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ORIGINAL ARTICLE

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Characterization of three new members of the *Arabidopsis thaliana* calmodulin gene family: conserved and highly diverged members of the gene family functionally complement a yeast calmodulin null

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Abstract Three genes encoding members of the EF-hand family of Ca^{2+}-binding proteins were identified from *Arabidopsis thaliana* (L.) Heynh. sequences deposited in the expressed sequence tag and genomic sequence databases. Full-length cDNAs for each of the genes, *Cam7*, *Cam8*, and *Cam9*, were sequenced. *Cam7* encodes a conventional 16.8-kDa, 148-amino-acid calmodulin protein (CaM). In contrast, *Cam8* and *9* encode highly diverged isoforms of the protein that share 73 and 49% amino acid sequence identity, respectively, with CaM7. RNA gel blot and reverse transcription-polymerase chain reaction experiments revealed that each of the genes is expressed in leaves, flowers and siliques. To test the functional properties of the polypeptides encoded by these genes, they were expressed in *Escherichia coli* and the yeast *Saccharomyces cerevisiae*. Each was purified by Ca^{2+}-dependent hydrophobic affinity chromatography. CaM7, but neither CaM8 nor CaM9, formed a complex with a basic amphiphilic helical peptide in the presence of Ca^{2+} that could be identified by gel electrophoresis. In spite of these in vitro differences, each of the sequences functionally substituted for yeast *CMD1* to maintain viability. Isolation of yeast strains complemented by *Cam9* required selection against the plasmid harboring wild-type yeast sequences, whereas complementation by *Cam7* and *Cam8* did not. These results suggest that the mechanism of action of CaM8 and CaM9 is similar to that of more conventional CaM sequences. CaM9, and to a lesser degree CaM8, however, appear to represent Ca^{2+}-binding sensor proteins that interact with a more limited set of target proteins than do more conventional CaM isoforms.

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Abbreviations Arg: arginine · Asp: aspartic acid · CaM: calmodulin protein · *Cam*: calmodulin gene · 5-FOA: 5-fluoro-orotic acid · Glu: glutamic acid · Gly: glycine · GST: glutathione *S*-transferase · His: histidine · Ile: isoleucine · Lys: lysine · RT-PCR: reverse transcription-polymerase chain reaction · Thr: threonine · WT: wild type

Introduction

All eukaryotic cells utilize changes in Ca^{2+} concentration as a second messenger to generate cellular responses to extracellular stimuli. In plants, Ca^{2+} signals arise in response to a diverse array of stimuli (Trewavas and Malho 1998). Ca^{2+} signals are transient because a series of Ca^{2+}-transporting pumps and energy-driven co-transporters, which are located on a variety of cellular membranes, quickly removes the ion from the cytosol (Trewavas and Malho 1998; Berridge et al. 2000). Not only are Ca^{2+} signals transient, but they also vary spatially. Different organelles or cytoplasmic loci may act as distinct compartments for Ca^{2+}-mediated signaling. Thus, receptors for Ca^{2+} must be widely distributed and bind the ion selectively and rapidly to transduce temporally and spatially complex signals and communicate them to other components of the cell.

The information encoded in Ca^{2+} signals is transduced by proteins that specifically bind the ion in the nanomolar to micromolar range in the presence of much greater concentrations of other metal ions. Ca^{2+}-binding proteins can be grouped into three very broad categories: enzymes, such as protein kinase C and the calmodulin-like domain protein kinase (Harmon et al. 2000), whose activities are directly regulated by Ca^{2+}-binding; proteins that bind Ca^{2+} and function as

buffers, such as parvalbumin; and sensor proteins, such as calmodulin (CaM), that act as intermediaries and couple Ca^{2+} signals to the activation of other cellular components (Zielinski 1998; Chin and Means 2000). CaM is the most highly expressed and broadly distributed Ca^{2+}-binding sensor protein characterized by the EF-hand structural motif. When Ca^{2+} is bound by the EF-hands of CaM, hydrophobic surfaces are exposed on the molecule. These hydrophobic regions, in concert with a flexible linker domain, facilitate the interaction of CaM with a wide array of target proteins (Crivici and Ikura 1995). Interaction with target proteins by CaM raises the affinity of CaM for Ca^{2+} (Peersen et al. 1997) and sensitizes the activity of the CaM-target protein complex to changes in Ca^{2+} concentration. Thus, CaM-target protein interaction couples changes in Ca^{2+} concentration to the regulation of biochemical activities in cells.

One mechanism by which cells transduce Ca^{2+} signals to elicit specific physiological responses involves the differential expression of EF-hand, Ca^{2+}-binding sensor proteins. In animal cells, numerous Ca^{2+} sensor proteins have been characterized. In plants, however, this protein family is much less well characterized and its functions are incompletely understood. In marked contrast to vertebrates, whose genomes encode multiple calmodulin gene (*Cam*) sequences that encode a single isoform of the protein, or yeasts and filamentous fungi, which contain single genes encoding CaM (Davis et al. 1986; Takeda and Yamamoto 1987; Toutenhoofd and Strehler 2000), higher plants encode and express a variety of CaM isoforms (Zielinski 1998). A growing body of in vitro (Lee et al. 1995; Liao et al. 1996; Reddy et al. 1999) and in vivo (Heo et al. 1999) evidence suggests that CaM isoforms may interact preferentially with certain subsets of target proteins and further diversify the responses to Ca^{2+} signals.

In this report, sequences encoding new members of the EF-hand family of Ca^{2+}-binding proteins from *Arabidopsis* are described and their molecular properties compared using a combination of biochemical and genetic approaches. These new sequences encode a highly conserved, *Cam7*, and two highly diverged, *Cam8* and *Cam9*, members of the *Cam* family. Evidence is provided by both experimental approaches that the highly diverged sequences are not as broadly functional as conventional CaMs. The results support and extend previous studies and are consistent with the idea that *Cam8* and *Cam9* are specialized members of the EF-hand family of Ca^{2+}-binding proteins that may interact with a more limited spectrum of target proteins in vivo.

