ARTICLE

Immunoelectron microscopic localization of calmodulin in corn root cells

LI Jiaxu, Jiewen* LIU , Daye SUN[1]

Department of Biology, Hebei Normal University, Shijiazhuang 050016, China

Institute of Hematology, Chinese Academy of Medical Sciences, Tianjin 300020, China

ABSTRACT

Methods for the localization of plant calmodulin by immuno-gold and immuno-peroxidase electron microscopy have been developed. In both corn root-cap cells and meristematic cells, calmodulin was found to be localized in the nucleus, cytoplasm, mitochondria as well as in the cell wall. In the meristematic cells, calmodulin was distinctly localized on the plasma membrane, cytoplasmic face of rough endoplasmic reticulum and polyribosomes. Characteristically, calmodulin was present in the amyloplasts of root-cap cells. The widespread distribution of calmodulin may reflect its pleiotropic functions in plant cellular activities.

Key words: *calmodulin, corn root cells, immunoelectron -microscopic localization.*

INTRODUCTION

Calmodulin (CAM) is a heat-stable, acidic and multifunctional calcium-binding protein which modulates a number of enzymic activities and many cellular processes in a calcium-dependent manner. In plants, CaM has been reported to regulate NAD kinase, quinate oxidoreductase, Ca^{2+}-ATPase, protein kinases and H^+-ATPase[1].

---

1. Corresponding author.

Abbreviations: CaM, calmodulin; DAB, 3.3ʹ- diaminobenzidine-4HCl; ELISA, enzyme-linked immunosorbant assay; PBS, phosphate buffered saline(10 m*M* sodium phosphate, 150 m*M* NaCl, pH 7.4); RIA, radioimmunoassay; SDS, sodium dodecyl sulfate.

Localization of calmodulin in corn root cells

In addition, plant CaM may be involved in gravitropic responses, phytochrome-induced responses, and hormone-mediated processes[2,3].

The studies of the cellular and subcellular distribution of CaM in situ are essential for understanding the function of CaM in cells. Precise knowledge of the subcellular localization of CaM may provide some clue to its possible function in the biochemical mechanisms where its actions have been demonstrated in vitro. Although many biochemical studies of plant CaM have been done, relatively little is known about its subcellular location in plant cells. In this paper, the experimental results of the subcellular distribution of CaM in corn root cells by immunoelectron microscopy are reported.

MATERIAIS AND METHODS

Chemicals

Peroxidase-labeled goat anti-rabbit IgG was purchased from Bio-Rad . 3,3'-diamino-benzidine-4HCl (DAB) from Sigma, paraformaldehyde and glutaraldehyde from Polysciences, sodium meta-periodate from Fisher. Protein A-gold conjugates (15 nm) was purchased from Institute of Medical Sciences, Chinese Academy of Military Medical Sciences(Beijing). All other chemicals were of reagent grade.

Plant material

Corn seeds (*Zea mays L.*) were surface- sterilized in 1 % sodium hypochlorite for 30 min and then rinsed at least 3 times with sterile distilled water. After soaked in the water for 24 h, seeds were placed on moist filter paper in a covered dish and germinated for 48 h in the dark at 20 °C. 1.5 mm terminals of corn roots were excised for immunoelectron microscopic observation.

Preparation and characterization of anti-CaM antibody

CaM was purified to homogeneity from wheat coleoptiles by the method of Biro et al[4]. Anti-CaM serum was raised in rabbits. Briefly, 0.5 ml wheat CaM (0.8 mg) in PBS was emulsified with equal volume of either complete Freund's ,adjuvant (initial injection) or incomplete Freund's adjuvant (subsequent injections). Male New Zealand white rabbits were injected subcutaneously into 6-8 sites on the back. The boost injections were repeated at 2 wk intervals. Ten days after the first boost, the serum was obtained and tested for immunoreaetivity with CaM by ELISA. A series of boosts and tests was performed until a sufficient titer was achieved.

