with different elements of this system during interphase and in the various stages of division.

The gross tissue patterns observed are consistent with the analytical data of Muto and Miyachi (1984) who show highest amounts of calmodulin in the apical growth regions of etiolated pea seedlings. This pattern is also consistent with the suggestion of Wick et al. (1985) and others that calmodulin may be involved in the regulation of cell division and development. Staining of meristematic regions, of the root cap, and of vascular tissues was also reported by Lin et al. (1983) in corn and spinach after localization with the immunoperoxidase procedure. The specific tissue patterns observed here that may have significance for tropic responses are the staining of epidermal cells and the staining in the columella of the rootcap. The strong fluorescence observed in leaf epidermal cell vacuoles is not likely to be the result of a penetration artifact, for this staining pattern is asymmetric with respect to leaf surface. The lack of stain in guard-cell vacuoles also indicates the specificity of this epidermal distribution pattern. The regulation of calcium may well be involved in tropic responses as has been indicated by work in our laboratory (Slocum and Roux 1983). Even more evidence has been presented to indicate that the fine control of calcium fluxes in rootcap cells may be essential for the sensing of gravitropic stimuli. The particularly high staining associated with the plastids in the rootcap columella could represent a special interaction with cytoskeletal elements that is important in signalling changes in root orientation. The general association of anti-calmodulin fluorescence with plastids has important implications for the cellular-level responses of plants to various environmental stimuli.

One further staining reaction deserves note: the

fluorescence pattern seen in the tracheary elements (Fig. 14). The staining is localized immediately adjacent to the wall thickenings, and shows a pattern that is strikingly similar to that obtained by Lloyd (1984) in xylem from onion roots stained with monoclonal antibodies to yeast α-tubulin. Preliminary staining of the samples with immunogold techniques shows clearly that the staining is not in the wall but is associated with it. Indeed none of the data we have obtained are consistent with a wall localization for calmodulin, although it could be in a form that is not available for staining.

Biro et al. (1984) used radioimmunoassay to quantitate calmodulin in various subcellular fractions of oat cells, and found substantial levels in their preparations of extracellular fluid. Based on these results, they proposed that calmodulin was present in wall spaces. The immunocytochemical results presented here indicate that the calmodulin found in extracellular preparations by Biro et al. may have originated in tracheary elements rather than in wall spaces.

Different control samples for the staining of tracheary elements gave different results. Staining was observed in the calmodulin-absorbed control, but not in samples treated with pre-immune serum. Although anti-mammalian calmodulin cross reacts with plant calmodulin (Biro et al. 1984), plant calmodulin is antigenically distinct from animal calmodulin (Watterson et al. 1984). Thus, pre-absorbing a solution of anti-rat calmodulin antibodies with oat calmodulin would not be expected to remove all anti-calmodulin antibodies from the solution. Apparently the anti-rat calmodulin antibodies remaining after pre-absorption treatment can efficiently detect a calmodulin-like antigen in the tracheary elements, but nowhere else in pea tissue. At least three explanations for this seem possible: there may be an especially high concentration of calmodulin in the tracheary elements; tracheary elements may have a tissue-specific subtype of calmodulin that is especially reactive with the antibodies remaining after the pre-absorption treatment; or, there may be a non-calmodulin antigen unique to the tracheary elements that reacts with the antibodies remaining after the pre-absorption treatment. Any one or combination of these explanations could account for the results we observed in the control samples of tracheary elements.

In *conclusion* both by biochemical and cytochemical evidence, calmodulin appears to be a fairly ubiquitous cytoplasmic component that is associated with different cellular organelles. The association with elements of the cytoskeleton system, with plastids and with rootcap columellar cells, might provide key control points in gravitropic responses. Experiments are currently in progress to determine if differences in the tissues staining patterns are seen with gravitropic stimulation, and to study the organelle interactions at the ultrastructural level.

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