Materials and methods

Materials

Bacteria harboring the *Cam7* (dbEST Clone Id. 240D7T7; GenBank Accession number N65851), *Cam8* (dbEST Clone Id. E4G7T7; GenBank Accession number AA042238), and *Cam9* (dbEST Clone Id. 163P20T7; GenBank Accession number R65197) cDNAs were obtained from the Arabidopsis Biological Resource Center (Ohio State University). TOPO-TA cloning kits and *Escherichia coli* strain TOP10F′ were from Invitrogen (Carlsbad, Calif., USA). Synthetic oligonucleotides for reverse transcription-polymerase chain reaction (RT-PCR) and PCR-based mutagenesis were obtained from Operon Technologies (Alameda, Calif., USA). Trp-3, a synthetic peptide encoding an idealized CaM-binding domain (O'Neil and Degrado 1990), was a gift of William DeGrado. *Taq* DNA polymerase, restriction enzymes, and DNA ligase were obtained from Life Technologies (Gaithersburg, Md., USA). Reagents for RT-PCR were from Amersham-Pharmacia Biotech (Piscataway, N.J., USA).

Plasmids

The plasmids used in this study are listed in Table 1. Plasmid DNA was isolated using a commercial kit (Qiagen, Chatsworth, Calif., USA). Sequencing of cDNA inserts and PCR-derived products of the cDNAs was carried out over the full lengths of both DNA strands at the W.M. Keck Center for Comparative and Functional Genomics in the University of Illinois Biotechnology Center. The GenBank Accession numbers for the complete nucleotide sequences are: (*Cam7*, AF178073); (*Cam8*, AF178074); (*Cam9*, AF178075). *Cam* cDNA clones were engineered by PCR to create *Nco*I restriction sites within the context of their initiation codons flanked upstream by a *Bam*HI restriction site, and *Sna*BI restriction sites within their 3′ untranslated regions. The forward and reverse primers used to create these mutations were:

- 5′-GGAT**CCATGG**CGGATCAGCTAACCGAT-3′ and
- 5′-GATG TAAAAGAAAAACAT**ACG TA**GGAT TTCGAG-3′ for *Cam7*;
- 5′-GGAT**CCATGG**AAGAAACAGCACTGACAAAAG-3′ and
- 5′-GTGAACAC**TACGTA**GTAACAACTAACAAG-3′ for *Cam8*;
- 5′-GGAT**CCATGG**CGGATGCTTTCACAGATG-3′ and
- 5′-**TACGTA**ATGCTAATAAGAGGCAGCAATCATCATTTTAG-3′ for *Cam9*,

where the nucleotides in bold represent the *Nco*I and *Sna*BI recognition sequences in the forward and reverse primers, respectively. After amplification, the PCR products were cloned and the fidelities of the amplified regions were verified by DNA sequencing. Engineered inserts from single isolates of each sequence were recovered by cleavage with *Nco*I and *Sna*BI for constructing the yeast complementation plasmids described in Table 1. To construct *E. coli* expression plasmids, the engineered cDNAs were cleaved with *Nco*I and *Xho*I to construct pRZ72 and pRZ82, or *Bam*HI and *Xba*I to construct pRZ92.

Analyses of gene expression

RNA gel blot assays were performed by fractionating total RNA in formaldehyde-agarose gels, transferring the RNAs to positively charged nylon membranes, and hybridizing the membranes to DNA probes labeled with ^{32}P-dATP by random priming using a commercial kit (Promega, Madison, Wis., USA). RT-PCR assays for *Cam* mRNAs were performed with oligo $(dT)_{12-18}$ as the primer for reverse transcription using Ready-to-Go RT-PCR reagents (Amersham-Pharmacia Biotech). Sequences of the forward and reverse primers, respectively, were:

Table 1 Plasmids used in this study

Plasmid	Parent vector	Relevant markers and construction	Reference or source
pCR2.1		TA-cloning site for PCR products, Amp^r, Kan^r	Invitrogen
pET24d		P_{T7} Kan^r	Novagen
pJG7		*CMD1*, *CEN4 ARS*, *ADE3*, *URA3*, Amp^r	a
pPHIL1		P_{tac} with gene encoding GST, Amp^r	Phil Wakeley
pRS415		*CEN6 ARS*, *LEU2*, Amp^r	b
pRS426		2-μm origin, *URA3*, Amp^r	c
pRZ30	pRS415	1.56-kb *Sal*I-*Bam*HI fragment of *CMD1*, Amp^r, *LEU2*, *CEN6 ARS*	This study
pRZ31	pRS426	1.56-kb *Sal*I-*Bam*HI fragment of *CMD1*, Amp^r, *URA3*, 2-μm origin	This study
pRZ70	pZL1	EST clone 240D7T7, Amp^r	d
pRZ71	pCR2.1	600-bp *Bam*HI/*Nco*I-*Sna*BI/*Xba*I PCR product of *Cam7*, Amp^r, Kan^r	This study
pRZ72	pET24d	P_{T7}::*Cam7*, Kan^r	This study
pRZ73	pJG7	P_{CMD1}::*Cam7*, Amp^r *URA3*, *CEN4 ARS*	This study
pRZ74	pRZ30	P_{CMD1}::*Cam7*, Amp^r, *LEU2*, *CEN6 ARS*	This study
pRZ80	pZL1	EST clone E4G7T7, Amp^r	d
pRZ81	pCR2.1	600-bp *Bam*HI/*Nco*I-*Sna*BI/*Xba*I PCR product of *Cam8*, Amp^r, Kan^r	This study
pRZ82	pET24d	P_{T7}::*Cam8*, Kan^r	This study
pRZ83	pJG7	P_{CMD1}::*Cam8*, Amp^r, *URA3*, *CEN4 ARS*	This study
pRZ84	pRZ30	P_{CMD1}::*Cam8*, Amp^r, *LEU2*, *CEN6 ARS*	This study
pRZ90	pZL1	EST clone 163P20T7, Amp^r	d
pRZ91	pCR2.1	600-bp *Bam*HI/*Nco*I-*Sna*BI/*Xba*I PCR product of *Cam9*, Amp^r, Kan^r	This study
pRZ92	pPHIL1	P_{tac}::GST::*Cam9*, Amp^r	This study
pRZ93	pJG7	P_{CMD1}::*Cam9*, Amp^r, *URA3*, *CEN4 ARS*	This study
pRZ94	pRZ30	P_{CMD1}::*Cam9*, Amp^r, *LEU2*, *CEN6 ARS*	This study
pTD56		*CMD1*, *LYS2,* Amp^r, 2-μm origin	e

[a]Geiser et al. (1991)
[b]Sikorski and Hieter (1989)
[c]Christianson et al. (1992)
[d]Arabidopsis Biological Resource Center, Ohio State University, http://www.aims.org/
[e]Davis and Thorner (1989)

- 5′-TCGAGAAAATCCGAAGAAGACAAAATGGCG-3′and
- 5′-CGTCACGGACAATAACAAAACCAAGAAGCA-3′ for *Cam7*;
- 5′-CTTCAAAATATTCTCAGAAAACAATGGAAG-3′ and
- 5′-TTTGTTCAACTTCTTCATCTGTTAGCTTTT-3′ for *Cam8*;
- 5′-CAGATCCAAGAGTTTTACGAAGCCTTCTGT-3′ and
- 5′-CTGTTATCTTCATCCCCATGTCCTTCATTC-3′for *Cam9*.