Specificity of the antiserum toward CaM was tested by immunostaining of Western blots of corn root proteins. Briefly, corn root proteins were separated on a 15% SDS-polyacrylamide gel using the procedure of Laemmli[5], and then electrophoretieally transferred to nitrocellulose membrane (0.45 μm pore size) by the method of Towbin et al[6] at 200 mA for 45 min at 4℃. Amido black staining of the control strip revealed the band pattern of the transferred proteins. The blotted nitrocellulose membrane was blocked with 2% (W /V) nonfat dry milk in PBS (pH 7.4) for 2 h as described in Johnson et al.[7], and then incubated with anti-CaM serum in PBS containing 0.05% Tween-20 (PBST) for 3 h. After being washed in PBST , the membrane was incubated with peroxidase-labeled goat anti-rabbit IgG in PBS for 1 h. After washing, the immunoblots were visualized in a

solution containing 0.05% (W/ V) DAB and 0.01% H₂0₂ in 0.05 M Tris-HCl buffer (pH 7. 6)

Immunoperoxidase labeling

Corn roots were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 6 h at 4℃. Longitudinal slices (30 μm) of the roots were prepared with a vibratome. The immunostaining procedures employed here were the same as those described by Lin et al.[8]. After immunostaining, the slices were post fixed in 1% OsO4 in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature, dehydrated through ethanol series and propylene oxide, and then fiat-embedded in Epon 812. The positive immunoreaction areas were marked under a light microscope and mounted on blank blocks. Superficial ultrathin(700 $\mathring{A}$) sections, cut Parallel to the tissue surface originally exposed to the immunological reagents, were examined in a Hitachi H-600 electron microscope without further staining. Control experiments included: a) the anti-CaM serum was omitted; b) use of preimmune rabbit serum instead of anti-CaM serum; c) anti-CaM serum absorbed with excess of purified wheat CaM.

Immunogold labeling

Corn roots were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 6 h at 4℃. Longitudinal slices (60 μm) of the roots were prepared with a vibratome . The slices were postfixed, dehydrated, and fiat-embedded as described above. Thin sections (700 $\mathring{A}$) were cut with glass knives and mounted on nickel grids (300 mesh). All the following operations were performed at room temperature, unless otherwise stated. The sections were immersed in distilled water for 10 min and then etched with a saturated aqueous solution of sodium meta periodate for 30 min to expose the antigenic sites[9]. The sections were then washed in distilled water 3 times and treated with 20 mM glycine in PBS for 15 min to inactivate the nonreacted aldehyde groups[10].

The sections were washed 3 times with PBS and then blocked in 1% nonfat dry milk in PBS for 1 h. The sections were incubated in anti- CaM serum diluted 1:30 with PBS for 24 h at 4℃, washed 4 times with PBS, and incubated with protein A-gold conjugates (15 nm) diluted 1:30 in PBS for 1 h. After the sections were washed in PBS 3 times and twice in distilled water, they were dried and poststained with saturated uranyl acetate in 50% (V / V) ethanol (2 min) and lead citrate (30 s). The sections were examined and photographed with a Hitachi H-600 electron microscope. Three control experiments were also performed as described in immunoperoxidase labeling.

Quantitation of gold labeling

The density of labeling of each cell compartment was obtained by determining the number of gold particles per μm² of the cellular compartment. 20 micrographs were analyzed[11] at a final magnification of 30,000 $\times$.

RESULTS

The specificity of the antibody probe is most crucial for immunocytochemical localization. We analyzed the specificity of anti-wheat CaM serum by Western blots. As shown in Fig 1, anti-wheat CaM serum reacted only with one protein band identified as Ca M (with an electrophoretic mobility in the range of corn CaM) in the

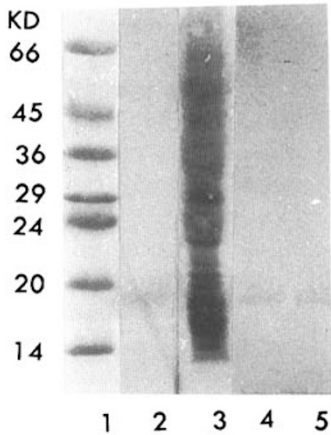# Localization of calmodulin in corn root cells

