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Primary Structures of *Arabidopsis* Calmodulin Isoforms Deduced from the Sequences of cDNA Clones[1]

Vincent Ling[2], Imara Perera, and Raymond E. Zielinski*

Department of Plant Biology, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Complementary DNA (cDNA) clones encoding calmodulin isoforms were isolated from an *Arabidopsis* leaf λgt10 library by screening with cloned barley calmodulin cDNA probes. Two cDNAs, one a 626-base pair partial-length clone (ACaM-1) and one a 1400-base pair full-length clone (ACaM-2), encode calmodulin polypeptides that differ by four conservative amino acid substitutions. None of the amino acid sequence differences occur within the four Ca^{2+}-binding domains of the proteins. Whereas the deduced amino acid sequences of the two *Arabidopsis* calmodulin isoforms share 97% identity, the nucleotide sequences encoding the two isoforms share 87% sequence identity. Most of these nucleotide sequence differences (80%) occur in codon wobble positions. ACaM-1 and ACaM-2 both hybridize with a distinct set of restriction fragments of *Arabidopsis* total DNA, indicating that they were derived from transcripts of separate genes; these genes are single- or very low-copy in the *Arabidopsis* genome. Both cDNAs hybridize to messenger RNA (mRNA) species of 0.8 kilobases that are expressed to a greater extent in developing siliques compared with leaves, flowers, and stems. Northern blot and polymerase chain reaction assays both indicate that ACaM-1 mRNA is more highly expressed than ACaM-2 mRNA in developing siliques. The steady-state levels of both isoform mRNAs increase as a result of touch stimulation; the kinetics and extent of increase are comparable for the two mRNAs.

Calcium is an essential plant macronutrient that has been implicated in regulating a diverse array of physiological processes, including cell proliferation and growth, organelle movement, photosynthesis, tropic and nastic responses, and secretion (10, 20). In many cases, calcium functions as an intracellular messenger and couples these physiological responses with the perception of external stimuli, such as light (24), gravity, or phytohormones (10, 20). An integral part of the calcium-mediated signal transduction pathway is a group of proteins that bind calcium reversibly with dissociation constants in the nanomolar to micromolar range, known as calcium-modulated proteins.

CaM[3] is a member of the calcium-modulated protein family and has been identified in all eukaryotes examined. One of the hallmarks of CaM is its highly conserved primary structure. Except for amidation states, CaM amino acid sequences are identical among mammals and avian species (23), and higher plant CaM shares 98% amino acid sequence similarity with vertebrate CaM (15, 16). Most of the differences among CaM proteins are conservative substitutions that could be explained by minimal nucleotide changes in their gene coding sequences. In rats and humans, CaM is encoded by a family of at least three genes (6, 19). Within either of these species, the CaM proteins encoded by the different gene family members possess identical amino acid sequences, but their respective nucleotide sequences are diverged by approximately 20%. Similar results have also been reported for CaM protein and gene sequences in *Xenopus* (5) and a trypanosome (27, 28). These observations have been interpreted to mean that there is strong selective pressure to maintain the primary structure of CaM, not only within a species but also across the spectrum of eukaryotic phylogeny.

In this paper, we characterize two cDNAs encoding CaM from *Arabidopsis,* which were isolated using a cloned barley CaM cDNA (15) as a probe. Nucleotide sequence analyses and genomic Southern blot assays using these clones demonstrated that they were derived from mRNAs transcribed from two distinct genes, and that they encode CaM isoforms, which differ by at least four conservative amino acid substitutions. The mRNAs encoding the two CaM isoforms were expressed to similar extents in all aerial tissues of *Arabidopsis* seedlings that were tested. In addition, we describe a PCR assay for detecting specific CaM mRNAs in tissues in which their abundance is very low. A portion of these data was presented previously in abstract form (14).

MATERIALS AND METHODS

Plaque Screening

An *Arabidopsis thaliana* (Heynh, ecotype Columbia) cDNA library, cloned in λgt10, was obtained from Dr. Chris Somerville (Michigan State University). Library screening was performed using a full-length barley CaM cDNA (pBcam-3Δ1) (15); plaque hybridizations were performed at 37°C in 40% (v/v) formamide, 5× SSPE (20× SSPE is 0.2 M $NaH_2PO_4 \cdot H_2O$, 20 mM $Na_2EDTA \cdot 2H_2O$, and 2.98 M NaCl, pH 7.4), 0.1% (w/v) SDS, 5× Denhardt's solution (100× Denhardt's solution is 2% [w/v] each of BSA, PVP, and ficoll), and 100 μg/mL denatured calf thymus DNA. Restriction fragment probes were labeled by the random hexamer priming method (11) to specific activities of 0.6 to 1.2 × 10^{10} Bq/μg

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[2] Current address: Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Ave., Cambridge, MA 02138.