Recombinant protein production and analysis

Plasmids pRZ72, pRZ82 and pRZ92 were transformed into *E. coli* strain BL21 (DE3) and used to produce recombinant proteins (Liao et al. 1996). The final purification step was by Ca^{2+}-dependent hydrophobic chromatography on phenyl-Sepharose (Liao and Zielinski 1995). Denaturing polyacrylamide gel electrophoresis was performed with the modification that 2 mM $CaCl_2$ or EDTA was added to samples prior to electrophoresis. CaM-peptide gel mobility shift electrophoresis assays were performed as described previously (Liao et al. 1996).

Yeast complementation

Saccharomyces cerevisiae strain TDY55-5D, which harbors a *TRP1* insertion in the *Cam* (*CMD1*) locus, was generously provided by Dr. Trisha Davis (Department of Biochemistry, University of Washington). The genotype of this haploid strain is: *MATa*, *ade2-1oc*, *ade3*Δ*-100*, *can1-100*, *cmd1*Δ::^*TRP1*, *his3-11*, *15*, *leu2-3*, *112*, *lys2*Δ::^*HIS3*, *trp1-1*, *ura3-1*. Viability of TDY55-5D is maintained by complementation by a 2-μm plasmid harboring *CMD1*, together with *ADE3* and *LYS2* markers (pTD56; Davis and Thorner 1989). Plant *Cam* isoforms were expressed in yeast by cloning their coding sequences in the unique *Nco*I and *Sna*BI restriction sites in the shuttle vector pJG7 (Geiser et al. 1991) in place of the *CMD1* coding sequences to construct pRZ73, pRZ83, and pRZ93. Thus, the plant *Cam* sequences in these plasmids were expressed under the direction of the wild-type (WT) *CMD1* promoter. Transformants harboring these stable CEN4 plasmids and losing pTD56 are expected to have a Ura^+, Lys^- phenotype and form white colonies as a result of their *ade2 ade3* genotype. Because this complementation method is passive, it is referred to here as "gratuitous" complementation to distinguish it from a complementation method that actively selects against cells harboring plasmids containing the WT *CMD1* sequences described below. Cells that do not retain *CMD1* or *Cam* sequences are not viable (Davis et al. 1986; Takeda and Yamamoto 1987). For a second set of selection experiments, additional expression vectors for *Cam8* and *Cam9* were constructed. The plasmids pRZ30 and pRZ31 were derived from pRS415 (Sikorski and Hieter 1989) and pRS426 (Christianson et al. 1992), respectively, by inserting a 1.56-kb *Sal*I-*Bam*HI restriction fragment harboring the *CMD1* sequences from pJG7. Plant *Cam* expression vectors pRZ84 and pRZ94 were derived from pRZ30 by substituting the coding sequences of *Cam8* and *Cam9* for that of *CMD1*. Transformants harboring pRZ84 or pRZ94 and lacking the *URA3* marker and the linked WT *CMD1* sequences were selected on media containing 5-fluoro-orotic acid (5-FOA) (Adams et al. 1997). Yeast transformations were carried out using reagents and methods from a commercially available kit (Zymo Research, Orange, Calif., USA). Rich (YPD) and synthetic drop-out media, methods for maintaining strains, and growth conditions were as described previously (Adams et al. 1997).

PCR assays to estimate the retention of WT *CMD1* and plant *Cam* sequences in transformants were carried out by transferring a small colony to a microfuge tube containing all components of the reaction except primers. After incubating the samples for 5 min at 96 °C, primers were added to the reaction tubes and *CMD1* sequences were amplified using a program consisting of 25 cycles of 30 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by 5 min at 72 °C. The primers were derived from the 5′ and 3′ flanking sequences of *CMD1*; the sequences of the sense and antisense primers were

- 5′-TTATTCTTGACCGGAAACTATCGAACTACA-3′ and
- 5′-TCTGCTGCTGACGATAACTTAAATCCTTTTC-3′, respectively.

Results

Identification of highly diverged sequences related to *Cam* in *Arabidopsis*

By searching the *Arabidopsis* database using either the nucleotide or amino acid sequence of *Arabidopsis Cam2* as query sequences in the programs BLASTn and tBLASTn (Altschul et al. 1997), respectively, a number of cDNAs were identified whose nucleotide sequences share substantial identity with those of plant or animal *Cam* sequences. Among these sequences were three not described previously in the literature. Expressed sequence tag (EST) clones encoding these sequences were obtained from the Arabidopsis Biological Research Center and their nucleotide sequences were determined. The deduced primary structures of these proteins are shown in Fig. 1 and compared with CaM sequences encoded by yeast *CMD1* (Davis et al. 1986). *Cam7* encodes a typical plant CaM sequence (Zielinski 1998) that differs by a single amino acid residue, a conservative Lys to Arg substitution at residue 126, from CaM2 (Ling et al. 1991). *Cam8* and *9*, on the other hand, encode unconventional, 150-residue CaM-like polypeptides. BLAST searches of the nucleotide sequence databases revealed that *Cam8* shares the highest level of nucleotide and amino acid sequence identity with the highly divergent soybean CaM isoforms, SCaM4 (83% at the amino acid level) and SCaM5 (81% at the amino acid level) (Lee et al. 1995). The nearest neighbors to *Cam9* were sequences encoding CaM from a variety of organisms not restricted to the plant kingdom; the closest match was *T. pyriformis* CaM (GenBank Accession number D10521), which shares 52% identity and 70% similarity with CaM9. The deduced amino acid sequences of the proteins encoded by *Cam8* and *Cam9* are 77% and 50% identical to *Cam7*, respectively.