Fig 1. ◁ The specificity of anti-CaM antibody to CaM from corn roots. Protein samples were subjected to SDS-PAGE and transferred to nitrocellulose (Western blot). The Western blots were either stained with 0.1% Amido black (lanes 1-3) or immunostained with anti-CaM antibody (lanes 4 and 5). Lane 1, Molecular-weight markers (Values in kD); lanes 2 and 4 purified corn CaM (5 μg); lanes 3 and 5, corn roots proteins (100 μg). In the lane 5. the only band that was immunostained by anti-CaM antibody was one with the same mobility as purified corn CaM.

Fig 2. ▷ Meristematic cell of corn root. Gold particles are localized on the plasma membranes (PM), polyribosomes (arrow), and cytoplasmic, face of rough endoplasmic reticulum (RER). Some gold particles are present in the cell wall (CW). × 30,000.
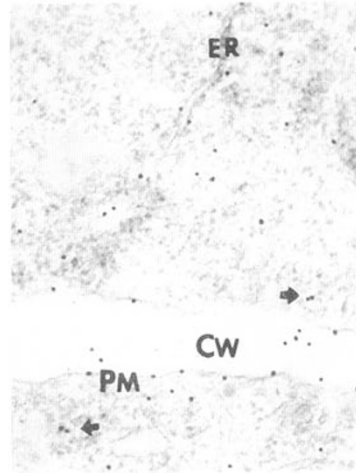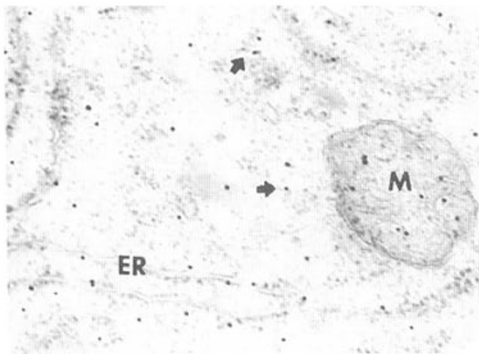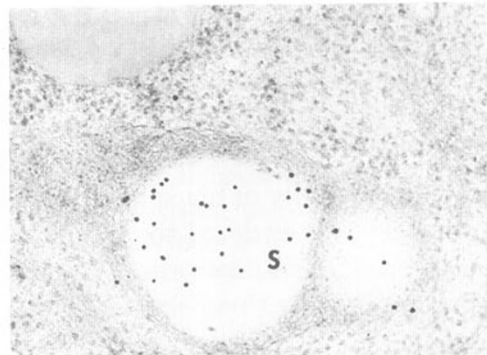

Fig 3. ◁ Meristematic cell of corn root. Gold particles are localized on the cytoplasmic face of RER. mitochondrion (M), and polyribosomes (arrow). × 30.000.

Fig 4 ▷ Corn root-cap cell. The starch grain (S) in the amyloplast is densely labeled with gold particles. × 35,000.
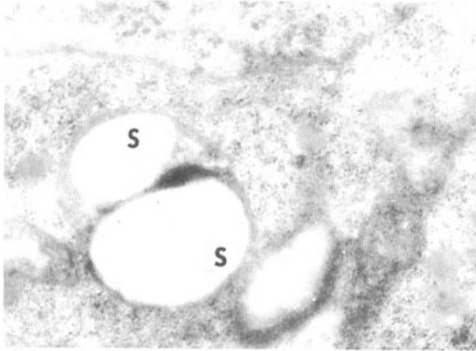
Fig 5. ◁ Control. Corn root-cap cell. No labeling is seen in the cell treated with CaM-absorbed antiserum. × 25,000.