[3] Abbreviations: CaM, calmodulin; bp, base pair(s); PCR, polymerase chain reaction; RT, reverse transcriptase.

and were included in the hybridization solutions at 20 ng/mL. Posthybridization washes were performed twice in 2× SSPE, 0.2% (w/v) SDS, and twice in 1× SSPE, 0.1% (w/v) SDS at hybridization temperature. Positively hybridizing phage were purified by three rounds of plaque hybridization, and the phage DNA inserts assayed by Southern blot hybridization using the same ^{32}P-labeled probes described above.

Subcloning and DNA Sequencing

Inserts from positively hybridizing phage were excised with *Eco*RI and subcloned into either pGEM-3Z (Promega-Biotec, Madison, WI) or pBS-SK$^+$ (Stratagene, LaJolla, CA). DNA sequencing was performed by the dideoxy chain termination method (25), using Sequenase (United States Biochemicals) and [^{35}S]dATP with double-stranded plasmid DNA templates (15). Progressive deletions for sequence analyses were made using either *Bal*31 nuclease or the exonuclease III/S1 nuclease method (9).

RNA Isolation and Northern Blot Assays

Total RNA was isolated from 4-week-old *Arabidopsis* seedling tissues by the hot borate extraction method (7). Seedlings were grown aseptically on Murashige-Skoog agar medium in Petri dishes held in a growth chamber maintained at 20°C on a 16-h light/8-h dark cycle at an irradiance level of 80 $\mu E \cdot s^{-1} \cdot m^{-2}$. Five-microgram samples of total RNA were fractionated in formaldehyde-agarose gels (30), transferred to nitrocellulose filters, and immobilized either by baking at 80°C or by UV irradiation using a commercially available cross-linking device (Stratagene). Hybridizations were carried out in 50% (v/v) formamide, 5× SSPE, 5× Denhardt's solution, 0.1% (w/v) SDS, and 100 μg/mL denatured calf thymus DNA. Hybridization probes consisted of a 626-bp *Eco*RI restriction fragment for ACaM-1 (40.7% G + C), and a 766-bp *Eco*RV-*Eco*RI restriction fragment for ACaM-2 (36.1% G + C), which were labeled with ^{32}P as described previously (11). Hybridization temperatures were 44°C for ACaM-1 and 41°C for ACaM-2; these temperatures were calculated to give Tm 25°C as described previously (2). As controls to test for the gene specificity of hybridization, CaM mRNAs were synthesized *in vitro* from the two CaM cDNAs using T7 RNA polymerase (30).

Southern Blotting

Blot hybridizations using restriction enzyme-cleaved *Arabidopsis* DNA were performed using hybridization solutions and ^{32}P-labeled restriction fragment DNA probes identical to those described above for Northern blots. Hybridization temperatures were 40 and 37°C for ACaM-1 and ACaM-2, respectively. Posthybridization washes were performed successively in 2× SSPE, 1× SSPE, 0.5× SSPE, and 0.1× SSPE, each containing 0.1% (w/v) SDS for 15 min at room temperature. Final washes were in 0.1× SSPE, 0.1% (w/v) SDS at 50°C.

PCR Amplification of mRNA Sequences

Arabidopsis total RNA (10 μg) was treated with 5 units of RNase-free DNase (RQ1 DNase, Promega) for 30 min at 37°C in the presence of 40 units of RNasin (Promega) to remove traces of contaminating genomic DNA. DNase digestions were terminated by incubating the samples in a boiling water bath for 2 min followed by quick-freezing in liquid N_2. Samples of total RNA (1 μg) or *in vitro* run-off RNA transcripts (1 pg–1 ng, diluted in 1 μg of *Escherichia coli* rRNA) were reverse transcribed in 20-μL reactions containing 10 mM Tris-HCl, pH 8.4 (at room temperature), 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg/mL BSA, 1 mM of each deoxynucleoside triphosphate, 100 pmol p(dN)$_6$, 10 units of RNasin, and 200 units of Moloney murine lukemia virus RT. Reactions were carried out for 10 min at room temperature, followed by 60 min at 42°C. As controls, identical reactions were incubated as described, but without RT. The reactions were terminated by diluting the mixture with 79 μL of the same Tris-KCl-Mg-BSA buffer (without dNTPs, random primers, or RT) containing 50 pmol each of the desired sense and antisense primers, and boiling the mixture for 2 min. The reactions were transferred to a thermal cycler set to 80°C, where 2.5 units of *Taq* DNA polymerase (Perkin-Elmer or BRL) and 50 μL of mineral oil were added. Amplification was carried out for 25 cycles using 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Following chloroform extraction, reaction products were fractionated by electrophoresis in 1% (w/v) agarose and 2% (w/v) NuSieve agarose gels in TBE buffer (100 mM Tris, 100 mM H_3BO_3, 2.5 mM Na_2 EDTA) and visualized by ethidium bromide staining.

