A Pollen-specific Novel Calmodulin-binding Protein with Tetratricopeptide Repeats*

Received for publication, March 30, 2000, and in revised form, August 23, 2000
Published, JBC Papers in Press, August 23, 2000, DOI 10.1074/jbc.M002720200

Farida Safadi, Vaka S. Reddy, and Anireddy S. N. Reddy‡

From the Department of Biology and Program in Cell and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523

Calcium is an essential constituent of in vitro pollen germination media and a potential chemotactic agent guiding pollen tube growth in the transmitting tissue of the pistil (1–6). High Ca$^{2+}$ concentrations have been reported in the vicinity of the germination apertures of hydrated pollen (7). Studies with $^{45}$Ca$^{2+}$ (3, 8) and later direct measurement of cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{cyt}$) using fluorescence ratio imaging of Ca$^{2+}$ indicator dyes established that, in pollen tubes, [Ca$^{2+}$]$_{cyt}$ accumulates at the growing tip and forms a steep-tip focused gradient with about 3.0 μM at the tip to about 0.2 μM within 20 or 65 μM from the tip (6, 9–16). Pollen tube elongation is inhibited by chemicals (e.g., ion channel blockers or ionophores) that interfere with Ca$^{2+}$ homeostasis in the growing pollen tubes (3, 11, 12, 14, 17–19). Disrupting the Ca$^{2+}$ gradient or blocking Ca$^{2+}$ influx inhibited pollen tube growth, dissipated the cytoplasmic streaming associated with the pollen tube elongation and eliminated the clear zone found at the tip of actively elongating pollen (7, 12, 20). Extracellular Ca$^{2+}$ influx at the pollen tube tip was reported and this influx was correlated with the intracellular Ca$^{2+}$ gradient and polarized growth of the tip (12, 21–23). Ca$^{2+}$ influx at the apex is speculated to be due to stretch-activated Ca$^{2+}$ channels at the tip (12, 22), while Ca$^{2+}$-ATPase pumps are implicated in maintaining the Ca$^{2+}$ gradients in the cytosol (7, 17). Recently, the relationship between [Ca$^{2+}$]$_{cyt}$ and pollen tube growth was further strengthened by the discovery that the tip-focused [Ca$^{2+}$]$_{cyt}$ gradients oscillate with tip-high [Ca$^{2+}$]$_{cyt}$ corresponding to peaks in pollen tube growth (24–27). Furthermore, Ca$^{2+}$ has been shown to influence the direction of pollen tube growth. Induced Ca$^{2+}$ fluctuation in the cytosol or modifying extracellular Ca$^{2+}$ influx redirected pollen tube growth toward the high Ca$^{2+}$ concentration (13, 27, 28), suggesting a role for pistil-derived Ca$^{2+}$ in the directed growth of pollen tubes toward the ovary. These studies showed that the establishment and maintenance of a precise tip-focused intracellular Ca$^{2+}$ gradient, possibly through regulating Ca$^{2+}$ influxes at the tip, is essential for pollen tube elongation and directional growth. How the tip-focused Ca$^{2+}$ gradient at the tip regulates pollen tube growth and direction is poorly understood. Some studies suggest that Ca$^{2+}$ interacts directly or indirectly, through Ca$^{2+}$-binding proteins, with the cytoskeleton to regulate cytoplasmic streaming, vesicle fusion, and the function of cytoskeletal elements required for tube emergence and growth (6, 12, 18, 19, 28–35).

Calcium has been implicated in regulating diverse physiological processes in plants (36–40). Calcium-regulated physiological responses are often mediated directly or indirectly by Ca$^{2+}$-modulated proteins of which CaM is ubiquitous in all eukaryotes. Calmodulin, a Ca$^{2+}$-modulated protein with four Ca$^{2+}$-binding EF-hands, is considered to be the primary intra-
cellular Ca\(^{2+}\) receptor in all eukaryotes. In plants, over the last 10 years CaM and CaM isoforms have been identified, and their involvement in transducing Ca\(^{2+}\) signals into a variety of cellular responses has been reported (41–43). Since CaM acts by modulating the activity of a variety of other proteins directly by interacting with them, research in recent years has focused on identifying the CaM-target proteins and analyzing the function of these proteins in cellular processes. Whereas a wide variety of CaM-activated proteins were described and characterized in animals only a few CaM-binding proteins have been identified in plants and their function in regulating plant growth and development in response to elevated Ca\(^{2+}\) signals is still in its infancy (42–44). Furthermore, very little is known about CaM-target proteins in pollen (37).

Considering the widespread association between CaM and Ca\(^{2+}\)-sensitive cellular processes, it is reasonable to expect CaM to mediate Ca\(^{2+}\) action in pollen tubes. Some reports suggest the involvement of CaM in mediating Ca\(^{2+}\) effect on pollen tube growth (37). Exogenous CaM enhances pollen germination and pollen tube growth (30, 45), whereas CaM antagonists and anti-CaM serum inhibit pollen germination and tube growth (18, 45, 46) and stop cytoplasmic streaming (17) in a concentration-dependent manner. In addition, upon CaM antagonist treatment of pollen tubes, Ca\(^{2+}\) remains in the tip membranes (45) or its level increases behind the tip (17), suggesting that CaM is involved in maintaining Ca\(^{2+}\) gradients in the pollen tubes through Ca\(^{2+}\) influx from the plasma membrane channels and/or sequestration of Ca\(^{2+}\) into the internal organelles by CaM-regulated Ca\(^{2+}\)-ATPases (34, 47). Ca\(^{2+}\)-ATPase activity has been detected in the plasma membrane of the pollen tube tip as well as in the endoplasmic reticulum and mitochondria behind the tip and these ATPases are stimulated by CaM (7, 43, 48). Calmodulin localization in the pollen during various stages of pollen growth has produced contradicting results. Calmodulin is localized to the region of germinal apertures of the hydrated pollen, the plasma membrane, and the cytoplasm in the vicinity of the germination bubble and in the plasma membrane and the cytosol of the growing pollen tube where it forms an apically focused gradient similar to the tip-focused Ca\(^{2+}\) gradient (7, 49). A similar localization of CaM is observed in the Fucus rhizoid tip pre-emergence location (37), suggesting a role for CaM in polar growth and tip extension. In other localization studies, however, CaM was found to be diffuse and uniformly distributed in the pollen tube (50, 51) and no tip high gradient of the protein was observed (51).