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                          * * * * * *
CaM7   ADQLTDDQISEFKEAFSLFDKDGDGCITTKELGTVMRSL  39
CaM8  EETA--K---T------C-----------VE--A--I---
CaM9   --AF--E--Q--Y---C-I---S--F--KEK-TK--K-M
CMD1p SSN--EE--A------A-----NN-S-SSS--A------

                          * * * * * *
CaM7      GQNPTEAELQDMINEVDADGNGTIDFPEFLNLMARK  75
CaM8      D-----Q--H-I-T-I-S-S----E-A-------K-
CaM9      -K--KAEQ--Q-MSD--IF---G-T-DD--YI--QN
CMD1p     -LS-S---VN-LM--I-V---HQ-E-S---A--S-Q

                          * * * * * *
CaM7     MKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNL 112
CaM8     LQES-A--------K--------Y---S--S---I--
CaM9     TSQESASD--I-V-----R-GD-L--QL--GEG-KDM
CMD1p    L-SN---Q--L---K----NGD-L------K--L-SI

                          * * * * * *
CaM7      GEKLTDEEVDEMIREADVDGDGQINYEEFVKVMMAK 148
CaM8      ---------EQ------L-----NV-D----M-INID
CaM9      -M-I-A--AEH-VR---L----FLSFH--S-M-I-ASY
CMD1p     ------A---D-LR-VΔS--S-E--IQQ-AALLSK
```

Fig. 1 Comparison of the deduced amino acid sequences of *Arabidopsis thaliana* CaM7, 8, and 9 with that of *Saccharomyces cerevisiae* CaM (CMD1p). Sequences are arranged to illustrate the relationships among the four Ca^{2+}-binding domains. Identical amino acids are indicated by a *dash* (–). Residues marked with *asterisks* (*) correspond to ones that act as Ca^{2+}-binding ligands in conventional CaM proteins. The symbol *delta* (Δ) in the CMD1p sequence indicates a deletion introduced for the purposes of alignment

The sequences of the putative Ca^{2+}-binding loops of CaM9 display differences from CaM7 and CaM8 that are likely to be functionally significant. First, residues 30 and 31 of CaM9 are inverted compared with conventional CaM sequences. Second, the Glu to Asp substitution at residue 67 in CaM9 is not generally found in conventional CaM sequences. The significance of these observations is that they both alter the critical 12th residue of the ion-binding loop (position –Z) in the first and second EF-hands of CaM9. This residue is critically important because it donates two oxygen ligands to the 7-fold chelation of Ca^{2+} by EF-hands (Peersen et al. 1997; Cates et al. 1999). A recent study on the structure of the Ca^{2+}-binding protein parvalbumin found that an Asp for Glu substitution in the –Z position makes the structure of the EF-hand more favorable for binding Mg^{2+} over Ca^{2+} (Cates et al. 1999).

Although the amino acid sequences of CaM8 and CaM9 diverge considerably from that of CaM2, it is noteworthy that the positions of strongly hydrophobic residues in these proteins are highly conserved functionally, if not in sequence identity. This observation is consistent with structural analyses of CaM-target protein complexes, which demonstrated that the preponderance of residue-residue interactions between CaM and its target proteins are hydrophobic rather than charge-charge interactions (Crivici and Ikura 1995). In addition, both CaM8 and CaM9 are predicted to have strongly acidic isoelectric points (pIs) (3.69 and 3.86, respectively, for CaM8 and CaM9 compared with 3.79 for CaM7) and they contain high proportions of methionine and phenylalanine residues; both properties are characteristics of CaM-like proteins.

During the course of this study, DNA sequences for the structural genes encoding each of these proteins were deposited in GenBank as part of the *Arabidopsis* genome project (GenBank accessions AL162691, Z97336 and AL049711). The genes encoding the sequences described in this report are dispersed in the *Arabidopsis* genome (*Cam7*, chromosome III; *Cam8*, chromosome IV; *Cam9*, chromosome II). In an arrangement that is typical of all plant *Cam* sequences described to date (Zielinski 1998), the codon encoding Gly25 is interrupted by an intron in each of the genes. The intron in this position is the only one found in *Cam7*. The coding sequences of *Cam8* and *Cam9*, on the other hand, are interrupted by two additional introns. In no case is there an obvious relationship between

intron placement and separation of functional domains in the proteins these genes encode.

Expression of *Cam7*, *8*, and *9* mRNAs

RNA gel blot and RT-PCR analyses of *Cam7*, *8*, and *9* mRNA accumulation were carried out to examine the expression of these genes at the whole-organ level, and to exclude the possibility that their cDNA sequences represent partial copies of much longer mRNAs. Figure 2a shows that the *Cam* sequences expressed in leaf total RNA fractions range between approximately 700 and 800 nucleotides. This size is slightly larger than the sums of the coding and untranslated regions of each of the sequences, a difference that could be explained by the presence of short poly(A)-tails on each of the mRNAs. The sizes of *Cam7*, *8*, and *9* mRNAs observed in the blots shown in Fig. 2a are consistent with the sizes of other *Cam* and *Cam*-like mRNAs in *Arabidopsis* (Ling et al. 1991; Perera and Zielinski 1992; Ling and Zielinski 1993). Therefore, the sequences described here are unlikely to represent the products of truncated cDNA clones derived from much larger mRNAs by incomplete reverse transcription or mRNA degradation prior to cDNA synthesis. It should also be noted that the RT-PCR products obtained in these experiments were of the predicted sizes based on the *Cam* gene sequences. Cloning and sequencing one randomly chosen clone for each sequence confirmed the identities of the RT-PCR products.