RESULTS

We previously reported the molecular cloning of CaM cDNA sequences from barley and showed that restriction fragments containing the coding region of barley CaM were suitable for use as DNA probes in Northern hybridization assays for detecting CaM mRNA from different plant species, including *Arabidopsis* (15). Different subclones of the barley CaM cDNA were used as probes against an *Arabidopsis* λgt10 library with the intent of isolating sequences encoding CaM cDNAs. Because we had reason to believe that a portion of the unusually long (255 bp) 5′ untranslated region of our full-length barley CaM cDNA (pBCaM-3) was an artifactual product of cDNA cloning (15), pBCaM-3 cDNA was digested with exonuclease *Bal*31, eliminating most of the 5′ untranslated region. The subcloned fragment, now termed pBCaM-3Δ1, contained the entire coding region along with 21 bp of 5′ untranslated sequences, and the 3′ untranslated region of barley CaM. The insert from this subclone was used to screen an *Arabidopsis* cDNA library cloned in phage λgt10 at a hybridization criterion that would allow for approximately 15 to 20% nucleotide sequence mismatch. Several positively hybridizing plaques were picked and subcloned into plasmids. Two of the cDNAs were subsequently sequenced; restriction maps and nucleotide sequencing strategies are shown in Figure 1. The deduced amino acid sequences of the two cDNAs were very similar, but not identical, to each other and to CaM characterized from other higher plants. Figure 2 shows that one of the cDNAs was a 626-bp partial length clone, which we termed ACaM-1. This clone contained a single long open reading frame, which encoded an amino acid sequence of 136 residues that corresponded to positions 13 through 148 of

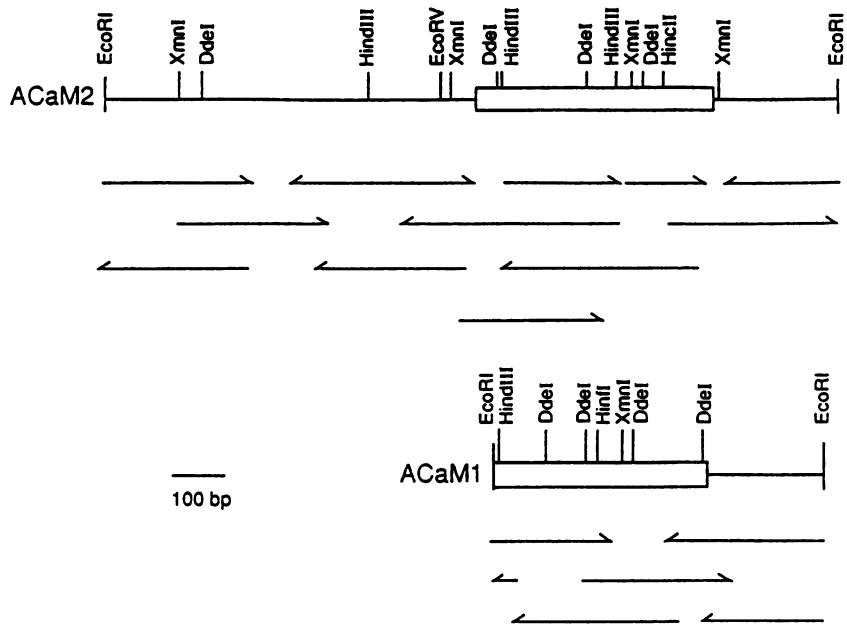

Figure 1. Restriction maps and sequencing strategies for the *Arabidopsis* CaM cDNAs. The open bars represent the protein coding regions. Restriction enzyme sites are indicated above the map of each clone. The overlapping arrows below the restriction maps indicate the direction and extent of nucleotide sequence determined using either subcloned restriction fragments or exonuclease III-generated deletion mutants. The bar to the left of the ACaM-1 map indicates the scale.

CaM, by comparison with previously published CaM amino acid sequences (1, 4, 15, 16). The second cDNA (ACaM-2) was 1400 bp in length and contained an open reading frame that encoded the complete amino acid sequence of CaM starting at position 718. We suspected, however, that the 5′ untranslated region of this cDNA might represent a cloning artifact due to the presence of eight methionine codons before the CaM initiation methionine. Also, there are several possible open reading frames in the opposite orientation with respect to the CaM reading frame within this region of the ACaM-2 cDNA.

Within the region in which ACaM-1 and ACaM-2 align, the deduced amino acid sequences differ at four residues, a lysine for an arginine at position 74, a glutamic acid for an aspartic acid at position 122, an arginine for a lysine at position 126, and an isoleucine for a valine at position 174. Figure 3 shows that of the two *Arabidopsis* CaM isoforms, the amino acid sequence of the protein encoded by ACaM-2 is the most similar to those of other higher plant CaMs. The deduced sequence of ACaM-2 differs from barley (15), alfalfa (1), and spinach (16) CaM at only two positions, and the CaM protein encoded by ACaM-1 differs from these sequences at three positions. In contrast, the amino acid sequences of all the above-mentioned higher plant CaMs differ from that of potato CaM (13) in at least 11 positions. It is not yet clear whether there are genes encoding a protein similar to potato CaM in *Arabidopsis*. Previous studies (15) suggested that there are multiple genes encoding CaM, or closely related proteins in barley.