Recent studies on effects of exogenous CaM on pollen tube germination and growth concluded that CaM acts extracellularly exerting its effect on pollen possibly through a signal transduction pathway involving a receptor-mediated stimulation of a G protein (30, 46). Although these studies implicate the involvement of CaM in pollen germination and tube growth, little is known about proteins that bind to CaM in pollen. Hence studies on pollen-specific CaM-binding proteins should help us understand the role of CaM in Ca\(^{2+}\)-mediated signal transduction pathways in pollen. Here we report the isolation of a gene encoding a maize pollen calmodulin-binding protein (MPCBP) using a protein-protein interaction-based screening of an expression library with radiolabeled CaM. The MPCBP is expressed specifically in mature and germinating pollen and binds CaM in a Ca\(^{2+}\)-dependent manner. The MPCBP contains three tetratricopeptide repeats (TPRs) that are known to function in protein-protein interaction. The region of the protein that binds to CaM is mapped to an 18-amino acid stretch between the TPR1 and TPR2. Pollen-specific expression of MPCBP and its CaM-binding ability suggest a role for this protein in pollen germination and tube growth.

**EXPERIMENTAL PROCEDURES**

**Materials—Maize (Zea mays L.) inbred lines KYS and A632 seeds were germinated on moist filter paper, and the tissues (roots, hypocotyls, and leaves) were collected after 10 days of germination. Mature pollen was collected from tassels of field or greenhouse-grown maize. Freshly collected maize pollen was germinated on a medium containing 12% sucrose, 300 mg/liter CaCl\(_2\), 100 mg/liter boric acid, and 0.7% agar. Triton X-100-free nitrocellulose filter discs were obtained from Millipore. Easy tag 35S-isotope labeling mixture was obtained from PerkinElmer Life Sciences. Exassist helper phage and Escherichia coli SOLR cells were obtained from Stratagene. Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, IPTG, and Trizol were obtained from Life Technologies, Inc. PET vectors and E. coli strain BL21(DE3) were purchased from Novagen. Gelatin and dianisobenzoicid were obtained from Sigma. Biochemicals from Roche Molecular Biochemicals. Phenyl-Sepharose CL-4B, bovine CaM Sepharose-4B, and CNBr-activated Sepharose-4B were obtained from Amerham Pharmacia Biotech. Vanadyl ribonucleoside RNase inhibitor was from BioLabs. Expression and purification of the carboxyl-terminal region of RCBP (1.4C) was described in Reddy et al. (52). All other chemicals were of reagent grade.

**Expression and Purification of Recombinant Arabidopsis thaliana CaM Isoforms—**PET expression vectors containing CaM-2, -4, or -6 isoforms were kindly provided by Dr. Raymond Zielinski (53). The expected molecular weights for AtCaM2, -4, and -6 are 16,808, 16,824, and 16,822, respectively. The AtCaM isoforms were induced and purified as described earlier with some modifications (53). The E. coli BL21(DE3) cells containing recombinant plasmids were grown to an OD\(_{600}\) of 0.6 and induced by 1 mM IPTG for 3 h at 37 °C in 1 l of NYZ medium containing 50 μg/ml ampicillin as described from Fromm and Chua (54). All the following steps were performed at 4 °C. The cells were harvested, washed in buffer A (50 mM Tris-HCl, pH 7.5), and resuspended in extraction buffer (Buffer A with 2 mM EDTA, 1 mM DTT, 200 μg/ml lysozyme, and complete protease inhibitor mixture). After centrifugation, the cell extract was clarified by centrifugation and the supernatant fraction was precipitated with 55% ammonium sulfate. The proteins in the supernatant were precipitated with 50% H\(_2\)SO\(_4\) (pH 4) for 30 min with stirring. After centrifugation, the pellet was resuspended in buffer A containing 1 mM DTT, dialyzed first in distilled water, and then in buffer A containing 100 mM NaCl, 0.5 mM EGTA, and 1 mM DTT. After adjusting CaCl\(_2\) concentration to 5 mM, the protein was loaded onto a phenyl-Sepharose column CL-4B (10 ml bed volume) pre-equilibrated with buffer B (buffer A containing 0.1 mM CaCl\(_2\) and 0.5 mM DTT). The column was washed with buffer B containing 5 mM NaCl and the AtCaM protein was eluted with elution buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, and 0.5 mM DTT. The eluates containing the proteins were dialyzed in water.

**Production and Purification of 35S-labeled AtCaM Isoforms—**The 35S-AtCaM isoforms were prepared and purified as described (54) with slight modifications. Initially, the cells were grown in M9 medium with 10 g/liter tryptone and 50 μg/ml ampicillin overnight and then the cells were concentrated in 10 ml of M9 medium with ampicillin. One mM IPTG was added to the cells (0.6 A\(_{600}\)), 2 ml of Easy tag 35S-labeling mixture was added after 15 min, and the cultures were grown for 3 h at 37 °C. The cells were pelleted, resuspended in 1.5 ml of buffer A (see above), lysed with lysozyme (0.2 mg/ml), and DNase-treated (50 units) in the presence of 3 mM MgCl\(_2\). After centrifuging the lysate at 30,000 rpm for 30 min, the supernatant was heated for 3 min at 90 °C, centrifuged, and the resulting supernatant was used to purify the radiolabeled CaM on a 1-ml phenyl-Sepharose CL-4B column as described above.