Fig 6. ▷ Meristematic cell of corn root. Peroxidase reaction deposits are shown to be attached to the inner surface of plasma membranes(PM). It seems reaction deposits are also on the plasmodesma (arrow). × 20,000.
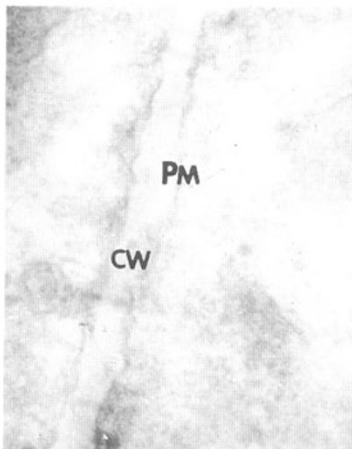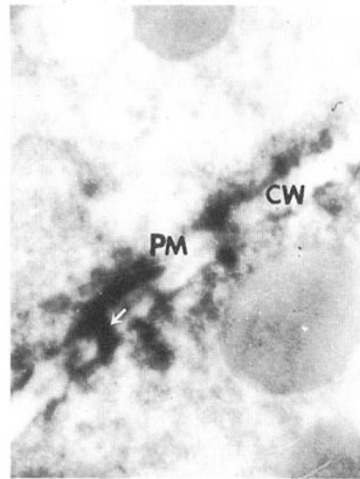

Fig 7. ◁ Control. Meristematic cell of corn root. No specific reaction deposits are seen in the cell treated with preimmune rabbit serum, × 25,000.

corn root proteins (Lane 5) and with purified corn CaM (Lane 4). No cross-reaction with other polypeptides was observed. In addition, anti-CaM serum absorbed with purified wheat CaM could not react with any protein from the corn roots on Western

blots (data not shown). These results implied that CaM in corn root cells can be specifically detected by the anti-wheat CaM serum.

Ultrathin sections of Epon 812-embedded corn roots were used to localize CaM by protein A-immunogold technique. Good ultrastructural preservation with the retention of antigenic determinants was obtained by the methods used. In the meristematic cells, gold particles were associated with the plasma membrane, cytoplasmic face of RER, polyribosomes, mitochondria as well as with the cell wall (Fig 2 and 3). Characteristically, the starch grains of the amyloplasts was densely labeled in the root-cap cells (Fig 4). Little gold labeling could be observed in the control (Fig 5).

The distribution of CaM in corn root cells was also investigated by immunoperoxidase technique. In the meristematic cells, the peroxidase reaction deposits were associated with the inner surface of plasma membrane and possibly with plasmodesmata (Fig 6). The deposits were also observed on the cytoplasmic face of ER in these cells (data not shown). No peroxidase reaction deposits could be seen in the control (Fig 7).

Since the immunoreactive sites for CaM were widely distributed in corn root cells, we have tried to evaluate the relative amount of CaM in different cellular compartments by calculating the labeling density of some cell compartments. As shown in Tab 1, the labeling density of amyloplasts was the highest among those of all cellular compartments tested in the root-cap cells. The density of labeling over the cell wall was the lowest among those cellular compartments tested in both the root-cap cells and the meristematic cells. However, since the background labeling obtained with CaM-absorbed antiserum was subtracted from the labeling obtained with anti-CaM serum, the cell wall labeling was very likely to represent *bona fide* CaM antigenic sites.

Tab 1.   Density of labeling over various cell compartments (n=20)

	Gold particles / μm^2 $\pm$ SE					
	Nucleolus	Nucleoplasm[a]	Mitochondrion	Amyloplsat* plastid	Cytoplasm[b]	Cell wall
Root-cap cells	3.8 $\pm$ 0.5	3.0 $\pm$ 0.3	4.5 $\pm$ 0.5	6.4 $\pm$ 0.7*	3.1 $\pm$ 0.3	0.6 $\pm$ 0.1
Meristematic cells	3.1 $\pm$ 0.3	2.3 $\pm$ 0.2	4.6 $\pm$ 0.4	3.9 $\pm$ 0.5	2.5 $\pm$ 0.2	0.8 $\pm$ 0.1

The background labeling obtained with CaM-absorbed antiserum was subtracted from the labeling obtained with anti-CaM serum.
a. The nuclear region not occupied by the nucleolus.
b. The cytoplasmic region not occupied by organelles.