To gain more information on the nature and number of genomic DNA sequences encoding CaM in *Arabidopsis*, genomic Southern blots were performed using both isoform cDNAs as probes. As is illustrated in Figure 4, ACaM-1 and ACaM-2 DNA probes hybridized to distinct sets of both *Eco*RI and *Hin*dIII restriction fragments. Single, major hybridizing *Eco*RI restriction fragments of 3.0 and 2.1 kb were observed for ACaM-1 and ACaM-2, respectively; these fragments hybridized with the equivalent signal of a single copy gene as determined in separate experiments (data not shown). In addition, several minor hybridizing *Eco*RI fragments appeared upon longer exposures of these autoradiographs. One of the weakly hybridizing fragments in each experiment most likely represented hybridization with the other CaM isoform coding sequence, *e.g.* the ACaM-2 probe weakly hybridized with a 3.0-kb *Eco*RI fragment. In addition, both probes hybridized weakly with fragments of 1.0 and 6.0 kb. It is not yet known whether these two weakly hybridizing restriction fragments represent portions of additional CaM genes or genes encoding other EF-hand homologs. The hybridization patterns of the *Hin*dIII digests shown in Figure 4 also suggest that ACaM-1 and ACaM-2 are encoded by distinct genes. ACaM-1 hybridizes strongly with two fragments of 5.5 and 2.3 kb. ACaM-2, on the other hand, hybridizes most strongly to a series of relatively small fragments, which are ≤1 kb. These results are consistent with our observation that the ACaM-1 and ACaM-2 cDNAs have one and two *Hin*dIII restriction sites in their coding regions, respectively (Fig. 1).

Northern blot hybridization experiments were performed to determine whether the CaM isoform mRNA sequences are expressed differentially or coordinately in *Arabidopsis* seedlings. Total RNA was isolated from stems, leaves, flowers, and developing siliques of 3-week-old plants, fractionated in formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized with the ACaM-1 and ACaM-2 cDNA inserts. Figure 5a shows that only very low levels of expression of both mRNAs were observed in stem, leaf, and flower tissues. RNA fractions isolated from developing siliques, however, contained substantial levels of both ACaM-1 and ACaM-2 mRNA. In the developing siliques, as well as in leaf poly A^+ RNA, hybridization signals to ACaM-1 mRNA appeared to be more intense than those to ACaM-2 mRNA under conditions of autoradiographic exposure in which equal amounts