**Screening of Maize Pollen Expression Library with 35S-CaM—**A cDNA library from maize pollen constructed in the EcoRI site of λ Zap II vector was used for screening. About 120,000 recombinants were screened with a mixture of 35S-labeled AtCaM isoforms 4 and 6. Approximately 9000 pfu per 15-em plate were plated on NZCYM plates using E. coli XL1-blue MRA (Stratagene) as the host strain. The plates were incubated at 42 °C until the plaques appeared, at which point the plates were overlaid with nitrocellulose filters that were previously soaked in 10 mM IPTG. Plaques were allowed to resume growth overnight at 37 °C. The plates were then placed at 4 °C for 1 h. The nitrocellulose filters were removed and washed briefly in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl\(_2\)). The filters were blocked in TBS containing 1% nonfat milk for 15 min with gentle shaking at room temperature and then incubated in a mixture of 35S-labeled At-
CaM isoforms at 5 µmol/l for 12 h. The membranes were rinsed in TBS/Ca²⁺ (TBS containing 5 mM CaCl₂) three times for 5 min each and dried between 2 sheets of 3MM paper for 24 h before exposing to a x-ray film. The putative positive plaques were plaque-purified by two additional rounds of screening. The cDNA inserts from λ Zap II were excised in HindIII/EcoRI form according to the manufacturer's recommendation. The infected E. coli SOLR cells with plaque recombinant. The insert was excised by digesting the plasmid DNA with EcoRI. The positive clones were confirmed for Ca²⁺ dependence in binding to CaM by expressing the purified clones as above, incubating the membrane in 325-S-CaM probe, and washing the membrane in TBS buffer containing either 2 mM or 5 mM EGTA, a Ca²⁺ chelator, as described above. To detect the proteins from the induced and uninduced cultures were separated on 12% SDS-polyacrylamide gels and blotted as described above. To detect the proteins, the gels were blotted onto a nitrocellulose membrane using a slot blot apparatus, and incubating the membrane in 35S-CaM (supernatant) from the insoluble (pellet) fractions. The pellet was dissolved in lysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM mercaptoethanol, 0.1% Triton X-100) on ice for 20 min to separate the soluble (supernatant) from the insoluble (pellet) fractions. The pellet was dissolved in 6.8, 30% glycerol, and 0.023% bromphenol blue) was added to the samples. The mixture was electrophoresed on 12% SDS-polyacrylamide gels. The gels were blotted onto a nitrocellulose membrane using a slot blot apparatus, and incubating the membrane in 35S-CaM (supernatant) from the insoluble (pellet) fractions. The pellet was dissolved in lysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM mercaptoethanol, 0.1% Triton X-100) on ice for 20 min to separate the soluble (supernatant) from the insoluble (pellet) fractions. The pellet was dissolved in 6.8, 30% glycerol, and 0.023% bromphenol blue) was added to the samples. The mixture was electrophoresed on 12% SDS-polyacrylamide gels containing 4 µmol/l or 7.5% glycerol, 0.375 µmol/l Tris-HCl, pH 8.8, and either 1 mM CaCl₂ or 5 mM EGTA. The gels were run at a constant voltage of 25 V per gel in an electrode buffer (25 mM Tris-HCl, pH 8.3, 192 mM glucose, and either 1 mM CaCl₂ or 5 mM EGTA). The gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 7.5% acetic acid and 50% methanol for 1 h and then destained with 30% methanol and 7% acetic acid.

Fluorescence Spectroscopy Assay—The tryptophan fluorescence spectra of free and CaM-bound synthetic peptide were recorded with a Hitachi-F-3010/4010 spectrofluorometer as described in Reddy et al. (52). The absorption of the fluorescence at 538 nm (as described in Reddy et al. (52)). Briefly, 1 g of CNBr-activated Sepharose-4B was rehydrated in 1 mM HCl and then washed with 1 mM HCl on sintered glass for 15 min to remove additives. Ten mg of each of the CaM isoforms 2, 4, and 6 were dialyzed in the coupling buffer (0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl) and incubated with the activated Sepharose-4B in 5 mM CaCl₂ for 2 h at room temperature for 2 h in a 15-ml tube after which it was centrifuged at low speed (1000 x g) and washed 3 times with 5 ml volumes of coupling buffer to remove unconjugated ligand. After centrifugation, the washing solution was replaced with blocking solution (0.1% Tritos-HCl, pH 8.0) and incubated for 1 h at room temperature to block remaining active groups. The beads were then washed three times, each.
time with 0.1 M acetate buffer containing 0.5 M NaCl followed by 0.1 M Tris-HCl, pH 8.0, containing 0.5 M NaCl. The solution was then replaced by CaM-Sepharose binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM CaCl$_2$) and the slurry was degassed and packed into a column. Insoluble fusion protein from P3 clone was dissolved in binding buffer containing 6 M urea. To purify these proteins, the column was equilibrated in the binding buffer with 6 M urea and the dissolved proteins were loaded. The unbound protein was washed with the same buffer, and the bound protein was eluted with binding buffer containing 6 M urea except that CaCl$_2$ was replaced with 7 mM EGTA. The fractions were analyzed on 12% SDS-polyacrylamide gel and detected with 35S-labeled AtCaM in the presence or absence of CaCl$_2$.

**Northern Blot Analysis**—Total RNA was extracted from maize pollen and kernels as described earlier (58). Pollen was ground in liquid nitrogen and sand, then homogenized in 3 volumes of lysis buffer (50 mM EGTA, 100 mM NaCl, 1% SDS, 100 mM Tris-HCl, pH 7.6, 50 mM β-mercaptoethanol containing 10 mM vanadyl ribonucleoside). The homogenate was extracted several times with an equal volume of phenol/ chloroform and centrifuged for 10 min at 10,000 rpm until there was no detectable interphase. RNA was precipitated with sodium acetate and ethanol, dissolved in diethyl pyrocarbonate-treated water, and reprecipitated twice with equal volume of 5 M LiCl on ice for 1 h. RNA from other maize tissues was extracted using the Trizol method according to manufacturer's instructions. RNA was loaded on denaturing 1% agarose containing formaldehyde and blotted and probed according to standard procedures (55).

**Southern Blot Analysis**—Maize genomic DNA was digested with different restriction enzymes, electrophoresed in 0.8% agarose gel, and transferred onto a Hybond nylon membrane. The DNA was fixed to the membrane by UV cross-linking. The blot was hybridized to the radio-labeled 1.2-kb cDNA at 65 °C and washed under high stringency conditions (55).

**Antibody Production**—About 150 μg of fusion protein induced from the 1.2-kb cDNA (P3) clone and purified on CaM-Sepharose column was electrophoresed on a preparative SDS-polyacrylamide mini-gel. The gel was washed 3 times with distilled water, stained in water-based 0.5% Coomassie R-250 for 30 min, and rinsed in several changes of water for 1 h. The protein band (32 kDa) was cut, rinsed briefly with 50 ml of water, and then overnight with 5 ml of buffer (50 mM Tris-HCl, pH 7.5, at 4 °C). The gel pieces were solubilized, mixed with Freud's incomplete adjuvant (1:1 ratio), and injected intradermally at multiple spots into New Zealand White rabbits. Booster injections were performed on 14, 28, 42, and 56 days after initial injection. Bleeds were performed before the first injection and on the day of each booster injection. The terminal bleed was collected on day 140. Serum from day 126-bleed was used for antibody purification and immunodetection studies.