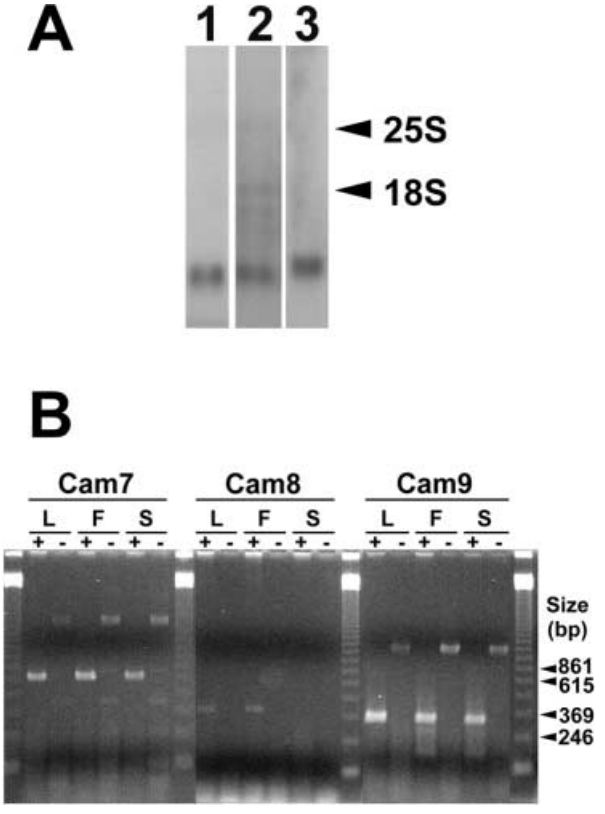

Fig. 2a, b Analyses of *Cam7*, *8*, and *9* mRNA accumulation. **a** RNA gel blot analyses of *Cam* isoform mRNA accumulation in *Arabidopsis* leaf RNA fractions. Five-μg aliquots of total leaf RNA were fractionated in an agarose-formaldehyde gel, transferred to a charged nylon filter, and fixed by UV-crosslinking. Triplicate regions of the filter were excised and hybridized with ^{32}P-labeled *Cam7* (*lane 1*), *Cam8* (*lane 2*), or *Cam9* (*lane 3*) DNA probes having approximately equal specific activities (±10%). Autoradiographic exposures were for either 20 h (*lanes 1, 3*) or 96 h (*lane 2*) under identical conditions. The approximate sizes of *Cam* mRNAs were deduced from *E. coli* rRNA and synthetic RNA (Life Technologies) markers run in parallel lanes of the gel, which were stained with ethidium bromide. **b** RT-PCR analysis of *Cam7*, *8*, and *9* mRNA expression in different *Arabidopsis* organs. Duplicate 1-μg portions of total RNA isolated from leaves (*L*), flowers (*F*), and green siliques (*S*) were analyzed in single-tube RT-PCR reactions in which the reverse transcriptase was untreated (+) or inactivated by heat denaturation (–), prior to adding the RNA. One-tenth of each reaction was fractionated in a 1% agarose/2% NuSeive gel and stained with ethidium bromide. The predicted sizes of the RT-PCR products were: 655 bp (*Cam7*), 400 bp (*Cam8*), and 332 bp (*Cam9*)

RT-PCR analyses of *Cam* mRNA accumulation are shown in Fig. 2b and reveal that each mRNA is expressed in leaf, flower, and silique total RNA fractions. Amplification conditions were not established that permit precise quantification of steady-state *Cam7*, *8*, and *9* mRNA levels in these assays. Nevertheless, they are consistent with RNA gel blots and suggest that *Cam8* mRNA is expressed at lower levels than are *Cam7* and *Cam9* mRNAs, particularly in siliques where *Cam8* mRNA accumulated near the limits of detection of this assay.

Production of recombinant CaM isoforms in *E. coli*

To investigate the molecular properties of CaM7, 8, and 9 in more detail, the respective cDNA sequences were engineered by the PCR to facilitate cloning in expression plasmids. CaM7 and CaM8 were expressed readily as unfused proteins using T7-based expression plasmids; these proteins were purified using methods employed previously to isolate recombinant plant CaM (Liao and Zielinski 1995; Liao et al. 1996). On the other hand, yields of CaM9 were poor in the T7 expression system. To recover CaM9, gene fusions consisting of the full-length *Cam9* reading frame fused to glutathione-*S*-transferase (GST) were constructed and the recombinant fusion protein was purified by affinity chromatography on glutathione-agarose. As a final purification step, after treating the fusion protein with thrombin, CaM9 was isolated by Ca^{2+}-dependent hydrophobic chromatography free of detectable levels of GST.

One of the hallmarks of CaM is its ability to bind Ca^{2+} in the presence of SDS, which increases its electrophoretic mobility relative to CaM in the absence of free Ca^{2+}. Figure 3a shows that CaM7 and CaM8 displayed this characteristic electrophoretic mobility shift when incubated with 2 mM $CaCl_2$ prior to electrophoresis. In contrast, the mobility of CaM9 did not change under these conditions but required at least 5 mM $CaCl_2$ in order to display altered mobility (Köhler and Neuhaus 2000; and data not shown). To further explore

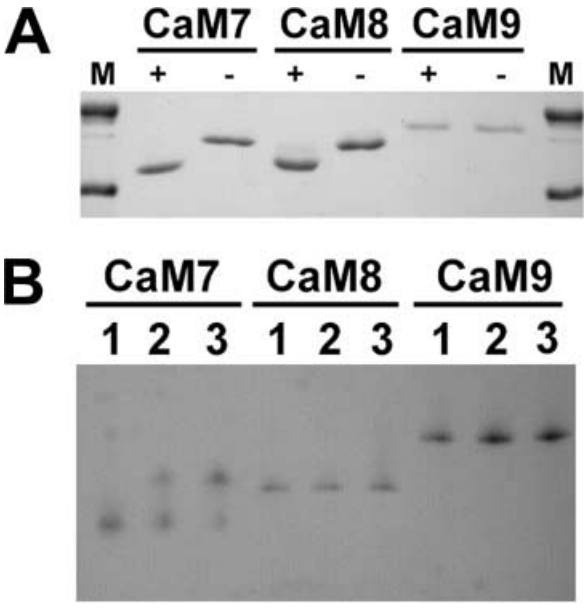

Fig. 3a, b CaM7, 8 and 9 differ in their apparent Ca^{2+} and peptide-binding properties in vitro. **a** Purity and Ca^{2+}-induced electrophoretic mobility shift analyses of recombinant CaM proteins. Three μg (CaM7 and CaM8) or 1.5 μg (CaM9) of protein was brought to 2 mM with either $CaCl_2$ (*lanes designated* +) or EDTA (*lanes designated* –), subjected to electrophoresis in a 15% polyacrylamide gel containing SDS, and detected by Coomassie blue staining. *Lanes marked M* contained molecular mass standard proteins (Bio-Rad, broad range). **b** Gel mobility shift analyses of CaM isoform interaction with a basic, amphiphilic peptide, Trp-3. *Lanes 1* No added peptide, *lanes 2* 0.5 mol equivalent, *lanes 3* 1 mol equivalent. After mixing, protein samples were fractionated in a 10% polyacrylamide gel containing 4 M urea and detected by Coomassie blue staining

the biochemical properties of the newly described proteins, their abilities to bind a synthetic, idealized CaM-binding peptide, Trp-3 (O'Neil and Degrado 1990), were assessed by a gel mobility shift assay. In this assay, fixed amounts of protein were incubated with different molar equivalents of Trp-3 in the presence of Ca^{2+} and the mixtures were fractionated in polyacrylamide gels in the presence of Ca^{2+} and 4 M urea to minimize spurious protein-protein interaction. Figure 3b shows that a peptide-protein complex whose electrophoretic mobility could be distinguished from that of free CaM7 was readily detected in these assays; CaM7 appeared to bind approximately 1 mol Trp-3 per mol protein. In contrast, under identical conditions neither CaM8 nor CaM9 formed complexes with Trp-3 that could be resolved from the free proteins by gel electrophoresis. Eliminating urea from the electrophoretic separation did not improve the recovery of CaM8 or CaM9 complexes with Trp-3.