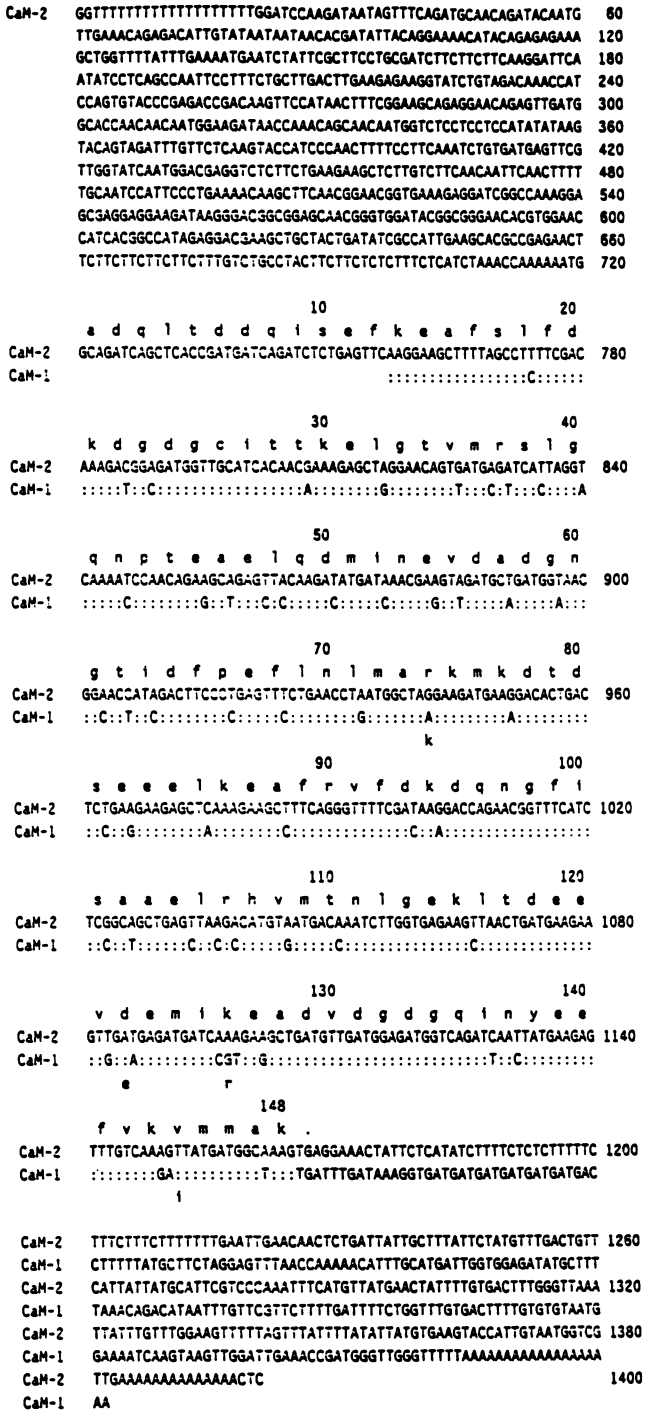

Figure 2. Comparison of the nucleotide and deduced amino acid sequences of cDNA clones encoding *Arabidopsis* CaM isoforms. The complete protein coding sequences are shown for the full-length ACaM-2 cDNA. Only the sequence differences for the partial-length ACaM-1 cDNA within the CaM coding region are noted in the figure. Nucleotides are numbered at the right in the 5′ to 3′ direction for the ACaM-2 sequence only. Amino acid residues are numbered above the ACaM-2 sequence beginning with the presumptive amino-terminal alanine residue and are given in the single letter IUPAC nomenclature.

of control ACaM-1 and ACaM-2 transcripts showed comparable hybridization intensity (Fig. 5a, lanes 6 and 7). The sense-strand control transcripts of the two CaM cDNA clones also showed that, under our hybridization conditions, the CaM cDNA probes hybridized at least 100-fold more strongly with their cognate RNA sequences than with heterologous CaM sequences.

It was recently shown that CaM mRNA steady-state levels increase when *Arabidopsis* seedlings are stimulated thigmomorphically, by water spray, wind, touch, or wounding (3); this result led to the cloning of cDNA sequences equivalent to the ACaM-2 cDNA described in this report. However, it was not recognized at that time that CaM is encoded by multiple genes in *Arabidopsis*. The effect of touch-stimulation on the steady-state levels of the CaM isoform mRNAs was tested using RNA isolated from 2-week-old seedlings whose leaves had been stimulated by rubbing. Figure 5b shows that the steady-state levels of both ACaM-1 and ACaM-2 mRNA increased dramatically within 30 min after touch-stimulation. Within 60 min after the stimulus was given, the levels of both mRNAs began to decrease. Densitometric scans of the autoradiographs shown in Figure 5b revealed that the levels of both CaM mRNAs increased approximately 10-fold during this experiment.

Because the expression of both CaM mRNA species was

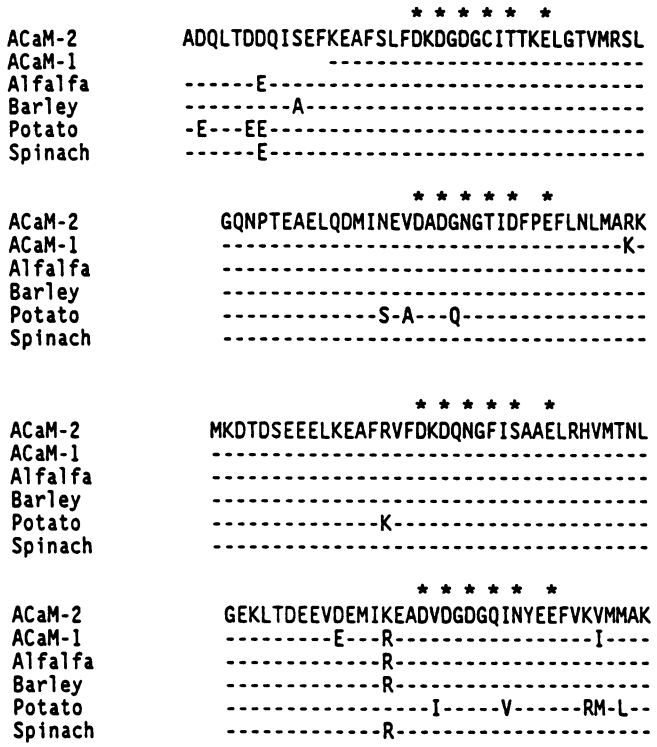

Figure 3. Amino acid sequence comparison of higher plant CaM polypeptides. Sequences are aligned to illustrate the relationships of the four Ca^{2+}-binding domains. Amino acids are indicated by single letter IUPAC nomenclature. Residues marked with asterisks are those which act as Ca^{2+}-binding ligands. Amino acid sequence data were taken from the following: alfalfa (1); barley (15); potato (13); and spinach (16).