Anti-MPCBP antibodies were affinity purified using a modified method of the Millipore technical protocol TP015 (Millipore Corp., Bedford, MA). About 1 mg of the purified MPCBP protein was electrophoresed on an SDS-polyacrylamide gel and transblotted onto a polyvinylidene difluoride membrane. The membrane was stained with Ponceau stain (0.1% w/v) in 1% (v/v) acetic acid, destained in distilled water, and a strip was cut with the band corresponding to MPCBP protein. The strip was cut into 1 × 2-cm pieces, equilibrated in 0.5 M potassium phosphate buffer, pH 7.4, rinsed in phosphate-buffered saline containing 0.1% Tween 20, and then incubated in 10% monoethanolamine in 1 M NaClO$_4$, for 2 h. Following two 30-min rinses in phosphate-buffered saline containing 0.1% Tween 20, the strips were incubated with 2 ml of serum for 3.5 h. The strips were rinsed three times and incubated in 0.9 ml of 100 mM glycine, pH 2.5, for 10 min. The solution was removed and neutralized with 0.1 ml of 1 M Tris, pH 8.0 (59).

**Immunodetection of MPCBP**—Proteins from pollen grains, germinating pollen grains, and other maize tissues were extracted by grinding tissues in liquid nitrogen and homogenizing in extraction buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 250 mM sucrose, 5 mM DTT, and complete protease inhibitor mixture). After centrifuging at 14,000 × g for 20 min, the supernatant was collected and used for electrophoresis. Microsomal and soluble fractions of the pollen extract were separated by centrifuging at 100,000 × g, and washing the pellet twice with extraction buffer. The microsomal fraction was dissolved in sample buffer. Proteins were separated on SDS-polyacrylamide gel and transblotted onto nitrocellulose membrane using a Bio-Rad transfer cell. After blocking with 3% gelatin in antibody buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) for 2 h, the membranes were incubated with the affinity-purified MPCBP antibody (1:5,000) in antibody buffer containing 1% gelatin for 2 h at 30 °C. The membrane was then washed with antibody buffer containing 0.05% Tween 20 followed by incubation for 1 h at 30 °C in 1:4000 dilution of goat-anti rabbit IgG conjugated to alkaline phosphatase (Strategene). Immunoreactive bands were detected colorimetrically by immersing the filter in substrate solution (0.3 mg/ml nitro blue tetrazolium and 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in AP buffer).
sequence data bases with the predicted amino acid sequence using BLAST searches revealed that the protein is highly similar to two hypothetical proteins found in the Arabidopsis genome data base. A protein with accession number AC006224 showed 54% identity and 68% similarity, and another protein (accession number AC00457) showed 39% identity and 56% similarity. Both of these proteins have similar gene structure with five exons and the CaM-binding domain in MPCBP is conserved in both of these proteins (Fig. 2C). Based on this high degree of sequence identity we named these APCBP1 (accession number AC006224) and APCBP2 (accession number AC00457). APCBP1 and APCBP2 are located on chromosome 2 and chromosome 1, respectively. In addition, the MPCBP showed limited sequence similarity to SPINDLY, a protein involved in GA signal transduction from Arabidopsis (U62135; 34% identity and 57% similarity) (60), and barley (AF035582; 19% identity and 35% similarity) (61), and to an O-linked GlcNAc transferase from Methanobacterium thermoautotrophicum (62) with 19% identity and 37% similarity. Fig. 2D shows the alignment of the deduced amino acid sequence of MPCBP with the amino acid sequence of APCBP1 and APCBP2, two hypothetical proteins identified in the Arabidopsis genome sequencing project. SPINDLY was not included in the alignment as it is a much larger protein (914 amino acids) and showed limited homology. Analysis of the predicted amino acid sequence of MPCBP using SMART (Simple Modular Architecture Research Tool), a program that predicts functional domains (63) revealed the presence of three TPRs, one in each of exons 3, 4, and 5 (Fig. 2, C and D). TPR domains consist of degenerate consensus sequences and are implicated in protein-protein interaction (64). The proteins that are similar to MPCBP contain one or more TPRs. The alignment of TPR1, -2, and -3 of MPCBP with TPRs from Arabidopsis proteins is shown in Fig. 2D. TPR1 of MPCBP showed 63% identity and 69% similarity with TPR1 of APCBP1. TPR2 of MPCBP showed 43% identity and 59% similarity with TPR2 of APCBP2. TPR3 of MPCBP has 79% identity and 85% similarity with TPR2 of APCBP1. A Blast search also revealed sequence similarities between MPCBP TPRs and TPRs from a variety of other proteins.

Mapping of the CaM-binding Domain—The protein encoded by the partial cDNA (1.2 kb) showed CaM-binding, suggesting that the CaM-binding domain is present in the region between 418 and 659 amino acids. To map the location of the CaM-binding domain, four truncated versions of the cDNA were made (Fig. 3A) and expressed in E. coli as His-Tag fusions using pET28 expression vector. Fig. 3B shows a Coomassie-stained gel of the fusion proteins and the corresponding blots detected with various probes. The presence of the fusion protein was detected with T7 tag antibody whereas the binding of the fusion protein to CaM was tested with either radiolabeled or biotinylated CaM (Fig. 3B). The expression of the expected size fusion proteins was verified by probing the blot with T7 tag antibody (Fig. 3B). Probing a duplicate blot with 35S-CaM showed that the protein (16 kDa) expressed from the N-terminal 168-bp region of the cDNA contained the putative CaM-binding domain (Fig. 3B, 35S-CaM/CaCl2, lane 6I). Truncated clones P3/BamHI and P3/NcoI which include the 168-bp region of the cDNA also produced CaM-binding peptide (see Fig. 3B, 35S-CaM/CaCl2, lanes 2 and 4I). Therefore, the protein encoded by the 168-bp truncated cDNA contains the CaM-binding domain. The protein was mainly detected in the insoluble inclusion bodies although under some conditions a small amount was expressed in the soluble fractions (data not shown). No binding to CaM was observed in the presence of EGTA confirming the Ca2+-dependence of the binding of MPCBP to CaM (Fig. 3B, 35S-CaM/EGTA). Interestingly, the protein did not bind to biotinylated CaM when a duplicate blot was probed with biotinylated CaM in the presence of Ca2+. This is probably related to the biotin moiety on CaM that may interfere with its binding to MPCBP. The protein expressed from clone 1 (P3) was solubilized in 6 M urea and successfully purified on a bovine CaM-Sepharose affinity column (Fig. 3C), further confirming the binding of the MPCBP to CaM.