DISCUSSION

Using immunoelectron microscopy, we have determined the subcellular distribution of CaM in corn root cells. Specific immunolabeling for CaM with good ultrastructural preservation was obtained with Epon 812-embedded corn root cells. Similarly, Hatase et al[12] also showed that there was specific immunogold labeling for CaM in Epon 812-embedded mitochondria isolated from rat liver. These results indicated that CaM antigenicity in Epon 812-embedded tissues could be retained. Western blot analysis showed that the anti-wheat CaM serum we raised did not recognize proteins other than CaM in corn roots. This result indicated the specificity of anti-wheat CaM serum towards CaM. Moreover, control experiments demonstrated the specificity of immunogold or immunoperoxidase labeling of CaM in corn root cells. Thus, the immunocytochemical methods employed here rendered us confident in the reliability of CaM antigenic site distribution.

CaM was shown *in situ* to be present in nuclei, cytoplasm, mitochondria, and cell wall(Tab 1). These results were in accord with other reports of CaM distribution by RIA in isolated subcellular fractions from several plants[4,13,14]. In addition, CaM was also precisely localized on the plasma membrane, cytoplasmic face of RER and poly-ribosomes of the meristematic cells. These findings are in agreement with the CaM localization reported in animal cells[15,16]. Using biochemical methods, Collinge and Trewavas[17] demonstrated that CaM was associated with pea plasma membrane preparations and that CaM may be projected from the inner surface of the membrane. Our immunoelectron microscopic studies (Fig 2 and 6) confirmed their findings. Membrane-bound CaM may play a role in controlling the intracellular calcium level through stimulating membrane Ca^{2+}-ATPase activity[1].

There are plenty of evidences that calcium plays an important role in the gravitropic response of roots and shoots[2,18]. And also there is some clue that CaM is implicated in the gravitropic mechanism[19]. In the immunocytochemical localization studies of CaM, Lin and Sun[20] found an intense staining in the root cap of corn, and Dauwalder et al.[21], observed a strong staining of amyloplasts in the columella of pea root-cap. Our immunogold labeling results showed that the starch grains in amyloplasts of root-cap cells were densely labeled (Fig 4). Generally speaking, the amyloplasts of root-cap cells are thought to be the gravity-sensing organelle in the gravitropic response of roots[22]. Moreover, CaM could regulate the phosphorylation of several proteins in amyloplasts[23]. Taking these data together, it is quite possible that CaM associated with amyloplasts in the root-cap cells plays a role in the sensing of plant roots to gravitropic stimuli.

The cell wall of root-cap cells and meristematic cells was lightly labeled for CaM (Tab 1). This result was consistent with the findings using ultralcentrifugation and RIA[4,14]. That Dauwalder et al.[21] could not observe the staining of cell wall may be due to the very limited resolution of the immunofluorescence technique used and the very low concentration of CaM in the cell wall. in out laboratory, cell wall CaM

Localization of calmodulin in corn root cells

was purified from wheat etiolated coleoptiles and several CaM-binding proteins in wheat cell wall were also identified[24, 25]. Further work is currently in progress to investigate the possible function of cell-wall CaM in plant cell regulation.

Finally, CaM may be associated with plasmodesmata in the meristematic cells (Fig 6). But this result requires further confirmatiom