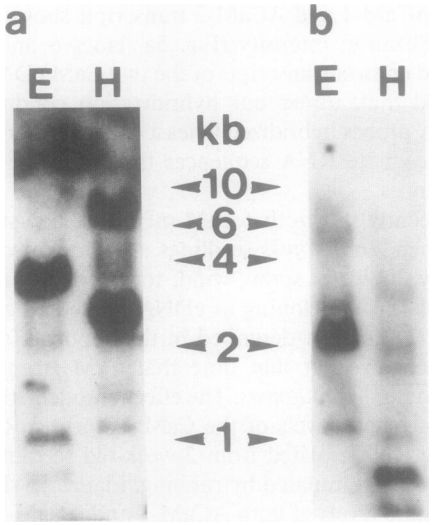

Figure 4. Genomic Southern blot analysis of calmodulin-related sequences encoded in the *Arabidopsis* genome. *Arabidopsis* total DNA (5 μg/lane) was restricted with either *Eco*RI (lanes E) or *Hin*dIII (lanes H), fractionated in a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled CaM cDNA probes. Panel a, hybridization pattern obtained with ACaM-1; panel b, hybridization pattern obtained with ACaM-2.

near the limits of detection in Northern blot experiments using leaf, stem, and root RNA samples, we confirmed that both are expressed in leaves by a PCR amplification assay. Samples of leaf and silique total RNA were treated with DNase to remove contaminating genomic DNA, reverse transcribed using random hexamer primers, and subjected to PCR amplification using primers specific for either ACaM-1 or ACaM-2 mRNA. For ACaM-2, 5′-GCTGATGGTA-ACGGAACCATAAG-3′ and 5′-CATGAAATTTGGGAC-GAATCGA-3′ were used as sense and antisense primers, respectively; they correspond to positions 889 to 910 and 1269 to 1290 in Figure 2. For ACaM-1, 5′-CAC-TATCGACTTCCCCGAGTTC-3′ and 5′-GCA-TATCTCCACCAATCATGCA-3′ were used as sense and antisense primers, respectively; they correspond to positions 903 to 924 and 1236 to 1257 in Figure 2. The predicted sizes of PCR amplification products of ACaM-1 and ACaM-2 mRNAs, therefore, were 355 and 402 bp, respectively. It should be noted that the antisense primers were constructed using sequences from the 3′ untranslated regions of ACaM-1 and ACaM-2 mRNAs, and the sense primers were chosen to be within regions of sequence that diverged by at least 20% between the two mRNAs. The control amplifications in Figure 6 (left and center panels) show that these primers were at least 10^3- to 10^4-fold more specific for the CaM mRNA sequences from which they were derived than they were for competing, heterologous CaM mRNAs. This specificity was confirmed by direct DNA sequence analyses of both PCR products (data not shown). Figure 6 also shows that both ACaM-1 and ACaM-2 mRNAs were detected in leaf as well as in silique RNA fractions. The PCR signals were not due to contaminating genomic DNA, because control reactions on samples that had not been reverse transcribed showed no amplification products (Fig. 6, right panel, lanes 5–8). It should be noted that these assays were not carried out under conditions in which quantitative comparisons could be made with a high degree of confidence. Note that the differences in yields of amplification products of 10-fold serial dilutions of *in vitro* transcripts (Fig. 6, left and center panels) were perceptible, but too small to permit accurate quantitation. Nevertheless, the PCR results support our Northern blot experiment (Fig. 5, panels a), which indicated that ACaM-1 mRNA is more highly expressed in developing siliques than is ACaM-2 mRNA. However, the PCR signal for ACaM-2 mRNA in leaf total RNA appears to be comparable to that in silique RNA (Fig. 6, right panel, lanes 2 and 4), even though the Northern hybridization signal for ACaM-2 mRNA in leaf poly A$^+$ is much less than that observed in siliques. This result is likely to represent a plateau artifact (12). Thus, our PCR assay is best interpreted on a qualitative rather than a quantitative basis.

DISCUSSION

Using a barley CaM cDNA as a heterologous hybridization probe, we isolated two cDNA clones encoding CaM from *Arabidopsis*. The nucleotide sequences of the two cDNAs revealed that they encoded isoforms of CaM, which differed in their deduced amino acid sequences by four conservative substitutions. The two *Arabidopsis* CaM coding sequences characterized in this report differ by 13% at the nucleotide sequence level. This divergence largely reflects sequence differences in codon wobble positions. However, we note that the differences between ACaM-1 and ACaM-2 are nearly as great as those between either *Arabidopsis* CaM cDNA and