A Synthetic Peptide Corresponding to Amino Acids 421 to 438 Binds to CaM—In many CaM target proteins in animals, the CaM-binding domain has been shown to reside in a stretch of 18–20 amino acid residues (42–44). Although the amino acid sequence in the CaM-binding domain of different CaM target proteins is not conserved, the binding region is predicted to form a basic, amphiphilic α-helix in which hydrophobic residues are segregated from hydrophilic residues along the helix (65). In addition, studies using synthetic peptides confirmed the speculation that CaM recognizes amphiphilic peptides (65). CaM binding studies with truncated proteins of MPCBP have shown that the CaM-binding domain is located in a 56-aa stretch (418–474). Analysis of this stretch of amino acids using a helical wheel program has revealed a region from 421 to 438 forms basic amphiphilic α-helical structure. To test if this 18–20 aa stretch binds CaM, a synthetic peptide containing these amino acids was synthesized and used for binding studies. As shown in Fig. 4A, the synthetic peptide (MP-1, VSKGWRLLA-LIL2AQCRF) bound to the bovine CaM and CaM isoforms 2, 4, and 6 from Arabidopsis at concentrations as low as 0.5 μg indicating that this region is indeed involved in CaM binding (Fig. 4A). Another synthetic peptide (MP2, AKLDQGSSLL-RVKKALKQAQQSPM) corresponding to a different region of MPCBP but lacking typical features of CaM-binding domains was used as a negative control in the binding studies. Neither MP2 nor BSA showed any binding to the labeled CaM isoforms (Fig. 4A, MP-2, and B). These results indicate the specificity of the synthetic peptide in binding to CaM. A previously characterized CaM-binding peptide of a microtubule motor protein (KCBP) bound to all three CaM isoforms (52) (Fig. 4A, K). To determine the stoichiometry of the CaM-peptide complex, we performed binding studies using the synthetic peptide (MP-1) and CaM in the presence of Ca2+ or EGTA. The binding of the synthetic peptide to CaM was detected by a gel mobility shift assay in polyacrylamide gels containing 4 M urea (Fig. 4B). At 4 M urea, low affinity and nonspecific complexes dissociate while high affinity complexes remain intact. In the presence of...
A Pollen-specific Novel Calmodulin-binding Protein

B

C

D
A Pollen-specific Novel Calmodulin-binding Protein

**Fig. 2.** Sequence analysis of MPCBP. **A**, nucleotide and deduced amino acid sequence of MPCBP gene. Exons are shown in uppercase letters and introns are presented in lowercase letters. The predicted amino acid sequence is shown under the nucleotide sequence. Numbers at the right correspond to nucleotides and deduced amino acids. Translation initiation and termination codons are shown in bold and the CaM-binding domain is underlined. **B**, schematic diagram of the MPCBP gene showing introns (lines) and exons (boxes). **C**, diagrammatic representation of different structural features of MPCBP. TPR sequences were identified using SMART program (63), CaM-binding domain was identified by testing the binding of truncated proteins of MPCBP and a synthetic peptide to CaM. **D**, alignment of MPCBP with two uncharacterized proteins in the Arabidopsis genome database (accession numbers AC006224 and AC004557). Identical amino acids are shown by reverse lettering. Dashes indicate gaps in alignment. The three TPRs and the CaM-binding domains are denoted by a single and a double line over their corresponding residues, respectively.
CaCl_2_ the synthetic peptide retarded the migration of CaM in the gel indicating the formation of a complex between the peptide and CaM (Fig. 4B). No change in CaM mobility was observed in the presence of EGTA (data not shown) suggesting that Ca^2+ is required for the formation of CaM-peptide complex. At a molar ratio of 1:1 (peptide:CaM) about 50% of CaM showed a shift (Fig. 4B, lane 2). At a molar ratio of 2:1 and 4:1 (peptide:CaM) the entire CaM migrated as a complex, and the band corresponding to the free CaM disappeared (Fig. 4B, lanes 3 and 4). The synthetic peptide retarded mobility of CaM similarly with bovine CaM and the three AtCaM isoforms, indicating that the peptide binds to these CaMs in the same stoichiometry (Fig. 4B). Peptide-CaM complexes that do not dissociate in 4 M urea have dissociation constants of less than 100 nM (57). These mobility assays suggest that the binding between the peptide and CaM is strong and does not dissociate in the presence of 4 M urea.

The binding of a peptide to CaM can also be tested by fluorescence spectroscopy since the peptide contains a tryptophan residue which is absent in CaM. Tryptophan-containing peptides have been shown to, upon binding to CaM, shift their fluorescence spectrum and change the intensity of fluorescence (52, 57, 66–68). In our study, The MP1 peptide contains a tryptophan and therefore was tested for fluorescence shift at equimolar ratios of peptide and CaM. As shown in Fig. 4C, the fluorescence of the synthetic peptide was shifted significantly in the presence of bovine CaM and the three isoforms of AtCaMs. The wavelength of peak emission shifted from about 350 to about 315 nm. In addition, the fluorescence intensity increased from about 0.1 to about 4.5, 6.1, and 8.4 in the presence of CaM4, bovine CaM/CaM6, and CaM2, respectively. The difference among the fluorescence intensity shifts of the different CaM isoform-peptide complexes indicates variation in the affinity of the CaM isoforms to MPCBP. Fig. 5 shows the purification of the MPCBP fusion protein (P3) on a bovine CaM-Sepharose column.
cation of the MPCBP fusion protein by affinity chromatography columns of Sepharose-4B conjugated to each of the AtCaM 2, 4, and 6 isoforms. The affinity column purified MPCBP fusion protein to a high degree of purity as indicated by the single band on Coomassie-stained gels as well as on blots probed with T7 tag antibody (see Fig. 5).