REFERENCES

[1] Poovaiah BW: Reddy ASN. Calcium messenger system in plants . CRC Crit Rev Plant Sci 1987; **47**-103.
[2] Evans ML, Moore R, Hasenstein KH. How does root respond to gravity. Sci Amer 1986; 255:100-7.
[3] Roux SJ, Wayne RC, Datta N. Role of calcium ions in phytochrome responses: an update. Physiol Plant 1986; **66:**344-8.
[4] Biro RL, Sun DY, Serlin BS, Terry ME, Datta N, Sopory SK et al. Characterization of oat calmodulin and radioimmunoassay of its subcellular distribution. Plant Physiol 1984; **75:**382-6.
[5] Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; **227:**680-5.
[6] Towbin H, Staehelin T, Gordon J. Electronphoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 1979; **76:**4350-4.
[7] Johnson DA, Gautsch JW, Sportsman CR, Elder JH. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal Tech 1984; **1:**3-8.
[8] Lin CT, Sun DY, Song GX, Wu JY. Calmodulin: Localization in plant tissues. J Histochem Cytochem 1986;**34:**561-7.
[9] Bendayan M, Zollinger M. Ultrastructural localization of antigenic sites on osmium fixed tissues applying protein A-gold technique. J Histochem Cytochem 1983; **31:** 101-9.
[10] Stafstrom JR, Stachelin LA. Antibody localization of extensin in cell walls of carrot storage roots. Planta 1988; **174:**321-32.
[11] Smart CC, Amrhein N. Ultrastructural localization by protein A-gold immunocytochemistry of 5-enolpyruvylshimic acid 3-phosphate synthase in a plant cell culture which overproduces the enzyme . Planta 1987; **170:**1-6.
[12] Hatase O, Doi A, Itano T, Matsui H, Chmuia Y. A direct evidence of the localization of mito-chondrial calmodulin. Biochem Biophys Res Commun 1985; **132:**63-6.
[13] Muto S. Distribution of calmodulin within wheat leaf cells. FEBS Lett. 1982; **147:**161-4.
[14] Hernandez-Nistal J, Rodriguez D, Nicolas G, Aldasoro, JJ. Abscisic acid and temperature modify the levels of calmodulin in embryonic axes of *Cicer arietinum.* Physiol Plant 1989; **75:**255-60.
[15] Lin CT, Dedman JR, Welsh M J, Brink ley BR, Means AR. Localization of calmodulin in rat cerebellum by immunoelectron microscopy. J Cell Biol 1980; **85:**473-80.
[16] Weinman S, Ores-carton C, Escaig F, Feinberg J, Puszkin S. Calmodulin immunoelectron mi-croscopy: Redistribution during ram spermatogenesis and epididymal maturation. J Histochem Cytochem 1986; **34:**1181-93.

[17] Collinge M, Trewavas AJ. The location of calmodulin in the pea plasma membrane. J Biol Chem 1989; **264**:8865-72.

[18] Sun DY, Biro R1, Roux SJ. Inhibition of gravitropism in oat coleoptiles by the calcium chelator, ethylene-glycol-bis-(2-aminoethylether)-N, N'-tetraacetic acid. Physiol Plant 1984; **61**:449-54.

[19] Stinemetz CL, Kuzmanoff KM, Evans ML, Jarrett HW. Correlation between calmodulin activity and gravitropic sensitivity in primary root of maize. Plant Physiol 1987; **84**:1337-42.

[20] Lin CT, DY Sun, JY Wu. Immunohistochemical localization of calmodulin in certain corn and spinach tissues. J Cell Biol 1983;**97**:40a.

[21] Dauwalder M, Roux S J, Hardison L. Distribution of calmodulin in pea seedlings: Immunocyto-chemical Localization in plumules arid root apices. Planta 1986; **168**:461-70.

[22] Chandra S, Chabot JF, Morrison GH, Leopold AC. Localization of calcium in amyloplasts of root-cap cells using ion microscopy. Science 1982; **216**:1221-3.

[23] Macherel D, Viale A, Akazawa T. Protein phosphorylation in amyloplasts isolated from suspension-cultured cells of sycamore. Plant Physiol 1986; 8**0**:1041-4.

[24] Ye ZH, DY Sun, JF Guo, Preliminary study on wheat cell wall calmodulin. CMna Sci. Bull 1989; **34**:158-61.

[25] Ye ZH, JF Guo, DY Sun. Studies on cell wall calmodulin and calmodulin-binding proteins of wheat etiolated coleoptiles. Acta Phytophysiol Sin 1989;**15**:223-9.