Complementation of a yeast *Cam* null by sequences encoding CaM7, 8 and 9

Biochemical measurements of the interaction of CaM7, 8, and 9 with Ca^{2+} and a synthetic target peptide suggested that these proteins might not function identically. To define further the functional relationships among the three *Cam* (or *Cam*-like) sequences, their abilities to complement a non-revertible yeast *Cam* null (*cmd1::^TRP1*) were tested. Two strategies were followed in these studies. In one set of experiments, a host whose viability is maintained by a 2-μm plasmid harboring *CMD1* as well as *ADE3* and *LYS2* markers, was transformed with a series of centromere-based (CEN) plasmids expressing the plant *Cam* sequences under the control of the *CMD1* promoter. In this case, the transformants were scored initially for the loss of *CMD1*, and thus functional complementation by the plant sequences, by loss of the pink phenotype associated with the *ade2 ADE3* genotype. This is referred to here as gratuitous complementation because there was no active selection for or against the host *CMD1* sequences. An example of this phenotypic change is shown in Fig. 4a, in cells transformed with *Cam8* coding sequences. Under these conditions, *Cam7* and *Cam8* functionally complemented *cmd1*, but *Cam9* did not. Although both *Cam7* and *Cam8* substituted for *CMD1*, *Cam7* did so more readily as evidenced by the recovery of a high proportion of white colonies (> 50%) upon plating the transformed *cmd1* host. In contrast, white transformants were obtained at a 10-fold lower frequency when identical batches of yeast were transformed with plasmids harboring *Cam8*. Typically, one or two cycles of streaking colonies on media selective for the plasmid harboring *Cam8* but non-selective for the plasmid harboring *CMD1* were required to observe the white phenotype indicative of the loss of the *CMD1*-harboring plasmid. PCR assays shown in Fig. 4b show that yeast transformed with plasmids pRZ73 and pRZ83, which express *Cam7* or *Cam8*, respectively, retained less than one copy per cell of plasmid harboring *CMD1*. This assessment is based on the assumption that plant *Cam*, *CMD1*, and the inactivated chromosomal *cmd1::^TRP1* sequences amplified with similar efficiencies.

The PCR assays used in this experiment were not tested rigorously to determine how accurately they reflect quantitative differences in the copy numbers of *Cam* sequences in different strains. Nevertheless, densitometry of the gel shown in Fig. 4b indicated that plasmids harboring *Cam8* appeared to accumulate to 3-fold higher levels than did those encoding *Cam7* in complemented yeast strains. Thus, gene dosage may play a role in the ability of *Cam8* to substitute for *CMD1*. In contrast, yeast transformed with pRZ93, which expresses *Cam9*, retained both the yeast and plant sequences. Repeated cycles of growth in liquid medium and streaking on agar medium, both of which were selective for the plasmid harboring *Cam9* but non-selective for the plasmid harboring *CMD1*, failed to yield white colonies. When strains transformed with *Cam7* or *Cam8* were grown in rich medium there were no observable differences in their growth rates compared with yeast harboring functional *CMD1* (data not shown).

In a second set of experiments, a host whose viability was maintained by a 2-μm plasmid harboring *CMD1*

and a *URA3* marker (pRZ31) was transformed with plasmids expressing *Cam7*, *Cam8*, or *Cam9* under the direction of the *CMD1* promoter together with a *LEU2* marker (plasmids pRZ74, pRZ84 and pRZ94, respectively). Leu^+ transformants harboring the plant *Cam* sequences were plated on media containing 5-FOA to select against cells retaining the plasmid bearing the *URA3* marker (Adams et al. 1997) and, as a consequence, the linked *CMD1* sequence. This selection method readily yielded viable Leu^+ and Ura^- colonies complemented by *Cam7* and *Cam8*. Colonies transformed with *Cam9* sequences, on the other hand, were recovered at a very low frequency. Nevertheless, Fig. 5a shows that yeast strains were recovered in which *Cam9*

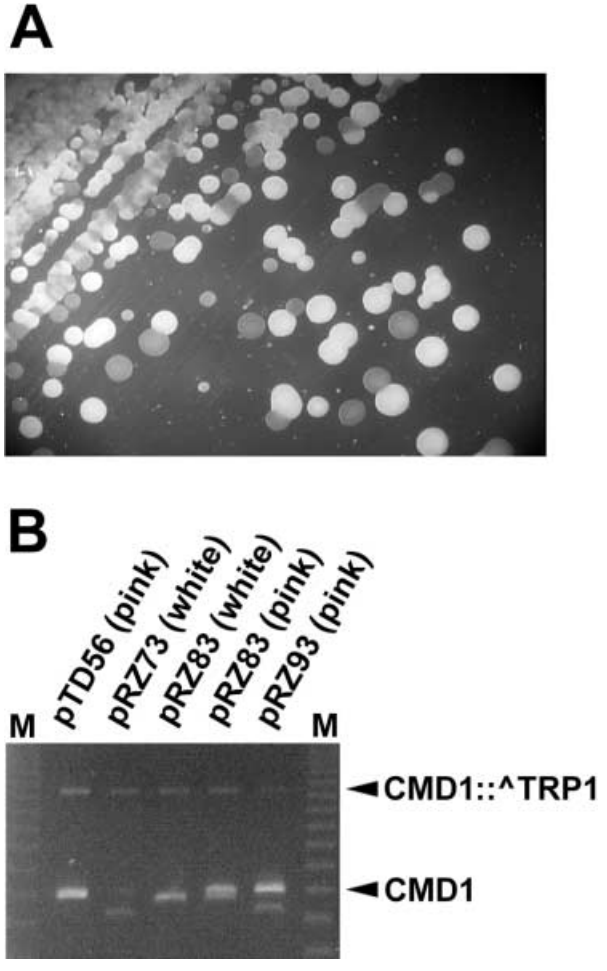

Fig. 4a, b Evidence for functional complementation of yeast *cmd1* by *Cam7* and *Cam8*. **a** Gratuitous complementation illustrated for *Cam8* transformants selected on synthetic medium lacking uracil. Darker (pink) colonies accumulate an intermediate of adenine biosynthesis from the *ade2 ADE3* genotype of the parent yeast strain and the complementing plasmid (pTD56) they harbor. Light (white) colonies have lost pTD56, resulting in an *ade2 ade3* genotype; CaM function in these colonies is provided by pRZ83, which expresses *Cam8*. **b** Colony PCR assays of *Cam* sequences harbored in yeast strains transformed with WT *CMD1* (pTD56), *Cam7* (pRZ73), *Cam8* (pRZ83), or *Cam9* (pRZ93) expression plasmids. The migration of amplification products of plasmid-derived *CMD1* and the *TRP1* insertional knock out of chromosomal *CMD1* sequences is indicated on the *right*. The phenotype (*pink* or *white*) of each colony used in the assay is indicated at the top of the figure. *Lanes designated M* contained DNA size standards