Expression of MPCBP in Maize—To determine the expression of MPCBP, total RNA from maize roots, shoots, kernels, and pollen was isolated and RNA gel blot analysis was performed. Single transcripts of about 2 kb was detected only in the pollen and the germinated pollen (Fig. 6). The transcript is absent in all other tissues. These results were further confirmed by reverse transcription-polymerase chain reaction where the MPCBP transcript was not detected in maize roots, shoots, and kernels (data not shown). To demonstrate the presence of first strand cDNA in reverse transcriptase-polymerase chain reaction, another maize gene (CBP-1) that is expressed in mature pollen and during germination, suggested a role for MPCBP in pollen germination and tube growth. These results may indicate that this protein is expressed in mature pollen and during germination, suggesting that the truncated C-terminal region of MPCBP binds to CaM in the presence of Ca2+ (Figs. 3 and 5). However, these results do not show that the full-length MPCBP binds CaM. To demonstrate that the native MPCBP interacts with CaM in a Ca2+-dependent manner, we isolated MPCBP from maize pollen extract either by using a pull-down assay with CaM-Sepharose beads or by passing protein through a CaM-Sepharose column as described under “Experimental Procedures.” The proteins that were isolated with both of these methods were blotted and probed with either affinity purified MPCBP-specific antibody or HRP-CaM (Fig. 9). As shown in Fig. 9A, pollen CaM-binding proteins bound to CaM-Sepharose column in the presence of Ca2+ and eluted in a buffer containing EGTA, a Ca2+-chelator. The spectral curves at 235 and 280 nm clearly show the elution of CaM-binding proteins with EGTA (Fig. 9A). We then analyzed the initial soluble protein extract, the flow-through, the wash, and the eluted proteins from the column as well as the pull-down assay with CaM-Sepharose beads. We separated these proteins along with the bacterially expressed truncated MPCBP (P3) and Arabidopsis KCBP 1.5C (70) on three gels. One gel was stained with Coomassie Blue. The other two were blotted onto nitrocellulose membranes. One blot was probed with MPCBP-specific antibody to detect MPCBP and the second one was subjected to HRP-CaM overlay assay to detect CaM-binding proteins. The majority of the pollen proteins did not bind CaM-Sepharose column (Fig. 9B, compare lane 1 to...
A Pollen-specific Novel Calmodulin-binding Protein

FIG. 5. Purification of MPCBP fusion protein on AtCaM isoform-Sepharose columns. Coomassie-stained SDS-polyacrylamide gels (top three gels) and corresponding blots below show the purification of the MPCBP (P3) fusion protein on Sepharose coupled to Arabidopsis CaM2 (CaM2), CaM4 (CaM4), and CaM6 (CaM6) isomers in the presence of 6 M urea. The blots were probed with T7 tag antibodies (T7-Tag). Lane 1 contains KCBP fusion protein, a known CaM-binding protein (for details see Fig. 3, legend); lanes 1–7 are different elution fractions. Expressed proteins in the inclusion bodies of the bacterial pellet were dissolved in 6 M urea and loaded on a CaM-Sepharose 4B column equilibrated with binding buffer containing 6 M urea and CaCl₂, and eluted in elution buffer containing EGTA.

FIG. 6. Expression of MPCBP. Northern analysis of MPCBP in different tissues of maize. Total RNA was electrophoresed on a formaldehyde-containing agarose gel, transferred to a Hybond N⁺ membrane, and hybridized with ³²P-labeled MPCBP cDNA. Left, ethidium bromide-stained gel showing the amounts of RNA loaded in each lane. Right, autoradiogram, germ-pollen, germinated pollen. Size markers (in kb) are shown on the right.

FIG. 7. Southern blot analysis of genomic DNA. Ten µg of genomic DNA was digested with different restriction enzymes. The digested DNA was electrophoresed through a 0.8% agarose gel, transferred onto a Hybond nylon membrane, and probed with ³²P-labeled MPCBP cDNA which contains the coding region for the CaM-binding domain. Size markers are shown on the left in kilobase pairs.

To understand calmodulin action in pollen germination and tube growth, we screened an expression library from maize with calmodulin, one of the primary calcium sensors in eukaryotes. This screening has resulted in isolation of a cDNA clone encoding a CaM-binding protein. Using several different approaches, we have demonstrated that the partial cDNA-encoded peptide binds to CaM with high affinity in a Ca²⁺-dependent manner. First, the cDNA-encoded protein bound CaM only in the presence of CaCl₂ but not in the presence of EGTA (Fig. 1). Second, in experiments using the truncated proteins of MPCBP in CaM binding studies, MPCBP bound to ³⁵S-CaM in a Ca²⁺-dependent manner in an SDS gel blot overlay assay (Fig. 3, A and B). The Ca²⁺-dependent CaM binding of this protein was further confirmed by purifying the bacterially expressed protein on a CaM-Sepharose column (Fig. 9).
Finally, the CaM-binding domain was narrowed to a stretch of 18 aa (Figs. 2A and 4). The synthetic peptide corresponding to the CaM-binding domain bound CaM in gel and fluorescence shift assays only in the presence of Ca$^{2+}$ (Fig. 4). This property of CaM-peptide complexes in gel shift assays and fluorescence spectrometry has been described for many CaM-binding proteins and has been used to determine the binding stoichiometry between CaM-binding proteins and CaM (57). Binding of the protein and the peptide to CaM at a concentration as low as 0.18 μM in mobility shifts indicates the high affinity between the MPCBP and CaM. The concentration of CaM used in screening and binding studies is within the physiological levels in the cell indicating that this protein plays a physiological role in pollen (71).

Slot blots, gel mobility, and fluorescence shift assays using the synthetic peptide corresponding to the putative CaM-binding domain from MPCBP and the three CaM isoforms CaM2, CaM4, and CaM6, indicated that MPCBP binds to the three CaM isoforms. Immunodetection of MPCBP in different tissues of maize (Fig. 8A) and pollen (Fig. 8B) showed that the protein is present in all tissues and at different levels. The molecular mass markers are shown on the left in kDa. Arrow indicates the expected size protein. Stars denote degraded polypeptides of MPCBP.

Fig. 8. Immunodetection of MPCBP in different tissues of maize. A, Western blot analysis of different maize tissues. Proteins from different tissues and membrane and soluble protein fractions from pollen were separated on SDS-containing gels. The gels were either stained with Coomassie blue (top) or blotted onto a polyvinylidene difluoride membrane and detected with an affinity purified antibody raised against MPCBP (bottom). Fusion protein from MPCBP (P3 clone) was loaded in lane P3 as a positive control. Immunoreactive bands were detected colorimetrically using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrates. Molecular mass markers are shown on the left in kDa. Arrow indicates the expected size protein. Stars denote degraded polypeptides of MPCBP.

Finally, the CaM-binding domain was narrowed to a stretch of 18 aa (Figs. 2A and 4). The synthetic peptide corresponding to the CaM-binding domain bound CaM in gel and fluorescence shift assays only in the presence of Ca$^{2+}$ (Fig. 4). This property

of CaM-peptide complexes in gel shift assays and fluorescence spectrometry has been described for many CaM-binding proteins and has been used to determine the binding stoichiometry between CaM-binding proteins and CaM (57). Binding of the protein and the peptide to CaM at a concentration as low as 0.18 μM in mobility shifts indicates the high affinity between the MPCBP and CaM. The concentration of CaM used in screening and binding studies is within the physiological levels in the cell indicating that this protein plays a physiological role in pollen (71).