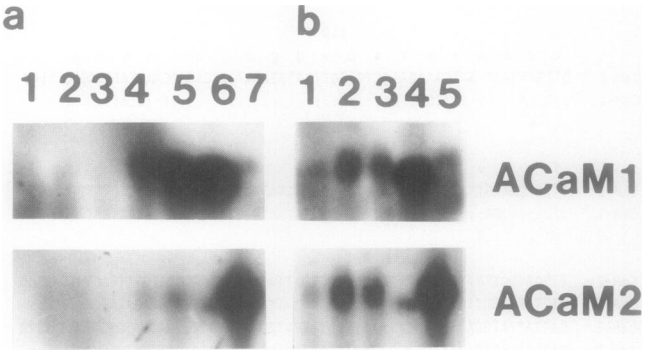

Figure 5. Expression of mRNA sequences encoding *Arabidopsis* calmodulin isoforms. *Arabidopsis* total RNA (5 μg/lane), poly A$^+$ RNA (0.5 μg/lane), or sense-strand transcripts produced by T7 RNA polymerase *in vitro* from ACaM-1 and ACaM-2 cDNA templates (50 pg/lane) were fractionated in formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled ACaM-1 (top panels) or ACaM-2 (bottom panels) cDNA inserts. RNA samples in panels a were obtained as follows: lanes 1, stem; 2, leaf; 3, flower; 4, developing siliques; 5, leaf poly A$^+$; 6, *in vitro* transcripts from ACaM-1; 7, *in vitro* transcripts from ACaM-2. In panels b are total RNAs derived from the leaves of thigmomorphogenically stimulated seedlings at 0 min (lanes 1), 30 min (lanes 2), and 60 min (lanes 3) after stimulation. Lanes 4 contain ACaM-1 *in vitro* transcripts, and lanes 5 contain ACaM-2 *in vitro* transcripts.