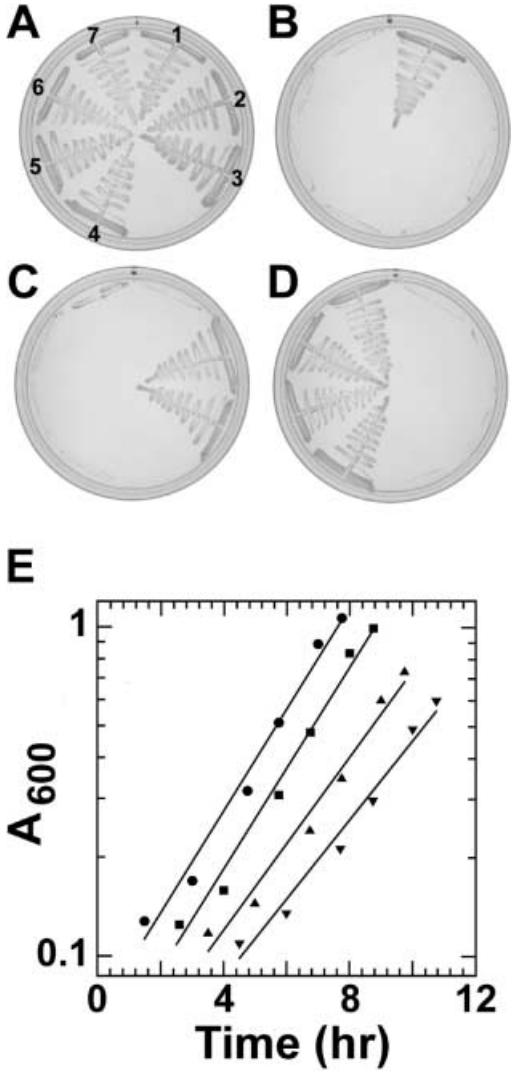

Fig. 5a–e Complementation of *S. cerevisiae cmd1* by conserved and divergent members of the *Arabidopsis Cam* family. The compositions of media on the plates are: **a** complete (YPD), **b** synthetic medium lacking lysine, **c** synthetic medium lacking uracil, **d** synthetic medium lacking leucine. Different sectors of each plate represent strains in which *cmd1* function is complemented by plasmids harboring different *Cam* sequences: *1* pTD56 (*CMD1*), *2* pJG7 (*CMD1*), *3* pRZ73 (*Cam7*), *4* and *5* pRZ84 (*Cam8*), *6* and *7* pRZ94 (*Cam9*). **e** Growth curves of complemented strains in synthetic medium lacking leucine containing 2 mM EGTA. Test cultures were inoculated by 1–100 dilutions into fresh medium of overnight cultures grown in rich medium. The growth of duplicate cultures was followed for each strain by measuring the A_{600}. Each point on the graph represents the average of these duplicate readings. The complementing plasmids, the *Cam* sequences they harbor, and the mean doubling times, respectively, in different strains are: *circles* pRZ30 (*CMD1*), 268 min; *squares* pRZ74 (*Cam7*), 271 min; *upward-pointing triangles* pRZ84 (*Cam8*), 317 min; and *downward-pointing triangles* pRZ94 (*Cam9*), 347 min. This experiment was performed twice with similar results

sequences functionally substituted for *CMD1* as inferred from their patterns of growth on synthetic medium. Plasmid rescue experiments confirmed this conclusion since only the expected plant *Cam* sequences were recovered from each of the complemented strains shown in Fig. 5a (data not shown).

Because *Cam9* was unable to gratuitously substitute for *CMD1*, it was expected that a strain harboring this plant sequence might differ in its growth rate from strains complemented by *Cam7* or *Cam8*. Figure 5b illustrates an experiment demonstrating that the growth rates of yeast strains complemented by *Cam9* and *Cam8* are modestly reduced from those of strains complemented by *CMD1* or *Cam7* in synthetic medium in which Ca^{2+} was depleted by the addition of 2 mM EGTA. A smaller but statistically significant difference in growth rate was also observed for *Cam9*-complemented strains in rich medium (data not shown). Neither *Cam8* nor *Cam9* transformants displayed obvious *ts* phenotypes when grown on rich or synthetic agar media, but both displayed moderate cold-sensitivities in their growth rates at 20–23 °C. On the other hand, under all conditions tested, the more conventional CaM encoded by *Cam7* substituted for *CMD1* with no measurable difference in function.

Discussion

This report characterizes the molecular properties of both conserved and highly divergent sequences encoding CaM-related proteins from *Arabidopsis thaliana*. As is the case for previously described *Cam* sequences in *Arabidopsis* (Ling et al. 1991; Perera and Zielinski 1992; Gawienowski et al. 1993), *Cam7*, *8*, and *9* mRNAs are expressed in a variety of organs. The polypeptides they encode contain recognizable EF-hand Ca^{2+}-binding motifs, predicted acidic pIs, and highly conserved clusters of hydrophobic amino acids. What is particularly novel about two of the new sequences, however, is that they encode highly diverged members of the *Cam* family. While CaM9 represents a polypeptide unlike any other characterized previously, CaM8 is very similar to the highly diverged CaM isoforms from soybean, SCaM4 and SCaM5, which have been extensively characterized (Lee et al. 1995, 2000). A combination of biochemical and genetic analyses presented here supports the idea that CaM7, 8, and 9 are Ca^{2+}-binding sensor proteins that function by similar mechanisms but which differ in the efficiency with which they can fulfill the role of CaM in the model eukaryote *S. cerevisiae*.

The broad significance of multiple CaM isoforms in plants is not understood. However, a frequently proposed hypothesis is that diverged CaM isoforms may activate selected subsets of target proteins involved in Ca^{2+}-mediated signal transduction (Liao et al. 1996; Snedden and Fromm 1998; Heo et al. 1999). There are a number of biochemical studies demonstrating differential regulation of target proteins by plant CaM isoforms that support this idea. The $K_{0.5}$ of NAD kinase activated by the *Arabidopsis* CaM2 isoform is approximately half that for the CaM4 or CaM6 proteins (Liao et al. 1996). The highly diverged soybean SCaM4 isoform (Lee et al. 1995) does not activate this enzyme, and is the presumptive ortholog of CaM8 described in this report. This approach was extended to a wider array of CaM-regulated enzymes, where activation by SCaM4 generally required higher levels of the EF-hand protein or higher Ca^{2+} concentrations than did activation by the more conventional SCaM1 (Lee et al. 2000). This result, in particular, is consistent with the findings reported here that *Cam8* and *Cam9* expression supported reduced rates of growth in media depleted of Ca^{2+} (Fig. 5b). Limited in vivo experiments also support the idea of different functional roles for CaM isoforms. SCaM1 and the diverged SCaM4 and SCaM5 mRNAs are differentially induced in response to pathogens, elicitors, and treatments that elevate cytosolic Ca^{2+}. Further, ectopic expression of SCaM4 in transgenic plants triggered the formation of spontaneous lesions reminiscent of hypersensitive cell death on leaves and induced the expression of several resistance-associated genes (Heo et al. 1999). While this work was in progress, Kohler and Neuhaus (2000) used the sequences reported here to construct two hybrid plasmids and test the abilities of CaM8 and CaM9 to interact with the CaM-binding domain of the cyclic nucleotide-gated channel CNGC2. In contrast to more conventional CaM isoforms, however, CaM8 and CaM9 showed no ability to bind the channel.