Slot blots, gel mobility, and fluorescence shift assays using the synthetic peptide corresponding to the putative CaM-binding domain from MPCBP and the three CaM isoforms CaM2, CaM4, and CaM6, indicated that MPCBP binds to the three
MPCBP showed significant similarities to proteins from Arabidopsis (APCBP1 and APCBP2). Although the function of the hypothetical proteins is not known, the close similarity between MPCBP and Arabidopsis hypothetical protein APCBP1 in regions other than TPR regions indicate that APCBP1 may be an orthologue of MPCBP. The sequence similarity between MPCBP and APCBP2 suggests that APCBP2 is also related to MPCBP. SPINDLY from plants and O-linked glucose N-acetyltransferase from bacteria have limited sequence similarities in certain TPRs and are diverse in functions indicating that they are not related to MPCBP. However, it is interesting to note that each of the 3 TPR domains of MPCBP aligned with specific TPRs from the four proteins suggesting functional specialization for each of the TPR motifs. This specificity of TPR function may be expressed by TPR binding to specific target proteins, or distinct combination of different TPRs may provide a specific binding site for a target protein. For example, only two out of 10 TPRs in SPINDLY share sequence similarities with MPCBP. This may indicate some specificity of substrate binding of an individual TPR.

The observation that TPRs are present in proteins with a wide array of functions and in various combinations and numbers has led researchers to assume (and prove in some cases) that TPR functions as a scaffold in binding to specific substrates depending upon the secondary structure assumed by the individual or combination of TPRs (64, 78, 80). The functions reported in the literature all require that the TPR-containing proteins form a complex with another component to regulate their function (64, 75, 76, 79–82). Genetic analysis of SPINDLY in Arabidopsis and barley predicted the protein to be a negative regulator of the GA signal transduction possibly by interacting with other proteins in the pathway or a protein factor that binds to GA-regulated promoter through its TPR motifs. Mutations in two of the TPR motifs activated the GA transduction pathway indicating the importance of these domains for the function of the protein (60, 61). MPCBP as well as SPINDLY showed sequence homology with N-acetylglucosamine transferases (O-linked GlcNAc transferase) from bacteria and animals. However, various O-linked GlcNAc transfersases have a range of 9–13 tandemly repeated TPR domains clustered in their NH2-terminal region while the catalytic domain of transference activity is in the COOH-terminal (83, 84). MPCBP TPR motifs that are homologous to O-linked GlcNAc transferase are COOH-terminal and no sequence similarity in the catalytic domain of the transference with MPCBP is observed. It is therefore unlikely that MPCBP has a similar enzyme activity.

Northern and Western analyses indicated accumulation of the transcript and the protein in the mature pollen grain of maize. Immunodetection with proteins from germinated pollen indicated that the protein may be utilized in the first half-hour of germination and then is resynthesized. It is known that the pollen develops it accumulates mRNA required for its rapid germination and then is resynthesized. It is known that as the pollen develops it accumulates mRNA required for its rapid germination and tube growth upon landing on the stigma (85). MPCBP may be one of the proteins that is required immediately after hydration especially since it is related to a Ca2+-mediated pathway that is implicated early in the germination process and tube growth (27).

Since the MPCBP protein homologues from Arabidopsis are newly identified in the Arabidopsis genome sequencing project, functional analysis of these proteins has not been studied. The presence of TPR domains implies a role of these proteins in regulating cellular processes by interacting with themselves or other proteins. The presence of a CaM-binding domain in MPCBP between TPR-1 and TPR-2 makes this protein unique and suggests that the function of MPCBP is regulated by Ca2+/
CaM is known that intracellular tip-focused Ca\(^{2+}\) gradients together with the tip-directed inward currents of extracellular Ca\(^{2+}\) are major events in the process of pollen tube growth and directionality of tip growth (47). It is speculated that Ca\(^{2+}\)-sensing proteins couple changes in Ca\(^{2+}\) levels to growth response. CaM, a ubiquitous receptor of Ca\(^{2+}\) that is involved in mediating Ca\(^{2+}\) action, is a strong candidate for this role. CaM has been localized in the tip region of growing pollen (49) and in the extracellular vicinity of the pollen tip (30). Tip extension relies upon vesicle migration to the pollen tube tip that is involved in the tip region of growing pollen (49) and in the extracellular vicinity of the pollen tip (30). Tip extension relies upon vesicle migration to the pollen tube tip that is driven by cytoplasmic streaming. Cytoplasmic streaming and tip growth are inhibited by reagents that interfere with Ca\(^{2+}\) homeostasis and antagonize CaM (17, 18, 45, 46). Hence, part of Ca\(^{2+}\) action in controlling tip growth is likely to be mediated by CaM and its target proteins. Cytoplasmic streaming is known to be accomplished by the actomyosin cytoskeletal system and the maintenance of this directional streaming has been reported to be controlled by microtubules (86). Several reports indicated that these two cytoskeletal elements and the associated proteins are regulated by Ca\(^{2+}\) and CaM (87). Calmodulin is known to stabilize the microtubules (43) and to bind to some of the microtubule-associated proteins. It is possible, therefore, that MPCBP after being modulated by CaM may be involved in binding to some component of the cytoskeletal system through its TPR proteins. TPR proteins were reported to bind to all four heavy chain myosin isoforms in a manner that is involved in signal transduction, interact with other membrane proteins and this association could be modulated by Ca\(^{2+}\)/CaM. The identification and isoforms across mitochondrial and peroxisomal membranes. The association of MPCBP with microsomes suggests that it may interact with other membrane proteins and this association should help elucidate the function of MPCBP in pollen.

Acknowledgments—We thank Dr. Patricia Bedinger for providing the pollen cDNA library, Dr. R. E. Zielinski for AtOCAm isoform constructs, Dr. Golovkin for help on this project, and Dr. Irene Day for comments on the manuscript.