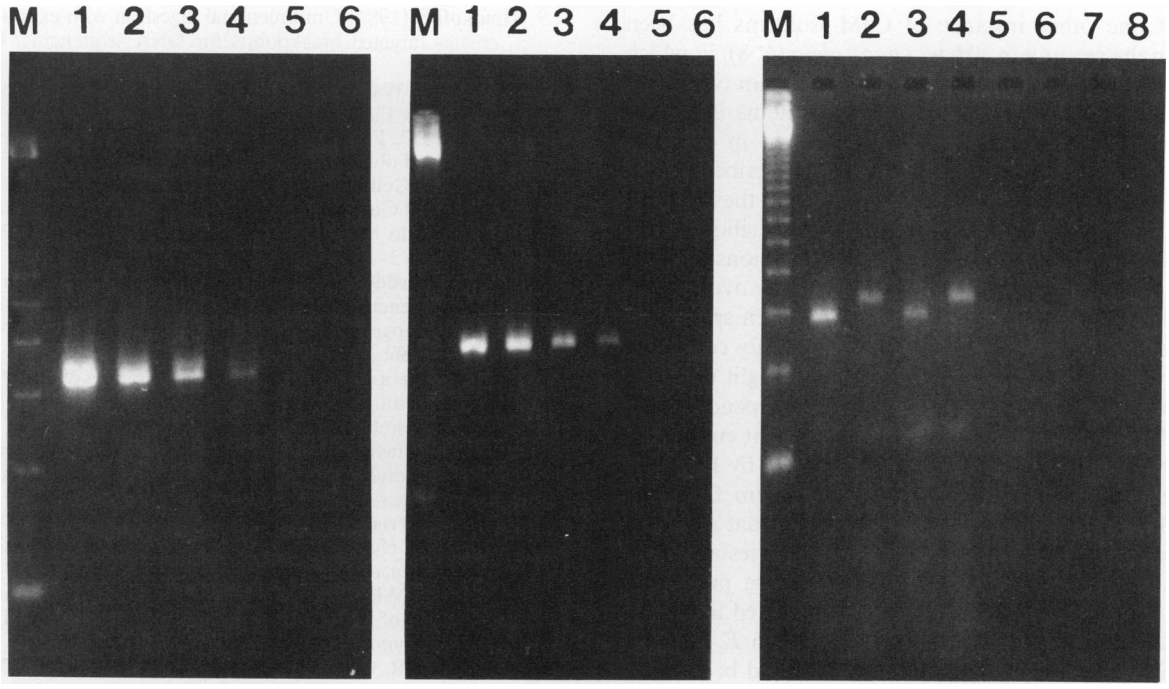

Figure 6. Expression of ACaM-1 and ACaM-2 mRNAs detected by PCR amplification. The left panel shows control amplifications using the ACaM-2 primers: *in vitro* run-off RNAs from ACaM-2 (10-fold serial dilutions from 1 ng to 1 pg, lanes 1–4); *in vitro* run-off RNA (1 ng) from ACaM-1 (lane 5); and no RT control (lane 6). The center panel shows control amplifications using the ACaM-1 primers: *in vitro* run-off RNAs from ACaM-1 (10-fold serial dilutions from 1 ng to 1 pg, lanes 1–4); *in vitro* run-off RNA from ACaM-2 (1 ng) (lane 5); and no RT control (lane 6). The right panel shows amplifications using silique (lanes 1, 2, 5, and 6) or leaf (lanes 3, 4, 7, and 8) total RNA, using ACaM-1 (lanes 1, 3, 5, and 7) or ACaM-2 (lanes 2, 4, 6, and 8) primers. Lanes 5 to 8 show the results of PCR amplification without prior reverse transcription. Identities of both ACaM-1 and ACaM-2 amplification products were confirmed by DNA sequencing. Lanes designated M show the migration of 123-bp ladder standards (Bethesda Research Labs, Inc.).

CaM-encoding nucleotide sequences previously isolated from alfalfa (1) (84 and 82% identities with ACaM-1 and ACaM-2, respectively) and barley (15) (84 and 80% identities with ACaM-1 and ACaM-2, respectively). This observation is consistent with the idea that duplication of CaM genes occurred early in the process of plant evolution. Gene families encoding CaM also have been reported in vertebrates. In contrast to the different isoforms described in this report, however, CaM proteins encoded by different members of vertebrate CaM gene families possess identical amino acid sequences (5, 6, 19). We suggest that this difference is a reflection of different evolutionary pressures in plants *versus* animals for conserving CaM amino acid sequences.

ACaM-2 encodes the full amino acid sequence of CaM plus an additional 720 bp of upstream sequences. Because ACaM-2 hybridizes with an mRNA species of about 0.85 kb, it is likely that most of the upstream sequences contained in this clone were added artifactually during cDNA cloning. A likely step in which this could have occurred was during the addition of synthetic *Eco*RI linkers, when the ACaM-2 sequence was ligated to an unrelated cDNA prior to being ligated to the linkers. ACaM-1 is a partial-length clone encoding 134 of the usual 148 amino acids of the CaM sequence, assuming that the protein encoded by ACaM-1 is typical of CaM proteins characterized previously from higher plants and vertebrates. However, it is possible that the mRNA from which ACaM-1 was derived encodes a form of CaM that differs at its amino terminus, similar to CaM proteins from *Chlamydomonas* (17) and *Dictyostelium* (18), which possess additional N-terminal amino acid residues. Final characterization of the protein encoded by ACaM-1 awaits isolation of a full-length cDNA or the corresponding genomic DNA sequences.

In contrast to the *Arabidopsis* CaM isoforms described here, mRNAs transcribed from different vertebrate CaM gene family members are expressed differentially (26). The *Arabidopsis* CaM isoform mRNAs characterized in this report appeared to be coordinately expressed when RNAs from whole plant organs are analyzed by Northern blotting or by PCR amplification, although ACaM-1 mRNA appears to be more highly expressed than ACaM-2 mRNA in leaves and developing siliques. Thigmomorphic stimulation resulted in similar changes in ACaM-1 and ACaM-2 mRNA accumulation. However, differential expression of ACaM-1 and ACaM-2 on a cell-type basis within a given organ would not have been detected in the analyses presented in this report. In addition, we do not yet know the consequence of ACaM-1 and ACaM-2 mRNA accumulation at the polypeptide level. However, because the two CaM isoforms whose sequences we deduced are so similar, it is unlikely that we will be able to directly address the question of whether expression of the CaM polypeptides follows a pattern similar to that of their respective mRNAs.

At least one other instance of CaM isoforms has been reported in the sea urchin *Arbacia punctulata* (4, 8), in which four amino acid sequence differences occur within two forms of the protein. The sea urchin isoform proteins and their respective mRNAs are differentially expressed in gametes. The *Arabidopsis* CaM isoform mRNAs we described in this report were not assayed in gametes; however, they are co-expressed in both whole flowers and developing siliques.

It is inferred from *in vitro* studies that interactions between CaM and its target enzymes involve structurally overlapping sites on the CaM molecule (29). It has also been speculated that selection pressure for CaM to remain highly conserved across phylogenetic lines is a consequence of tight substrate specificity for CaM-binding sites within CaM-dependent proteins. Differential activation of a CaM-dependent enzyme by vertebrate and plant CaM proteins (which differ by 13 of 148 amino acid residues) has been observed *in vitro* for NAD kinase (21). The demonstration in this report that sequence variants in CaM exist within a single plant species raises the possibility that different CaM isoforms may be present to optimize interactions with specific CaM-regulated target enzymes. Because it is possible to express CaM in *E. coli* and recover biologically active protein (22), it should be possible to use a similar approach to ask whether there are significant biochemical differences between ACaM-1 and ACaM-2, once a full-length clone is available for ACaM-1.

Note Added in Proof

The sequences reported here have been registered in the GenBank/EMBL Data Bank with accession numbers M38379 and M38380.

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