Structural studies have elucidated at the atomic level a primary mechanism by which CaM interacts with target proteins, which includes the exposure of hydrophobic patches in globular domains within the N- and C-terminal portions of the molecule and the flexible tethering of these domains by the central region of the protein (Crivici and Ikura 1995). One of the most striking features of the CaM and CaM-like proteins described in this report is the functional conservation of hydrophobic residues among the proteins. The positions occupied by the strongly hydrophobic amino acids in CaM7 are 90% conserved by function in both CaM8 and CaM9; the amino acid sequence identities in the corresponding positions, on the other hand, are only 71% and 61% conserved, respectively. Within regions of clustered hydrophobic amino acid residues, CaM9 diverges from CaM7 and CaM8, however. This is most easily observed in regions bordering the third EF-hand Ca^{2+}-binding loop domain, in which CaM9 has the non-conservative substitutions Lys 86 to Ile, Arg 106 to Gly, His 107 to Glu, and Thr 110 to Lys compared with CaM7. The observation that Ca^{2+}-dependent hydrophobic affinity chromatography could be used successfully to purify each of the CaM isoforms examined in this study supports the idea that the diverged and conventional proteins function by similar mechanisms: metal-ion binding induces the exposure of hydrophobic surfaces on the proteins which facilitate interaction with other hydrophobic surfaces. In contrast, Ca^{2+}-dependent mobility

shifting during SDS-PAGE and the ability to bind the idealized basic, amphiphilic peptide, Trp-3, differs among the proteins. These results suggest that CaM9, in particular, probably binds Ca^{2+} with a lower affinity than CaM7 or CaM8, and is consistent with amino acid sequences substitutions for Glu residues in the EF-hands in the amino-terminal half of CaM9 that are critically required for Ca^{2+}-binding (Cates et al. 1999).

The strategy of complementing yeast *cmd1* used in this work is essentially an extension of the approaches taken previously by several other groups to analyze CaM function in yeast. These studies demonstrated that CaM is essential for cell viability (Davis et al. 1986; Ohya and Anraku 1989), and that vertebrate CaM can substitute functionally for yeast CMD1p (Davis and Thorner 1989). They also elegantly demonstrated the functional importance of hydrophobic interaction between CaM and its target proteins (Ohya and Botstein 1994). Based on the similar biochemical properties of vertebrate and conventional plant CaM isoforms in many enzyme activation assays (Zielinski 1998), it is not surprising that *Cam7* readily substitutes for yeast CaM to maintain cell viability and growth. More surprising is the finding that CaM8, which differs considerably from CaM7 and CMD1p in its amino acid sequence, substituted gratuitously for CMD1p. CaM8 also supported WT growth rates in rich medium and robust growth rates in synthetic medium depleted of Ca^{2+} (Fig. 5b). Although the findings of this work are new, this is not the first use of yeast complementation to examine the function of a plant *Cam* sequence. Petunia *Cam53*, a 184-amino-acid protein with a 150-residue, N-terminal CaM domain and a 34-residue, prenylated C-terminal domain was shown to complement the same *CMD1* knock out used in this study (Rodriguez-Concepcion et al. 1999). Unfortunately, *Cam53* transformants were selected with 5-FOA and no information on the growth rates of the complemented strains was presented, nor was the ability of *Cam53* to gratuitously complement yeast mutants tested.

Taken together, the results presented in this work support the idea that, although there are likely to be some specialized in vivo functions for different members of the gene family, there is a level of functional redundancy among the CaM isoforms. This conclusion is consistent with the results of other studies that have primarily taken an in vitro biochemical approach. This study demonstrates that yeast complementation can be used to define differences among plant CaM isoforms and it opens avenues for further genetic analyses of plant CaM function. While a yeast genetic approach provides a more sensitive and subtle means for dissecting CaM and CaM-like protein function than do biochemical assays, it cannot be viewed as a total substitute for in planta assays of CaM function. This is because the spectrum of target proteins regulated by CaM overlaps but is not identical in different organisms (Snedden and Fromm 1998; Zielinski 1998).

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