REFERENCES

1. Brewbaker, J. L., and Kwack, B. H. (1963) Am. J. Bot. 50, 859–865
2. Mascarénhas, J. P., and Machlis, L. (1964) Plant Physiology 39, 70–77
3. Bednarska, E., and Buttott, R. (1992) Politycheskoe Cytobioz 33, 43–52
4. Pichon, J. M., and Steer, M. W. (1988) Protoplasma 151, 11–17
5. Steer, M. W., and Gilroy, S. (1997) Planta 207, 495–505
6. Schultz, J., Marshall-Carlson, L., and Carlson, M. (1999) Plant Physiol. 120, 995–1007
7. Smith, D. R., Doucette-Stamm, L. A., Deloughery, C., Lee, H., Dubois, J., Blanchard, J. A., and Jaffe, L. F. (1975) J. Cell Biol. 67, 488–492
8. Rathore, K. S., Corthier, M., and Anderson, A. R. (1983) J. Biol. Chem. 258, 7135–7155
9. Oshino, S., and Kato, H. (1993) Ann. Rev. Plant Physiol. Plant Mol. Biol. 44, 317–337
10. Smith, D. R., Doucette-Stamm, L. A., Deloughery, C., Lee, H., Dubois, J., Blanchard, J. A., and Jaffe, L. F. (1975) J. Cell Biol. 67, 488–492
11. Hirth, W., Reiss, H., and Hartmann, E. (1990) In Tip Growth in Plant and Fungal Cells (Heath, I. B., ed) pp. 91–118, Academic Press, New York
12. Pinion, J. M., and Steer, M. W. (1988) Politycheskoe Cytobioz 33, 43–52
13. Sambrook, J., Fritsch, F. F., and Maniatis, T. (1989) Methods Cell Biol. 32, 1–21
14. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Lee, S. H., Kim, M. C., Heo, W. D., Kim, J. C., Chung, W. S., Park, C. Y., Mimura, H., and Cho, M. J. (1999) Biochim. Biophys. Acta 1433, 56–67
16. Franklin-Tong, V. E. (1999) Curr. Opin. Plant Biol. 2, 131–139
17. Schmidt, B., Klopstein, S., and DeGrado, W. F. (1987) Methods Enzymol. 139, 455–478
18. Grantcharov, R., Gruys, E., and DeGrado, W. F. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 163–193
19. O’Neil, K. T., and DeGrado, W. F. (1990) Sci. U. S. A. 87, 93–100
20. Schultz, J., Marshall-Carlson, L., and Carlson, M. (1999) Plant Physiol. 120, 995–1007
21. Smith, D. R., Doucette-Stamm, L. A., Deloughery, C., Lee, H., Dubois, J., Blanchard, J. A., and Jaffe, L. F. (1975) J. Cell Biol. 67, 488–492
22. Hirth, W., Reiss, H., and Hartmann, E. (1990) In Tip Growth in Plant and Fungal Cells (Heath, I. B., ed) pp. 91–118, Academic Press, New York
23. Kuhlthaler, W. M., and Jaffe, L. F. (1990) J. Cell Biol. 110, 1565–1573
24. Holdaway-Clarke, T. L., Feijo, J. A., Hackett, G. R., Kunkel, J. M., and Hepler, P. K. (1997) Plant Cell 9, 1999–2010
25. Cresti, M., and Hepler, P. K. (1994) Ann. Rev. Plant Physiol. Plant Mol. Biol. 45, 461–491
26. Misra, M., and Cho, M. J. (1999) Biochim. Biophys. Acta 1433, 56–67
27. Erickson-Vitanen, S., and DeGrado, W. F. (1987) Methods Enzymol. 139, 455–478
28. Oshino, S., and Kato, H. (1993) Ann. Rev. Plant Physiol. Plant Mol. Biol. 44, 317–337
29. Oshino, S., and Kato, H. (1993) Ann. Rev. Plant Physiol. Plant Mol. Biol. 44, 317–337
30. Ma, L., Xu, X., Cui, S., and Sun, D. (1999) Plant Cell 11, 303–313
31. CaI, G., Moscatelli, A., and Cresti, M. (1997) Trends Plant Sci. 2, 86–91
32. Taylor, L. P., and Hepler, P. K. (1997) Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 37–414
33. Sanders, D., Brownlee, C., and Harper, J. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 697–725
34. Reddy, A. S. N., and Reddy, V. S. (2000) in Handbook of Plant and Crop Physiology (Pessarakli, M., ed) Marcel Dekker Inc., New York, in press
35. Gillmor, S. H., and Gilroy, S. (1997) Trends Plant Sci. 2, 130–134
36. Cai, G., Moscatelli, A., and Cresti, M. (1997) Trends Plant Sci. 2, 86–91
37. Taylor, L. P., and Hepler, P. K. (1997) Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 461–491
38. Sanders, D., Brownlee, C., and Harper, J. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 697–725
39. Reddy, A. S. N., and Reddy, V. S. (2000) in Handbook of Plant and Crop Physiology (Pessarakli, M., ed) Marcel Dekker Inc., New York, in press
40. Gillmor, S. H., and Gilroy, S. (1997) Trends Plant Sci. 2, 130–134
41. Sambrook, J., Pritsch, F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
42. Lee, S. H., Kim, M. C., Heo, W. D., Kim, J. C., Chung, W. S., Park, C. Y., Park, H. C., Cheong, Y. H., Kim, C. Y., Lee, S. H., Lee, J. K., Bahk, J. D., Lee, S. Y., and Cho, M. J. (1999) Biochim. Biophys. Acta 1433, 56–67
A Pollen-specific Novel Calmodulin-binding Protein

78. Das, A. K., Cohen, P. W., and Barford, D. (1998) EMBO J. 17, 1192–1199
79. Prodromou, C., Siligardi, G., O’Brien, R., Woolfson, D. N., Regan, L., Panaretou, B., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1999) EMBO J. 18, 754–762
80. Venolia, L., Ao, W., Kim, S., Kim, C., and Pilgrim, D. (1999) Cell Motil. Cytoskeleton 42, 163–177
81. Vurich, V. A., and Gasser, C. S. (1996) Mol. Gen. Genet. 252, 510–517
82. Hernandez Torres, J., Chatellard, P., and Stutz, E. (1995) Plant Mol. Biol. 27, 1221–1226
83. Kreppel, L. K., Blomberg, M. A., and Hart, G. W. (1997) J. Biol. Chem. 272, 9308–9315
84. Lubas, W. A., Frank, D. W., Krause, M., and Hanover, J. A. (1997) J. Biol. Chem. 272, 9316–9324
85. Rozwadowski, K., Zhao, R., Jackman, L., Huebert, T., Burkhart, W. E., Hemmingsen, S. M., Greenwood, J., and Rothstein, S. J. (1999) Plant Physiol. 120, 787–798
86. Bibikova, T. N., Blancaflor, E. B., and Gilroy, S. (1999) Plant J. 17, 657–665
87. Reddy, A. S. N. (2001) Int. Rev. Cytol., in press